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Feeding a sustainable blue revolution: The physiological consequences of novel ingredients on farmed fish

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Feeding a sustainable blue revolution: The physiological consequences of novel ingredients on farmed fish

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Editorial: Feeding a sustainable blue revolution: The physiological consequences of novel ingredients on farmed fish

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Editorial on the Research Topic
Feeding a sustainable blue revolution: The physiological consequences of novel ingredients on farmed fish

Increased reliance on the use of alternative ingredients in the formulations of diets for farmed fish has long been a priority to sustain the unparalleled growth of aquaculture. This need which has led the feed industry to explore many alternative protein and oil sources, has also led to many physiological impacts on those species being fed those alternatives. The intensification of aquaculture under a global climate change scenario also contributes as another major challenge to feed the blue revolution, since there is a need to develop diets that enhance fish robustness. Recent research on farmed fish species has resulted in the development of a range of innovative novel ingredients that are able to sustain fish growth performance, improve fish welfare and resilience, and still assure a safe, nutritious and tasty product for human consumption. Since the suitability and sustainability of vegetable ingredients in diets for carnivorous fish has increasingly being questioned, other resources need to be explored and increasingly we need to identify and develop alternatives that do not compete directly with human food supply. Insects and agrofood byproducts have great nutritional value and may contribute towards a circular economy concept. Biotechnology also offers a wide range of possibilities, including microbial biomass and single cell products, but scale-up is still required for many of these initiatives to realize any serious benefit. Micro and macroalge are also receiving attention, not only as sources of macronutrients, but also for their richness in bioactive compounds. However, the potential of each new feed ingredient has to be thoroughly

evaluated before their wide acceptance by the feed industry. There is little to gain by simply transferring risk from one ingredient to another, when ideally what the feed sector needs is greater security of supply, quality and on a cost-effective basis.

This Research Topic on "Feeding the blue revolution" represents a Research Topic of 14 original research articles, highlighting the latest findings related to the evaluation of the physiological consequence of novel ingredients on farmed fish. In the following collection of papers, the readers will note that such an evaluation relies on classical methodological approaches, but also on some cutting edge tools associated with the use of omics to unravel the impact of nutritional clues on the physiology of farmed fish species that are emerging.

Growing interest in the use of insect protein since the inclusion of this ingredient in aquafeed was authorized by the European Union (EU) in 2017 presents new opportunities. In the present volume, Basto et al. have explored the impact of partial and total replacement of fish meal by defatted *Tenebrio molitor* larvae in a comprehensive approach focused on European sea bass (*Dicentrarchus labrax*) growth performance and nutrient utilization, but also further addressed the underlying physiological mechanisms involved in nutrient metabolism. In this carnivorous species, the authors concluded it was feasible to replace up to 80% fish meal without growth impairment, and still assuring fillet nutritional value for consumers. Another important insect species, black soldier fly, *Hermetia illucens*, was assessed by Li et al. as protein source for tongue sole (*Cynoglossus semilaevis*). The replacement of 25% of the fish meal by defatted black soldier fly larvae meal had positive effects on growth, but with some drawback effects on feed conversion. They also noticed intestinal structural damage and abnormal liver function at higher replacement levels. These studies highlighted the potential of insect meals to be included in aquafeeds, but identify that there are maximal inclusion limits depending on the fish species considered. It is also clear that further studies are warranted to fully validate such nutritional approaches at farm scale considering the full production cycle.

Kousoulaki et al. considered a series of low trophic ingredients (*Schizochytrium limacinum*, the diatom *Phaeodactylum tricornutum*, *Hermetia illucens* and tunicate meal from *Ciona intestinalis*) in fishmeal and fish oil free diets when fed to Atlantic salmon (*Salmo salar*). Extensive raw material and dietary chemical characterisation was undertaken to identify constraints and opportunities for using such novel ingredients. Overall, each of the studied ingredients were well accepted by the fish, resulting in high performance and efficiency. It was highlighted that the relevance and impact of the quality of fishmeal and fish oil on comparative studies with such novel ingredients is an important consideration.

Insights in fish feeds for increased circularity and resource utilization were provided by Naya-Català et al. who evaluated processed animal proteins (PAPs), insect meal, yeast, and microbial biomasses in diets for gilthead sea bream (*Sparus aurata*) juveniles. This study focused on the interaction between intestinal microbiota and host transcriptomics to aid the identification of fish diets promoting gut health and metabolic homeostasis, and ultimately, the overall health of farmed fish. Using a circular economy concept, Terova et al. assessed crude glycerol, a primary by-product of biodiesel production, for heterotrophic cultivation of *Schizochytrium limacinum*. The resulting biomass, a rich source of docosahexaenoic acid, was further shown to be a useful alternative to marine-derived raw materials for European sea bass feeds, providing a much needed additional source of essential omega-3 fatty acids for indirect human consumption. However, the feed cost-effectiveness for that resource still needs to be improved.

Algae were examined in several papers in this Research Topic. García-Márquez et al. demonstrated the capacity of the microalga *Chlorella fusca* (15% inclusion level) to promote growth, metabolism and digestive functionality in a low trophic omnivorous fish species, the thick-lipped grey mullet (*Chelon labrosus*). In another paper, Fang et al., showed that a newly isolated strain of Haematococcus pluvialis GXU-A23 improved the growth performance, antioxidant and anti-inflammatory status, metabolic capacity and mid-intestine morphology of juvenile *Litopenaeus vannamei*. A 50 g/kg *Haematococcus pluvialis* was proposed to be used as a green additive in aquafeeds due to its rich astaxanthin and polyunsaturated fatty acid content. Ferreira et al. also explored a range of natural highly nutritious alternatives to replace fishmeal in gilthead sea bream diets. They found that microalgae were a good source of protein and lipids, able to promote the accumulation of LC-PUFAs in muscle, whilst selenised yeast was a good vehicle to fortify selenium levels in fish. However, the use of *Laminaria digitata* as strategy to increase iodine muscle content still requires further evaluation.

Functional diets continue to develop as strategies to boost fish health status. Hydrolyzed *Debaryomyces hansenii* yeasts were shown to modulate physiological responses in plasma and immune organs of Atlantic salmon (Morales-Lange et al.) counteracting possible consequences of hypoxic stress. Balbuena-Pecino et al. used an *in vivo* and *in vitro* approach to evaluate hydroxytyrosol-rich extract from olive juice (major phenolic compounds found in olives) as an additive in gilthead sea bream juveniles fed a high-fat diet. That study highlighted the beneficial use of hydroxytyrosol as a way to improve fish muscle-skeletal condition. The metabolic effects of flavonoids included in diets for hybrid grouper (*Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) was evaluated by Luo et al. Classical feeding trials combined with extensive tissue metabolomics analysis, helped to shed light on metabolic adaptations induced by such functional feeds. The use of glycerol, a by-product of biodiesel, was also evaluated in diets for rainbow trout (*Oncorhynchus mykiss*) and European sea bass. Viegas et al. suggested that these fish are able to catabolize glycerol and incorporate it into carbohydrates, but species metabolic differences were identified. An additional

study by the same authors recommended a dietary glycerol supplementation of up to 2.5%, as it had no major effects on fish lipid metabolism or fat accumulation and therefore had some potential as a useful energy source (Viegas et al.). Finally, Selvam et al. provided new insights into intracellular trafficking of fatty acids using a rainbow trout intestinal epithelial cell line (RTgutGC) as an *in vitro* model. This model provided comparable results to similar mammalian cell lines as well as *in vivo* studies in fish or mammals. It was concluded that carbon chain length and saturation level of fatty acids differently regulate their intracellular fate during fatty acid absorption.

Overall, the "Research Topic" highlights a variety of current research in the area. This set of studies also shows how classical and novel research strategies are increasingly being used in tandem to underpin future directions to increase the range of alternative ingredient options for use in feeding farmed fish species. Beyond the theoretical aspects of these studies, increasing use of novel research strategies is providing an objective understanding of the physiological responses by fish to a growing range of alternative ingredients.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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The Use of Defatted *Tenebrio molitor* Larvae Meal as a Main Protein Source Is Supported in European Sea Bass (*Dicentrarchus labrax*) by Data on Growth Performance, Lipid Metabolism, and Flesh Quality

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Objective: This study aims to determine the maximal inclusion level of defatted (*d*-) *Tenebrio molitor* larvae meal (TM) able to replace dietary fishmeal (FM) without compromising growth performance, general metabolism, and flesh quality traits in European sea bass, and to evaluate the major underlying physiological mechanisms.

Materials and Methods: Fish (55 ± 2 g) were fed with diets containing increasing levels of *d*TM: 0, 40, 80 and 100% (CTRL, TM40, TM80, and TM100, respectively) to replace FM. After 10 weeks of feeding, the growth performance, nutrient and energy balance, intestinal integrity, plasma metabolites and the expression of genes related to growth and nutrient metabolism, in liver and muscle were determined. The fatty acids (FA) profile, textural properties and color were also evaluated in muscle.

Results: Protein and lipids digestibility remained unaltered up to 80% *d*TM inclusion. Growth performance parameters were similar among dietary treatments. The *d*TM inclusion increased the hepatosomatic index in fish fed TM100. Muscle eicosapentaenoic acid, docosahexaenoic acid and n-3 long-chain polyunsaturated FA levels were maintained up to 80% *d*TM inclusion, but total cholesterol and non-esterified FA increased with dietary *d*TM inclusion. In liver, the expression of elongation of very long-chain FA protein 6 (*elovl6*) and FA desaturase 2 (*fads2*) did not change in fish fed TM40 and TM80, but *elovl6* decreased whilst *fads2* increased in fish fed TM100 when compared to those fed CTRL. The expression of cholesterol 7 alpha-monooxygenase (*cyp7a1*) decreased with dietary *d*TM inclusion. In muscle, the expression of myoblast determination protein-2 (*myod2*) decreased in fish fed TM80 and TM100.

Conclusion: It is feasible to substitute dietary FM by *d*TM up to 80% in European sea bass without detrimental effects on nutrient digestibility, growth performance and associated genetic pathways, whilst assuring fillet nutritional value for human consumption.

Keywords: alternative protein sources, fatty acids profile, feedstuffs, insect meal, muscle cell proliferation and differentiation, nutrient utilization

INTRODUCTION

The use of insect protein as sustainable alternative to animal and plant protein sources has been encouraged for direct human consumption and incorporation into animal feeds (Nogales-Mérida et al., 2018; Salter, 2019). Insects have several advantages when compared with the conventional protein sources, as they grow fast and reproduce easily, have low feed conversion ratio (FCR) and small need of arable land and water (Gasco et al., 2020). Additionally, insects are rich protein sources, with a well-balanced essential amino acid (EAA) profile; being also rich sources of fat and some vitamins and minerals (Koutsos et al., 2019). Insects can also be valuable sources of healthy compounds, such as chitin and antioxidant and antimicrobial peptides. Recent studies showed that insect-based diets modulate fish microbiota and improve the immune system, which may reduce the use of antibiotics in aquaculture (Bruni et al., 2018: Henry et al., 2018; Antonopoulou et al., 2019; Rimoldi et al., 2019; Stenberg et al., 2019; Li et al., 2021). In 2017, European Union (EU) authorized the use of insect meal (IM) from seven species, including yellow mealworm (*Tenebrio molitor*), in aquafeeds (European Commission, 2017). Basto et al. (2020) have recently demonstrated that defatted (*d*-) *T. molitor* larvae meal (TM) is a highly digestible protein source able to meet European sea bass (*Dicentrarchus labrax*) EAA requirements (Basto et al., 2020). The potential of TM to partially replace fishmeal (FM) in aquafeeds has been previously assessed in various marine fish species, such as European sea bass or sparids (Gasco et al., 2016; Piccolo et al., 2017; Henry et al., 2018; Antonopoulou et al., 2019; Iaconisi et al., 2019; Ido et al., 2019), whilst total replacement was only evaluated in red sea bream (*Pargus major*) (Ido et al., 2019). Results among authors are controversial, and the maximal replacement level of FM by TM are extremely variable and never exceeding 71% in diets for European sea bass (Gasco et al., 2016).

The main nutritional limitation of including insects in aquafeeds is their low content of long-chain polyunsaturated fatty acids (LC-PUFA), such as the eicosapentaenoic (EPA; 20:5n−3) and docosahexaenoic (DHA; 22:6n−3) acids. Fish muscle is the main dietary source of these two n-3 LC-PUFA for humans, which are associated with beneficial health effects (Lands, 2014). Thus, IM may compromise not only flesh quality, but also growth performance when included at high levels in diets for marine fish species. Fish oil (FO) and FM are the main dietary sources of n-3 LC-PUFA, such as EPA and DHA. Thus, high FM replacement levels, or the concomitant replacement of FM and FO, have to be carefully addressed to not compromise the recommended dietary levels of n-3 LC-PUFA for marine fish species (approximately 0.7% on dry matter (DM) basis) (Skalli and Robin, 2004). In blackspot sea bream (*Pagellus bogaraveo*), full-fat TM did not affect fillet EPA or DHA content, when included at 21% of feed, but decreased the EPA content when included at 40% (Iaconisi et al., 2017). This suggest that dietary IM may induce changes in lipid metabolism, depending on its dietary inclusion level. Besides, IM also has a limited content of phosphorus (P), which may affect not only lipid metabolism, but also fish growth performance (Sugiura et al., 2004; Prabhu et al., 2014).

The present study aimed to explore the impact of partial and total replacement of FM by *d*TM in a comprehensive approach focusing not only on growth performance and nutrient utilization, but also on the underlying mechanisms involved in nutrient metabolism, namely lipid metabolism, in European sea bass. This is one of the most important fish species in Mediterranean aquaculture, where production surpassed 194,000 tons in 2018 (Eurostat, 2020). Muscle quality traits and fillet nutritional value for human consumption were also evaluated.

MATERIALS AND METHODS

Diets

Extruded practical diets were formulated and produced by SPAROS Lda. (Portugal) to meet all the known nutritional requirements of European sea bass (NRC, 2011). All diets contained FO (12–13% inclusion level) as the main dietary lipid source. In the control diet (CTRL), FM was added at 45% and was progressively replaced in experimental diets (40%, TM40; 80%, TM80; 100%, TM100) by *d*TM from Entomo Farm (France). The inclusion level of *d*TM increased up to 60% in TM100, and was due to the concomitant replacement of FM and plant proteins, resulting in increased protein and lipid content (**Table 1**). All experimental diets were properly supplemented with DL-methionine and mono-calcium phosphate. For determination of the apparent digestibility coefficients (ADC), 1% chromium oxide (Cr_2O_3) was added, as an inert marker, to each experimental diet. Details of dietary fatty acid (FA) and amino acid (AA) profile are shown in **Table 2** and **Supplementary Table 1**, respectively.

Fish Husbandry

Juvenile fish of Atlantic origin were obtained from Acuinuga S. L. (Spain). Fish, for both the digestibility and the growth trials, were held in quarantine (2000 L tanks) for 2 weeks and hand-fed with a commercial diet (AQUASOJA, Portugal – 50% crude protein and 20% crude fat on DM basis). During quarantine period, fish were held in a recirculation aquaculture system (RAS) at 24 ± 1°C, 35 ± 0.5 ‰ and 6 L/min $^{-1}$. Total ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and pH levels were maintained within

TABLE 1 | Ingredients and chemical composition of the experimental diets.

	CTRL	TM40	TM80	TM100
Ingredients, g/kg				
Fishmeal[1]	450	270	90	–
Defatted *Tenebrio molitor* larvae meal[2]	–	180	360	600
Soy protein concentrate[3]	80	80	80	–
Soybean meal[4]	100	100	100	–
Rapeseed meal[5]	50	50	50	–
Wheat meal[6]	170	168	155	240
Sardine oil[7]	130	130	130	121
Vitamin and mineral premix[8]	10	10	10	10
Binder[9]	10	10	10	10
Mono-calcium phosphate	–	–	11	13
DL-Methionine[10]	–	2	4	6
Chemical composition, g/100 g DM				
Dry matter	93.5	94.3	93.2	94.2
Protein	47.8	47.7	47.0	50.6
Lipids	18.7	19.2	20.4	22.1
Energy, kJ/g DM	22.0	22.6	23.4	24.5
Phosphorus	1.4	1.1	1.0	1.0
Ash	11.7	9.0	7.4	6.5

CTRL, control diet; DM, dry matter; TM40, TM80 and TM100, diet with 40, 80 and 100% of fishmeal replacement by defatted Tenebrio molitor larvae meal, respectively.
[1]Peruvian fishmeal super prime: 71.0% protein, 11.0% lipids, Exalmar S.A.A, Peru.
[2]Defatted Tenebrio molitor larvae meal: 65% protein, 12% lipids, 4.8% chitin, Entomo, France.
[3]Soy protein concentrate: 65% protein, 0.7% lipids, ADM Animal Nutrition™, The Netherlands.
[4]Soybean meal 48: 47.7% protein, 2.2% lipids, Cargill, Spain.
[5]Rapeseed meal: 36% protein, 2.7% lipids, Premix Lda., Portugal.
[6]Wheat meal: 10.2% protein, 1.2% lipids, Casa Lanchinha Lda., Portugal.
[7]Sardine oil: Sopropêche, France.
[8]Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal.
[9]Kielseguhr (natural zeolite): LIGRANA GmbH, Germany.
[10]DL-Metionine 99%: Evonik Degussa GmbH, Germany.

TABLE 2 | Fatty acid profile of the experimental diets.

	CTRL	TM40	TM80	TM100
Fatty acids, g/100 g DM				
14:00	1.1	1.1	1.1	1.2
16:00	3.1	3.3	3.4	4.2
18:00	0.7	0.7	0.7	0.9
Σ SFA[1]	5.3	5.5	5.6	6.8
16:1n-7	1.2	1.1	1.1	1.1
18:1n-9	1.6	2.2	2.8	4.7
18:1n-7	0.5	0.4	0.4	0.5
20:1n-9	0.4	0.3	0.2	0.2
22:1n-11	0.06	0.04	0.03	0.02
Σ MUFA[2]	4.4	4.7	4.8	6.8
18:2n-6	0.5	1.2	1.9	3.6
18:3n-3	0.2	0.2	0.2	0.3
18:4n-3	0.3	0.3	0.3	0.3
20:4n-6	0.1	0.1	0.1	0.1
20:5n-3, EPA	2.0	1.8	1.8	1.8
22:5n-3	0.1	0.1	0.1	0.1
22:6n-3, DHA	1.4	1.3	1.1	1.1
EPA + DHA	3.4	3.1	2.9	2.9
Σ PUFA[3]	5.4	5.6	6.2	7.9
Σ n-3 LC-PUFA[4]	4.3	3.9	3.7	3.8
Σ n-6 LC-PUFA[5]	0.7	1.4	2.1	3.8
Σ n-3/Σ n-6	5.9	2.8	1.8	1.0

DHA, docosahexaenoic acid; DM, dry matter; EPA, eicosapentaenoic acid: LC-PUFA, long-chain PUFA; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
[1]Includes 12:0, 13:0, 15:0, 17:0, 20:0, 20:0, 22:0, 24:0.
[2]Includes 14:1n-5, 16:1, 17:1n-7, 20:1n-7, 20:1n-11, 22:1n-9, 24:1n-9.
[3]Includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-4, 18:3n-6, 18:4n-1, 20:2n-6, 20:3n-3, 20:4n-3, 20:3n-6, 20:3n-3, 22:2n-6, 21:5n-3 22:4n-6.
[4]Includes 20:3n-3, 20:4n-3, 21:5n-3.
[5]Includes 18:3n-6, 20:3n-6, 22:4n-6.

the recommended ranges for marine species ($NH_4^+ \leq 0.05$ mg L^{-1}; $NO_2^- \leq 0.5$ mg L^{-1}; $5 \leq$ mg L^{-1}; $7.5 \leq$ pH ≥ 8.5). Dissolved oxygen level was kept above 90% saturation and an artificial photoperiod of 12 h' light/12 h' dark cycle was fixed.

Digestibility Trial

Fish (58 ± 1 g) from the initial stock were randomly distributed in 8 tanks of 50 L (18 fish per tank) equipped with feces sedimentation columns (Cho and Slinger, 1979). Fish were fed the experimental diets (with 1% chromium oxide (Cr_2O_3) until apparent satiation, once a day, during 5 days for adaptation to the diets, before the feces collection began. Approximately 30 min after feeding, every tank was carefully cleaned to assure that no remains of uneaten feed were left in the bottom of the tank or in the sedimentation column. Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged at 3000 *g*, to eliminate water excess, and kept at −20°C until chemical analysis. Daily collection of the feces was performed for 10 consecutive days. In order to test each diet in quadruplicate and since the RAS was only constituted by 12 tanks equipped with feces sedimentation columns, this procedure was repeated over two consecutive rounds. In each round, each diet was tested in duplicate. In the second round, diets were allocated to different tanks and fed to new groups of 18 fish per tank from the same initial stock and with the same size range (58 ± 1 g) used in the first round.

Growth Trial

Twelve homogeneous groups of 25 fish (55 ± 2 g) from the initial stock were randomly distributed in 160 L tanks. Each diet was distributed to triplicate groups of fish, by automatic feeders until visual satiation, three times daily, for 10 weeks. The amount of feed supplied to each tank was adjusted daily, and when some uneaten feed remained in the bottom of the tank, the total amount of feed distributed each day was reduced by 5%, until no feed losses were recorded. When no feed losses were observed, the amount of feed was maintained for 2 days, and then augmented by 5%.

Fish Sampling

After overnight fasting, ten fish from the initial stock, and five fish from each tank at the end of growth trial were sampled, sacrificed by anesthetic overdose (2-phenoxyethanol, 500 μL/L),

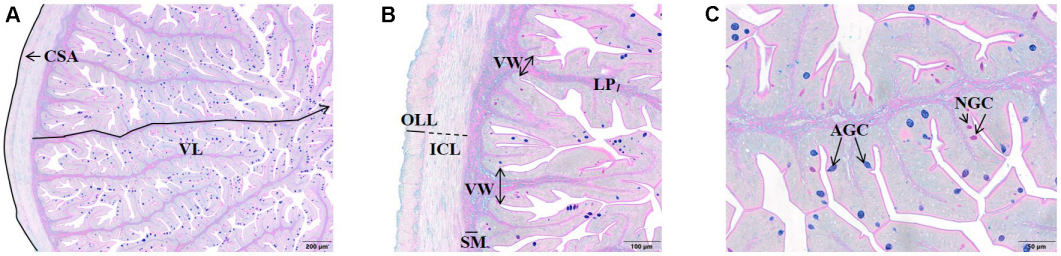

FIGURE 1 | Histological sections (Alcian blue/PAS staining, pH = 2.5) of the anterior intestine of European sea bass. **(A)** CSA, cross-sectional area, VL, *villus* length (40×). **(B)** ICL, inner circular layer, LP, lamina propria, OLL, outer longitudinal layer, SM, submucosa VL, *villus* width (100×). **(C)** AGC, acid goblet cell, NGC, neutral goblet cell (200×).

and kept at −20°C until whole-body composition analysis. Also, at the end of the growth trial, five additional fish per tank were slightly anesthetized with 2-Phenoxyethanol (200 μL/L) for blood and tissue sampling. Blood was collected from the caudal vein with heparinized syringes, centrifuged at 10,000 *g* for 5 min at 4°C, and the collected plasma was stored at −80°C until metabolite analysis. Prior to sampling the remaining tissues, fish were sacrificed by a sharp blow on the head. Liver and white skeletal muscle (dorsal right side) portions (100 mg) were then collected, frozen in liquid nitrogen and stored at −80°C until RNA extraction. Sections of anterior intestine after the pyloric caeca (5 mm thick) were excised, washed, fixed in phosphate-buffered formalin 4% (pH 7) for 24 h, and posteriorly preserved in ethanol 70% until histological analysis. A cross-sectional slab with skin (5 mm thick) was taken just before the dorsal fin, and photographed (with a scale reference) for determination of the cross-sectional area (CSA). A portion from the left part of the fillet (1 × 1 cm) was frozen in isopentane, cooled by dry ice, and stored at −80°C for later histological analysis. Two other muscle samples, from the left part of the fillet, were taken, frozen in liquid nitrogen and stored at −80°C for DM, total lipid content and FA profile analyses. Additionally, the right dorsal fillet was collected with skin for instrumental color and texture analysis.

Proximate Composition Analysis

Freeze-dried fish, diets and feces were ground and homogenized prior to proximate composition analysis. Ash, DM, crude protein (N × 6.25), lipids, P and energy were determined according to AOAC methods (AOAC, 2006) as described by Basto et al. (2020). The Cr_2O_3 content in diets and feces was determined according to Bolin et al. (1952).

Total Lipids and Fatty Acids Analyses

Muscle total lipids were extracted and quantified gravimetrically according to Folch et al. (1957), using dichloromethane instead of chloroform. The muscle FA methyl esters (FAME) were obtained through transesterification of lipid extracts, whilst in diets FAME were obtained by direct transesterification. The identification and quantification of FAME was performed as described by Campos et al. (2017), using the tricosaenoic acid (23:0) as internal standard.

Histological Analysis

Samples of anterior intestine were embedded in paraffin and processed according to standard histological procedures. Intestinal cross-sections (3 μm) were stained with specific Alcian Blue/PAS staining (pH 2.5) and examined under a light microscope (Olympus BX51, GmbH, Germany) coupled with a camera (Olympus DP50, GmbH, Germany). An imaging software (Olympus cellSens Dimension Desktop) was used to determine the following parameters: intestine CSA (mm^2); width of *muscularis* externa (μm), outer longitudinal and inner circular layers (OLL and ICL, respectively); submucosa and lamina propria width (μm); *villus* length and width (μm); number of neutral and acid goblet cells (NGC and AGC, respectively) per *villus*. The OLL, ICL, and submucosa width were measured in eight points of each transverse section analyzed, and the mean was considered. The lamina propria width (measured in the middle of the *villus*), and *villus* length (measured from the top to the bottom following their natural curves) and width (measured at the base) were measured in the eight highest *villus*. Goblet cells were counted on these selected highest *villi*. All measurements are exemplified in **Figure 1**.

Frozen cross-sections of muscle (12 μm) were stained with hematoxylin-eosin and examined using the previously mentioned imaging software. The following parameters were calculated: muscle CSA (mm^2); total number of fibers per CSA; fiber number per unit of area (density; $n°/mm^2$); and frequency of fibers classes according to their diameter (≤20 μm and >140 μm) as reported in Lopes et al. (2017).

Instrumental Texture and Color Analysis

Muscle color measurements were done with a CR-400 chromameter (Konica Minolta Inc., Japan) with an aperture of 8 mm, with respect to CIE standard illuminant D65. The chromameter was applied onto each sample and three replicates of lightness, redness and yellowness (L*, a*, and b*, respectively) values were recorded. From a* and b* values the hue angle [$H° = \tan^{-1}(b^*/a^*)$] and the chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] were calculated (Choubert et al., 1997). Muscle texture was analyzed using a TA.XT.plus Texture Analyser with a 5 kg load cell and a 2.0 mm diameter probe (Stable Micro systems Inc., United Kingdom). Texture profile parameters [hardness (N), adhesiveness (J), springiness (-), cohesiveness (-), chewiness (J),

TABLE 3 | PCR-array layout for hepatic and white skeletal muscle gene expression profiling.

Function	Gene	Symbol	Accession number*
PERFORMANCE	[1,2]Growth hormone receptor-type I	*ghr-i*	AF438177
GH/IGF system	[1,2]Growth hormone receptor-type II	*ghr-ii*	AY642116
	[1,2]Insulin-like growth factor I	*igf-i*	AY800248
	[1,2]Insulin-like growth factor II	*igf-ii*	AY839105
	[1]Insulin-like binding-protein 1b	*igfbp1a*	(LG10:13787250-13788417)
	[1]Insulin-like binding-protein 2b	*igfbp2b*	EU526670
	[2]Insulin-like binding-protein 3a	*igfbp3a*	(LG4:1920612-1938180)
	[1]Insulin-like binding-protein 4	*igfbp4*	MN045298
	[2]Insulin-like binding-protein 5b	*igfpb5b*	(LG15:3836279-3847001)
	[2]Insulin-like binding-protein 6b	*igfbp6b*	(LG22-25:348158-350835)
LIPID METABOLISM	[1]Elongation of very long chain fatty acids 1	*elovl1*	KF857295
FA elongases,	[1]Elongation of very long chain fatty acids 4	*elovl4*	KF857296
FA desaturases,	[1]Elongation of very long chain fatty acids 5	*elovl5*	FR717358
Lipases,	[1]Elongation of very long chain fatty acids 6	*elovl6*	KF857297
Transcription factors	[1,2]Stearoyl-CoA desaturase 1b	*scd1b*	FN868643
	[1]Fatty acid desaturase 2	*fads2*	EU647692
	[1]Lipoprotein lipase	*lpl*	AM411614
	[1]Hepatic lipase	*hl*	KF857289
	[1]Adipose triglyceride lipase	*atgl*	KF857294
	[1]Hormone sensitive lipase	*hsl*	KF857293
	[1]Peroxisome proliferator-activated receptor α	*pparα*	AY590300
	[1]Peroxisome proliferator-activated receptor γ	*pparγ*	AY590303
ENERGY	[1,2]Carnitine palmitoyltransferase 1a	*cpt1a*	KF857302
METABOLISM-	[1,2]Citrate synthase	*cs*	KF857304
OXPHOS,	[1]NADH dehydrogenase subunit 5	*nd5*	KF857307
Cholesterol metabolism,	[1]Succinate dehydrogenase cytochrome b560 subunit	*sdhc*	KF857305
Energy sensing,	[1]Cytochrome b	*cyb*	EF427553
Respiration uncoupling	[1]Cytochrome c oxidase subunit I	*coxi*	KF857308
	[1]Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KF857306
	[1,2]Sirtuin 1	*sirt1*	MH138004
	[1,2]Sirtuin 2	*sirt2*	MK983171
	[1]Mitochondrial respiratory uncoupling protein 1	*ucp1*	MH138003
	[2]Mitochondrial respiratory uncoupling protein 3	*ucp3*	(LG14:12134586-12136013)
MUSCLE CELL	[2]Myoblast determination protein 1	*myod1*	(LG6:934633-937237)
PROLIFERATION	[2]Myoblast determination protein 2	*myod2*	(LG5:26406310-26408511)
& DIFFERENTIATION	[2]Myogenic regulatory factor 4	*mrf4*	(LGx:14305213-14306264)
	[2]Myogenic factor 5	*myf5*	(LGx:14298644-14300040)
	[2]Myogenin	*myog*	(LG1A:13290583-13292182)
	[2]Myostatin	*mstn*	AY839106
	[2]Follistatin	*fst*	MK983166
	[2]Fibroblast growth factor 4	*fgf4*	(LG5:29962695-29967359)
	[2]Fibroblast growth factor 6	*fgf6*	AY831723
	[2]Muscle RING-finger protein 1	*murf1*	(UN:85299200-85300236)
	[2]Muscle atrophy F-box	*mafbx/atrogin-1*	MK983167
	[2]Myomaker	*mymk*	(LG20:21064893-21067975)
PROTEIN	[2]Calpain 1	*capn1*	FJ821591
TURNOVER	[2]Calpain 2	*capn2*	MK983168
	[2]Calpain 3	*capn3*	MK983169
	[2]Calpastatin	*cpst*	MK983170

**Obtained from GenBank database or the European sea bass genome project (http://seabass.mpipz.mpg.de). Accession number of sea bass genome is shown in parentheses.*
[1]liver PCR-array.
[2]white skeletal muscle PCR-array.

TABLE 4 | Apparent digestibility coefficients and nutrient balances of European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
ADC, %					
Dry matter	79.0 ± 0.4^{a}	76.4 ± 0.5^{b}	78.3 ± 0.3^{a}	78.9 ± 0.3^{a}	< 0.01
Protein	91.7 ± 0.1^{a}	91.3 ± 0.4^{a}	90.1 ± 0.3^{ab}	89.1 ± 0.9^{b}	< 0.01
Lipids	97.4 ± 0.2^{b}	97.0 ± 0.4^{b}	97.3 ± 0.2^{b}	98.0 ± 0.1^{a}	0.02
Energy	87.5 ± 0.6^{a}	85.7 ± 0.4^{b}	86.6 ± 0.3^{ab}	87.9 ± 0.4^{a}	< 0.01
Phosphorus	62.4 ± 1.0^{c}	71.8 ± 0.6^{b}	78.6 ± 1.0^{a}	80.1 ± 0.5^{a}	< 0.01
Nitrogen balance, mg/100 g ABW/day					
Digestible N intake	119.4 ± 1.4^{a}	115.9 ± 1.5^{a}	111.5 ± 3.1^{ab}	106.5 ± 2.7^{b}	0.02
N gain	39.1 ± 0.4	40.9 ± 1.2	39.7 ± 0.8	40.0 ± 0.7	0.49
N retention efficiency,% DN	32.8 ± 0.2^{b}	35.3 ± 1.0^{ab}	35.6 ± 0.6^{ab}	37.6 ± 1.7^{a}	0.04
Fecal N losses	10.9 ± 0.1^{c}	11.1 ± 0.1^{c}	12.2 ± 0.4^{b}	13.1 ± 0.3^{a}	< 0.01
Metabolic N losses	80.3 ± 1.1^{a}	74.9 ± 1.7^{ab}	71.9 ± 2.6^{ab}	66.5 ± 3.4^{b}	0.02
Total N losses	91.2 ± 1.2	86.0 ± 1.8	84.1 ± 2.9	79.6 ± 3.7	0.08
Phosphorus balance, mg/100 g ABW/day					
Digestible P intake	14.5 ± 0.2^{a}	12.7 ± 0.2^{bc}	13.2 ± 0.4^{b}	12.0 ± 0.3^{c}	< 0.01
P gain	9.3 ± 1.8	9.1 ± 1.1	9.2 ± 1.0	7.8 ± 1.6	0.86
Phosphorus retention efficiency,% DP	64.2 ± 12.1	71.6 ± 9.0	70.6 ± 9.6	64.5 ± 12.6	0.94
Fecal P losses	8.8 ± 0.1^{a}	5.0 ± 0.1^{b}	3.2 ± 0.1^{c}	3.0 ± 0.1^{d}	< 0.01
Metabolic P losses	5.2 ± 1.8	3.6 ± 1.2	3.9 ± 1.4	4.2 ± 1.5	0.89
Total P losses	13.9 ± 1.7^{a}	8.6 ± 1.2^{b}	7.5 ± 1.5^{b}	7.2 ± 1.4^{b}	0.04
Lipid balance, g/kg ABW/day					
Digestible L intake	3.1 ± 0.04	3.1 ± 0.04	3.3 ± 0.1	3.2 ± 0.1	0.29
L gain	2.0 ± 0.1^{b}	2.0 ± 0.1^{b}	2.1 ± 0.1^{b}	2.6 ± 0.1^{a}	0.01
Lipids retention efficiency,% DL	63.8 ± 3.8^{b}	65.0 ± 2.8^{b}	65.5 ± 0.3^{b}	80.6 ± 2.7^{a}	< 0.01
Fecal L losses	0.08 ± 0.001^{b}	0.10 ± 0.001^{a}	0.09 ± 0.003^{a}	0.07 ± 0.002^{c}	< 0.01
Metabolic L losses	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.1 ± 0.02^{a}	0.6 ± 0.1^{b}	< 0.01
Total L losses	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.2 ± 0.02^{a}	0.7 ± 0.1^{b}	< 0.01
Energy balance, kJ/kg ABW/day					
Digestible E intake	327.5 ± 3.8	322.0 ± 4.0	334.6 ± 9.5	318.2 ± 8.1	0.41
E gain	123.4 ± 2.3^{b}	127.3 ± 5.2^{b}	128.6 ± 3.6^{b}	148.6 ± 6.7^{a}	0.02
Energy retention efficiency,% DE	37.7 ± 1.2^{b}	39.5 ± 1.8^{b}	38.5 ± 0.8^{b}	46.7 ± 1.1^{a}	< 0.01
Metabolizable Energy	307.5 ± 3.6	303.3 ± 3.8	316.7 ± 8.8	301.6 ± 3.7	0.39
Fecal E losses	46.9 ± 0.6^{b}	53.9 ± 0.7^{a}	51.5 ± 1.5^{a}	43.9 ± 1.1^{b}	< 0.01
Branchial + urinary E losses	20.0 ± 0.3^{a}	18.7 ± 0.4^{ab}	17.9 ± 0.4^{ab}	16.6 ± 0.9^{b}	0.02
Total E losses	250.9 ± 6.7^{a}	248.7 ± 7.8^{a}	257.5 ± 8.8^{a}	213.4 ± 3.7^{b}	0.01
Total heat production	184.0 ± 5.9^{a}	176.1 ± 6.9^{a}	188.1 ± 6.8^{a}	153.0 ± 2.6^{b}	< 0.01

Values are means ± SEM; ADC: n = 4 (pools of feces from 18 fish/replicate); Nutrient balance: n = 3 (pools of 5 fish/replicate). Labeled means without a common superscript letter differ significantly, p < 0.05. ADC, apparent digestibility coefficient; DE, digestible energy; DL, digestible lipids; DN, digestible nitrogen; DP, digestible phosphorus; E, energy; L, Lipids; N, nitrogen; P, phosphorus.

and resilience (-)] were obtained by double penetration (probe speed of 1 mm/s; probe penetration depth of 4 mm; wait time between penetrations of 5 s) on the thickest part of each raw fillet according to Batista et al. (2020).

Plasma Biochemistry

Glucose, total protein, triglycerides, total cholesterol and non-esterified FA (NEFA) levels were determined enzymatically using commercial kits (1001190, 1001291, 1001313, and 1001090, Spinreact, Spain; 434-91795 NEFA-HR (2) R1 and 436-91995 NEFA-HR (2) R2, Wako Chemicals, Germany, respectively), adapting manufacturer's instructions to microplate format.

Gene Expression

Total RNA from liver and skeletal muscle was extracted using the MagMAX-96 total RNA isolation kit (Life Technologies, United States) after tissue homogenization in TRI reagent following manufacturers' instructions. The RNA quantity and purity were determined by Nanodrop (Thermo Fisher Scientific, United States) with absorbance ratios (A260/A280) of 1.9–2.1. Reverse transcription (RT) of 500 ng of total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems, United States) following manufacturer's instructions. The RT reactions were incubated for 10 min at 25°C and 2 h at 37°C. Negative control reactions were run

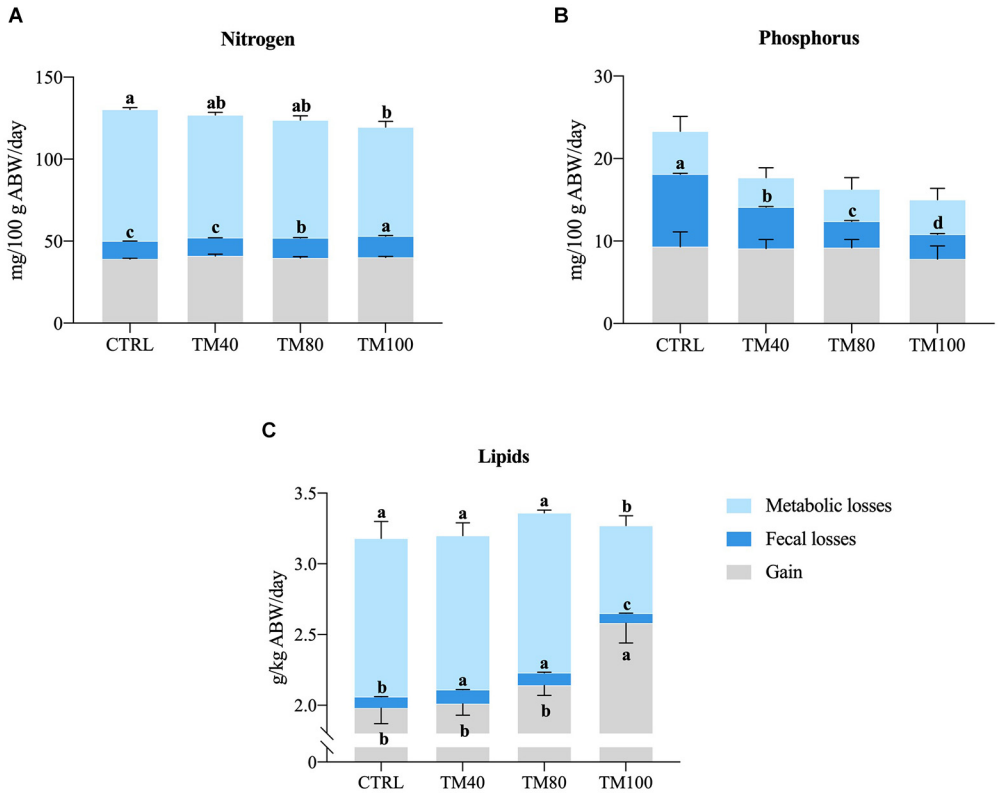

FIGURE 2 | Daily nitrogen **(A)**, phosphorous **(B),** and lipids **(C)** balance of European sea bass fed experimental diets. Bars represent means ± SD; $n = 3$ (pools of 5 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$.

TABLE 5 | Growth performance, somatic indexes and whole-body composition of European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
Growth performance					
Initial body weight, g	55.6 ± 0.01	55.7 ± 0.03	55.7 ± 0.03	55.7 ± 0.02	0.31
Final body weight, g	159.4 ± 3.9	161.0 ± 3.7	159.2 ± 3.5	162.9 ± 2.2	0.86
Final K	1.3 ± 0.01	1.3 ± 0.01	1.3 ± 0.01	1.3 ± 0.02	0.09
DGI,%/day	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.5 ± 0.04	0.87
VFI,%/day	1.7 ± 0.02^{a}	1.7 ± 0.02^{a}	1.6 ± 0.1^{a}	1.5 ± 0.04^{b}	< 0.01
FCR	1.2 ± 0.02^{a}	1.1 ± 0.03^{a}	1.1 ± 0.02^{a}	1.0 ± 0.03^{b}	0.01
PER	1.8 ± 0.03	1.9 ± 0.1	1.9 ± 0.04	2.0 ± 0.1	0.13
Somatic indexes					
HSI,%	1.2 ± 0.03^{b}	1.1 ± 0.1^{b}	1.1 ± 0.03^{b}	1.5 ± 0.01^{a}	0.02
VSI,%	8.1 ± 0.2	8.1 ± 0.3	7.9 ± 0.3	9.0 ± 0.3	0.06
Whole body composition, g/100 g WW					
Moisture	66.3 ± 0.2^{a}	66.6 ± 0.5^{a}	66.3 ± 0.1^{a}	63.9 ± 0.9^{b}	0.02
Protein	16.9 ± 0.1	17.4 ± 0.3	17.1 ± 0.03	17.0 ± 0.2	0.34
Lipids	11.4 ± 0.6^{b}	11.6 ± 0.3^{b}	12.2 ± 0.3^{b}	14.1 ± 0.7^{a}	0.02
Energy, kJ/g WW	7.7 ± 0.1^{b}	7.9 ± 0.2^{b}	8.0 ± 0.1^{b}	8.8 ± 0.3^{a}	0.02
Phosphorus	0.6 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	0.6 ± 0.1	0.81
Ash	4.2 ± 0.5	3.9 ± 0.2	4.1 ± 0.3	4.1 ± 0.1	0.89

Values are means ± SEMs; growth performance: n = 75 (25 fish/replicate); somatic indexes and whole-body composition: n = 15 (5 fish/replicate). Labeled means without a common superscript letter differ significantly, p < 0.05. DGI, daily growth index; FCR, feed conversion ratio; HSI, hepatosomatic index; K, condition factor; PER, protein efficiency ratio; VFI, voluntary feed intake; VSI, viscerosomatic index; WW, wet weight.

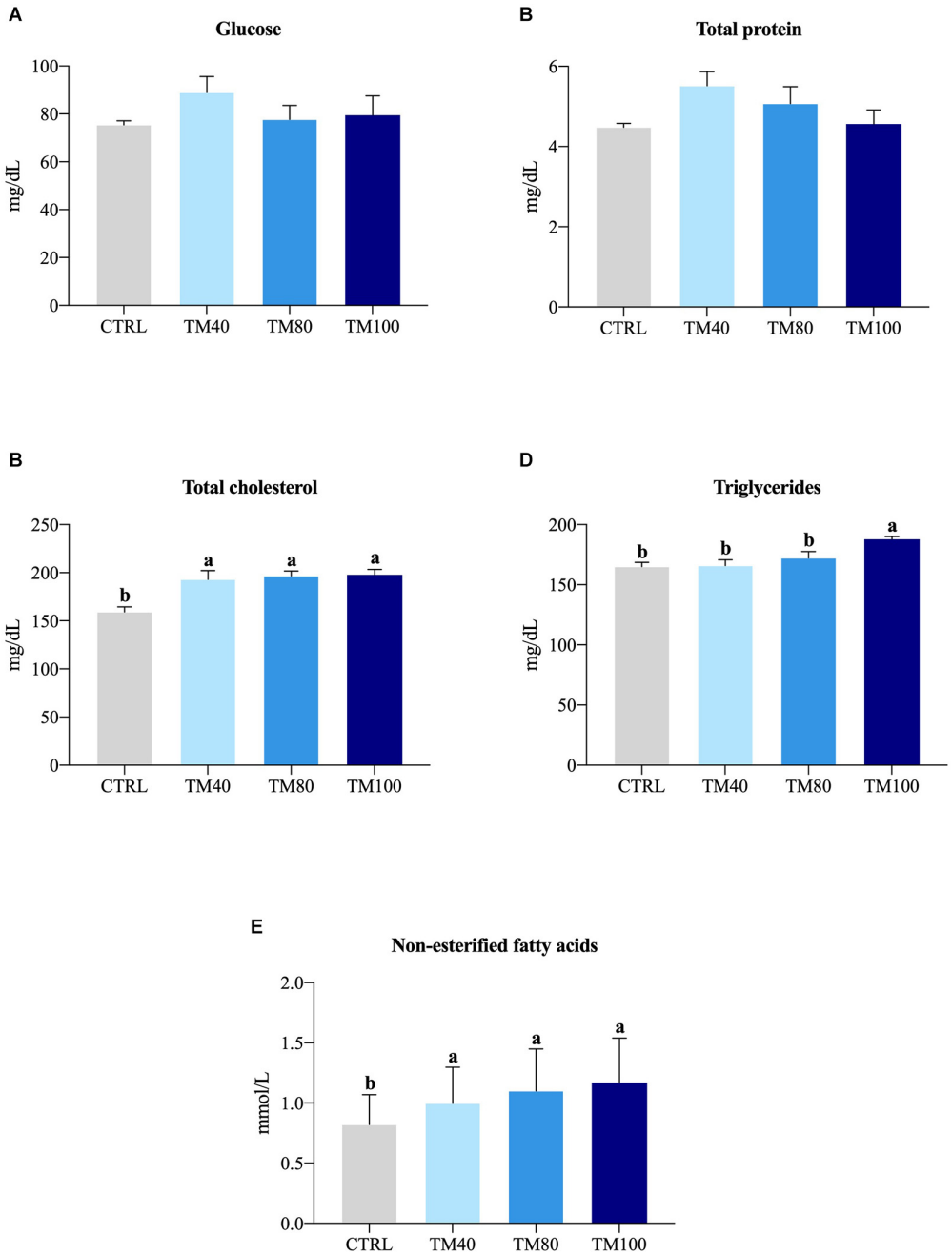

FIGURE 3 | Plasma glucose **(A)**, total protein **(B)**, total cholesterol **(C)**, triglycerides **(D)** and non-esterified fatty acids **(E)** of European sea bass fed experimental diets. Bars represent means ± SEM; $n = 15$ (5 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$.

without reverse transcriptase. The synthetized cDNA was used for PCR quantification with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States), and specific primers at a final concentration of 0.9 μM (**Supplementary Table 2**). Two customized PCR-array layouts were designed for the simultaneous gene expression profiling of 49 genes covering a number of markers of growth-hormone/insulin-growth-factors (GH/IGF) system (10), lipid metabolism (12), energy metabolism (11), muscle cell proliferation and differentiation (12), and protein turnover (4) (**Table 3**). The program used for qPCR reactions included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Germany) to improve data reproducibility. The efficiency of qPCRs was checked, and the specificity of

reactions was verified by analysis of melting and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the extension phase were normalized by the delta-delta CT method (Livak and Schmittgen, 2001), using β-*actin* as housekeeping gene due to its stability among different experimental conditions (average CT among experimental groups varied less than 0.2).

Calculations

The ADC of the experimental diets were calculated according to Maynard et al. (1979): dry matter ADC (%) = 100 × [1 - (dietary Cr_2O_3 level/feces Cr_2O_3 level)] and nutrients or energy ADC (%) = 100 × [1 - (dietary Cr_2O_3 level/feces Cr_2O_3 level) × (feces nutrient or energy level/dietary nutrient or energy)]; Average body weight (ABW) = (final body weight + initial body weight)/2; Digestible nitrogen (N), lipids (L), P or energy (E) intake = (dry feed consumption × N, L, P (%) or E (kJ/g) in the diet × ADC N, L, P, or E)/ABW/days; N, L, P, or E gain = (final carcass N, L, P or E content - initial carcass N, L, P, or E content)/ABW/days; N, L, P, or E retention efficiency (NRE, LRE, PRE, or ERE) = (N, L, P, or E gain/digestible N, L, P or E intake) × 100; Fecal N, L, P, or E losses = crude N, L, P, or E intake × [1 - (ADC N, L, P, or E/100)]. Metabolic N, L, or P losses = digestible N, L, or P intake−N, L or P gain; Branchial and urinary E losses = non-fecal N losses × 25 kJ/N; Total N, L, P, or L losses = crude N, L, or P intake−N, L, or P gain; Metabolizable energy (ME) = digestible E intake - branchial and urinary E losses; Total heat loss = E intake – E gain; Total heat production = ME - energy gain. Condition factor (K) = [final body weight/(final body length)^3] × 100; Daily growth index (DGI) = 100 × [(final body weight)$^{1/3}$ - (initial body weight)$^{1/3}$]/days; Voluntary feed intake (VFI) = 100 × dry feed intake/average body weight/day; Feed conversion ratio (FCR) = dry feed intake/weight gain; Protein efficiency ratio (PER) = weight gain/crude protein intake; Hepatosomatic index (HSI) = 100 × liver weight/body weight; Viscerosomatic index (VSI) = 100 × weight of viscera/body weight.

Statistical Analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required before submitted to a one-way ANOVA using IBM SPSS Statistics 26.0 (IBM corporation, United States). When one-way ANOVA showed significance ($p < 0.05$), individual means were compared using Student-Newman-Keuls post-test. Fold-changes of genes were submitted to a student's t-test with significance set at $p < 0.05$. A bivariate spearmen's rank correlation coefficient (*rs*) test was applied to all variables. Significant correlations were considered at the bilateral level of 0.05.

RESULTS

Digestibility and Nutrient Balance

Overall, all diets were well digested (DM ADC > 76%; protein ADC ≥ 89%; lipids ADC ≥ 97%; energy ADC > 86%; P ADC > 62%), but differences on nutrient digestibility were found among groups in the digestibility trial (**Table 4**). Fish fed TM100 had the lowest protein apparent digestibility, and the highest lipid and P digestibility. As a result, N intake and metabolic N losses decreased with total FM replacement, and the opposite pattern was found for N retention efficiency. Fecal N losses increased both in fish fed TM100 and in those fed TM80. Digestible P intake decreased with FM replacement, being significantly lower in fish fed TM80 and TM100 compared to fish fed CTRL. Fecal P losses decreased significantly with the concomitant increase of dietary *d*TM. Total P losses of fish fed *d*TM decreased significantly compared to fish fed CTRL, regardless of the dietary *d*TM level. Conversely, in comparison to fish fed CTRL and other experimental groups, both lipid and energy gain and retention efficiency were consistently higher in fish fed TM100, whilst lower branchial and urinary E losses, total E losses and total heat production were observed. Fecal E losses increased significantly in fish fed TM40 and TM80. The net balance (total gain, fecal and metabolic losses) of N, P, and L is summarized in **Figure 2**.

Growth Performance and Blood Biochemistry

As shown in **Table 5**, fish almost tripled their initial weight, and no mortality was registered during the entire growth trial. All groups grew at the same rate and a significant decrease of FCR from 1.2 in fish fed CTRL to 1.0 in those fed TM100 was observed. Final K remained similar among dietary treatments. The whole-body lipids and energy content increased in fish fed TM100 with a concomitant decrease of moisture. This group also showed a statistically significant increase of HSI that was positively correlated with whole-body lipid content (0.59; $p = 0.04$) and lipid gain (0.59; $p = 0.04$). The VSI also displayed a tendency to increase in fish fed TM100 fish, being this biometric index negatively correlated with metabolic (−0.64; $p = 0.03$) and total lipid (−0.64; $p = 0.03$) losses (**Supplementary Table 3**). Regarding blood analysis, plasma levels of total cholesterol and NEFA increased with the replacement of FM by *d*TM, regardless of the inclusion level. Plasma triglycerides were only significantly affected by TM100, resulting in the highest values (**Figure 3**). Cholesterol and/or triglycerides showed positive correlations with HSI and VSI (**Supplementary Table 3**). Concerning the fish-in:fish-out ratio (Fi:Fo), the calculated values were reduced by 20% in fish fed TM100 in comparison to those fed CTRL or the two other experimental groups (**Figure 4**).

Muscle Total Lipids and Fatty Acids Analyses

Despite the increase of lipids in TM100 diet, the muscle total lipids content was similar among groups (**Table 6**). The muscle saturated (SFA) and monounsaturated FA (MUFA) profile reflected the dietary FA profile (**Table 2**). Up to 80% of FM replacement by *d*TM, the muscle PUFA profile also reflected the dietary FA profile. On the other hand, and despite the highest percentage of PUFA in TM100 diet, muscle PUFA levels in fish fed TM100 were comparable to those of all other treatments.

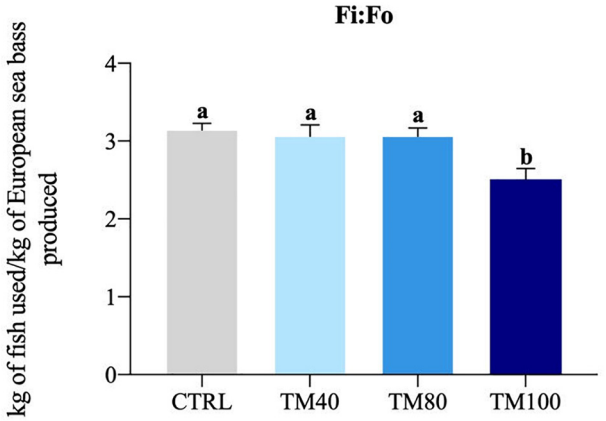

FIGURE 4 | Fi:Fo ratio. Bars represent means ± SD; $n = 3$ (pools of 5 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$. Fi:Fo was calculated according to Tacon and Metian (2008), assuming yield from forage fish of 24% for FM and 4.8% for FO.

The relative percentage of muscle EPA, DHA, and n-3 LC-PUFA of fish fed TM40 and TM80 was similar to those fed CTRL, despite their dietary percentage being decreased with the *d*TM inclusion. Although fish fed TM100 had the lowest relative percentage of EPA and DHA, when expressed in wet weight (g/100 g WW), both muscle EPA and EPA + DHA final contents were similar among all fish. As observed in the experimental diets, the percentage of muscle n-6 LC-PUFA increased with *d*TM inclusion and consequently the n-3/n-6 ratio decreased.

Intestine and Muscle Histological Analysis

The morphology of anterior intestine was well-preserved in all fish, with no signs of *villus* fusion or enterocyte vacuolization. Intestine CSA varied between 11 and 15 mm^2. The OLL and ICL varied between 32 and 41 μm, and 58 and 69 μm, respectively. The lamina propria width varied between 27 and 30 μm. The *villus* length and width ranged from 1749 to 2001 μm and 161 to 180 μm, respectively. The number of AGC and NGC per *villus* ranged from 147 to 192, and 70 to 113, respectively. The morphometric parameters analyzed in the anterior intestine did not vary significantly among dietary treatments, with the exception of submucosa thickness that increased concomitantly with dietary *d*TM inclusion level: the submucosa thickness increased from 27 μm in fish fed CTRL to 29, 30, and 33 μm in those fed TM40, TM80 and TM100, respectively (**Supplementary Table 4**). White muscle CSA varied between 459 and 496 mm^2, total number of fibers ranged from 104 to 108 thousand, and fiber density from 217 to 228 (**Supplementary Table 5**). Fiber diameter varied between 65 and 67 μm, with 5–6% of the fibers having below 20 μm and 2–3% above 140 μm. None of the morphometric parameters analyzed in muscle had significant differences among dietary treatments.

Muscle Instrumental Color and Texture Analysis

Texture and color parameters were similar among dietary treatments (**Supplementary Table 6**). Hardness ranged from 0.9 to 1 N, adhesiveness from −0.01 to −0.004 J, springiness from 1.2 to 1.6, cohesiveness from 0.4 to 0.5, chewiness from 0.5 to 0.8 J, and resilience was 0.4 irrespectively of the dietary treatment. In the muscle, L^* varied from 41 to 42, a^* from −0.5 to −0.2, b^* from −0.7 to 0.1, H° from 183 to 250 and C° from 1 to 1.2.

Gene Expression

The relative gene expression profile of key selected markers in liver and muscle is shown in **Supplementary Tables 7, 8**, with arbitrarily assigned values of 1 for hormone sensitive lipase (*hsl*) in liver of fish fed CTRL, and calpain 2 (*capn2*) in muscle of fish fed CTRL. Among all the analyzed genes, up to three markers of hepatic lipid metabolism and four markers of muscle growth were differentially expressed with the FM replacement, and the corresponding $\log_2$ fold-changes (*d*TM/CTRL) were graphically represented. In liver, the expression of elongation of very long-chain FA protein 6 (*elovl6)* was significantly down-regulated in fish fed TM100, but not in those fed TM40 and TM80 (**Figure 5A**). Conversely, FA desaturase 2 (*fads2)* was markedly up-regulated in fish fed TM100, remaining almost unaltered in those fed TM40 and TM80 (**Figure 5B**). A strong nutritional regulation was also found for cholesterol 7-alpha-monooxygenase (*cyp7a1*) with a significant down-regulation in all *d*TM groups (**Figure 5C**). A similar trend was observed for the expression of stearoyl-CoA desaturase 1b (*scd1b*), although this down-regulation was not statistically significant (**Figure 5D**). The expression of lipolytic peroxisome proliferator-activated receptor α (*pparα*) also evidenced a non-significant down-regulation trend for fish fed TM100 (**Figure 5E**). The expression of e*lovl6*, *cyp7a1* and *pparα* was negatively correlated with plasma cholesterol levels (−0.64 ($p < 0.01$), −0.46 ($p < 0.01$), and −0.37 ($p = 0.02$), respectively) (**Supplementary Table 9**). Myoblast determination protein 2 (*myod2*) was down-regulated in TM80 and TM100 (**Figure 6A**), whereas a significant down-regulation of muscle atrophy F-box (*mafbx/atrogin-1*) was only found in fish fed TM100 (**Figure 6B**). Contrarily, the expression of myostatin (*mstn*) (**Figure 6C**) and myoblast fusion factor (*mymk*) (**Figure 6D**) tended to increase with FM replacement level, but this increase was only statistically significant in TM100.

DISCUSSION

Since the use of IM in aquafeeds was authorized by EU in 2017, increasing efforts have been made to identify the best insect species to substitute FM, and its highest dietary inclusion level (Gasco et al., 2020). Despite the current high market prices of insect meal compared to conventional protein sources (Arru et al., 2019), the insect production is a fast-growing sector, and several companies or start-ups have been recently founded in Europe. This may lead to increased price competitiveness in the near future. The present study clearly shows that the total

TABLE 6 | Muscle total lipid content and fatty acid composition of the European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
Total lipids, g/100 g WW	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.3	2.8 ± 0.2	0.73
Fatty acids, g/100 g total fatty acids					
14:0	4.2 ± 0.1	4.1 ± 0.04	4.0 ± 0.3	3.6 ± 0.04	0.05
16:0	17.8 ± 0.03	17.6 ± 0.1	17.6 ± 0.2	17.8 ± 0.1	0.74
18:0	3.5 ± 0.06	3.7 ± 0.05	3.7 ± 0.05	3.9 ± 0.02	0.08
Σ SFA[1]	27.4 ± 0.1	27.3 ± 0.1	27.1 ± 0.4	26.9 ± 0.1	0.81
16:1n-7	6.1 ± 0.2^{a}	5.6 ± 0.02^{a}	5.6 ± 0.2^{a}	4.5 ± 0.03^{b}	0.01
18:1n-9	17.5 ± 0.5^{b}	17.3 ± 0.1^{b}	18.0 ± 0.5^{b}	22.4 ± 0.1^{a}	0.01
18:1n-7	2.9 ± 0.1^{a}	2.7 ± 0.02^{ab}	2.6 ± 0.1^{ab}	2.2 ± 0.004^{b}	0.04
20:1n-9	2.0 ± 0.2	1.9 ± 0.02	1.7 ± 0.2	1.2 ± 0.003	0.08
22:1n-11	1.3 ± 0.2	1.2 ± 0.01	1.1 ± 0.2	0.5 ± 0.004	0.07
Σ MUFA[2]	31.7 ± 0.3	30.6 ± 0.1	30.8 ± 0.3	32.2 ± 0.2	0.05
18:2n-6	4.6 ± 0.04^{d}	7.1 ± 0.1^{c}	9.9 ± 0.005^{b}	13.5 ± 0.2^{a}	< 0.01
18:3n-3	1.1 ± 0.01	1.1 ± 0.01	1.1 ± 0.02	1.1 ± 0.01	0.23
18:4n-3	1.3 ± 0.1^{a}	1.2 ± 0.01^{a}	1.2 ± 0.1^{a}	0.9 ± 0.003^{b}	0.01
20:4n-6	0.9 ± 0.02^{a}	1.0 ± 0.02^{a}	0.9 ± 0.01^{a}	0.7 ± 0.01^{b}	< 0.01
20:5n-3, EPA	10.2 ± 0.2^{a}	10.0 ± 0.05^{a}	9.9 ± 0.2^{a}	7.9 ± 0.1^{b}	< 0.01
22:5n-3	1.8 ± 0.04^{a}	1.8 ± 0.02^{a}	1.8 ± 0.02^{a}	1.3 ± 0.2^{b}	< 0.01
22:6n-3, DHA	11.6 ± 0.3^{a}	12.6 ± 0.1^{a}	11.7 ± 0.1^{a}	9.0 ± 0.2^{b}	< 0.01
EPA + DHA	21.8 ± 0.5^{a}	22.6 ± 0.1^{a}	21.6 ± 0.1^{a}	16.9 ± 0.1^{b}	< 0.01
Σ PUFA[3]	35.0 ± 0.6^{b}	37.9 ± 0.1^{a}	39.7 ± 0.5^{a}	37.3 ± 0.2^{ab}	< 0.01
Σ n-3 LC-PUFA[4]	27.1 ± 0.6^{a}	27.7 ± 0.2^{a}	26.7 ± 0.4^{a}	21.1 ± 0.3^{b}	< 0.01
Σ n-6 LC-PUFA[5]	6.4 ± 0.1^{d}	9.0 ± 0.1^{c}	11.7 ± 0.02^{b}	15.2 ± 0.2^{a}	< 0.01
Σ n-3/Σ n-6	4.2 ± 0.1^{a}	3.1 ± 0.05^{b}	2.3 ± 0.04^{c}	1.4 ± 0.03^{d}	< 0.01
Fatty acids, g/100 g WW					
20:5n-3, EPA	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.46
22:6n-3, DHA	0.25 ± 0.01^{a}	0.24 ± 0.01^{a}	0.25 ± 0.02^{a}	0.18 ± 0.01^{b}	< 0.01
EPA + DHA	0.47 ± 0.02	0.44 ± 0.01	0.46 ± 0.04	0.38 ± 0.01	0.38

Values are means ± SEMs; Total lipids: n = 15 (5 fish/replicate); Fatty acids: n = 6 (2 pools/replicate). Labeled means without a common superscript letter differ significantly, p < 0.05. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long-chain PUFA; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; WW, wet weight.

[1]Includes 12:0, 13:0, 15:0, 17:0, 20:0, 20:0, 22:0, 24:0.

[2]Includes 14:1n-5, 16:1, 17:1n-7, 20:1n-7, 20:1n-11, 22:1n-9, 24:1n-9.

[3]Includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-4, 18:3n-6, 18:4n-1, 20:2n-6, 20:3n-3, 20:4n-3, 20:3n-6, 20:3n-[3]22:2n-6, 21:5n-3; 22:4n-6.

[4]Includes 20:3n-3, 20:4n-3, 21:5n-3.

[5]Includes 18:3n-6, 20:3n-6, 22:4n-6.

substitution of FM by *d*TM can be achieved in European sea bass without impairing growth performance or condition factor and even improving FCR after 10 weeks of feeding. To the best of our knowledge, the maximum replacement of FM by full-fat IM, successfully tested in European sea bass, varied between 36% and 50%, in trials of similar duration (Gasco et al., 2016; Abdel-Tawwab et al., 2020). Skalli and Robin (2004) pointed out that growth impairment at higher FM replacement levels is primarily due to nutrient deficiencies in n-3 LC-PUFA, which are fixed for marine fish close to 0.7% on DM basis (Skalli and Robin, 2004). In fact, a previous study in sea bass with 71% FM replacement by TM did not fulfil this requirement, impairing growth (Gasco et al., 2016), whilst all experimental diets tested in the present study contained 3.8–4.3% DM n-3 LC-PUFA, probably due to the high quality of FO, and did not affect growth performance. In blackspot sea bream and gilthead sea bream (*Sparus aurata*), it was possible to replace FM by full-fat TM up to 49% and 74%, respectively, without affecting growth performance or FCR, after 18–23 weeks of feeding (Iaconisi et al., 2017; Piccolo et al., 2017). In red sea bream, the total replacement of FM by *d*TM was successfully achieved without affecting FCR and even increasing growth after 4 weeks of feeding (Ido et al., 2019). Contrarily, the use of defatted black soldier fly (*Hermetia illucens)* to replace FM at levels above 18% in turbot (*Psetta maxima*) and 35% in meagre (*Argyrosomus regius*), for 7–9 weeks, did not affect FCR, but impaired growth performance (Kroeckel et al., 2012; Guerreiro et al., 2020). Thus, optimal FM substitution levels could vary among fish, insect species, rearing conditions, processing methods of IM, and duration of the experimental trials making comparisons difficult (Osimani et al., 2016; Iaconisi et al., 2019). Therefore, standardization of insect protein production is needed to assure the correct formulation of diets not only for fish, but also for other animal species.

In the present study, up to 80% of FM replacement did not alter N intake, N retention or metabolic N losses. However,

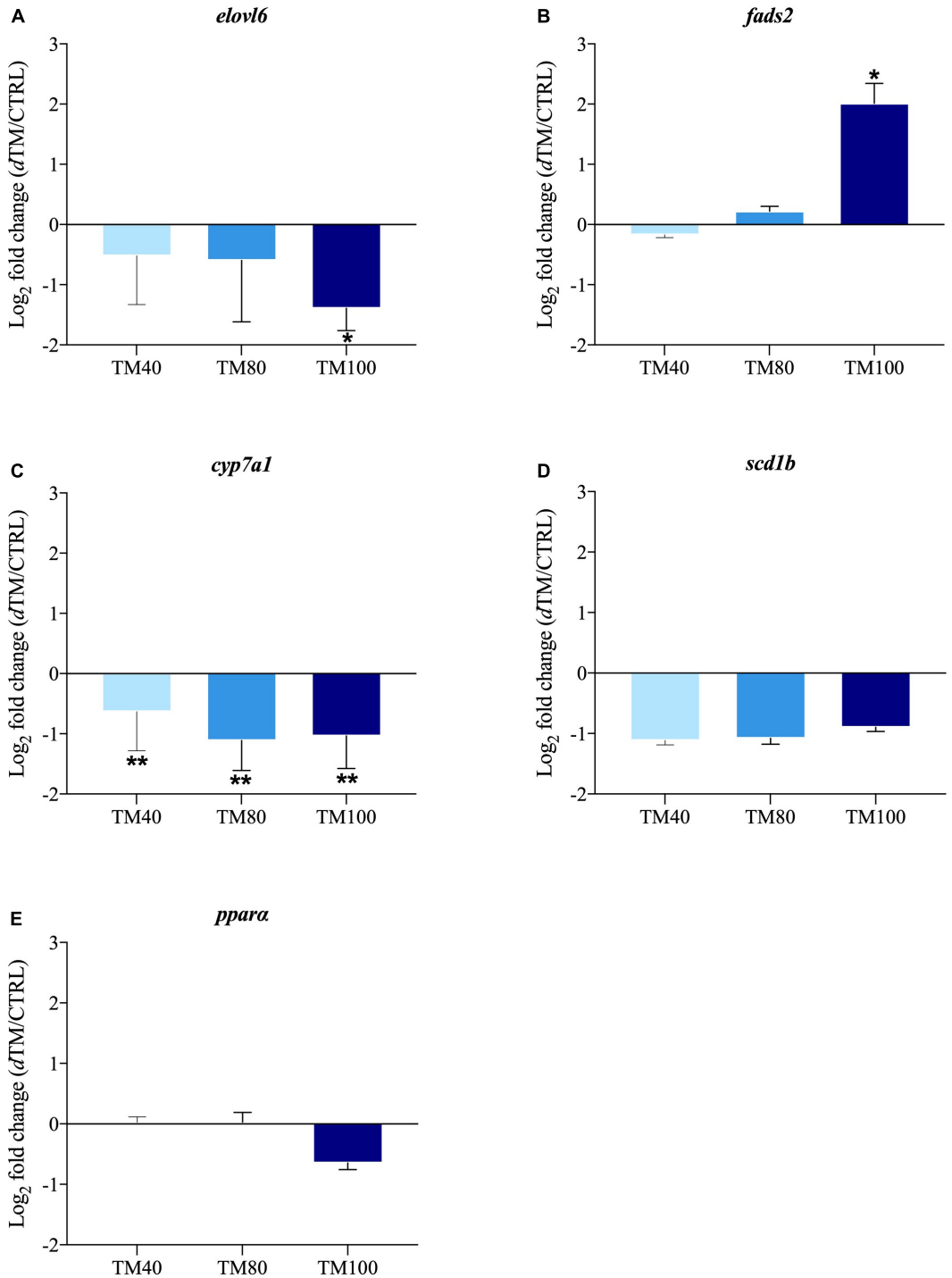

FIGURE 5 | Fold-changes (*d*TM/CTRL) of differentially expressed genes in liver tissue: *elovl6* **(A)**, *fads* **(B)**, *cyp7a1* **(C)**, *scd1b* **(D)** and *pparα* **(E)**. The asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$; t-student) between European sea bass fed *d*TM and CTRL diets. Values >1 indicate up-regulated genes in *d*TM fish; values <1 indicate down-regulated genes in *d*TM fish; $n = 9$ (3 fish/replicate).

this trade-off was apparently altered in fish fed TM100; these fish conserved a higher N retention compared to control by reducing metabolic N losses. Digestibility disturbances with the use of IM are attributed to the chitin, which has been negatively correlated with protein digestibility in *in vitro* digestibility studies (Marono et al., 2015). Although chitinolytic activity was reported in some fish species (Gutowska et al., 2004; Krogdahl et al., 2005; Kawashima et al., 2016), there are evidences that high

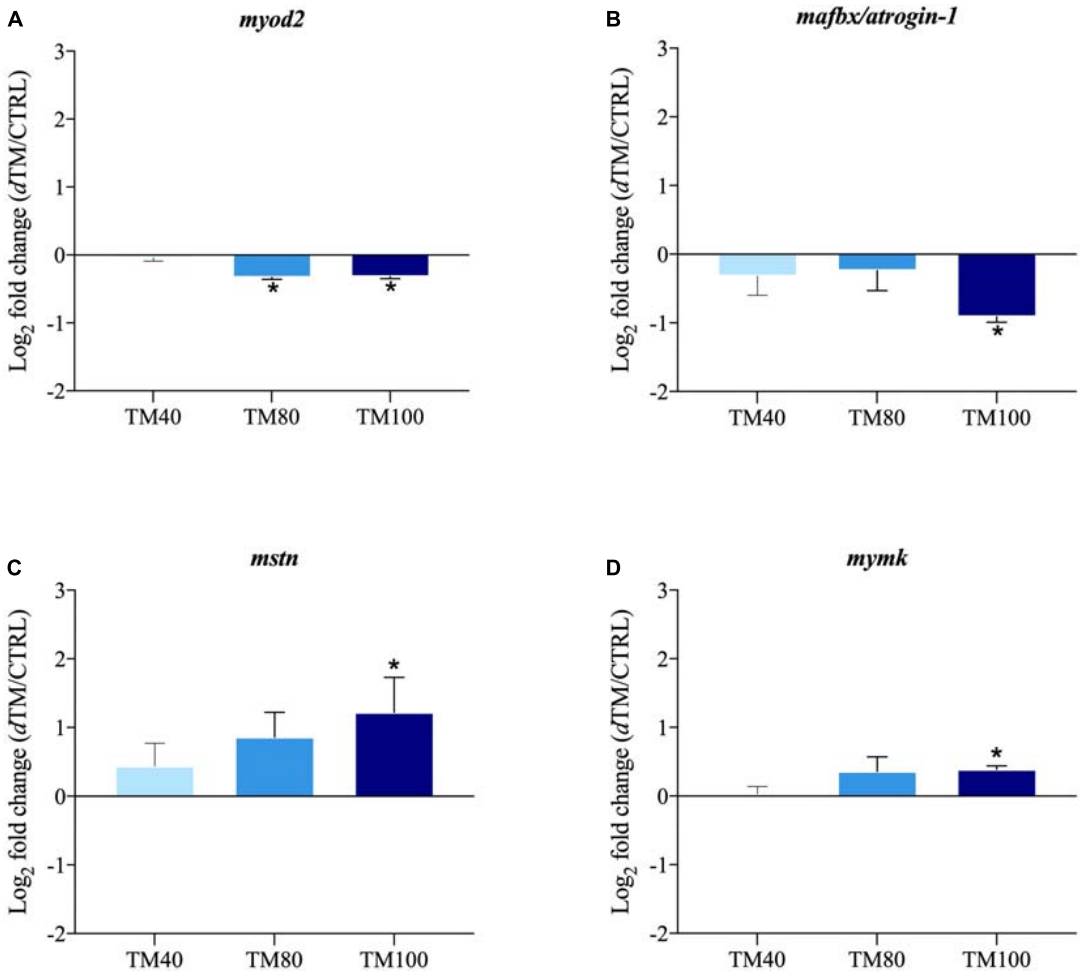

FIGURE 6 | Fold-changes (*d*TM/CTRL) of differentially expressed genes in muscle tissue: *myod2* **(A)**, *mstn* **(B)**, *mafbx/atrogin* 1 **(C)**, and *mymk* **(D)**. The asterisks indicate statistically significant differences (*$p < 0.05$; t-student) between European sea bass fed *d*TM and CTRL diets. Values >1 indicate up-regulated genes in *d*TM fish; values <1 indicate down-regulated genes in *d*TM fish. $n = 9$ (3 fish/replicate).

dietary chitin levels have negative impacts on fish growth and nutrient utilization (Alegbeleye et al., 2012; Kroeckel et al., 2012). In the present study, this negative impact seems to have apparently been mitigated by the low chitin content (4.8% DM) in *d*TM. Moreover, the integrity of anterior intestine was well preserved in all fish, without major morphological changes, with the exception of submucosa thickening in fish fed *d*TM. Li et al. (2020) have observed a higher degree of submucosa cellularity in the anterior intestine of seawater-phase Atlantic salmon (*Salmo salar*) fed a *H. illucens* based diet, but the submucosa of mid and posterior intestine remained unaltered after 8 weeks of feeding such diets to freshwater-phase fish (Li et al., 2019). According to Sitjà-Bobadilla et al. (2005), the increase in thickness of submucosa is usually due to infiltrations of granular eosinophil cells, but this could not be confirmed in the present study. In contrast to the present results, in rainbow trout (*Oncorhynchus mykiss*), the replacement of FM by 25–50% full-fat or 100% defatted *H. illucens* reduced *villus* length in the anterior intestine after 12–14 weeks of feeding (Dumas et al., 2018; Cardinaletti et al., 2019). These controversial results may be associated to different species, feeds and processing methods used for IM production, leading to different nutritional profiles.

It was also observed that P ADC increased from 62% in fish fed CTRL to 72–80% in those fed the *d*TM diets. This higher P digestibility resulted in a pronounced reduction of total P losses, which suggests that the use of *d*TM as a FM replacer in European sea bass could help reducing P output into the environment. Likewise, Wang et al. (2017) demonstrated in Nile tilapia that FM replacement by increasing levels of *M. domestica* from 18% to 100% resulted in lower concentrations of total phosphate in the water. This metabolic feature might be part of the adaptive response of fish facing a reduced dietary P supply. Indeed, P deficiencies are a common nutritional disturbance of FM replacement by alternative proteins in aquafeeds (Sugiura et al., 2004; Prabhu et al., 2014). In the case of IM, this is a relevant issue because the mineral content of this feedstuff is markedly lower than FM or other conventionally FM substitutes,

such as plant proteins. Indeed, ash content was reduced herein from 11.7% in CTRL diet to 6.5% in TM100 diet.

With total FM replacement, the increase of dietary lipids and energy content was concomitant with an improved digestibility of lipids that would favor increased whole-body lipid content by the end of the growth trial. This trend was not found in previous European sea bass studies where FM was replaced by IM from 25% to 71% (Gasco et al., 2016; Magalhães et al., 2017; Abdel-Tawwab et al., 2020). Likewise, in rainbow trout, Rema et al. (2019) did not observe any impact of *d*TM on lipid digestibility and retention. Regardless of this, our data of tissue body fat depots suggest an enhanced flux of lipids toward liver and secondly mesenteric fat when FM is totally replaced by *d*TM. This assumption is supported by data on muscle lipid content, and circulating lipid levels that were positively correlated with HSI and VSI. This is not surprising since European sea bass is a fish with low to moderate lipid deposition rates in the fillet (Ballester-Lozano et al., 2016). Although lipid content of liver has not been assessed, the strong and positive correlation between circulating triglycerides and HSI, and the moderate and positive correlation between final whole-body lipids content, lipid gain and HSI may indicate liver steatosis which is a sign of deficiencies in minerals and n-3 LC-PUFA (Ballester-Lozano et al., 2015). In contrast, enhanced deposition rate in mesenteric fat depots is highly informative of P deficiencies (Sugiura et al., 2004). In any case, both mineral and lipid metabolism seem to be highly sensitive to FM replacement by IM, which might be exacerbated at long-term through the production cycle, especially in the case of lipid-enriched diets (see below).

Liver plays a major role in fish lipid metabolism, and its gene expression profile is greatly influenced by dietary composition and feeding levels (Monroig et al., 2018). In the present study, all diets were formulated with 12–13% of FO and the n-3 LC-PUFA levels (3.8–4.3% DM) were above recommended level for marine fish species (approximately 0.7% DM) (Skalli and Robin, 2004). When revisiting the gene expression profile of gilthead sea bream fed isolipidic diets with a maximal replacement of FO by vegetable oils or semisynthetic diets formulated to be deficient in n-3 LC-PUFA, the most responsive hepatic enzymes of lipid metabolism are *scd1* and *elovl6,* and secondly *fads2* (Benedito-Palos et al., 2016). This will contribute to mitigate the signs of deficiencies in n-3 LC-PUFA, though it is very important to limit hepatic lipogenesis to avoid the lipotoxic effects of excessive fat accumulation (Perera et al., 2019). Indeed, *scd1* is the rate limiting enzyme in the synthesis of MUFA, especially oleic acid (OA, 18:1n-9) and palmitoleic acid (16:1n-7) from stearoyl-CoA and palmitoyl-CoA, respectively. Likewise, *elovl6* is responsible for the elongation of SFA and MUFA of 12, 14 and 16 carbons to form 18-carbon FA (Weiss-Hersh et al., 2020). Therefore, the up-regulation of *scd1b* and *elovl6* enhances the biosynthesis of MUFA, which in turn increases the unsaturation index of FA membrane phospholipids. This is reinforced by the recent observation of Perera et al. (2019) that *scd1a* is epigenetically regulated in gilthead sea bream by broodstock nutrition with alpha-linolenic acid (ALA, 18:3n-3) enriched diets. In the present study, no changes in ALA were found among diets, whilst both OA and linoleic acid (LA, 18:2n-6) were 3–7 times higher in TM100 than in the CTRL diet. The observed down-regulation of *elovl6* in fish fed TM100, accompanied by a global decreasing trend of *scd1b* expression with FM replacement, support a reduced *de novo* lipogenic activity. In this scenario, the simultaneous up-regulated expression of *fads2* in fish fed TM100 will serve to further desaturate PUFA, but the capacity of *de novo* synthesis of n-3 LC-PUFA is limited in marine fish and European sea bass in particular, due to the lack of *elovl2* and reduced delta 5 desaturase activity (Monroig et al., 2018). Thus, although all dietary treatments provided European sea bass recommended n-3 LC-PUFA levels, TM100 diet may compromise the muscle n-3 LC-PUFA content at long term.

Cholesterol metabolism is also affected by FM replacement, and the hypocholesterolemic effect of plant proteins as FM replacers has been observed in a wide range of fish species, including gilthead sea bream (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2016), European sea bass (Messina et al., 2013), rainbow trout (Romarheim et al., 2008) and salmon (Hartviksen et al., 2014). Likewise, feeding trials conducted with semisynthetic diets highlighted that FO replacement with vegetal oils acts lowering triglycerides and cholesterol in gilthead sea bream (Ballester-Lozano et al., 2015). In the same study, diets formulated for deficiencies in phospholipids and vitamins also showed a hypocholesterolemic effect, whereas P deficiency acted as a hypercholesterolemic factor. Since this same trend was found herein with FM replacement by *d*TM, the availability of bioactive P becomes again a limiting factor for the successful replacement of FM, though signs of growth and health impairment were mostly masked at short-term. The precise mechanism remains unknown, but it would be favored by the down-regulated expression of hepatic *cyp7a1,* the rate limiting enzyme of the bile acid synthesis pathway (Jelinek et al., 1990). This assumption was supported by the negative correlation between circulating cholesterol and the expression level of hepatic *cyp7a1*, as *cyp7a1* deficiency has been reported to lead to hypercholesterolemia (Pullinger et al., 2002; Erickson et al., 2003; Qayyum et al., 2018). The ultimate physiological consequences of these findings remain elusive, but intriguingly the suppression of the hepatic *cyp7a1* expression in mice fed cholesterolemic diets mainly involves the activation of inflammatory cytokines (Henkel et al., 2011).

Growth of fish is intrinsically related to protein deposition in muscle, which is regulated by systemic and local signaling pathways involving the GH/IGF system (Mommsen, 2001). In this study, no differences in the expression of *gh* receptors or *igfs* transcripts were observed among diets, which is in line with growth performance results. Nonetheless, *myod2* plays a pivotal role in myoblast proliferation and differentiation (Valente et al., 2013), and its down-regulated expression in fish fed TM80 and TM100 should be indicative of some muscle growth derangement. Moreover, *mstn* is a negative regulator of muscle growth through inhibition of proliferation and differentiation of myogenic progenitor cells (Bonnieu et al., 2007), and its up-regulation coupled to the down-regulated expression

of *myod2* highly supports muscle growth inhibition at the transcriptional level. The extent to which this negative feature is counter-regulated by compensatory growth mechanisms remains uncertain, but the expression of key transcripts of the ubiquitin proteasome pathway, with a main role in the protein turnover of skeletal muscle (Foletta et al., 2011), remained unaltered (muscle RING-finger protein-1; *murf1*) or were even down-regulated (*mafbx/atrogin-1*) with the total FM replacement. Furthermore, a recent study in zebrafish demonstrated that *mymk* plays a pivotal role in myoblast fusion and consequently in muscle growth (Shi et al., 2018), and interestingly the expression of this growth-promoting factor was consistently up-regulated with the total FM replacement. The net result at short-term would be, thereby, the preservation of muscle growth with the use of IM as a main protein source in European sea bass. This was supported by the lack of changes in skeletal muscle cellularity and instrumental texture properties. To our best knowledge, the muscle cellularity of fish fed IM diets was never assessed before, but previous studies in different farmed fish species have evidenced the preservation of textural properties with high levels of FM replacement by IM (Lock et al., 2016; Borgogno et al., 2017; Iaconisi et al., 2017; Wang et al., 2017; Secci et al., 2019; Bruni et al., 2020).

In conclusion, the results of the present study demonstrate that FM replacement by *d*TM is largely feasible in European sea bass without detrimental effects on growth performance, nutrient utilization, intestinal integrity, and flesh nutritional and textural quality. Indeed, FM replacement by *d*TM resulted in fair levels of EPA and DHA in muscle of European sea bass (0.38–0.46 g/100 g WW), which are above those recommended by EFSA (2010) for human consumption to decrease the risk of cardiovascular diseases (0.25 g of EPA plus DHA per 100 g of fish). Also, most of the possible negative effects on P and lipid metabolism are largely mitigated at this high replacement level. Nonetheless, further research is needed to fully validate such nutritional approach at farm level throughout the production cycle, giving special attention to the total energy content of the diet since lipid-energized diets can exacerbate the disturbance of lipid metabolism. It is also important to mention that the reduction of P emissions to the environment, coupled with the reduction in the Fi:Fo ratio, prove that *d*TM is an environmentally sustainable alternative to FM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IATS-CSIC and CSIC Review Boards, European (2010/63/EU) Animal Directives, and Spanish Laws (Royal Decree RD53/2013) on the handling of experimental animals.

AUTHOR CONTRIBUTIONS

LV, JP-S, JC-G, and EM conceived and designed the study. AB, BO, LP, TS, MM, and SF conducted research, analyzed data, and performed statistical analysis. AB, JP-S, and LV wrote the article and had primary responsibility for final content. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.659567/full#supplementary-material

REFERENCES

Abdel-Tawwab, M., Khalil, R. H., Metwally, A. A., Shakweer, M. S., Khallaf, M. A., and Abdel-Latif, H. M. R. (2020). Effects of black soldier fly (*Hermetia illucens* L.) larvae meal on growth performance, organs-somatic indices, body composition, and hemato-biochemical variables of European sea bass, *Dicentrarchus labrax*. *Aquaculture* 522, 735136. doi: 10.1016/j.aquaculture.2020.735136

Alegbeleye, W. O., Obasa, S. O., Olude, O. O., Otubu, K., and Jimoh, W. (2012). Preliminary evaluation of the nutritive value of the variegated grasshopper (*Zonocerus variegatus* L.) for African catfish *Clarias gariepinus* (Burchell. 1822) fingerlings. *Aquacult. Res.* 43, 412–420. doi: 10.1111/j.1365-2109.2011.02844.x

Antonopoulou, E., Nikouli, E., Piccolo, G., Gasco, L., Gai, F., Chatzifotis, S., et al. (2019). Reshaping gut bacterial communities after dietary *Tenebrio molitor* larvae meal supplementation in three fish species. *Aquaculture* 503, 628–635. doi: 10.1016/j.aquaculture.2018.12.013

AOAC, (2006). *Official Methods of Analysis*. Gaithersburg, MD: Association of Official Analytical Chemists.

Arru, B., Furesi, R., Gasco, L., Madau, F., and Pulina, P. (2019). The introduction of insect meal into fish diet: the first economic analysis on European sea bass farming. *Sustainability* 11:1697.

Ballester-Lozano, G. F., Benedito-Palos, L., Estensoro, I., Sitja-Bobadilla, A., Kaushik, S., and Perez-Sanchez, J. (2015). Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in

gilthead sea bream fed semi-purified diets. *Br. J. Nutr.* 114, 713–726. doi: 10.1017/S0007114515002354

Ballester-Lozano, G. F., Benedito-Palos, L., Mingarro, M., Navarro, J. C., and Pérez-Sánchez, J. (2016). Up-scaling validation of a dummy regression approach for predictive modelling the fillet fatty acid composition of cultured European sea bass (*Dicentrarchus labrax*). *Aquacult. Res.* 47, 1067–1074. doi: 10.1111/are.12563

Basto, A., Matos, E., and Valente, L. M. P. (2020). Nutritional value of different insect larvae meals as protein sources for European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 521:735085. doi: 10.1016/j.aquaculture.2020.735085

Batista, S., Pereira, R., Oliveira, B., Baião, L. F., Jessen, F., Tulli, F., et al. (2020). Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. *J. Appl. Phycol.* 32, 2041–2059. doi: 10.1007/s10811-020-02118-z

Benedito-Palos, L., Ballester-Lozano, G. F., Simó, P., Karalazos, V., Ortiz, Á, Calduch-Giner, J., et al. (2016). Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454, 8–18. doi: 10.1016/j.aquaculture.2015.12.008

Bolin, D. W., King, R. P., and Klosterman, E. W. (1952). A simplified method for the determination of chromic oxide (Cr_2O_3) when used as an index substance. *Science* 116, 634–635. doi: 10.1126/science.116.3023.634

Bonnieu, A., Carnac, G., and Vernus, B. (2007). Myostatin in the pathophysiology of skeletal muscle. *Curr. Genomics* 8, 415–422. doi: 10.2174/138920207783591672

Borgogno, M., Dinnella, C., Iaconisi, V., Fusi, R., Scarpaleggia, C., Schiavone, A., et al. (2017). Inclusion of *Hermetia illucens* larvae meal on rainbow trout (*Oncorhynchus mykiss*) feed: effect on sensory profile according to static and dynamic evaluations. *J. Sci. Food Agric.* 97, 3402–3411. doi: 10.1002/jsfa.8191

Bruni, L., Belghit, I., Lock, E. J., Secci, G., Taiti, C., and Parisi, G. (2020). Total replacement of dietary fish meal with black soldier fly (*Hermetia illucens*) larvae does not impair physical, chemical or volatile composition of farmed Atlantic salmon (*Salmo salar* L.). *J. Sci. Food Agric.* 100, 1038–1047. doi: 10.1002/jsfa.10108

Bruni, L., Pastorelli, R., Viti, C., Gasco, L., and Parisi, G. (2018). Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source. *Aquaculture* 487, 56–63. doi: 10.1016/j.aquaculture.2018.01.006

Campos, I., Matos, E., Marques, A., and Valente, L. M. P. (2017). Hydrolyzed feather meal as a partial fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 476, 152–159. doi: 10.1016/j.aquaculture.2017.04.024

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., et al. (2019). Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). *Animals (Basel)* 9:5. doi: 10.3390/ani9050251

Cho, C. Y., and Slinger, S. J. (1979). "Apparent digestibility measurement in feedstuffs for rainbow trout," in *Finfish Nutrition and Fishfeed Technology*, eds J. E. Halver, and K. Tiews, (Berlin: Heenemann), 239–247.

Choubert, G., Blanc, J. M., and Vallée, F. (1997). Colour measurement, using the CIELCH colour space, of muscle of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed astaxanthin: effects of family, ploidy, sex, and location of reading. *Aquacult. Res.* 28, 12–22.

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E., and Weltzien, E. (2018). The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 492, 24–34. doi: 10.1016/j.aquaculture.2018.03.038

EFSA, (2010). Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J.* 8:1461. doi: 10.2903/j.efsa.2010.1461. European

Erickson, S. K., Lear, S. R., Deane, S., Dubrac, S., Huling, S. L., Nguyen, L., et al. (2003). Hypercholesterolemia and changes in lipid and bile acid metabolism in male and female cyp7A1-deficient mice. *J. Lipid Res.* 44, 1001–1009. doi: 10.1194/jlr.M200489-JLR200

European Commission, (2017). Commission Regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on processed animal protein. *Off. J. Eur. Union*. 60, 92–116.

Eurostat, (2020). *Production from Aquaculture Excluding Hatcheries and Nurseries (from 2008 Onwards)*. Available online at: https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do (acessed January 5, 2020).

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226, 497–509.

Foletta, V. C., White, L. J., Larsen, A. E., Leger, B., and Russell, A. P. (2011). The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflugers Arch. Eur. J. Physiol.* 461, 325–335. doi: 10.1007/s00424-010-0919-9

Gasco, L., Acuti, G., Bani, P., Dalle Zotte, A., Danieli, P. P., De Angelis, A., et al. (2020). Insect and fish by-products as sustainable alternatives to conventional animal proteins in animal nutrition. *Ital. J. Anim. Sci.* 19, 360–372. doi: 10.1080/1828051x.2020.1743209

Gasco, L., Henry, M., Piccolo, G., Marono, S., Gai, F., Renna, M., et al. (2016). *Tenebrio molitor* meal in diets for European sea bass (*Dicentrarchus labrax* L.) juveniles: growth performance, whole body composition and in vivo apparent digestibility. *Anim. Feed Sci. Technol.* 220, 34–45. doi: 10.1016/j.anifeedsci.2016.07.003

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan, D. F., et al. (2004). Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* 232, 493–510. doi: 10.1016/s0044-8486(03)00532-5

Guerreiro, I., Castro, C., Antunes, B., Coutinho, F., Rangel, F., Couto, A., et al. (2020). Catching black soldier fly for meagre: growth, whole-body fatty acid profile and metabolic responses. *Aquaculture* 2:516. doi: 10.1016/j.aquaculture.2019.734613

Gutowska, M. A., Drazen, J. C., and Robison, B. H. (2004). Digestive chitinolytic activity in marine fishes of Monterey Bay, California. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 139, 351–358. doi: 10.1016/j.cbpb.2004.09.020

Hartviksen, M., Bakke, A. M., Vecino, J. G., Ringo, E., and Krogdahl, A. (2014). Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (*Salmo salar* L.): digestive and metabolic investigations. *Fish Physiol. Biochem.* 40, 1621–1637. doi: 10.1007/s10695-014-9953-4

Henkel, A. S., Anderson, K. A., Dewey, A. M., Kavesh, M. H., and Green, R. M. (2011). A chronic high-cholesterol diet paradoxically suppresses hepatic CYP7A1 expression in FVB/NJ mice. *J. Lipid Res.* 52, 289–298. doi: 10.1194/jlr.M012781

Henry, M. A., Gasco, L., Chatzifotis, S., and Piccolo, G. (2018). Does dietary insect meal affect the fish immune system? The case of mealworm, *Tenebrio molitor* on European sea bass, *Dicentrarchus labrax*. *Dev. Comp. Immunol.* 81, 204–209. doi: 10.1016/j.dci.2017.12.002

Iaconisi, V., Marono, S., Parisi, G., Gasco, L., Genovese, L., Maricchiolo, G., et al. (2017). Dietary inclusion of *Tenebrio molitor* larvae meal: effects on growth performance and final quality treats of blackspot sea bream (*Pagellus bogaraveo*). *Aquaculture* 476, 49–58. doi: 10.1016/j.aquaculture.2017.04.007

Iaconisi, V., Secci, G., Sabatino, G., Piccolo, G., Gasco, L., Papini, A. M., et al. (2019). Effect of mealworm (*Tenebrio molito*r L.) larvae meal on amino acid composition of gilthead sea bream (*Sparus aurata* L.) and rainbow trout (*Oncorhynchus mykiss* W.) fillets. *Aquaculture* 513:734403. doi: 10.1016/j.aquaculture.2019.734403

Ido, A., Hashizume, A., Ohta, T., Takahashi, T., Miura, C., and Miura, T. (2019). Replacement of fish meal by defatted yellow mealworm (*Tenebrio molitor*) larvae in diet improves growth performance and disease resistance in red seabream (*Pargus major*). *Animals* 9:3. doi: 10.3390/ani9030100

Jelinek, D. F., Andersson, S., Slaughter, C. A., and Russell, D. W. (1990). Cloning and regulation of cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* 265, 8190–8197.

Kawashima, S., Ikehata, H., Tada, C., Ogino, T., Kakizaki, H., Ikeda, M., et al. (2016). Stomach chitinase from Japanese sardine *Sardinops melanostictus*:

purification, characterization, and molecular cloning of chitinase Isozymes with a long linker. *Mar. Drugs* 14:22. doi: 10.3390/md14010022

Koutsos, L., McComb, A., Finke, M., and Stull, V. (2019). Insect composition and uses in animal feeding applications: a brief review. *Ann. Entomol. Soc. Am.* 112, 544–551. doi: 10.1093/aesa/saz033

Kroeckel, S., Harjes, A. G. E., Roth, I., Katz, H., Wuertz, S., Susenbeth, A., et al. (2012). When a turbot catches a fly: evaluation of a pre-pupae meal of the black soldier fly (*Hermetia illucens*) as fish meal substitute - growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). *Aquaculture* 36, 345–352. doi: 10.1016/j.aquaculture.2012.08.041

Krogdahl, Å, Hemre, G. I, and Mommsen, T. P. (2005). Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquacult. Nutr.* 11, 103–122. doi: 10.1111/j.1365-2095.2004.00327.x

Lands, B. (2014). Historical perspectives on the impact of n-3 and n-6 nutrients on health. *Prog. Lipid. Res.* 55, 17–29. doi: 10.1016/j.plipres.2014.04.002

Li, Y., Bruni, L., Jaramillo-Torres, A., Gajardo, K., Kortner, T. M., and Krogdahl, A. (2021). Differential response of digesta- and mucosa-associated intestinal microbiota to dietary insect meal during the seawater phase of Atlantic salmon. *Anim. Microbiome* 3:8. doi: 10.1186/s42523-020-00071-3

Li, Y., Kortner, T. M., Chikwati, E. M., Belghit, I., Lock, E. J., and Krogdahl, A. (2020). Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*). *Aquaculture* 520:734967. doi: 10.1016/j.aquaculture.2020.734967

Li, Y., Kortner, T. M., Chikwati, E. M., Munang'andu, H. M., Lock, E. J., and Krogdahl, A. (2019). Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal. *Fish Shellfish Immunol.* 86, 1106–1113. doi: 10.1016/j.fsi.2018.12.057

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lock, E. R., Arsiwalla, T., and Waagbø, R. (2016). Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon *(Salmo salar)* postsmolt. *Aquacult. Nutr.* 22, 1202–1213. doi: 10.1111/anu.12343

Lopes, G., Castro, L. F. C., and Valente, L. M. P. (2017). Total substitution of dietary fish oil by vegetable oils stimulates muscle hypertrophic growth in Senegalese sole and the upregulation of fgf6. *Food Func.* 8, 1869–1879. doi: 10.1039/c7fo00340d

Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., and Peres, H. (2017). Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture* 476, 79–85. doi: 10.1016/j.aquaculture.2017.04.021

Marono, S., Piccolo, G., Loponte, R., Di Meo, C., Attia, Y. A., Nizza, A., et al. (2015). *In vitro* crude protein digestibility of *Tenebrio molitor* and *Hermetia illucens* insect meals and its correlation with chemical composition traits. *Ital. J. Anim. Sci.* 14:3889. doi: 10.4081/ijas.2015.3889

Maynard, L. A., Loosli, J. K., Hintz, H. F., and Warner, R. G. (1979). *Animal Nutrition*. New York, USA.

Messina, M., Piccolo, G., Tulli, F., Messina, C. M., Cardinaletti, G., and Tibaldi, E. (2013). Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. *Aquaculture* 37, 6–14. doi: 10.1016/j.aquaculture.2012.11.005

Mommsen, T. P. (2001). Paradigms of growth in fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 129, 207–219. doi: 10.1016/S1096-4959(01)00312-8

Monroig, O., Tocher, D. R., and Castro, L. F. C. (2018). "Polyunsaturated fatty acid biosynthesis and metabolism in fish," in *Polyunsaturated Fatty Acid Metabolism*, ed. G. C. Burdge, (Urbana, IL: AOCS Press), 31–60.

Nogales-Mérida, S., Gobbi, P., Józefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., et al. (2018). Insect meals in fish nutrition. *Rev. Aquac.* 2018, 1–14. doi: 10.1111/raq.12281

NRC, (2011). *Nutrient Requirements of Fish and Shrimp*. Washington, DC: The National Academy Press.

Osimani, A., Garofalo, C., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., et al. (2016). Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union. *Eur. Food Res. Technol.* 243, 1157–1171. doi: 10.1007/s00217-016-2828-4

Perera, E. A.-O., Turkmen, S. A.-O., Simó-Mirabet, P. A.-O., Zamorano, M. A.-O., Xu, H. A.-O., Naya-Català, F. A.-O. X., et al. (2019). Stearoyl-CoA desaturase (*scd1a*) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). *Epigenetics* 19, 1559–2308. doi: 10.1080/15592294.2019.1699982

Piccolo, G., Iaconisi, V., Marono, S., Gasco, L., Loponte, R., Nizza, S., et al. (2017). Effect of *Tenebrio molitor* larvae meal on growth performance, i*n vivo* nutrients digestibility, somatic and marketable indexes of gilthead sea bream (*Sparus aurata*). *Anim. Feed Sci. Technol.* 226, 12–20. doi: 10.1016/j.anifeedsci.2017.02.007

Prabhu, P. A. J., Schrama, J. W., and Kaushik, S. J. (2014). Mineral requirements of fish: a systematic review. *Rev. Aquac.* 8, 172–219. doi: 10.1111/raq.12090

Pullinger, C. R., Eng, C., Salen, G., Shefer, S., Batta, A. K., Erickson, S. K., et al. (2002). Human cholesterol 7alpha-hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype. *J. Clin. Invest.* 110, 109–117. doi: 10.1172/JCI15387

Qayyum, F., Lauridsen, B. K., Frikke-Schmidt, R., Kofoed, K. F., Nordestgaard, B. G., and Tybjćrg-Hansen, A. (2018). Genetic variants in CYP7A1 and risk of myocardial infarction and symptomatic gallstone disease. *Eur. Heart J.* 39, 2106–2116. doi: 10.1093/eurheartj/ehy068

Rema, P., Saravanan, S., Armenjon, B., Motte, C., and Dias, J. (2019). Graded incorporation of defatted yellow mealworm (*Tenebrio molitor*) in rainbow trout (*Oncorhynchus mykiss*) diet improves growth performance and nutrient retention. *Animals (Basel)* 9:4. doi: 10.3390/ani9040187

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., and Terova, G. (2019). The effects of dietary insect meal from *Hermetia illucens* prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). *Animals (Basel)* 9:4. doi: 10.3390/ani9040143

Romarheim, O. H., Skrede, A., Penn, M., Mydland, L. T., Krogdahl, Å, and Storebakken, T. (2008). Lipid digestibility, bile drainage and development of morphological intestinal changes in rainbow trout (*Oncorhynchus mykiss*) fed diets containing defatted soybean meal. *Aquaculture* 274, 329–338. doi: 10.1016/j.aquaculture.2007.11.035

Salter, A. M. (2019). Insect protein: a sustainable and healthy alternative to animal protein? *J. Nutr.* 149, 545–546. doi: 10.1093/jn/nxy315

Secci, G., Mancini, S., Iaconisi, V., Gasco, L., Basto, A., and Parisi, G. (2019). Can the inclusion of black soldier fly (*Hermetia illucens*) in diet affect the flesh quality/nutritional traits of rainbow trout (*Oncorhynchus mykiss*) after freezing and cooking? *Int. J. Food Sci. Nutr.* 70, 161–171. doi: 10.1080/09637486.2018.1489529

Shi, J., Cai, M., Si, Y., Zhang, J., and Du, S. (2018). Knockout of myomaker results in defective myoblast fusion, reduced muscle growth and increased adipocyte infiltration in zebrafish skeletal muscle. *Hum. Mol. Genet.* 27, 3542–3554. doi: 10.1093/hmg/ddy268

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., and Pérez-Sánchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (Sparus aurata). *Aquaculture* 249, 387–400. doi: 10.1016/j.aquaculture.2005.03.031

Skalli, A., and Robin, J. H. (2004). Requirement of n-3 long chain polyunsaturated fatty acids for European sea bass (*Dicentrarchus labrax*) juveniles: growth and fatty acid composition. *Aquaculture* 240, 399–415. doi: 10.1016/j.aquaculture.2004.06.036

Stenberg, O. K., Holen, E., Piemontese, L., Liland, N. S., Lock, E. J., Espe, M., et al. (2019). Effect of dietary replacement of fish meal with insect meal on in vitro bacterial and viral induced gene response in Atlantic salmon (*Salmo salar*) head kidney leukocytes. *Fish Shellfish Immunol.* 91, 223–232. doi: 10.1016/j.fsi.2019.05.042

Sugiura, S. H., Hardy, R. W., and Roberts, R. J. (2004). The pathology of phosphorus deficiency in fish – a review. *J. Fish Dis.* 27, 255–265. doi: 10.1111/j.1365-2761.2004.00527.x

Tacon, A. G. J., and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285, 146–158. doi: 10.1016/j.aquaculture.2008.08.015

Valente, L. M. P., Moutou, K. A., Conceição, L. E. C., Engrola, S., Fernandes, J. M. O., and Johnston, I. A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Rev. Aquac.* 5, S168–S193. doi: 10.1111/raq.12020

Wang, L., Li, J., Jin, J. N., Zhu, F., Roffeis, M., and Zhang, X. Z. (2017). A comprehensive evaluation of replacing fishmeal with housefly (*Musca domestica*) maggot meal in the diet of Nile tilapia (*Oreochromis niloticus*): growth performance, flesh quality, innate immunity and water environment. *Aquacult. Nutr.* 23, 983–993. doi: 10.1111/anu.12466

Weiss-Hersh, K., Garcia, A. L., Marosvolgyi, T., Szklenar, M., Decsi, T., and Ruhl, R. (2020). Saturated and monounsaturated fatty acids in membranes are determined by the gene expression of their metabolizing enzymes SCD1 and ELOVL6 regulated by the intake of dietary fat. *Eur. J. Nutr.* 59, 2759–2769. doi: 10.1007/s00394-019-02121-2

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frontiers in Marine Science

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Using Glycerol to Produce European Sea Bass Feed With Oleaginous Microbial Biomass: Effects on Growth Performance, Filet Fatty Acid Profile, and FADS2 Gene Expression

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Using a circular economy concept, the present study investigated the use of crude glycerol, a primary by-product of biodiesel production, as a low-priced nutrient source for heterotrophic cultivation of the fungus-like protist *Schizochytrium limacinum* SR21 strain. The whole biomass of this oleaginous microorganism, rich in docosahexaenoic acid (DHA) and high-quality proteins, was then paired with a vegetable oil (VO) source and used to replace fish oil (FO) in European sea bass (*Dicentrarchus labrax*) feeds. Four nutritionally balanced diets were formulated: diet FO (a FO-based diet), diet VO + 0 (a VO-based diet without *S. limacinum*), and diets VO + 5 and VO + 10 that were VO-based feeds supplemented with 5 and 10% of *S. limacinum*, respectively. After a 3-month feeding trial, fish of all dietary groups tripled their initial weight, but growth and feeding efficiencies of *D. labrax* were not significantly different among treatments. Although the formulated diets were balanced for polyunsaturated fatty acids (PUFAs), fish fed with feeds containing either VO or VO plus 5 and 10% of *S. limacinum* biomass had significantly higher levels of PUFAs in the flesh than fish fed the FO-based diet. Values of health-related lipid indexes, such as atherogenicity index, thrombogenicity index, and flesh lipid quality as well as n-6/n-3 and PUFAs/SFAs ratios confirmed the high nutritional value of sea bass filet, thus representing a healthy product for human consumption. Although the PUFAs/SFAs ratio showed a significantly higher value in fish fed with VO-based diets supplemented with *S. limacinum* than in those fed with FO diet, suggesting a better filet quality, the n-6/n-3 ratio clearly indicated that filet quality of dietary group FO was best (value of 0.55) and that of group VO + 10 second best (value of 0.98). We also evaluated the nutritional regulation of Δ6-desaturase (or *fads2*) gene expression in European sea bass liver. European sea bass fed the VO + 0 diet

had the highest number of mRNA copies for Δ6-desaturase (or *fads2*), fish fed with diet VO + 10 the lowest. Our study adds to the growing body of literature concerning the use of thraustochytrid biomass as a valid alternative to marine-derived raw materials for European sea bass feeds.

Keywords: aquaculture, aquafeed, *Dicentrarchus labrax*, *Schizochytrium limacinum*, Δ6-desaturase, gene expression, fish filet quality, fatty acid profile

INTRODUCTION

In the last few decades, the global demand for aquatic food products has been steadily increasing and aquaculture has been playing a major role in meeting this demand by boosting fish production. However, like all other food production practices, the aquaculture sector must expand in a sustainable way, as awareness of potential environmental issues of harvesting fish for feed has increased significantly. Therefore, most aqua farmers are continuously improving their production practices to make them more sustainable, efficient, and cost-effective. For an aquaculture system to develop in harmony with environmental goals, feed represents one limiting factor as it traditionally includes ocean-derived raw materials such as fishmeal (FM) and fish oil (FO) that provide high-quality proteins and lipids–mostly long-chain (C 20–C 22) polyunsaturated fatty acids (PUFAs)–to cultured fish (FAO, 2018). PUFAs, mainly eicosapentaenoic acid (EPA) (C 20:5n-3), and docosahexaenoic acid (DHA) (C 22:6n-3) have unique roles in controlling and regulating cellular metabolism and animal physiology. In fish, they are required early during ontogeny, principally for neural development, but also after this period, as they are involved in cell membrane fluidity, cellular metabolism, immune response, pigmentation, stress resistance, reproduction, and, finally, in schooling behavior (Sargent et al., 1997; Masuda and Tsukamoto, 1999; Montero et al., 2003; Tocher, 2003, 2010; Glencross, 2009). For these reasons, FO has constituted a major dietary lipid source in aquafeeds, but its limited availability and high cost have promoted the search for more affordable and sustainable alternatives.

Among alternative oils, those derived from seed plants [vegetable oils (VOs)], such as soybean, rapeseed, palm, linseed, and canola, have received much attention because of their competitive price, good catabolic potential to obtain dietary energy, and lower level of toxic pollutants, such as dioxins (Sales and Glencross, 2011; Carvalho et al., 2020). Little or no effects were reported on fish growth performance at low FO/VO replacement levels when minimum omega-3 and omega-6 intake requirements were met (Sargent et al., 1997; Martins et al., 2012), but reduced performance and decreased omega-3 content in fish filets were reported when high levels of VOs were included (Özşahinoğlu et al., 2013; Monge-Ortiz et al., 2018; Ayisi et al., 2019). Therefore, high FO/VO replacement levels may constrain production efficiency while also compromising many health benefits of fish consumption related to human diseases (Calder and Yaqoob, 2009; Siscovick et al., 2017; Layé et al., 2018). Human beings cannot synthesize EPA and DHA (humans are able to synthesize a small quantity of DHA, not *de novo* but from ingested α-linolenic acid (ALA, C 18:3n-3)) because they lack some of the enzymes needed for synthesis. This means that EPA and DHA are "essential" and should be taken through diet, principally by fish consumption (Erdman et al., 2012; Ross et al., 2012). Indeed, irrefutable evidence links an adequate dietary intake of fish (especially fatty fish) rich in EPA and DHA with a significant reduction in coronary risk (Innes and Calder, 2020).

A possible strategy to increase the content of PUFAs in fish feeds with no FO or low FO levels might be to include oleaginous microorganisms (bacteria, filamentous fungi, microalgae, yeast, and thraustochytrids) that are capable of accumulating high levels of lipids in their cellular compartments. Certain oleaginous species can accumulate up to 70% w/w of lipids on cell dry matter when cultivated in medium with a high carbon/nitrogen (C/N) ratio (Ratledge and Cohen, 2008; Beopoulos and Nicaud, 2012; Patel et al., 2020). These lipids, known as single-cell oils or microbial oils (MOs), are similar to VOs but it is easier to scale up their production (Becker, 2007; Moreira et al., 2011; Nasseri et al., 2011; Khot et al., 2012; Vanthoor-Koopmans et al., 2013). There are several advantages in using microorganisms to produce MOs. Indeed, microorganisms can be easily cultured in a controlled environment without depending on seasonal and climatic conditions can tolerate different cultivation conditions, can be genetically modified in order to obtain specific products and, finally, they do not compete with food crops for arable land and freshwater (Patel et al., 2020). However, the costs for producing MOs are still higher than for FOs, due to the nature of the substrates used for heterotrophic fermentation, the investment in lighting and electricity for phototrophic algae, and the refinement of products (Ratledge and Cohen, 2008; Sprague et al., 2015, 2017). Industrial wastes, which represent a threat to the environment, have been suggested as a solution to reduce such production costs; for instance, crude glycerol, which is the major by-product of the biodiesel industry, has been proposed (Mata et al., 2014; Katiyar et al., 2017).

Crude glycerol represents a burden for waste disposal in biodiesel production due to the different properties of this blend in comparison with pure glycerol (Johnson and Taconi, 2007; Checa et al., 2020). Therefore, different methods for valorizing crude glycerol have been developed; for instance, by using it as a nutrient source for the cultivation of oleaginous microorganisms (Athenaki et al., 2018; Patel et al., 2020). Among these, Thraustochytrids (heterotrophic fungus-like protists), such as *Schizochytrium* spp., are becoming increasingly important to produce oils rich in PUFAs. *Schizochytrium* is an excellent producer of DHA, when growing on glycerol. It synthesizes DHA through "anaerobic" polyketide synthases; therefore, low dissolved oxygen (DO) is used for DHA production. However, since low DO cannot facilitate cell proliferation, DHA

concentration is improved by firstly optimizing cell proliferation at high DO and then shifting to low DO for lipid accumulation (Chang et al., 2013).

Although several studies have examined the replacement of dietary FO with MO from *Schizochytrium* spp. in fish and shrimp feeds (Harel et al., 2002; O'Grady and Morgan, 2011; Lung et al., 2016; Katiyar et al., 2017; Allen et al., 2019; Glencross et al., 2020b), only very few studies have reported using the whole of cell biomass of this strain in fish feeds (Kousoulaki et al., 2015; Katerina et al., 2020; Lee Chang et al., 2020; Hart et al., 2021). By using the harvested *Schizochytrium* spp. biomass as a whole in fish feeds, it is possible to take advantage of all major nutrients synthesized by this microorganism (e.g., lipids, proteins, minerals, vitamins, and carbohydrates), thus maximizing its nutritional value in fish feed. Moreover, in this way, from an economic standpoint, the cost related to extracting omega-3 from the biomass is spared, and these nutraceuticals are protected in whole cell form from possible oxidation during the extraction process.

In this view, the aim and novelty of the present study was to assess the effects of using whole-cell *Schizochytrium limacinum* biomass, cultivated on crude glycerol waste from a palm oil biodiesel plant, as nutritional supplement in European sea bass (*Dicentrarchus labrax*) feed. The investigation focused on fish growth performance, nutritional quality of filets, and expression levels of Δ6-desaturase (or fatty acid desaturase 2, *fads2*) gene. Four fish diets that included different percentages of *S. limacinum* biomass were tested in a 3-month feeding trial. The objective for including DHA-rich biomass in European sea bass feed was twofold: (1) to achieve optimal growth and development of the fish and (2) to provide a vector for the delivery of essential fatty acids to filets for human consumption.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The Italian Ministry of Health approved the animal experiments [REF: 483/2017-PR (response of Prot. Nr. 344C6.6 of 13/03/2017)] in accordance with Art.31 of D.lgs. 26/2014.

Schizochytrium limacinum Fermentation, Lipid Production, and Analysis

The ability of *S. limacinum* to accumulate PUFAs in a heterotrophic culture, using crude glycerol, was tested in 2-L bioreactors. *S. limacinum* SR21 was purchased from the American Type Culture Collection (ATCC, United States) and stored at −80°C in glycerol stocks (20% v/v). Starting cultures for bioreactor inoculation were obtained in 250-mL shake flasks with 50 mL of synthetic medium (glucose 20 g/L, peptone 20 g/L, and yeast extract 10 g/L): cultures were maintained in a shaker at 30°C and 160 rpm for 72 h. The exponential phase cultures were then used to inoculate bioreactors to reach a final optical density (OD_{600}) of 0.5. Briefly, cells were centrifuged at 2621 × *g* for 10 min, washed twice with water, and finally resuspended in 10 mL of sterilized water. The batch experiments were conducted in 2 and 10 L stirred tank bioreactors (BIOSTAT® B plus, and BIOSTAT® C Sartorius Stedim Biotech GmbH, Göttingen, Germany) equipped with Visiferm DO ECS 225 for measuring pO_2 and Easyferm Plus K8 200 for determining pH (both from Hamilton Bonaduz AG, Bonaduz, Switzerland). As growth medium for the batch fermentations By + medium (Chi et al., 2007) was modified as follows: 20 g/L glycerol, 1.5 g/L yeast extract, 1.5 g/L tryptone, and 30 g/L seawater salts (20.758 g/L NaCl, 0.587 g/L KCl, 0.17 g/L $NaHCO_3$, 0.0746 g/L NaBr, 0.0225 g/L H_3BO_3, 0.0027 g/L NaF, 3.477 g/L Na_2SO_4, 9.395 g/L $MgCl_2 \cdot 6\ H_2O$, 1.316 g/L $CaCl_2 \cdot 2\ H_2O$, and 0.0214 g/L $SrCl_2 \cdot 6\ H_2O$). The medium was adjusted to pH 7 with HCl before sterilization, except for seawater salts, which were adjusted to pH 8 (using NaOH e HCl), filtrated with a 0.22-μm polytetrafluoroethylene (PTFE) filter, and added under sterile conditions to the final medium after bioreactor sterilization to prevent salt precipitation in the autoclave. After 28 h of growth, a crude glycerol supplement was added to the growth medium to reach a final concentration of 75 g/L, according to the method previously reported by Signori et al. (2016) for other oleaginous microbes, and the fermentation was carried out for 144 h to reach maximum lipid accumulation in the cells. Briefly, the crude extract consisted of 80% content of glycerol was centrifuged at 2621 × *g* for 20 min and then, the top dark red layer (mainly consisting of free fatty acids), was removed.

The parameters used during fermentation were 300 rpm of agitation in cascade to 25% of DO (to obtain a 1-vvm aeration rate) and a temperature of 30°C. A pH value of 7 was maintained during the entire fermentation process with NaOH 4 M and H_2SO_4 25%. All the reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, United States), except for the yeast extract, which was from Biolife Italia S.r.l. (Milan, Italy). Crude glycerol was derived from industrial biodiesel production out of palm oil.

After fermentation, *S. limacinum* cells were harvested by centrifugation at 7885 × *g* for 20 min (Sorvall Evolution RC, Thermo Fisher Scientific, Waltham, MA, United States); then, cells were washed twice with distilled water. Dry cell weight (DCW) was determined gravimetrically by drying 1 mL of cell culture (Concentrator 5301, Eppendorf AG, Germany).

To determine the lipid content in the cells, lipids were extracted from 1 g of biomass, based on the method of Bligh and Dyer (1959). Briefly, after drying in an oven at 60°C for 12 h, cells were resuspended in 5 mL of HCl 2 M (1:1) and chemically disrupted in a thermostatic bath at 75°C for 40 min. Then, lipids were collected by adding 5 mL of n-hexane to the mixture. The organic phase at the bottom was collected and left overnight under a chemical hood to evaporate hexane and subsequently weighed. To assess the fatty acid profiles, methyl esters from fatty acids (FAMEs) were obtained by esterification: 5 mL of KOH-methanol 0.4 M were added to the lipids; the mixture was then vortexed and placed in a thermobath at 60°C for 1 h. Subsequently, 5 mL of BF3-methanol (14% w/w) (1:1) were added, mixed by vortex, and placed in a thermobath at 60°C for 1 h. Finally, 10 mL of n-hexane were added to extract FAMEs for gas chromatography (GC) analysis (Savi Laboratori

and Service S.r.l., Italy) according to the following methods: UNI EN ISO 12966-1:2015 + UNI EN ISO 12966-2:2011 + UNI EN ISO 12966-4:2015.

To obtain *S. limacinum* biomass for producing fish feed after bioreactor fermentation, the broth culture was centrifuged in a Sorvall Evolution RC (Thermo Fisher Scientific, Waltham, MA, United States) at 7885 × *g* and 4°C for 10 min and the supernatant was discarded. The biomass was then frozen in liquid nitrogen and lyophilized (LIO5P, 5Pascal, Milan, Italy).

Experimental Diets

The four experimental diets, named FO, VO + 0, VO + 5, and VO + 10, were prepared at the University of Insubria (Varese, Italy), starting from a non-oil-based commercial feed manufactured by VRM S.r.l. Naturalleva feed company (Verona, Italy). Diets were formulated according to the nutritional requirements of *D. labrax* (Lupatsch, 2005; Tibaldi and Kaushik, 2005) and were isonitrogenous, isolipidic, and isoenergetic. In particular, diet FO was a FO-based diet, diet VO + 0 was a VO-based, whereas diets VO + 5 and VO + 10 were VO-based feeds supplemented with 5 and 10% of *S. limacinum*, respectively. Proximate composition of each feed is shown in **Table 1**. Feeds were prepared by using a food blender to mix and homogenize the ingredients that were commercial feed (without oil), soybean oil, and *S. limacinum* biomass at 5 and 10% for diets VO + 5 and VO + 10, respectively, and commercial feed (without oil) plus FO for diet FO or soybean oil for diet VO + 0. The final mixture was transferred to a meat grinder to obtain a 2 mm pellet. At the end of the process, the pellet was dried overnight in an oven at 50°C and then stored at room temperature until use (within a week). The feeds were prepared on a weekly basis. Amino acid and fatty acid composition of the four experimental diets are shown in **Tables 2, 3**, respectively.

Fish Feeding and Sampling

The fish feeding trial was conducted at the University of Insubria (Varese, Italy) in an indoor recirculating saltwater facility. At the beginning of the experiment, 280 European sea bass with an initial mean body weight of 22.38 ± 0.3 g and mean length of 10.13 ± 0.42 cm were randomly distributed in 8 rectangular tanks of 500 L in volume, with 35 fishes per tank (initial density: 1.56 kg/m^3), and allowed to acclimatize for 1 week. The photoperiod was set at 12 h: 12 h light: dark cycle. The temperature (19 ± 1.5°C) and pH (8.3 ± 0.4) were continuously monitored during the trial. DO concentration (maintained over 85%), total ammonia nitrogen, and salinity were measured periodically. Fish mortality was recorded daily.

After acclimation, fish were fed for 21 weeks with the experimental diets and each of the experimental feeds was given to duplicate populations (2 tanks/diet). The fish-feeding rate was maintained at 1.5% of the total biomass. To calculate growth indexes, all the specimens were weighed and their length measured on a 3-weekly basis. Data were used to calculate the feed conversion ratio [FCR = (feed intake × dry matter)/(100 × wet weight gain)], specific growth rate [SGR = 100 × [ln (final body weight)–ln (initial body weight)]/days], condition factor [CF = 100 × (body weight/standard length^3)], and feed efficiency [FE = wet weight gain/(feed intake × dry matter)].

At the end of the trial, fish were sacrificed to collect tissues and organs for subsequent analysis. For this, 12 fish/diet were euthanized with an overdose (320 mg/L at 22°C) of anesthetic (tricaine-methasulfonate MS-222, Sigma-Aldrich, Italy) and liver and white dorsal muscle were aseptically removed and then stored at −80°C until analysis.

Fatty Acid Extraction and Quantification

To assess the fatty acid profiles, six filets /dietary group and an aliquot from each diet were analyzed. The total fat content was extracted using acid hydrolysis (UNI 22605-1992). For analysis, fatty acids in methyl esters (FAMEs) were converted by esterification and determined by capillary GC, according to ISO 12966-2:2011 and ISO 12966-4:2015.

Indexes of Nutrition Quality of Filets

To evaluate the effects of replacing FO with VO and *Schizochytrium* biomass, the following indexes based on the ratios of constituent fatty acids were calculated (Ulbricht and Southgate, 1991; Abrami et al., 1992; Chen and Liu, 2020).

(1) Atherogenicity index [AI = (C 12:0 + (4 × C 14:0) + C 16:0)/(∑ Monounsaturated Fatty Acids (MUFAs) + n-6 PUFAs + n-3 PUFAs)];
(2) Thrombogenicity index [TI = (C 14:0 + C 16:0 + C 18:0)/(0.5 × ∑ MUFAs + 0.5 × n-6 PUFAs + 3 × n-3 PUFAs) + (n-3 PUFAs/n-6 PUFAs)];
(3) Flesh lipid quality [FLQ = C 20:5n-3 + C 22:6n-3/% Total FAs].

Gene Expression Analysis

The Δ6-desaturase transcript copies in fish liver were quantified using One-step TaqMan® quantitative real-time RT-PCR.

RNA Extraction and One-Step TaqMan® Real-Time RT-PCR

Total RNA was extracted from 100 mg of liver dissected out from each fish (6 fish/diet) using the kit Maxwell® 16 LEV simplyRNA Tissue (Promega, Italy) and Maxwell® 16 Instrument (Promega, Italy). The extracted RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific). One hundred nanograms of total RNA from each biological sample were then amplified via One-step TaqMan® quantitative real-time RT-PCR in the same plate with tenfold-diluted, defined amounts of standard mRNAs. The sequences of primers and TaqMan® probes used for target gene amplification are shown in **Table 4**. TaqMan® PCR reactions were performed on a Bio-Rad® CFX96™ System using the following run parameters: one cycle of 10 min at 50°C and 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Data from the TaqMan® PCR runs were collected with CFX™ Software. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values obtained

TABLE 1 | Proximate composition of the four different experimental diets.

Proximate composition (%)	Experimental diets			
	FO	VO + 0	VO + 5	VO + 10
Dry matter	92.50	92.50	92.50	92.50
Crude protein	52.80	52.80	52.83	52.73
Crude fat	15.58	15.58	15.53	15.56
Crude fibers	1.00	1.00	1.00	1.00
NFE[1]	16.12	16.12	16.14	16.21
Ash	7.00	7.00	7.00	7.00
Gross Energy[2] (MJ Kg^{-1})	21.39	21.39	21.38	21.38

[1]NFE = Nitrogen free extracted calculated by difference.
[2]Calculated based on 23.6, 39.5, and 17.2 KJ g^{-1} of protein, fat and carbohydrate, respectively.

TABLE 2 | Amino acids composition of the four different experimental diets.

Amino acid (g/100g of feed)	Experimental diets			
	FO	VO + 0	VO + 5	VO + 10
Alanine	2.68	2.68	2.70	2.72
Arginine	2.58	2.58	2.59	2.60
Aspartic acid	3.72	3.72	3.64	3.56
Glutamic acid	10.04	10.04	9.91	9.76
Glycine	2.20	2.20	2.27	2.33
Histidine	1.18	1.18	1.21	1.24
Isoleucine	2.03	2.03	2.02	2.01
Leucine	4.31	4.31	4.30	4.28
Lysine	2.47	2.47	2.48	2.49
Methionine	1.34	1.34	1.37	1.39
Phenylalanine	2.40	2.40	2.39	2.37
Proline	3.32	3.32	3.31	3.29
Serine	2.38	2.38	2.42	2.45
Threonine	1.81	1.81	1.82	1.83
Tyrosine	1.84	1.84	1.83	1.82
Tryptophan	0.52	0.52	0.51	0.49
Valine	2.37	2.37	2.37	2.36
Cystine	0.04	0.04	0.03	0.01

by amplifying serial dilutions of the mRNAs were used to create a standard curve, which was then used to calculate the mRNA copies in the biologic samples.

Statistical Analysis

Data were analyzed using GraphPad Prism8 software (GraphPad Software, Inc., La Jolla, CA, United States). Statistical analyses for multiple comparisons were tested by one-way ANOVA followed by Tukey's test. The differences were considered as statistically significant at p-value < 0.05.

RESULTS

S. limacinum Culture

Schizochytrium limacinum biomass was obtained by fermentation in 2 and 10 L bioreactors. This microorganism was fed with crude glycerol as carbon and energy source to obtain a suitable biomass for subsequent use as a fish feed ingredient. The ratio between the carbon and the nitrogen source was chosen in order to trigger lipid accumulation. After 144 h of fermentation, a DCW of 8.0 ± 1.1 g/L was obtained with a biomass yield of 0.1 ± 0.09 $g/g_{glycerol}$ (10% w/w). The amount of lipids from the biomass was 4.8 ± 0.51 g/L, with a yield of 0.6 ± 0.41 g/L $g/g_{biomass}$ (60% w/w). **Table 5** shows the lipidogram of *S. limacinum* obtained from the fermentation. DHA constituted 22.82% of the total lipids in the biomass, being the second most represented after palmitic acid (56.35%). Therefore, the DHA titer was 1.1 ± 0.28 g/L with a yield of 0.13 ± 0.25 g/L $g/g_{biomass}$ (13% w/w).

Fish Growth Performance and Feed Utilization

At the end of the trial, the total mortality of European sea bass was 2.14% and it was not correlated to a specific diet. Regarding the growth, after 21 weeks of feeding, fish of all dietary groups (**Table 6**) tripled their initial weight (from

TABLE 3 | Total fatty acid content and fatty acids profile of the four different experimental diets.

Fatty acid (% of the total fat fraction)	Experimental diets			
	FO	**VO + 0**	**VO + 5**	**VO + 10**
Total Fatty Acids (g/kg feed)	164.80	169.60	156.90	151.70
Lauric acid (C 12:0)	0.07	0.03	0.05	0.04
Myristic acid (C 14:0)	3.66	1.01	1.51	1.24
Myristoleic acid (C 14:1)	0.03	0.01	n.d.	n.d.
Pentadecanoic acid (C 15:0)	0.3	0.11	0.41	0.61
Pentadecanoic acid branched (C 16:0)	0.13	0.03	0.05	0.03
Palmitic acid (C 16:0)	15.16	13.00	14.76	15.61
Palmitoleic acid (C 16:1)	3.96	1.22	1.91	1.66
Heptadecanoic acid (C 17:0)	0.27	0.15	0.36	0.49
Heptadecenoic acid (C 17:1)	0.14	0.08	0.09	0.02
Heptadecanoic acid branched (C 18:0)	0.15	0.04	0.06	n.d.
Stearic acid (C 18:0)	3.53	4.03	3.61	3.47
Oleic acid (n-9) (C 18:1)	21.68	20.74	19.5	16.53
Linoleic acid (n-6) (C 18:2)	15.81	37.11	24.35	26.52
Alfa-linolenic acid (n-3) (C 18:3)	2.91	4.57	3.45	3.05
Octadecatetraenoic acid (n-3) (C 18:4)	0.91	0.24	0.38	0.26
Arachidic acid (C 20:0)	0.34	0.35	0.32	0.34
Eicosenoic acid (n-9) (C 20:1)	2.51	1.14	1.46	1.22
Eicosadienoic acid (n-6) (C 20:2)	0.34	0.08	0.14	n.d.
Eicosatetraenoic acid (n-3) (C 20:4)	0.40	n.d.	n.d.	0.15
Eicosatetraenoic acid (n-6) (C 20:4)	0.39	n.d.	n.d.	0.16
Eicosapentenoic acid (n-3) EPA (C 20:5)	5.38	1.46	1.87	1.54
Behenic acid (C 22:0)	0.20	0.33	0.28	0.30
Erucic acid (n-9) (C 22:1)	0.34	0.19	0.27	0.22
Docosapentaenoic acid (n-3) DPA (C 22:5)	0.75	0.31	0.31	0.23
Docosahexaenoic acid (n-3) DHA (C 22:6)	5.69	2.1	3.18	4.47
Not identified	5.35	5.00	2.62	4.97
Total fatty acids	**90.90**	**93.33**	**80.94**	**83.13**
Saturated fatty acids (SFAs)	23.85	19.08	21.41	22.13
Monounsaturated fatty acids (MUFAs)	28.66	23.38	23.23	19.65
Polyunsaturated fatty acids (PUFAs)	33.04	45.87	33.68	36.38
Omega 3 fatty acids	16.22	8.68	9.19	9.70
Omega 6 fatty acids	16.82	37.19	24.49	26.68

n.d. = not detected.

TABLE 4 | Primers and probe sequences used for the real time PCR quantification of Δ6-desaturase (or *fads2*) gene transcripts.

Gene	GeneBank Accession number	Primer	Nucleotide sequence (5′-3′)
Δ6-desaturase	EU647692	Forward	ATGATTTCCCGCCGTGACT
		Reverse	ACAGGTAGCGAAGGTAGTAAGACAT
		Probe	ACCAAGCCAGATCCAC

22.38 ± 0.30 g to 78.11 ± 1.39 g final mean body weight). Moreover, nutrient intake was not affected by dietary treatment and fish appeared healthy throughout the feeding trial. Sea bass fed with diet VO + 5 achieved the highest performance not only for weight (82.02 ± 5.37 g) and length (16.71 ± 0.1 cm), but also for the growth indexes, such as SGR (0.93 ± 0.02) and FCR (1.50 ± 0.12) (please see the dedicated session in section "Materials and Methods" for the calculations). Nevertheless, no significant differences between dietary groups were recorded for any of the performance indexes considered (**Table 6**).

Assimilation of Fatty Acids in Fish Filets

The fatty acid compositions of fish filets from the four dietary groups are shown in **Table 7** and **Figure 1**. Levels of different fatty acids were similar between dietary groups, ranging from 17.18 ± 0.02 to 18.62 ± 0.06. There were no

TABLE 5 | Fatty acids profile of *S. limacinum* biomass.

Fatty acid	Fraction (%)
Lauric acid (C 12:0)	3.33
Myristic acid (C 14:0)	6.78
Myristoleic acid (C 14:1)	n.d.
Pentadecanoic acid (C 15:0)	4.3
Pentadecanoic acid branched (C 16:0)	n.d.
Palmitic acid (C 16:0)	56.35
Palmitoleic acid (C 16:1)	n.d.
Heptadecanoic acid (C 17:0)	1.11
Heptadecenoic acid (C 17:1)	0.13
Heptadecanoic acid branched (C 18:0)	n.d.
Stearic acid (C 18:0)	1.24
Oleic acid (n-9) (C 18:1)	2.54
Linoleic acid (n-6) (C 18:2)	0.59
Alfa-linolenic acid (n-3) (C 18:3)	n.d.
Octadecatetraenoic acid (n-3) (C 18:4)	n.d.
Arachidic acid (C 20:0)	0.13
Eicosenoic acid (n-9) (C 20:1)	n.d.
Eicosadienoic acid (n-6) (C 20:2)	n.d.
Eicosatetraenoic acid (n-3) (C 20:4)	n.d.
Eicosatetraenoic acid (n-6) (C 20:4)	n.d.
Eicosapentenoic acid (n-3) EPA (C 20:5)	0.38
Behenic acid (C 22:0)	n.d.
Erucic acid (n-9) (C 22:1)	n.d.
Docosapentaenoic acid (n-3) (C 22:5)	n.d.
Docosahexaenoic acid (n-3) DHA (C 22:6)	22.82
Saturated fatty acids (SFAs)	73.2
Monounsaturated fatty acids (MUFAs)	2.67
Polyunsaturated fatty acids (PUFAs)	23.8

Levels of fatty acids are expressed as g/100g of the total fat fraction; n.d. = not detected.

significant differences in the total MUFAs and SFAs between fish dietary groups, whereas PUFAs were significantly higher in fish fed diets VO + 0, VO + 5, and VO + 10 than in fish fed the FO diet. In particular, fish fed with diet VO + 0 showed the highest level of n-6 fatty acids (20.81 ± 0.98), mostly constituted by linoleic acid (C 18:2n-6) (93%), but the lowest level of n-3 fatty acids (12.01 ± 0.52). Sea bass fed with diet VO + 10 showed significantly higher levels of n-3 fatty acids than fish fed diets VO + 0 and VO + 5 and similar levels in comparison to fish fed diet FO (**Table 7** and **Figure 1**). If compared to dietary group FO, group VO + 10 showed significantly higher levels of n-6 fatty acids, but lower than group VO + 0 and similar to group VO + 5 (**Figure 1**).

With regard to EPA, filets of fish fed with diet FO had the highest level (5.43 ± 0.07), whereas fish fed with diets VO + 0, VO + 5, and VO + 10 had less than half of that level and groups VO + 5 and VO + 10 showed similar levels.

Concerning DHA content, although the percentage of DHA was higher in feed FO (**Table 3**), DHA levels in fish filets increased from 9.53 ± 0.4 in group VO + 5 to 12.34 ± 0.36 in VO + 10, reflecting the increase in *S. limacinum* inclusion levels from 5 to 10% (**Table 7** and **Figure 1**). However, only DHA levels in group VO + 10 were significantly higher than those recorded in group FO.

Nutritional Quality of Filets

The ratios and the nutritional quality indexes of fish filets are shown in **Table 8** and **Figure 2**. Although the PUFAs/SFAs ratio showed a significantly higher value in fish fed with VO + 0, VO + 5, and VO + 10 diets than in those fed with FO-based diet, suggesting a better filet quality, the n-6/n-3 ratio clearly indicated that the filet quality of group FO was the best (value of 0.55 ± 0.01) and that of group VO + 10 the second best, followed in a decreasing fashion by filets of groups VO + 5 and VO + 0.

The AI was significantly higher in fish fed with diet FO (0.46 ± 0.001) than in fish fed diets VO + 0, VO + 5, and VO + 10, with no differences between the latter three groups.

No significant differences in TI were shown between groups FO and VO + 10, which presented the lowest values (0.31 ± 0.002 and 0.30 ± 0.01, respectively), whereas there were significant differences between groups FO and VO + 0, VO + 0 and VO + 10, and VO + 5 and VO + 10.

The highest FLQ levels were recorded in groups FO and VO + 10 (14.06 ± 0.17 and 14.38 ± 0.42, respectively), followed in a decreasing fashion by groups VO + 5 and VO + 0. In the latter two, a significantly higher value was recorded in fish fed diet VO + 5 containing 5% of *Schizochytrium* biomass than in fish fed diet VO + 0, with only VO.

Gene Expression

As shown in **Figure 3**, the highest number of mRNA copies for Δ6-desaturase (Δ6D or *fads2*) were determined for fish fed with diet VO + 0, the lowest copy number for fish fed with diet VO + 10. There were no significant differences between expression levels found in fish fed with diet FO and diets VO + 5 and VO + 10 containing VO and 5 and 10% of *Schizochytrium* biomass, respectively.

DISCUSSION

Unlike a linear economic model, which is based on a take-make-consume-dispose pattern, circular economy represents a model of production and consumption that implies reusing and recycling existing raw materials and products, thereby extending their life cycle as long as possible and reducing waste to a minimum (Yuan et al., 2006; Geissdoerfer et al., 2017; Sariatli, 2017). In a circular economy, products are not designed to be used once and then discarded, but are repeatedly reused, and those that cannot be reused are recycled by chemical or mechanical processes or through biological agents that convert wastes into value-added products, thereby providing alternative routes of exploitation in an environmentally friendly way.

The present research was framed in a circular economy, using crude glycerol derived from industrial biodiesel production as a low-cost carbon source for heterotrophic fermentation of the *S. limacinum* SR21 strain, which is a high-value biological product rich in DHA and high-quality proteins. For each ton

TABLE 6 | Growth performance of European sea bass fed the experimental diets for 21 weeks.

	IBW[1]	FBW[2]	CF[3]	FCR[4]	SGR[5]	FE[6]	Survival
FO	23.08 ± 0.28	76.76 ± 2.12	1.82 ± 0.01	1.67 ± 0.05	0.84 ± 0.01	0.59 ± 0.02	97.14%
VO + 0	22.18 ± 0.38	75.63 ± 3.27	1.82 ± 0.02	1.69 ± 0.10	0.85 ± 0.01	0.59 ± 0.03	100%
VO + 5	21.65 ± 0.65	82.02 ± 5.37	1.75 ± 0.08	1.50 ± 0.12	0.93 ± 0.02	0.66 ± 0.05	95.71%
VO + 10	22.58 ± 0.15	78.04 ± 6.61	1.76 ± 0.06	1.64 ± 0.20	0.86 ± 0.05	0.61 ± 0.07	95.57%

Data are means ľ SEM. Statistical differences were determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
[1]Initial body weight (g).
[2]Final body weight (g).
[3]Condition factor = 100 × (body weight/standard length[3]).
[4]Feed conversion ratio = (feed intake × dry matter)/(100 × wet weight gain).
[5]Specific growth rate = 100 × [ln (final body weight)–ln (initial body weight)]/days.
[6]Feed efficiency = wet weight gain/(feed intake × dry matter).

of biodiesel produced, 100 kg of crude glycerol is generated as by-product (Pyle et al., 2008). Crude glycerol has a low economic value and discarding it can have negative consequences for the environment; therefore, biodiesel producers must seek new uses for this waste stream. A method of valorizing crude glycerol is to use it as a substrate for cultivating oleaginous microorganisms, such as marine protist *Schizochytrium* spp., which accumulate significant amounts of triacylglycerols with a high proportion of PUFAs. Producing DHA on crude glycerol represents a great opportunity to utilize a large quantity of this by-product, converting it into a value-added product.

In our study, *S. limacinum* SR21 strain was able to grow well on crude glycerol and to accumulate fatty acids for almost 60% of its DCW. This result is aligned with findings from Sun et al. (2014) and Bouras et al. (2020), who reported that this strain could accumulate lipids for up to 50% of its cell dry matter, in a short time, with a DHA output of 34% of the total lipid concentration. Our analysis of fatty acid profiles showed that palmitic acid and DHA were the major fatty acids produced by the strain. Similar results were obtained by Ethier et al. (2011), who described that *S. limacinum* had a relatively simple fatty acid profile, with palmitic acid and DHA being the major fatty acids and myristic, stearic, and docosapentaenoic acid (C 22:5) being the minor fatty acids. Data derived from GC are also in line with previous analyses of the fatty acid composition of *S. limacinum* biomass (Pyle et al., 2008; Abad and Turon, 2015), confirming that this strain is a promising high-lipid, DHA−rich ingredient for fish feed formulations.

The *S. limacinum* whole biomass paired with a terrestrial plant-derived oil source was then used to replace FO in European sea bass (*D. labrax*) feed. Four nutritionally balanced diets were formulated: diet FO (a FO-based diet), diet VO + 0 (a VO-based diet), and diets VO + 5 and VO + 10 that were VO-based feeds supplemented with 5 and 10% of *S. limacinum*, respectively. From an economic standpoint, *S. limacinum* whole cell biomass is preferable to extracted oil because extraction constitutes a costly processing step. Furthermore, lipids are less susceptible to oxidation in a whole-cell form and this can positively affect a product's quality and shelf life (Hart et al., 2021).

All the feeds in our experiment were well accepted and there were no indications of feed rejection when sea bass were fed with diets without FO. After a 3-month feeding trial, fish of all dietary groups tripled their initial weight, but growth and feeding efficiencies of *D. labrax* were not significantly different among treatments. As is known, VOs contain some types of n-3 fatty acids, but they lack the n-3 PUFAs (EPA and DHA), which are found almost exclusively in FO and marine microalgae. Over the recent decades, VOs have replaced about half of the FO in fish feed formulations (Tacon and Metian, 2008). Although these levels of substitution have had no major effect on fish growth performance, they have detrimental effects to filet quality, reducing the proportions of health-beneficial fatty acids EPA and DHA in fish white muscle. Therefore, the challenge for aquaculture industry is to maintain healthful levels of EPA and DHA in cultured fish products as the percentage of FO in aquafeed formulations decreases (Glencross et al., 2020a). One of the ways this can be achieved is to add high DHA ingredients, such as products from thraustochytrids, to the VO-based diets.

So far, only few studies have reported the effects of whole *S. limacinum* cell biomass in fish diet on fish growth performance and most of them were focused on Atlantic salmon (*Salmo salar*) (Carter et al., 2003; Kousoulaki et al., 2015; Katerina et al., 2020; Lee Chang et al., 2020; Hart et al., 2021). In agreement with our data, results from those studies commonly indicated normal fish growth, demonstrating that *S. limacinum* is a source of highly digestible (98%) PUFAs and proteins for farmed fish. For instance, in the study of Lee Chang et al. (2020), no significant differences in specific growth rate, survival, feed consumption, and FCR were recorded after replacing FO (tuna oil) with 5% thraustochytrid biomass in salmon fingerlings diet. Using higher inclusion percentages, i.e., 10–13% of whole-cell thraustochytrids in canola oil-based diets for salmon, (Carter et al., 2003) reported similar data with no significant differences in weight gain, final weight, feed consumption, and feed efficiency ratio between the dietary groups. Nor did (Sprague et al., 2015) find any adverse effects on the growth of Atlantic salmon post-smolt fed for 133 days with diets in which 11 and 5.5% of FO was replaced with thraustochytrid biomass. Sea bass in our study behaved much like salmon in response to *S. limacinum* feeding, showing that feed formulations with this microbial biomass met the nutritional needs and growth potential of this farmed fish species, with no need for oil extraction or cell disruption.

TABLE 7 | Fatty acids profile of filets in fish fed the four experimental diets.

Fatty acid	Experimental diets			
	FO	VO + 0	VO + 5	VO + 10
Total Fatty Acids (g/kg feed)	17.18 ± 0.02	17.95 ± 0.1	17.55 ± 0.04	18.62 ± 0.06
Lauric acid (C 12:0)	0.04 ± 0.004^{a}	0.02 ± 0.003^{b}	0.03 ± 0.005^{ab}	0.02 ± 0.001^{b}
Myristic acid (C 14:0)	1.77 ± 0.04^{a}	0.91 ± 0.03^{b}	0.98 ± 0.06^{b}	0.98 ± 0.03^{b}
Myristoleic acid (C 14:1)	0.03 ± 0.002^{a}	0.02 ± 0.004^{b}	0.02 ± 0.001^{b}	0.02 ± 0.001^{b}
Pentadecanoic acid (C 15:0)	0.2 ± 0.002^{a}	0.12 ± 0.005^{b}	0.23 ± 0.01^{c}	0.3 ± 0.006^{d}
Pentadecanoic acid branched (C 16:0)	0.04 ± .003^{a}	0.02 ± 0.003^{b}	0.02 ± 0.001^{b}	0.02 ± 0.001^{b}
Palmitic acid (C 16:0)	15.3 ± 0.23	15.31 ± 0.29	15.61 ± 0.43	15.55 ± 0.33
Palmitoleic acid (C 16:1)	2.83 ± 0.04^{a}	1.57 ± 0.07^{b}	1.8 ± 0.12^{b}	2.19 ± 0.09^{c}
Hexadecatetraenoic acid (n-1) (C 16:4)	0.15 ± 0.008^{a}	0.17 ± 0.02^{a}	0.13 ± 0.003^{ab}	0.11 ± 0.003^{b}
Heptadecanoic acid (C 17:0)	0.2 ± 0.003^{a}	0.15 ± 0.003^{b}	0.24 ± 0.01^{c}	0.29 ± 0.004^{d}
Heptadecenoic acid (C 17:1)	0.13 ± 0.002^{a}	0.09 ± 0.003^{b}	0.11 ± 0.01^{a}	0.12 ± 0.001^{a}
Heptadecanoic acid branched (C 18:0)	0.09 ± 0.02^{a}	0.05 ± 0.004^{ab}	0.05 ± 0.005^{ab}	0.05 ± 0.009^{b}
Stearic acid (C 18:0)	4.19 ± 0.07^{a}	5.13 ± 0.12^{b}	4.69 ± 0.09^{c}	4.41 ± 0.1^{a}
Oleic acid (n-9) (C 18:1)	18.67 ± 0.31	19.19 ± 0.71	18.7 ± 0.75	17.96 ± 0.62
Linoleic acid (n-6) (C 18:2)	7.72 ± 0.12^{a}	19.37 ± 0.89^{b}	16.11 ± 0.5^{c}	14.9 ± 0.34^{c}
Gamma-linoleic acid (n-6) (C 18:3)	0.22 ± 0.003^{a}	0.4 ± 0.03^{b}	0.3 ± 0.01^{c}	0.28 ± 0.004^{c}
Alfa-linolenic acid (n-3) (C 18:3)	1.21 ± 0.02^{a}	2.08 ± 0.1^{b}	1.66 ± 0.06^{c}	1.52 ± 0.03^{c}
Octadecatetraenoic acid (n-3) (C 18:4)	0.46 ± 0.008^{a}	0.18 ± 0.006^{b}	0.19 ± 0.01^{b}	0.19 ± 0.007^{b}
Arachidic acid (C 20:0)	0.15 ± 0.004	n.d.	0.15 ± 0.004	0.14 ± 0.007
Eicosenoic acid (n-9) (C 20:1)	1.55 ± 0.04^{a}	1.01 ± 0.06^{b}	1.02 ± 0.05^{b}	1.03 ± 0.02^{b}
Eicosadienoic acid (n-6) (C 20:2)	0.53 ± 0.006^{a}	0.73 ± 0.02^{b}	0.61 ± 0.02^{c}	0.55 ± 0.01^{ac}
Eicosatrienoic acid (n-3) (C 20:3)	0.09 ± 0.003	n.d.	n.d.	n.d.
Eicosatetraenoic acid (n-3) (C 20:4)	n.d.	0.09 ± 0.004	n.d.	0.13 ± 0.004
Eicosatetraenoic acid (n-6) (C 20:4)	0.65 ± 0.01^{a}	0.38 ± 0.02^{b}	0.38 ± 0.02^{b}	0.42 ± 0.02^{b}
Eicosapentenoic acid (n-3) EPA (C 20:5)	5.43 ± 0.07^{a}	2.47 ± 0.06^{b}	2.09 ± 0.07^{c}	2.04 ± 0.06^{c}
Behenic acid (C 22:0)	n.d.	n.d.	n.d.	0.09 ± 0.004
Erucic acid (n-9) (C 22:1)	0.19 ± 0.005^{a}	0.14 ± 0.01^{b}	n.d.	0.14 ± 0.003^{b}
Docosapentaenoic acid (n-3) DPA (C 22:5)	0.77 ± 0.01^{a}	0.4 ± 0.02^{b}	0.32 ± 0.02^{c}	0.3 ± 0.01^{c}
Docosahexaenoic acid (n-3) DHA (C 22:6)	8.62 ± 0.11^{a}	6.66 ± 0.49^{b}	9.53 ± 0.4^{a}	12.34 ± 0.36^{c}
Not identified	1.29 ± 0.17^{a}	1.92 ± 0.14^{b}	2.77 ± 0.12^{c}	3.08 ± 0.15^{c}
Identified fatty acids	72.16 ± 0.74^{a}	78.42 ± 1.9^{b}	77.65 ± 1.94^{ab}	78.93 ± 1.37^{b}
Saturated fatty acids	21.92 ± 0.29	21.72 ± 0.39	21.92 ± 0.54	21.77 ± 0.48
Monounsaturated fatty acids	23.40 ± 0.36	21.97 ± 0.85	21.69 ± 0.91	21.39 ± 0.72
Polyunsaturated fatty acids	25.81 ± 0.14^{a}	32.81 ± 1.3^{b}	31.28 ± 0.68^{b}	32.69 ± 0.36^{b}
Omega 3 fatty acids	16.55 ± 0.15^{a}	12.01 ± 0.52^{b}	13.88 ± 0.5^{c}	16.51 ± 0.44^{a}
Omega 6 fatty acids	9.08 ± 0.14^{a}	20.81 ± 0.98^{b}	17.4 ± 0.5^{c}	16.17 ± 0.33^{c}

Levels of total fatty acids are expressed as g/kg of flesh, whereas the single fatty acids are expressed as g/100g of the total fat fraction.
Data are means ± SEM (n = 6).
Different superscript letters indicate significant differences between experimental diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$); n.d. = not detected.

Docosahexaenoic acid-rich biomass supplied in the diet ensured optimal fish growth, and also provided a vector for the accumulation of n-3 LC-PUFA in order to pass on to human consumers.

Fatty acid composition of the flesh generally mirrored the composition of the experimental diets, which is in accordance with results obtained in other fish species (Sprague et al., 2015; Katerina et al., 2020). Nevertheless, although the four formulated diets were balanced for PUFAs, fish fed with diets containing either VO (VO + 0) or VO plus 5 and 10% of *S. limacinum* biomass (diets VO + 5 and VO + 10) had significantly higher levels of PUFAs in the flesh than fish fed the FO diet. This could suggest that fatty acids are better accumulated and retained in fish muscle. Similar results were obtained using alternative lipids sources in different fish species, such as in salmon (Sargent et al., 1997), European sea bass (Monteiro et al., 2018), tilapia (Sarker et al., 2016), and greater amberjack (Monge-Ortiz et al., 2018).

To assess the nutritional value of sea bass filets, we used data on the fatty acid composition to calculate health-related lipid indexes, such as AI, TI, and FLQ, as well as n-6/n-3, and PUFAs/SFAs ratios. These indexes have become some of the most important markers for evaluating the potential effects of lipid

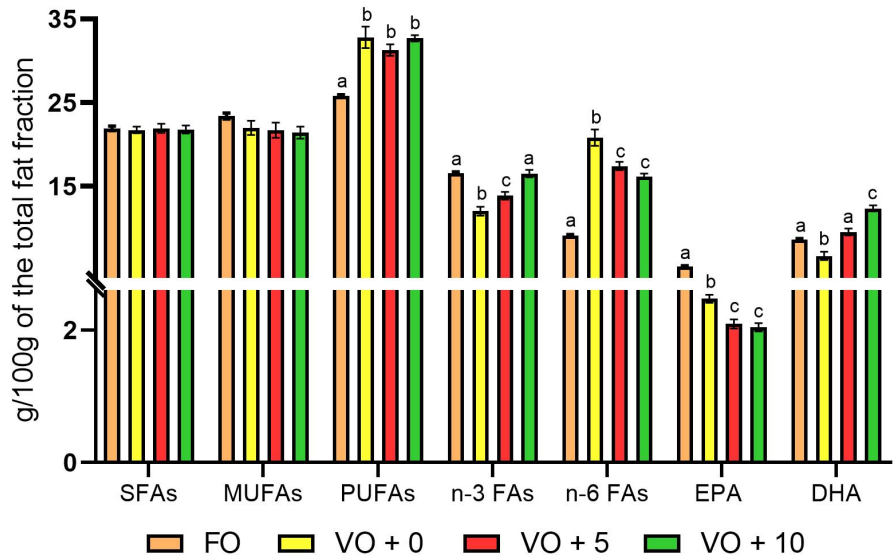

FIGURE 1 | Profile of the main FAs in filets of fish fed with the four different experimental diets. The values are expressed as g/100g of the total fat fraction and are reported as mean ± SEM (n = 6). Different letters indicate significant differences between experimental diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).

TABLE 8 | Fatty acids ratios and nutritional indices of filets from fish fed with the four experimental diets.

Ratios	Experimental diets			
	FO	VO + 0	VO + 5	VO + 10
PUFAs/SFAs	1.18 ± 0.01^{a}	1.51 ± 0.06^{b}	1.43 ± 0.03^{b}	1.50 ± 0.03^{b}
n-6 FAs/n-3 FAs	0.55 ± 0.01^{a}	1.74 ± 0.08^{b}	1.26 ± 0.05^{c}	0.98 ± 0.04^{d}
Indices				
AI	0.46 ± 0.001^{a}	0.35 ± 0.01^{b}	0.37 ± 0.005^{b}	0.36 ± 0.004^{b}
IT	0.31 ± 0.002^{ac}	0.37 ± 0.01^{b}	0.34 ± 0.01^{bc}	0.30 ± 0.01^{a}
FLQ	14.06 ± 0.17^{a}	9.13 ± 0.54^{b}	11.62 ± 0.46^{c}	14.38 ± 0.42^{a}

Data are expressed as mean ± SEM.
Different superscript letters indicate significant differences between experimental diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).

consumption on human health and are used as a tool to compare the lipid fractions of different foods in terms of health outcome.

Atherogenicity index and TI consider the different effects that individual fatty acids might have on human health and, in particular, on the probability of increasing the incidence of cardiovascular diseases. It is widely recognized that dietary fatty acids can play an important role in increasing or preventing the risk of atherosclerosis and coronary thrombosis because of their effect on blood cholesterol and low-density lipoprotein concentrations (Siscovick et al., 2017).

Atherogenicity index indicates the relationship between the sum of the main SFAs and the sum of the main unsaturated fatty acids (UFAs). The main classes of SFAs, *i.e.,* lauric (C12:0), myristic (C14:0), and palmitic (C16:0), but not stearic (C18:0), are considered proatherogenic, as they favor the retention of lipids in the walls of blood vessels and the subsequent plaque development (Chen and Liu, 2020). Moreover, myristic acid is considered four times more atherogenic than lauric and palmitic acids. UFAs (in particular, n-3 PUFAs, such as ALA, DHA, and EPA) are considered anti-atherogenic as they inhibit the accumulation of plaque and reduce the production of proinflammatory and adhesion proteins by the arterial wall, thereby providing primary protection against cardiovascular diseases (Siscovick et al., 2017; Abdelhamid et al., 2020). The IA values for sea bass white dorsal muscle were in the range of 0.35–0.46. In agreement with published reports reviewed by Chen and Liu (2020), these are to be considered as low levels, thus representing a healthy product with potential cardioprotective effects on human health.

The TI, defined as the ratio of the prothrombogenic (saturated) to anti-thrombogenic (unsaturated) fatty acids, indicates the tendency to form clots in the blood vessels. In particular, there is a very high correlation between the sum of three saturated acids, myristic, palmitic, and stearic acid, and thrombus formation (Ulbricht and Southgate, 1991). The TI value for fish ranges from 0.14 to 0.87 (Chen and Liu, 2020) and for *D. labrax* from 0.19 to 0.63 (Monteiro et al., 2018). In our study, the TI values were between 0.30 and 0.37, without showing any significant difference between the values for filets of sea bass

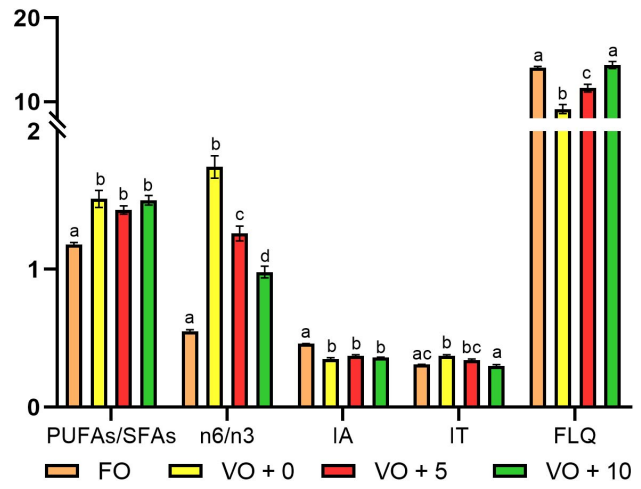

FIGURE 2 | Fatty acids ratios and nutritional indices of filets from fish fed with the four experimental diets. Data are expressed as mean ± SEM. Different letters indicate significant differences between experimental diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).

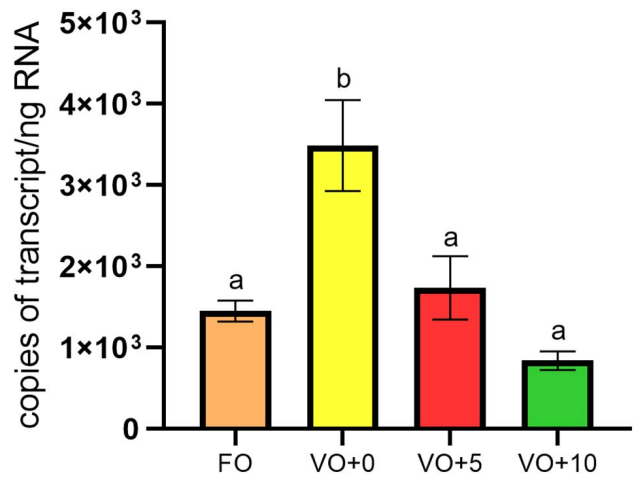

FIGURE 3 | Expression levels of Δ6-desaturase gene in fish liver. Data are means ± SEM ($n = 6$). Different letters indicate significant differences between experimental diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).

fed with diets FO and VO + 10, which were lowest. While there were significant differences in the TI of filet from sea bass fed with the other diets, these values did not exceed 0.37. According to the scientific literature, potential beneficial health impact of filets with AI and TI values higher than 1.0 are minor (Monteiro et al., 2018); therefore, the AI and TI values in our study confirmed the high nutritional value of sea bass filet.

Flesh lipid quality calculates the sum of DHA and EPA as a percentage of total fatty acids. This index is suitable for marine products (fish or shellfish), which are rich in n-3 PUFA and, so far, it has only been used to assess the quality of lipid fractions in fish. The higher the value of this index, the better the quality of the dietary lipid source (Abrami et al., 1992). According to Chen and Liu (2020), in fish, the FLQ value ranges from 13.01 to 36.37. The FLQ value found in our study suggested a high nutritional value of *D. labrax* filet. The highest levels were recorded in fish fed with diets FO and VO + 10 (14.06 and 14.38, respectively), followed by fish fed with diets VO + 5 and VO + 0. In the latter two, a higher value was found in fish fed with diet VO + 5, containing VO and 5% of *Schizochytrium* biomass, than in fish fed with diet VO + 0, containing only VO.

The PUFAs/SFAs ratio is normally used to assess the impact of diet on cardiometabolic health. It hypothesizes that dietary PUFAs can reduce low-density lipoprotein (sometimes called "bad" cholesterol), and serum cholesterol levels, whereas all SFAs increase serum cholesterol. Thus, the higher this ratio, the more beneficial the effect. PUFAs/SFAs ratio of European sea bass filets was in the range of 1.18–1.51, which is much higher than the minimum suggested (0.45) for a human healthy diet (Elmadfa and Kornsteiner, 2009). This finding is also in agreement with the literature reviewed by Chen and Liu (2020) that reports fish PUFA/SFA ratios in the range of 0.5–1.79.

Although the PUFAs/SFAs ratio showed a significantly higher value in fish fed with VO + 0, VO + 5, and VO + 10 than in those fed with FO diet, suggesting a better filet quality, the n-6/n-3 ratio clearly indicated that the quality of filet for group FO was best (value of 0.55) and that of group VO + 10 fed with 10% of *S. limacinum* second best (value of 0.98). Evidence from intense scientific scrutiny suggests that beyond individual n-6 and n-3 intake, the n-6/n-3 PUFA ratio also affects human health. Although an optimum intake ratio has yet to be determined, it was recommended that the ratio should be about 4:1 to 1:1 (Kris-Etherton et al., 2000). In general, a close balance of the n-6:n-3 ratio in our diets may have protective cardiometabolic effects and reduce the risk of developing age-related cognitive decline and dementia (Simopoulos, 2002), whereas a ratio higher than 4:1 can lead to inflammatory disorders, which play a significant role in initiating atherosclerosis (Simopoulos, 2002). Thus, there is a potential to enrich the human diet with n-3 PUFAs by modifying fish feed composition to satisfy nutritional requirements in humans because both type and ratio of dietary oils affect the accumulation of fatty acids in fish filet.

The presence of VOs and *S. limacinum* in fish diets also prompted our interest in evaluating the nutritional regulation of Δ6-desaturase (or *fads2*) gene expression in European sea bass liver. FADS2 acts as the first enzyme in the biosynthesis of long-chain (≥C20) PUFAs (LC-PUFAs), such as EPA (C 20:5n-3), DHA (C 22:6n-3), and arachidonic acid (C 20:4n-6), starting from the shorter-chain PUFAs α-linolenic acid (ALA, C 18:3n-3) and linoleic acid (LA, C 18:2n-6) (Tocher, 2003; Izquierdo et al., 2008; Montero et al., 2012). In particular, Δ6-desaturase catalyzes the initial, rate−limiting desaturation of LA to γ−linolenic (C 18:3) and ALA to stearidonic acid (C 18:4) (Tocher, 2003). LC-PUFAs are considered as essential in fish and other vertebrates, as their endogenous production, if present, is insufficient to meet demand. However, unlike freshwater fish, which have maintained a certain degree of Δ6-desaturase and elongase activity and produce EPA and DHA if their precursors LA and ALA are

present in the diet, marine fish do not produce LC-PUFA at a significant level as a consequence of living in LC-PUFA-rich marine environments (Izquierdo et al., 2008; Tocher et al., 2019). Vital to this process is providing LC-PUFA precursors LA and ALA, which cannot be synthesized *de novo* in hardly any living animal as their genomes do not encode for enzymes capable of synthesizing them (Bláhová et al., 2020). Hence, fish are usually dependent on the primary producers, such as phytoplankton, for providing the two major C_{18} PUFA precursors LA and ALA.

We noted that European sea bass fed the VO + 0 diet had the highest number of mRNA copies for Δ6-desaturase (or *fads2*), whereas fish fed with diet VO + 10 the lowest. A decreasing trend of expression was evident in fish that did not receive FO (diets VO + 0, VO + 5, and VO + 10). To assess these data, we first considered the fact that the content of DHA (C 22:6n-3), DPA (C 22:5n-3), and EPA (C 20:5n-3) in the flesh were higher than in the feeds, suggesting that these particular fatty acids accumulate and are retained in the muscle. Therefore, we assume that the DHA increase in filets of fish fed with *S. limacinum* (**Table 7**) could be related to the high content of this long-chain PUFA in *S. limacinum*, whereas the higher level of DHA found in the filet of fish fed with diet VO + 0 in comparison to the DHA level in the feed VO + 0 could be due to the over-expression of the Δ6-desaturase gene.

In vivo upregulation of *fads2* has been previously reported in marine fish (including sea bass) fed with VO diets in comparison to fish fed FO diets (Izquierdo et al., 2008; Geay et al., 2010; Betancor et al., 2016; Houston et al., 2017; Carvalho et al., 2018). Although *fads2* gene overexpression was shown to be correlated with an increase in FADS2 enzymatic activity in anadromous fish such as salmon fed with VO diets (Zheng et al., 2004, 2005), this is less straightforward and the results are controversial in marine fish. Previous studies, indeed, have shown that the partial or total substitution of FO with VO did not increase hepatic desaturase activity in sea bass (Geay et al., 2010), whereas a Δ6-desaturase increased activity in response to a deficiency in essential fatty acids has been suggested in gilthead sea bream (*Sparus aurata*) (Houston et al., 2017; Carvalho et al., 2021) as well as in meager (*Argyrosomus regiu*) (Carvalho et al., 2018). In these species, high retention of C 18:2n-6 and C 18:3n-6 and of chain-elongation products of LA and ALA, namely, C 20:2n-6 and C 20:3n-3, suggested that these PUFAs are endogenously synthesized through Δ6-desaturases and Elovl5 (fatty acid elongase 5), respectively.

Taken together our data suggest the value of microbial oils as a source of high value lipids, for use in aquaculture. Oil from thraustochytrid strains grown heterotrophically should be considered a sustainable alternative to partially replace dietary FO in marine fish diets contributing to a circular economy concept, as thraustochytrids can be reared on industrial by-products. Although further technological scale up of microbial oil production is needed, the experimental scenarios tested here provide different perspectives for the feed industry in the context of sustainable aquaculture development, as the environmental impacts connected with aquaculture and the increase in fish demand by a continuously growing global population require changes in fish feed formulations.

CONCLUSION

The present research was framed in a circular economy context, using crude glycerol derived from industrial biodiesel production as a low-cost carbon source for the heterotrophic fermentation of *S. limacinum* SR21 strain. This oleaginous microorganism was able to grow well on crude glycerol and to accumulate fatty acids for almost 60% of its DCW, with DHA being one of the major fatty acids produced by the strain. The *S. limacinum* whole biomass paired with a terrestrial plant-derived oil source was then successfully used to replace FO in European sea bass (*D. labrax*) feed. Based on fish filet fatty acid profiles and nutritional value indexes, *S. limacinum* whole-cell biomass appears to provide benefits for filet quality and n-3 LC-PUFA retention efficiency without any indications of negative impact on fish growth. Therefore, *S. limacinum* biomass can be used in feed for European sea bass as a good alternative source of marine-derived omega-3 fatty acids. However, considering the relatively high cost of *Schizochytrium* spp. biomass, future studies need to be carried out for the application of our protocol at larger scale, focusing into optimal ratios with VO to improve feed cost-effectiveness.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Italian Ministry of Health [REF: 483/2017-PR (response of Prot. Nr. 344C6.6 of 13/03/2017)] in accordance to the Art.31 of D.lgs. 26/2014.

AUTHOR CONTRIBUTIONS

GT: conceptualization, experimental investigation, data curation, and writing. FM and MA: experimental investigation, methodology, data curation, and editing. SB and CP: experimental investigation, methodology, formal analysis, and editing. PB and ML: conceptualization and writing—review and editing. DP: review and editing. CC: experimental investigation and data curation. SR: experimental investigation, data curation, and writing—review and editing. All authors read and approved the final manuscript.

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REFERENCES

Abad, S., and Turon, X. (2015). Biotechnological production of docosahexaenoic acid using aurantiochytrium limacinum: Carbon sources comparison and growth characterization. *Mar. Drugs* 13, 7275–7284. doi: 10.3390/md13127064

Abdelhamid, A. S., Brown, T. J., Brainard, J. S., Biswas, P., Thorpe, G. C., Moore, H. J., et al. (2020). Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. *Cochr. Database Syst. Rev.* 2020:3177. doi: 10.1002/14651858.CD003177.pub5

Abrami, G., Natiello, F., Bronzi, P., McKenzie, D., Bolis, L., and Agradi, E. (1992). A comparison of highly unsaturated fatty acid levels in wild and farmed eels (Anguilla Anguilla). *Compar. Biochem. Physiol. Biochem.* 101, 79–81. doi: 10.1016/0305-0491(92)90161-J

Allen, K. M., Habte-Tsion, H. M., Thompson, K. R., Filer, K., Tidwell, J. H., and Kumar, V. (2019). Freshwater microalgae (Schizochytrium sp.) as a substitute to fish oil for shrimp feed. *Sci. Rep.* 9, 1–10. doi: 10.1038/s41598-019-41020-8

Athenaki, M., Gardeli, C., Diamantopoulou, P., Tchakouteu, S. S., Sarris, D., Philippoussis, A., et al. (2018). Lipids from yeasts and fungi: physiology, production and analytical considerations. *J. Appl. Microbiol.* 124, 336–367. doi: 10.1111/jam.13633

Ayisi, C., Zhao, J., and Apraku, A. (2019). Consequences of Replacing Fish Oil with Vegetable Oils in Fish. *J. Anim. Res. Nutr.* 4, 1–11. doi: 10.21767/2572-5459.100053

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnol. Adv.* 25, 207–210. doi: 10.1016/j.biotechadv.2006.11.002

Beopoulos, A., and Nicaud, J. M. (2012). Yeast: A new oil producer? *OCL - Oleagineux Corps Gras Lipides* 19, 22–28. doi: 10.1684/ocl.2012.0426

Betancor, M. B., Sprague, M., Sayanova, O., Usher, S., Metochis, C., Campbell, P. J., et al. (2016). Nutritional Evaluation of an EPA-DHA Oil from Transgenic Camelina sativa in Feeds for Post-Smolt Atlantic Salmon (Salmo salar L.). *PLoS One* 11:e0159934. doi: 10.1371/journal.pone.0159934

Bláhová, Z., Harvey, T. N., Pšenièka, M., and Mráz, J. (2020). Assessment of fatty acid desaturase (Fads2) structure-function properties in fish in the context of environmental adaptations and as a target for genetic engineering. *Biomolecules* 10:20206. doi: 10.3390/biom10020206

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/y59-099

Bouras, S., Katsoulas, N., Antoniadis, D., and Karapanagiotidis, I. T. (2020). Use of biofuel industry wastes as alternative nutrient sources for DHA-yielding Schizochytrium limacinum production. *Appl. Sci.* 10, 1–16. doi: 10.3390/app10124398

Calder, P. C., and Yaqoob, P. (2009). Omega-3 polyunsaturated fatty acids and human health outcomes. *BioFactors* 35, 266–272. doi: 10.1002/biof.42

Carter, C. G., Bransden, M. P., Lewis, T. E., and Nichols, P. D. (2003). Potential of Thraustochytrids to Partially Replace Fish Oil in Atlantic Salmon Feeds. *Mar. Biotechnol.* 5, 480–492. doi: 10.1007/s10126-002-0096-8

Carvalho, M., Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., and Izquierdo, M. (2020). Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (Sparus aurata) fingerlings. *Aquaculture* 529:735696. doi: 10.1016/j.aquaculture.2020.735696

Carvalho, M., Montero, D., Torrecillas, S., Castro, P., Zamorano, M. J., and Izquierdo, M. (2021). Hepatic biochemical, morphological and molecular effects of feeding microalgae and poultry oils to gilthead sea bream (Sparus aurata). *Aquaculture* 532:736073. doi: 10.1016/j.aquaculture.2020.736073

Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., et al. (2018). Dietary requirement for n-3 long-chain polyunsaturated fatty acids for fast growth of meagre (Argyrosomus regius, Asso 1801). fingerlings. *Aquaculture* 488, 105–113. doi: 10.1016/j.aquaculture.2018.01.028

Chang, G., Luo, Z., Gu, S., Wu, Q., Chang, M., and Wang, X. (2013). Fatty acid shifts and metabolic activity changes of Schizochytrium sp. S31 cultured on glycerol. *Bioresour. Technol.* 142, 255–260. doi: 10.1016/J.BIORTECH.2013.05.030

Checa, M., Nogales-Delgado, S., Montes, V., and Encinar, J. M. (2020). Recent advances in glycerol catalytic valorization: A review. *Catalysts* 10, 1–41. doi: 10.3390/catal10111279

Chen, J., and Liu, H. (2020). Nutritional indices for assessing fatty acids: A mini-review. *Internat. J. Mole. Sci.* 21, 1–24. doi: 10.3390/ijms21165695

Chi, Z., Pyle, D., Wen, Z., Frear, C., and Chen, S. (2007). A laboratory study of producing docosahexsaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Proc. Biochem.* 42, 1537–1545. doi: 10.1016/j.procbio.2007.08.008

Elmadfa, I., and Kornsteiner, M. (2009). Fats and fatty acid requirements for adults. *Ann. Nutr. Metab* 55, 56–75. doi: 10.1159/000228996

Erdman, J. W., MacDonald, I. A., and Zeisel, S. H. (2012). *Present Knowledge in Nutrition: Tenth Edition*. Hoboken, NJ: Wiley-Blackwell, doi: 10.1002/9781119946045

Ethier, S., Woisard, K., Vaughan, D., and Wen, Z. (2011). Continuous culture of the microalgae Schizochytrium limacinum on biodiesel-derived crude glycerol for producing docosahexaenoic acid. *Bioresour. Technol.* 102, 88–93. doi: 10.1016/j.biortech.2010.05.021

FAO (2018). *World Fisheries and Aquaculture*. Rome: FAO.

Geay, F., Santigosa, I., Culi, E., Corporeau, C., Boudry, P., Dreano, Y., et al. (2010). Regulation of FADS2 expression and activity in European sea bass (Dicentrarchus labrax, L.) fed a vegetable diet. *Compar. Biochem. Physiol. B Biochem. Mole. Biol.* 156, 237–243. doi: 10.1016/j.cbpb.2010.03.008

Geissdoerfer, M., Savaget, P., Bocken, N. M. P., and Hultink, E. J. (2017). The Circular Economy – A new sustainability paradigm? *J. Clean. Product.* 143, 757–768. doi: 10.1016/j.jclepro.2016.12.048

Glencross, B. D. (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev. Aquacult.* 1, 71–124. doi: 10.1111/j.1753-5131.2009.01006.x

Glencross, B. D., Baily, J., Berntssen, M. H. G., Hardy, R., MacKenzie, S., and Tocher, D. R. (2020a). Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. *Rev. Aqua.* 12, 703–758. doi: 10.1111/RAQ.12347

Glencross, B. D., Huyben, D., and Schrama, J. W. (2020b). The application of single-cell ingredients in aquaculture feeds—a review. *Fishes* 5, 1–39. doi: 10.3390/fishes5030022

Harel, Z., Gascon, G., Riggs, S., Vaz, R., Brown, W., and Exil, G. (2002). Supplementation with omega-3 polyunsaturated fatty acids in the management of recurrent migraines in adolescents. *J. Adolesc. Health* 31, 154–161. doi: 10.1016/S1054-139X(02)00349-X

Hart, B., Schurr, R., Narendranath, N., Kuehnle, A., and Colombo, S. M. (2021). Digestibility of Schizochytrium sp. whole cell biomass by Atlantic salmon (Salmo salar). *Aquaculture* 533:736156. doi: 10.1016/j.aquaculture.2020.736156

Houston, S. J. S., Karalazos, V., Tinsley, J., Betancor, M. B., Martin, S. A. M., Tocher, D. R., et al. (2017). The compositional and metabolic responses of gilthead seabream (Sparus aurata) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. *Br. J. Nutr.* 118, 1010–1022. doi: 10.1017/S0007114517002975

Innes, J. K., and Calder, P. C. (2020). Marine omega-3 (N-3) fatty acids for cardiovascular health: An update for 2020. *Internat. J. Mole. Sci.* 21, 1–21. doi: 10.3390/ijms21041362

Izquierdo, M. S., Robaina, L., Juárez-Carrillo, E., Oliva, V., Hernández-Cruz, C. M., and Afonso, J. M. (2008). Regulation of growth, fatty acid composition and delta

6 desaturase expression by dietary lipids in gilthead seabream larvae (Sparus aurata). *Fish Physiol. Biochem.* 34, 117–127. doi: 10.1007/s10695-007-9152-7

Johnson, D. T., and Taconi, K. A. (2007). The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environ. Prog.* 26, 338–348. doi: 10.1002/ep.10225

Katerina, K., Berge, G. M., Turid, M., Aleksei, K., Grete, B., Trine, Y., et al. (2020). Microalgal Schizochytrium limacinum Biomass Improves Growth and Filet Quality When Used Long-Term as a Replacement for Fish Oil, in Modern Salmon Diets. *Front. Mar. Sci.* 7:57. doi: 10.3389/fmars.2020.00057

Katiyar, R., Gurjar, B. R., Bharti, R. K., Kumar, A., Biswas, S., and Pruthi, V. (2017). Heterotrophic cultivation of microalgae in photobioreactor using low cost crude glycerol for enhanced biodiesel production. *Renew. Ener.* 113, 1359–1365. doi: 10.1016/j.renene.2017.06.100

Khot, M., Kamat, S., Zinjarde, S., Pant, A., Chopade, B., and Ravikumar, A. (2012). Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microb. Cell Fact.* 11, 1–13. doi: 10.1186/1475-2859-11-71

Kousoulaki, K., Østbye, T. K. K., Krasnov, A., Torgersen, J. S., Mørkøre, T., and Sweetman, J. (2015). Metabolism, health and fillet nutritional quality in Atlantic salmon (Salmo salar) fed diets containing n-3-rich microalgae. *J. Nutrit. Sci.* 4:14. doi: 10.1017/jns.2015.14

Kris-Etherton, P. M., Taylor, D. S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., et al. (2000). Polyunsaturated fatty acids in the food chain in the United States. In: *American Journal of Clinical Nutrition*. *Am. Soc. Nutr.* 2000, 179S–188S. doi: 10.1093/ajcn/71.1.179s

Layé, S., Nadjar, A., Joffre, C., and Bazinet, R. P. (2018). Anti-inflammatory effects of omega-3 fatty acids in the brain: Physiological mechanisms and relevance to pharmacology. *Pharm. Rev.* 70, 12–38. doi: 10.1124/pr.117.014092

Lee Chang, K. J., Parrish, C. C., Simon, C. J., Revill, A. T., and Nichols, P. D. (2020). Feeding Whole Thraustochytrid Biomass to Cultured Atlantic Salmon (Salmo salar) Fingerlings: Culture Performance and Fatty Acid Incorporation. *J. Mar. Sci. Eng.* 8:207. doi: 10.3390/jmse8030207

Lung, Y. T., Tan, C. H., Show, P. L., Ling, T. C., Lan, J. C. W., Lam, H. L., et al. (2016). Docosahexaenoic acid production from crude glycerol by Schizochytrium limacinum SR21. *Clean Technol. Env. Policy* 18, 2209–2216. doi: 10.1007/s10098-016-1126-y

Lupatsch, I. (2005). Protein and energy requirements in Mediterranean species. *Cahiers Option. Méditerrané.* 63, 9–18.

Martins, D. A., Rocha, F., Martínez-Rodríguez, G., Bell, G., Morais, S., Castanheira, F., et al. (2012). Teleost fish larvae adapt to dietary arachidonic acid supply through modulation of the expression of lipid metabolism and stress response genes. *Br. J. Nutr.* 108, 864–874. doi: 10.1017/S0007114511006143

Masuda, R., and Tsukamoto, K. (1999). School formation and concurrent developmental changes in carangid fish with reference to dietary conditions. *Environ. Biol. Fishes* 56, 243–252. doi: 10.1023/A:1007565508398

Mata, T. M., Santos, J., Mendes, A. M., Caetano, N. S., and Martins, A. A. (2014). Sustainability Evaluation of Biodiesel Produced from Microalgae Chlamydomonas sp Grown in Brewery Wastewater. *Chem. Eng. Trans.* 37, 823–828. doi: 10.3303/CET1437138

Monge-Ortiz, R., Tomás-Vidal, A., Rodriguez-Barreto, D., Martínez-Llorens, S., Pérez, J. A., Jover-Cerdá, M., et al. (2018). Replacement of fish oil with vegetable oil blends in feeds for greater amberjack (Seriola dumerili) juveniles: Effect on growth performance, feed efficiency, tissue fatty acid composition and flesh nutritional value. *Aqua. Nutr.* 24, 605–615. doi: 10.1111/anu.12595

Monteiro, M., Matos, E., Ramos, R., Campos, I., and Valente, L. M. P. (2018). A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass. *Aquaculture* 487, 22–31. doi: 10.1016/j.aquaculture.2017.12.043

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., and Caballero, M. J. (2003). Vegetable lipid sources for gilthead seabream (Sparus aurata): effects on fish health. *Aquaculture* 225, 353–370. doi: 10.1016/S0044-8486(03)00301-6

Montero, D., Terova, G., Rimoldi, S., Tort, L., Negrin, D., Zamorano, M. J., et al. (2012). Modulation of adrenocorticotrophin hormone (ACTH)-induced expression of stress-related genes by PUFA in inter-renal cells from European sea bass (Dicentrarchus labrax). *J. Nutr. Sci.* 4, 1–9. doi: 10.1017/jns.2012.2

Moreira, L. M., Da Silva, A., Rocha, R., Lúcia, C., Ribeiro, G., Da, R., et al. (2011). Nutritional evaluation of single-cell protein produced by Spirulina platensis. *Afr. J. Food Sci.* 5, 799–805. doi: 10.5897/AJFS11.184

Nasseri, A. T., Rasoul-Ami, S., Morowvat, M. H., and Ghasemi, Y. (2011). Single Cell Protein: Production and Process. *Am. J. Food Technol.* 6, 103–116. doi: 10.3923/ajft.2011.103.116

O'Grady, J., and Morgan, J. A. (2011). Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol. *Bioproc. Biosyst. Eng.* 34, 121–125. doi: 10.1007/s00449-010-0474-y

Özşahinoğlu, I., Eroldogan, T., Mumoğullarinda, P., Dikel, S., Engin, K., Yílmaz, H. A., et al. (2013). Partial Replacement of Fish Oil with Vegetable Oils in Diets for European Seabass (Dicentrarchus labrax): Effects On Growth Performance and Fatty Acids Profile. *Turkish J. Fish. Aquat. Sci.* 2013, 819–825. doi: 10.4194/1303-2712-v13_3_05

Patel, A., Karageorgou, D., Rova, E., Katapodis, P., Rova, U., Christakopoulos, P., et al. (2020). An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3 fatty acid-based industries. *Microorganisms* 8:8030434. doi: 10.3390/microorganisms8030434

Pyle, D. J., Garcia, R. A., and Wen, Z. (2008). Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. *J. Agr. Food Chem.* 56, 3933–3939. doi: 10.1021/jf800602s

Ratledge, C., and Cohen, Z. (2008). Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? *Lipid Technol.* 20, 155–160. doi: 10.1002/lite.200800044

Ross, A. C., Caballero, B., Cousins, R. J., Tucker, K. L., and Ziegler, T. R. (2012). *Modern nutrition in health and disease: Eleventh edition*. Netherlands: Wolters Kluwer Health Adis, doi: 10.1097/01.ccm.0000236502.51400.9f

Sales, J., and Glencross, B. (2011). A meta-analysis of the effects of dietary marine oil replacement with vegetable oils on growth, feed conversion and muscle fatty acid composition of fish species. *Aqua. Nutr.* 17:761. doi: 10.1111/j.1365-2095.2010.00761.x

Sargent, J. R., McEvoy, L. A., and Bell, J. G. (1997). Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* 155, 117–127. doi: 10.1016/S0044-8486(97)00122-1

Sariatli, F. (2017). Linear Economy Versus Circular Economy: A Comparative and Analyzer Study for Optimization of Economy for Sustainability. *Visegr. J. Bioecon. Sust. Dev.* 6, 31–34. doi: 10.1515/vjbsd-2017-0005

Sarker, P. K., Kapuscinski, A. R., Lanois, A. J., Livesey, Bernhard, K. P., and Coley, M. L. (2016). Towards sustainable aquafeeds: Complete substitution of fish oil with marine microalga Schizochytrium sp. improves growth and fatty acid deposition in juvenile Nile tilapia (Oreochromis niloticus). *PLoS One* 11, 1–17. doi: 10.1371/journal.pone.0156684

Signori, L., Ami, D., Posteri, R., Giuzzi, A., Mereghetti, P., Porro, D., et al. (2016). Assessing an effective feeding strategy to optimize crude glycerol utilization as sustainable carbon source for lipid accumulation in oleaginous yeasts. *Microb. Cell Fact.* 15:467. doi: 10.1186/s12934-016-0467-x

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharm.* 56, 365–379. doi: 10.1016/S0753-3322(02)00253-6

Siscovick, D. S., Barringer, T. A., Fretts, A. M., Wu, J. H. Y., Lichtenstein, A. H., Costello, R. B., et al. (2017). Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory from the American Heart Association. *Circulation* 135, e867–e884. doi: 10.1161/CIR.0000000000000482

Sprague, M., Betancor, M. B., and Tocher, D. R. (2017). Microbial and genetically engineered oils as replacements for fish oil in aquaculture feeds. *Biotechnol. Lett.* 39, 1599–1609. doi: 10.1007/s10529-017-2402-6

Sprague, M., Walton, J., Campbell, P. J., Strachan, F., Dick, J. R., and Bell, J. G. (2015). Replacement of fish oil with a DHA-rich algal meal derived from Schizochytrium sp. on the fatty acid and persistent organic pollutant levels in diets and flesh of Atlantic salmon (Salmo salar, L.) post-smolts. *Food Chem.* 413–421. doi: 10.1016/j.foodchem.2015.03.150

Sun, L., Ren, L., Zhuang, X., Ji, X., Yan, J., and Huang, H. (2014). Differential effects of nutrient limitations on biochemical constituents and docosahexaenoic acid production of Schizochytrium sp. *Bioresour. Technol.* 159, 199–206. doi: 10.1016/j.biortech.2014.02.106

Tacon, A. G. J., and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* 285, 146–158. doi: 10.1016/j.aquaculture.2008.08.015

Tibaldi, E., and Kaushik, S. J. (2005). *Amino acid requirements of Mediterranean fish species*. Zaragoza: CIHEAM.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* 11, 107–184. doi: 10.1080/713610925

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquacul. Res.* 41, 717–732. doi: 10.1111/j.1365-2109.2008.02150.x

Tocher, D. R., Betancor, M. B., Sprague, M., Olsen, R. E., and Napier, J. A. (2019). Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: Bridging the gap between supply and demand. *Nutrients* 11:10089. doi: 10.3390/nu11010089

Ulbricht, T. L., and Southgate, D. A. (1991). Coronary heart disease: seven dietary factors. *Lancet* 338, 985–992. doi: 10.1016/0140-6736(91)91846-m

Vanthoor-Koopmans, M., Wijffels, R. H., Barbosa, M. J., and Eppink, M. H. M. (2013). Biorefinery of microalgae for food and fuel. *Bioresour. Tech.* 135, 142–149. doi: 10.1016/j.biortech.2012.10.135

Yuan, Z., Bi, J., and Moriguichi, Y. (2006). The circular economy: A new development strategy in China. *J. Industr. Ecol.* 10, 4–8. doi: 10.1162/10881980677554532 1

Zheng, X., Tocher, D. R., Dickson, C. A., Bell, J. G., and Teale, A. J. (2004). Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (Salmo salar). *Aquaculture* 236, 467–483. doi: 10.1016/j.aquaculture.2004.02.003

Zheng, X., Torstensen, B. E., Tocher, D. R., Dick, J. R., Henderson, R. J., and Bell, J. G. (2005). Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (Salmo salar). *Biochim. Biophys. Acta Mole. Cell Biol. Lipids* 1734, 13–24. doi: 10.1016/j.bbalip.2005.01.006

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Cross-Talk Between Intestinal Microbiota and Host Gene Expression in Gilthead Sea Bream (*Sparus aurata*) Juveniles: Insights in Fish Feeds for Increased Circularity and Resource Utilization

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New types of fish feed based on processed animal proteins (PAPs), insect meal, yeast, and microbial biomasses have been used with success in gilthead sea bream. However, some drawback effects on feed conversion and inflammatory systemic markers were reported in different degrees with PAP- and non-PAP-based feed formulations. Here, we focused on the effects of control and two experimental diets on gut mucosal-adherent microbiota, and how it correlated with host transcriptomics at the local (intestine) and systemic (liver and head kidney) levels. The use of tissue-specific PCR-arrays of 93 genes in total rendered 13, 12, and 9 differentially expressed (DE) genes in the intestine, liver, and head kidney, respectively. Illumina sequencing of gut microbiota yielded a mean of 125,350 reads per sample, assigned to 1,281 operational taxonomic unit (OTUs). Bacterial richness and alpha diversity were lower in fish fed with the PAP diet, and discriminant analysis displayed 135 OTUs driving the separation between groups with 43 taxa correlating with 27 DE genes. The highest expression of intestinal *pcna* and *alpi* was achieved in PAP fish with intermediate values in non-PAP, being the pro-inflammatory action of *alpi* associated with the presence of *Psychrobacter piscatorii*. The intestinal *muc13* gene was down-regulated in non-PAP fish, with this gene being negatively correlated with anaerobic (Chloroflexi and *Anoxybacillus*) and metal-reducing (*Pelosinus* and *Psychrosinus*) bacteria. Other inflammatory markers (*igm*, *il8*, *tnfα*) were up-regulated in PAP fish, positively correlating the intestinal *igm* gene with the inflammasome activator *Escherichia/Shigella*, whereas the systemic expression of *il8* and *tnfα* was negatively correlated with the Bacilli class in PAP fish and positively correlated with *Paracoccus yeei* in non-PAP fish. Overall changes in the expression pattern of *il10*, galectins (*lgals1*, *lgals8*), and toll-like receptors (*tlr2*, *tlr5*, *tlr9*) reinforced the anti-inflammatory profile of fish fed with the non-PAP diet, with these gene markers being associated with a wide range of OTUs. A gut microbiota-liver axis was also established, linking the microbial generation

of short chain fatty acids with the fueling of *scd1*- and *elovl6*-mediated lipogenesis. In summary, by correlating the microbiome with host gene expression, we offer new insights in the evaluation of fish diets promoting gut and metabolism homeostasis, and ultimately, the health of farmed fish.

Keywords: fish meal, processed animal proteins, insect proteins, algae meal, gut microbiota, host transcriptomics, inflammatory markers, lipid metabolism

INTRODUCTION

Fish meal (FM) is the gold dietary protein in aquafeeds (Tacon and Metian, 2015; Ytrestøyl et al., 2015), but the global increase in aquaculture production needs to be supported by alternative feed ingredients, which very often, have a negative impact on growth, intestinal health, and immuno-competence of farmed marine fish (Conceição et al., 2012; Krogdahl et al., 2015; Estensoro et al., 2016; Aragão et al., 2020). Traditionally, plant proteins have been considered as the most obvious FM alternative, but high levels of replacement can induce different signs of enteritis, including the shortening of mucosal folds, thickening of the lamina propia and submucosa, and infiltration of the distal intestine by inflammatory cells (Urán et al., 2009; Romarheim et al., 2013, Booman et al., 2018). These drawback effects are differentially exacerbated in each farmed fish species, but the supplementation of plant-based diets with fish protein hydrolysates or short-chain fatty acids (SCFAs) helps to mitigate most gut health detrimental effects in salmonid and non-salmonid fish (Estensoro et al., 2016; Piazzon et al., 2017; Egerton et al., 2020). However, novel ingredients and formulations need to be investigated to produce improved, efficient, and sustainable fish aquafeeds.

Seaweed polysaccharides have the capacity to regulate the non-specific host immunity, and supplementation of practical diets, with *Gracilaria* sp. improving the resistance of European sea bass (*Dicentrarchus labrax*) to *Photobacterium damselae* (Peixoto et al., 2019). In the same line, antioxidant extracts of *Gracilaria* protected against oxidative stress, improving the stress resilience of gilthead sea bream during acute hypoxia (Magnoni et al., 2017). Moreover, the supplementation of FM-free diets with marine diatomeas (*Phaeodactylum tricornutum*) stimulated the immune system of gilthead sea bream, which might be relevant as a prophylactic measure before predictable stressful events (Reis et al., 2021). Additionally, algae or algae-based products are currently used to replace or reduce the inclusion level of fish oil (FO) in aquafeeds, assuring the dietary supply of n-3 polyunsaturated fatty acids to meet the nutritional requirements of fish, as well as the healthy value of seafood products for human consumption (Maldonado-Othón et al., 2020; Pereira et al., 2020).

Processed animal proteins (PAPs), such as poultry meal, blood meal, and feather meal, are also used as a replacement for FM in practical fish diets (Wu et al., 2018; Ferrer Llagostera et al., 2019; Solé-Jiménez et al., 2021). Likewise, insect meal products, such as black soldier fly meal and defatted *Tenebrio* meal, did not compromise gut health and fish performance (Welker et al., 2007; Sánchez-Muros et al., 2016; Magalhães et al., 2017; Basto et al., 2021) and in some cases, even improved performance. Microbial biomasses have also been proposed as an effective replacement for marine feedstuffs in fish and shrimp diets (Delamare-Debouteville et al., 2019; Simon et al., 2019, 2020). Such new and emerging ingredients have, in fact, been on the agenda of formulators for some time, but they are still available in low amounts, and their costs are often too high in comparison with most conventional feed ingredients. Moreover, many of these ingredients have been tested on a one-by-one basis rather than with different formulation combinations, although by this way important advances were achieved. Thus, a recent study on gilthead sea bream pointed out that sustainable PAP- and non-PAP-based feed formulations are able to support high growth rates when theoretical nutrient requirements are met by the diet (Fernandes et al., 2021). However, the use of PAP-based feed formulations was related to a slight impairment in feed conversion ratio (FCR) in association with a progressive down-regulated expression of hepatic insulin-like growth factor-I, a well-known marker of growth performance in fish, gilthead sea bream in particular (Pérez-Sánchez et al., 2018). Additionally, fish fed with the highest amount of PAP feed ingredients showed a pro-inflammatory status, supported by the up-regulated expression in the head kidney of several cytokines (*il1β*, *tnfα*), chemokines (*ck8*), and T-cell markers (*cd3ζ*, *cd4-1*, *cd8α*).

In this study, we focused on gut health indicators using samples from the study of Fernandes et al. (2021) to address the main effects of PAP- and non-PAP feed formulations on the composition of mucosal adherent bacteria from the anterior intestine (AI) in association with changes in the host transcriptomic profile of the intestine liver, and head kidney. Over the course of the last 5 years, the number of studies on fish, gilthead sea bream in particular, addressing the nutritional regulation of gut microbiota has increased exponentially (e.g., Piazzon et al., 2017, 2020; Rimoldi et al., 2019, 2020; Firmino et al., 2021; Moroni et al., 2021; Pelusio et al., 2021; Solé-Jiménez et al., 2021). Although our study did not include probiotics, previous studies on fish supported the gut-to-brain communication, and interestingly, the dietary administration of *Lactobacillus rhamnosus* interfered in shoaling behavior and brain expression levels of genes involved in serotonin signaling and metabolism (Borrelli et al., 2016). Recently, significant differences in the intestinal expression pattern of key genes involved in innate and acquired immunity were reported when gilthead sea bream juveniles fed with probiotic (*Lactococcus lactis*) and control diets (Moroni et al., 2021) were compared. However, to our knowledge, this is the first report to link fish alternative feed formulations with changes in gut microbiota and host transcriptomics at the local and/or systemic level in typical farmed marine fish.

MATERIALS AND METHODS

Ethics

The feeding trial was conducted by trained scientists (following the Federation of European Laboratory Animal Science Associations, FELASA, category C recommendations), according to the animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at the experimental facilities of RIASEARCH/SPAROS (Murtosa, Portugal).

Diets

Three diets were formulated and manufactured by SPAROS Lda (Olhão, Portugal) according to the known nutritional requirements for gilthead sea bream (NRC 2011) (**Table 1**). The control diet (CRTL) contained 20% FM and high levels of traditional vegetable proteins following current industry formulations. Both the PAP- and non-PAP-based feed formulations contained insect meal, fish by-products, and microbial and yeast biomasses as replacements for FM and vegetable protein sources. The PAP-based diet also comprised poultry meal, feather meal hydrolysate, and porcine blood meal as main protein sources. Alternatively, the non-PAP-based diet included *Spirulina* and *Chlorella* meals as novel protein sources in fish aquafeeds. FO was replaced in both the PAP and NoPAP diets by an aquaculture by-product (salmon oil), DHA-rich algae biomass (*Schizochytrium*), and rapeseed oil. Additionally, both diets were adequately supplemented with rapeseed lecithin, phosphorous, L-tryptophan, DL-methionine, and L-taurine.

TABLE 1 | Ingredients and chemical composition of the experimental diets.

Ingredient (%)	CTRL	NoPAP	PAP
Fishmeal LT70	20		
Fishmeal 60 (by-products)		5	
Fish hydrolysate (by-products)		5	5
Insect meal		5	5
Microbial protein meal		5	5
Yeast protein meal		2.5	2.5
Feather meal hydrolysate			5
Porcine blood meal			3
Poultry meal 65	5		20
Microalgae meal (*Spirulina*)		5	
Microalgae meal (*Chlorella*)		0.5	
Soy protein concentrate	9		
Pea protein concentrate		4.1	
Wheat gluten	4	4	
Corn gluten meal	10	15	4.5
Soybean meal 48	12		
Rapeseed meal	4	11.5	5.7
Wheat meal	8.47		
Pea starch	3	7.9	6
Yellow peas	6.2	3	14.58
Fish oil	6		
Salmon oil		3	3
DHA-rich algae (*Schizochytrium*)		3.2	3.6
Rapeseed oil	8.26	8.5	6
Rapeseed lecithin	0.6	1	1
Vitamin and mineral premix	1	1	1
Vitamin C (35%)	0.1	0.1	0.1
Brewer's yeast		4	4
Macroalgae MIX		2	2
Antioxidant	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1
Monocalcium phosphate	2	2.5	1.9
L-Tryptophan	0.05	0.18	0.15
DL-Methionine		0.2	0.15
L-Taurine		0.5	0.5
Yttrium oxide	0.02	0.02	0.02
Chemical Composition			
Crude Protein (%)	44.2	44.88	44.71
Crude Lipid (%)	17.84	17.62	16.29
EPA +DHA (%)	1.9	1.5	1.5

Experimental Setup and Sampling

A total of 880 juveniles of gilthead sea bream (initial body weight 55–56 g on average) from a commercial hatchery (Sonrionansa, Santander, Spain) were reared at RIASEARCH/SPAROS trial facilities (Murtosa, Portugal) with the CTRL and experimental diets as reported in Fernandes et al. (2021). In short, the trial was run for 77 days in replicated 500-L tanks, in a flow-through system with brackish water (18 ppt) and constant photoperiod (12 h ligh:12 h dark). During that time, the fish were hand-fed *ad libitum* to satiation three times a day, water temperature was $22.03 \pm 1.4^{\circ}$C, and water oxygen concentration remained higher than 5.5 mg/L.

At the end of the trial, 48-h fasted fish were sampled for both gut bacterial microbiota and gene expression analyses (nine fish per feed formulation from three randomly selected tanks, three fish per tank). All the fish were euthanised by overdose of anesthetic 3-aminobezoic acid ethyl ester (MS-222, 0.1 g/L), according to the good practices of fish health and welfare. The skin surface of the abdomen was cleaned with ethanol 70%, and a cut from the anus to the esophagus was made to remove the intestine, which was sampled and placed on a sterile Petri dish. Tissue portions (∼0.4 cm) of the anterior intestine (AI; immediately after the pyloric caeca) were put in RNA later for subsequent gene expression analysis. The remaining part of the AI was then opened and washed with phosphate-buffered saline (PBS) to remove non-adherent materials and bacteria. The tissue was transferred to a clean Petri dish, and the internal mucus was scraped out with the blunt end of a sterile scalpel. The sampled mucus was then placed into sterile cryotubes and stored at -80°C until bacterial DNA extraction for microbiota analysis using the High Pure PCR Template Preparation Kit (Sigma-Aldrich, St. Louis, MO, United States) and including a lysozyme lysis step, as previously described (Piazzon et al., 2019). In this study, the AI was used because of its importance in nutrient absorption and metabolism. The procedure targeted mucosa-colonizing autochthonous bacteria, which have a more direct impact on fish physiology. Allochthonous bacteria (not resident) cannot colonize these habitats under normal conditions and have

a more transient impact on the host (Hao and Lee, 2004). The 48-h fasting period was chosen to ensure sample stability and avoid contamination by allochthonous bacteria, which are more difficult to eliminate when the intestines are filled with fecal matter. We are aware that fasting can affect intestinal microbiota (Xia et al., 2014; Nebo et al., 2017; Mekuchi et al., 2018), but these effects have been described for longer fasting periods or allochthonous populations, whereas autochthonous populations are more stable. In any case, we followed the standard procedure used in other gilthead sea bream gene expression and microbiota studies (Estensoro et al., 2016; Piazzon et al., 2019, 2020; Solé-Jiménez et al., 2021), allowing for comparisons to be made.

Illumina MiSeq Sequencing and Bioinformatics Analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341-805 nt) was sequenced using the Illumina MiSeq System (Illumina, San Diego, CA, United States) (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM). The details on the PCR and sequencing of amplicons are described elsewhere (Piazzon et al., 2019). Regarding bioinformatics analysis, raw forward and reverse reads were quality-filtered using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and pre-processed using Prinseq (Schmieder and Edwards, 2011). Terminal N bases were trimmed in both ends, and sequences with >5% of total N bases were discarded. Reads that were <150-bp long, with Phred quality score <28 in both of the sequence ends, and with a Phred average quality score <26 were excluded. Forward and reverse reads were merged using VSEARCH (Rognes et al., 2016). Raw sequence data from six samples did not display enough quality standards to be included in the analysis and were removed from further analysis. The remaining 21 samples (7 from the PAP, 9 from non-PAP, and 5 from CTRL groups) were uploaded to the Sequence Read Archive (SRA) under Bioproject accession number PRJNA745265 (BioSample accession numbers: SAMN20157689-709).

Bacteria taxonomy assignment was performed using the Ribosomal Database Project (RDP) release 11 as a reference database (Cole et al., 2014). The reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥90% sequence identity, ≥90% query coverage). Taxonomic assignment results were filtered, and data were summarized in an operational taxonomic units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth following the recommendations described by McKnight et al. (2019).

Inferred Metagenome and Pathway Analysis

Piphillin was used to normalize the amplicon data with 16S rRNA gene copy number and to infer metagenomic contents (Iwai et al., 2016). This analysis was performed using only the OTUs that significantly drove the separation by diet in the supervised partial least-squares discriminant analysis (PLS-DA) (see Statistics section). For the analysis, a sequence identity cut-off of 97% was implemented, and the inferred metagenomic functions were assigned using the Kyoto Encyclopedia of Genes and Genomes (KEGG, Oct 2018 Release) database. The raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters after fractional counts were floored to the nearest integer (Love et al., 2014; Bledsoe et al., 2016; Piazzon et al., 2020).

Gene Expression

Total RNA from AI (9 fish/diet) was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, United States). The RNA yield per sample was higher than 3.5 μg with absorbance measures (A260/280) of 1.9–2.2. Complementary DNA (cDNA) was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States), using random decamers and 500 ng of total RNA in a final volume of 100 μl. Reverse transcription (RT) reactions were incubated for 10 min at 25°C and for 2 h at 37°C. Negative control reactions were run without the enzyme. As reported elsewhere (Estensoro et al., 2016), a customized PCR array layout was designed for the simultaneous profiling of a panel of 43 selected genes, including markers of epithelial integrity (11), nutrient transport (3), mucins (3), cytokines (9), immunoglobulins (2), cell markers, chemokines, and chemokine receptors (7), and pattern recognition receptors (PRRs) (8) (**Table 2**). Quantitative PCR (qPCR) reactions were performed using the iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, United States). Diluted RT reactions (×6) were used for qPCR assays in a 25-μl volume, in combination with SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States) and specific primers at a final concentration of 0.9 μM (**Supplementary Table 1**). The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C, and annealing/extension for 60 s at 60°C. All the pipetting operations were executed by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analyzing the melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C) and linearity of serial dilutions of RT reactions ($r^2 > 0.98$). Fluorescence data acquired during the extension phase were normalized with the delta-delta CT method (Livak and Schmittgen, 2001), using beta-actin as a housekeeping gene because of its stability under different experimental conditions (average CT between experimental groups varied less than 0.2).

Statistical Analysis

Biometric and gene expression data were analyzed by one-way ANOVA using the R stats package and SigmaPlot v.14.5 (Systat Software Inc., San Jose, CA, United States). The data have been checked previously for normal distribution (Shapiro–Wilk test) and homogeneity of variances (F test). Following ANOVA, if

TABLE 2 | Polymerase chain reaction (PCR)-array layout for intestine gene expression profiling in sea bream.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krueppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
	Intestinal mucin	*i-muc*	JQ277712
Cytokines	Tumor necrosis factor-alpha	*tnfα*	AJ413189
	Interleukin-1 beta	*il1β*	AJ419178
	Interleukin-6	*il6*	EU244588
	Interleukin-7	*il7*	JX976618
	Interleukin-8	*il8*	JX976619
	Interleukin-10	*il10*	JX976621
	Interleukin-12 subunit beta	*il12*	JX976624
	Interleukin-15	*il15*	JX976625
	Interleukin-34	*il34*	JX976629
Cell markers, chemokines and chemokine receptors	Cluster of differentiation 4-1	*cd4-1*	AM489485
	Cluster of differentiation 8 beta	*cd8β*	KX231275
	C-C chemokine receptor type 3	*ccr3*	KF857317
	C-C chemokine receptor type 9	*ccr9*	KF857318
	C-C chemokine receptor type 11	*ccr11*	KF857319
	C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ cl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Immunoglobulins	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T	*igt*	KX599201
Pattern recognition receptors (PRRs)	Galectin-1	*lgals1*	KF862003
	Galectin-8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	C-type lectin domain family 10 member A	*clec10a*	KF857329
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

appropriate, multiple comparisons between fish groups were computed by Dunn's post-test. The statistical significance of growth parameters was tested at $P < 0.05$, whereas the threshold for differentially expressed (DE) genes was established at $P < 0.1$. Fish specimens sampled and used for gene expression analyses (nine fish/diet) were the same as those used in Fernandes et al. (2021) and in 16S sequencing (nine fish/diet before quality filter). Rarefaction curves (observed taxonomic assignations vs. number of sequences), species richness estimates, and alpha diversity indexes were obtained using the R package phyloseq (McMurdie and Holmes, 2013). To determine the coverage for microbial communities, the ratio between observed and expected OTUs (determined by the Chao1 index) was calculated. Differences in species richness, diversity indexes, and phylum abundance were determined by Kruskal–Wallis test followed by Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity across groups was tested by permutational multivariate ANOVA (PERMANOVA), using the non-parametric method *adonis* from the Vegan R package with 10,000 random permutations.

To study the separation among the groups, supervised PLS-DA and hierarchical clustering of samples were sequentially performed using EZinfo v3.0 (Umetrics, Umeå, Sweden) and R package ggplot2, respectively. Values of normalized counts of OTUs present in five or more of the samples were included in the analyses. The contribution of the different genes to the group separation was determined by the minimum variable importance in the projection (VIP) values achieving the complete clustering of the conditions with a VIP value ≥ 1, considered to be an adequate threshold to determine discriminant variables in the PLS-DA model (Wold et al., 2001; Li et al., 2012; Kieffer et al., 2016). Hotelling's T2 statistic was calculated with the multivariate software package Ezinfo v3.0 to detect outliers in the model and reported. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting of 500 random permutations was performed using the Bioconductor R package ropls (Thévenot et al., 2015). In order to determine the OTUs most likely to explain differences between and among the feed formulations, a linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) was used with the online tool Galaxy v1.2 (Afgan et al., 2018). OTUs with VIP ≥ 1 were included in this analysis, and statistically significant differences were retrieved by the factorial Kruskal–Wallis test, followed by the pairwise Wilcoxon post-test with a significance threshold of $\alpha = 0.05$.

The inferred metagenomics pathways were considered differentially represented using an FDR-corrected significance threshold of 0.05. For the correlation analysis, only 16S sequencing samples that passed the quality filter and remained after the T2 Hotelling outlier test (20 samples in total; 9 from non-PAP, 7 from PAP, 4 from CTRL) were included. The outliers were excluded from further analysis. Spearman correlation was calculated between the normalized values of discriminant OTUs abundances in the 20 samples and normalized values of DE gene expression in its corresponding sampled specimen (Weiss et al., 2016). The corresponding *P*-values were calculated using the *cor.test* function of the corrplot R package, with a two-sided alternative hypothesis. Significant gene-OTU correlations were considered at $P < 0.01$, and visualized with the corrplots R

package and Cytoscape v3.8.2 (Smoot et al., 2011). For the same fish, the correlation analysis was also performed for the DE genes of the liver (12) and head-kidney (9), retrieved from the study of Fernandes et al. (2021).

RESULTS

Growth Performance

Data on body weight, feed intake, and FCR from the whole population are taken from Fernandes et al. (2021), and reported in **Supplementary Table 2**. These parameters did not change significantly ($P < 0.05$) between CTRL and fish fed with the NoPAP diet. The equality was less evident for fish fed with the PAP diet, and slight changes in weight gain and feed intake resulted in a statistically significant increase in FCR. Concerning the individuals sampled for microbiota and gene expression analyses, the recorded body weight did not deviate from the expected values, with the trend being lowest in the body weight of fish fed with the PAP-based diet.

Alpha Diversity and Microbiota Composition

A total of 2,632,361 high-quality reads from the 21 sequenced samples were assigned to 1,281 OTUs at a 97% identity threshold, ranging from 70,301 to 164,133 reads per sample (in average 125,350 reads per sample) (**Supplementary Table 3**). Rarefaction curves approximated saturation, showing an optimal number of sequences obtained for the analysis (**Supplementary Figure 1**). Results of the coverage ratio in terms of richness also confirmed the adequateness of the samples, with an average value of 61.44%. Out of the 1,281 OTUs, 84.6% was classified up to the level of species, 93.2% to the level of genus, 97.4% to the level of family, 98.7% to the level of order, 99.4% to the level of class, and 99.9% to the level of phylum.

Figure 1 shows that the non-PAP diet induced a significant decrease in richness and alpha diversity in comparison with the CTRL group, when the ACE and Shannon estimators ($P < 0.05$), respectively, were considered. A total of 747, 621, and 539 OTUs were assigned to the CTRL, non-PAP, and PAP fish (**Figure 2A**). From them, 176 OTUs were present in all the dietary groups, representing more than 60% of the overall bacterial composition in all the groups, whereas 385 (16.2% of the total microbiota), 262 (11.8%), and 184 (7.3%) were present exclusively in the CTRL, non-PAP, and PAP fish, respectively. No significant differences among the groups (Kruskal–Wallis test, followed by Dunn's post-test, $P < 0.05$) were detected when taxonomic assignations were collapsed to the phylum level (**Figure 2B**). Proteobacteria was the most abundant phylum, reaching values from 35 to 50% of the total bacterial composition, followed by Firmicutes (19–29%), Actinobacteria (11–18%), and Bacteroidetes (2.5–3%). Verrucomicrobia was relatively abundant in the control diet (5.7%), but decreased in the non-PAP and PAP fish (<0.5%). The most abundant genera (>1% in at least one dietary group) are depicted in **Supplementary Figure 2**.

Beta Diversity and Discriminant Analyses

Regarding beta diversity, statistically significant differences among the dietary groups were found (PERMANOVA $P =$

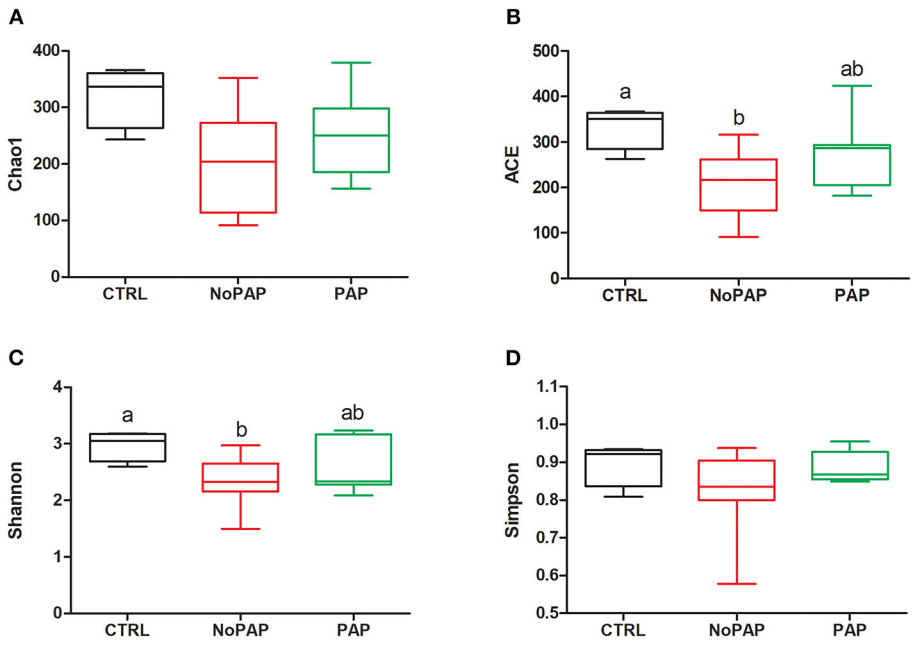

FIGURE 1 | Box plots representing the mean (min-max) of richness estimates [**(A)** Chao1 and **(B)** ACE] and diversity indexes [**(C)** Shannon and **(D)** Simpson] of the intestinal microbial populations found in fish fed with the CTRL ($n = 4$), non-PAP ($n = 9$), and PAP ($n = 7$) diets. Different letters indicate significant differences among the groups (Kruskal–Wallis with Dunn's post-test, $P < 0.05$).

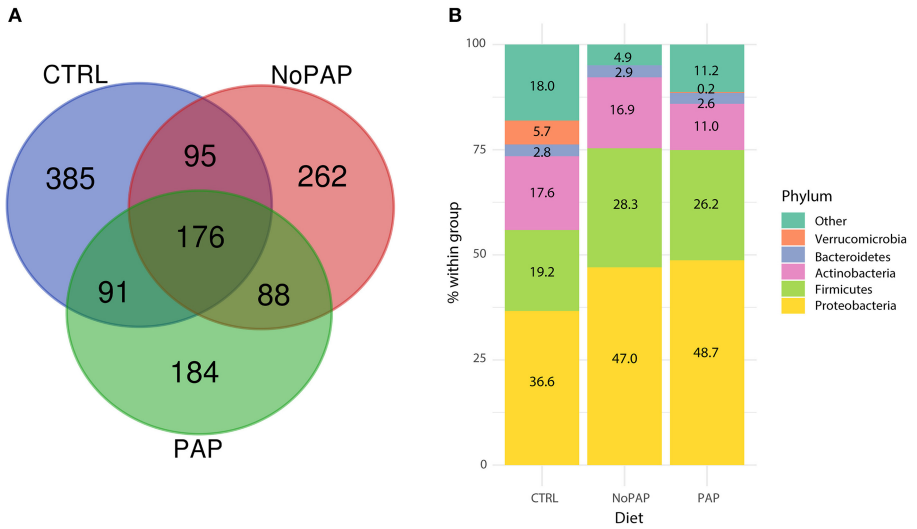

FIGURE 2 | (A) Venn diagram showing unique and shared operational taxonomic units (OTUs) in the intestines of fish fed with the three experimental diets. The 176 common OTUs represent 63.5, 69.4, and 60.5% of the overall microbiota in the CTRL, non-PAP, and PAP groups, respectively. Unique OTUs for the CTRL, non-PAP, and PAP groups represent 16.25, 11.8, and 7.3% of the overall bacterial composition, respectively. **(B)** Stacked bar chart representing the relative abundance of bacterial phyla in the three dietary groups. Only the phyla that are present in at least 1% in one of the groups are represented. No significant differences were found among the groups (Kruskal–Wallis + Holm–Sidak tests, $P > 0.05$).

0.049, $F = 1.0514$, $R^2 = 0.1101$). To further evaluate differences in the bacterial composition among the groups, a partial least squares discriminant analysis (PLS-DA) was performed. The discriminant model was based on four components, which explained 98% [R2Y(cum)] and predicted 47% [Q2Y(cum)] of the total variance (**Figure 3A**). During the statistical processing to construct the model, one fish from the CTRL group appeared as an outlier and was excluded from the model. The fit of the resulting PLS-DA model was validated by a permutation test (**Supplementary Figure 3**). The final model clearly separated the CTRL from the NoPAP and PAP fish in the first component (43% explained variance), whereas the second component mainly separated the fish fed with the PAP diet from the other two groups (>46% explained variance). According to this, the hierarchical clustering grouped together the CTRL and PAP fed fish, and all the samples were properly classified in their respective experimental group (**Figure 3B**). Filtering by VIP ≥ 1, a total of 135 OTUs mainly drove the separation among the experimental groups. The discriminant OTUs constituted 29.4, 10.6, and 46.5% of the overall microbial composition of fish fed with the CTRL, non-PAP, and PAP diets, respectively (**Supplementary Table 4**).

The LEfSe analysis revealed a total of 20 strong positive associations (Log_{10}LDA Score > 4) between the discriminant OTUs of the PLS-DA analysis and the experimental groups (**Supplementary Figures 4A,B**). In particular, the analysis revealed that the Pseudomonadaceae family and their genus *Pseudomonas*, as well as *Sphingomonas oligophenolica* and *Paracoccus koreensis*, were more represented in the CTRL group. In the non-PAP fish, a higher representation of the Flavobacteriaceae, Streptococcaceae, and Clostridiaceae families and the *Sphingomonas* and *Staphylococcus* (in particular *S. petrasii*) genera was found. In the PAP fish, the family of Lactobacillales and the *Clostridium* (*C. aciditolerans*),

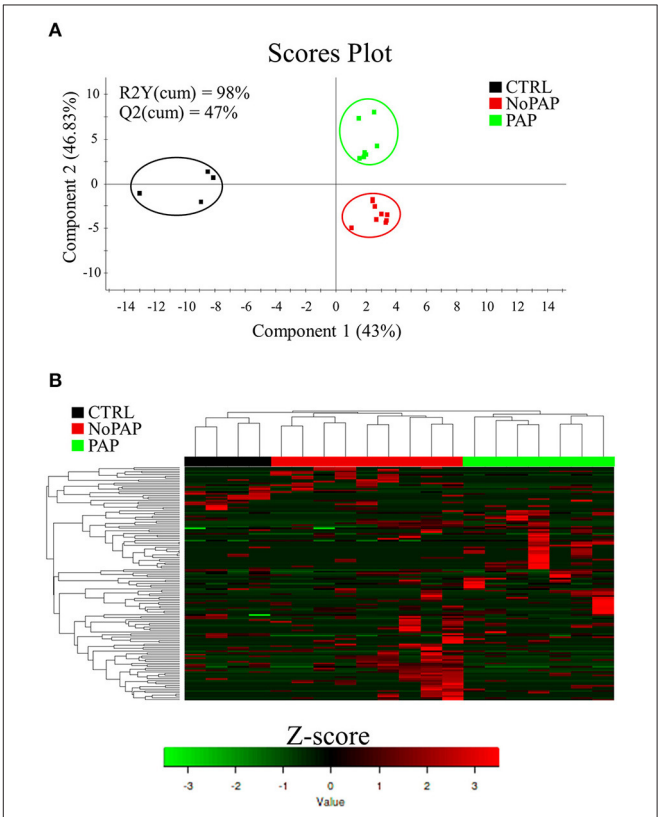

FIGURE 3 | (A) Two-dimensional partial least-squares discriminant analysis (PLS-DA) score plot constructed using the variable diet representing the distribution of the samples between the first two components in the model. The validation by permutation test can be found in **Supplementary Figure 3**. **(B)** Heatmap representing the abundance distribution (Z-score) of the OTUs identified to drive the separation by diet (VIP ≥ 1).

Maritimibacter (*M. alkaliphilus*), *Pelosinus*, and *Psychrosinus* genera were overrepresented.

Inferred Pathways

The sequences of the 135 OTUs, driving the separation of dietary groups, were used to discern the potential implication of microbiota in KEGG pathways by an inferred metagenome analysis. The analysis displayed a total of 38 OTUs (VIP $\geq$ 1) whose genomes were potentially associated with the expression of genes involved in the differentially represented pathways (FDR $<$ 0.05). When compared with the CTRL fish, 20 and 24 pathways showed to be potentially changing in the non-PAP and PAP fish, respectively (**Figure 4**). Ten of these pathways were common to NoPAP and PAP fish, with an up-regulation of the routes tailoring immune response and inflammation (C-type lectin receptor, VEGF, TNF, and NFκ-B signalling pathways), that was significantly lower (FDR = 0.01) in fish fed the NoPAP diet. Cholesterol metabolism and the neuroactive ligand-receptor interaction pathways were also over-represented to a similar extent in both conditions. Fish fed with the non-PAP and PAP-based feed formulations displayed an exclusive type of response at this level, with the differential representation of 10

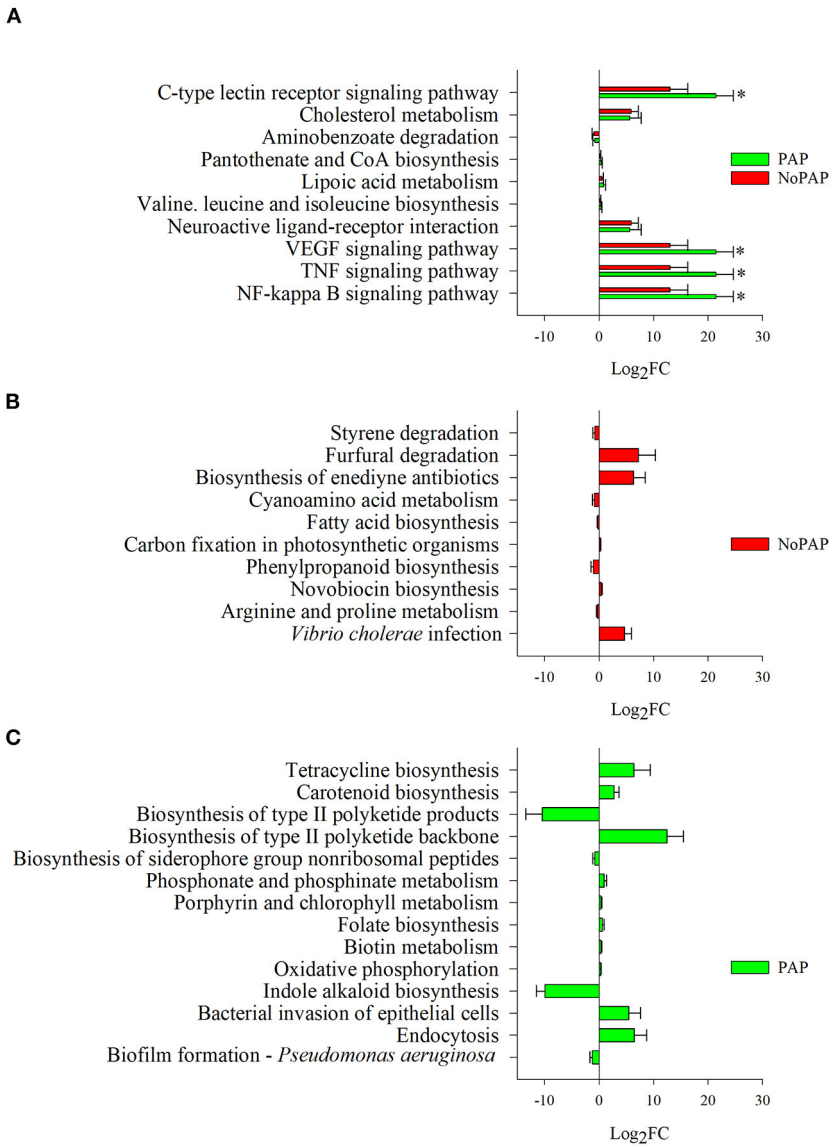

FIGURE 4 | Results from the pathway analysis performed with the predicted metagenome obtained from the discriminant OTUs with VIP $\geq$ 1. **(A)** Differentially and common represented pathways (padj $<$ 0.05) when comparing the PAP or non-PAP diets with the CTRL diet. The asterisk represents the result of a differential pathway representation (padj $<$ 0.05) between the NoPAP and PAP diets. **(B)** Exclusively differentially represented pathways in the non-PAP vs. CTRL comparison. **(C)** Exclusively differentially represented pathways in the PAP vs. CTRL comparison. Bars show the Log_2 fold change of differentially over- or under-represented pathways (± standard error of the calculated fold change).

and 14 inferred pathways, respectively. The list of bacteria related to each pathway can be found in **Supplementary Table 5**. This list is obtained from *in silico* inference and only reflects what could be hypothetically occurring, but it is still of value to assess the metabolic capability of bacterial populations.

Intestinal Gene Expression Profiling

All the genes included in the intestinal PCR-array were found at detectable levels, and dietary intervention significantly altered the expression pattern of 13 out of 43 genes (**Supplementary Table 6**), with PAP and non-PAP fish being clustered together in a heatmap expression pattern (**Figure 5A**). In comparison with the CTRL fish, galectin-8 (*lgals8*) was significantly down-regulated in fish fed with the non-PAP- and PAP-based diets (**Figures 5B,C**), whereas mucin 13 (*muc13*) and toll-like receptor 5 (*tlr5*) were only down-regulated in the non-PAP fish and immunoglobulin T (*igt*) in the PAP fish (**Figures 5B,C**). Toll-like receptor 2 (*tlr2*) was up-regulated in both the non-PAP and PAP fish, but the proliferating cell nuclear antigen (*pcna*) was significantly up-regulated in the PAP fish but not in the fish fed with the non-PAP diet (**Figures 5B,C**). Likewise, interleukin-10 (*il10*), C-C chemokine receptor type 9 (*ccr9*), toll-like receptor 9 (*tlr9*), and galectin-1 (*lgals1*) were up-regulated in the non-PAP fish (**Figure 5B**). Conversely, the expression of intestinal-type alkaline phosphatase (*alpi*), interleukin-8 (*il8*), and immunoglobulin M (*igm*) was only up-regulated in the fish fed with the PAP diet (**Figure 5C**).

Linking Gut Microbial Population and Host Transcriptome

The DE genes at the intestinal level, together with those that came from the study of Fernandes et al. (2021) (liver, 12 DE; head kidney, 9 DE) were correlated with the normalized counts of the 135 discriminant OTUs (VIP $\geq$ 1). Gene expression patterns of selected markers of liver and head kidney with a high representation of markers of growth, lipid, and energy metabolism, antioxidant defense, and immune response are shown in **Supplementary Tables 7, 8**. In total, 4,590 correlations were performed, establishing 69 significant associations ($P < 0.01$) between 43 discriminant OTUs and 27 DE genes (**Figure 6A**). The 43 correlated bacteria represented ~8% in CTRL group, with the predominant OTUs being *Nocardioides alpinus* (2.24%) and *Sphingomonas oligophenolica* (1.11%). The percentage increased by up to 16.23% in fish fed with the non-PAP diet, with dominance of Clostridiales (8.6%), *Skermanella aerolata* (2.9%), Bacilli (1.62%), and *Serratia nematodiphila* (1%). In fish fed with the PAP diet, the associated OTUs represented 16.4%, with OTUs assigned to the *Escherichia/Shigella* (1.6%) and *Pelosinus* (1.12%) genera, and the *Psychrobacter piscatorii* (1.6%), *Haemophilus pittmaniae* (1.4%), and *Paracoccus yeei* (1.03%) species (**Figure 6B**). As a result of all this complex interplay, a remarkable number of DE genes at the intestinal level (12 out of 13) were involved in 38 significant correlations with 28 out of the 43 discriminant OTUs (VIP $\geq$ 1). Within these taxa, 10 OTUs disclosed a significant correlation with five DE genes of the liver and two of the head kidney (**Figure 7**). In a comparative manner, the correlation tests displayed eight discriminant OTUs that showed a significant correlation with seven hepatic DE genes (**Supplementary Figure 5A**), whereas seven discriminant OTUs

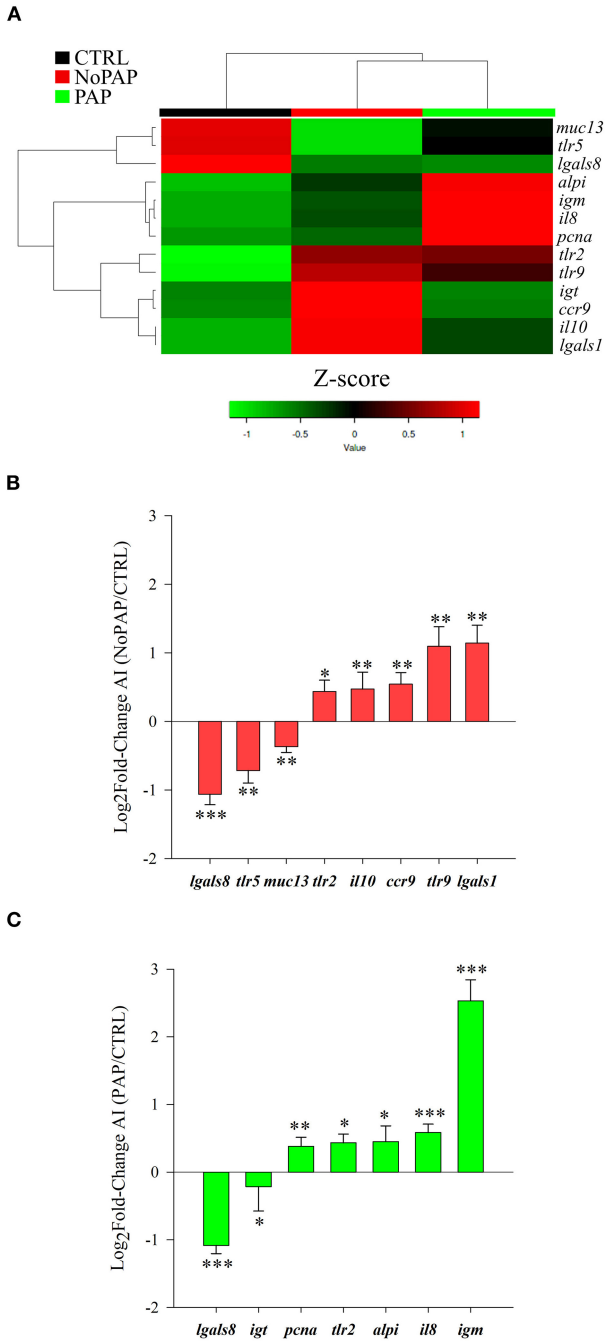

FIGURE 5 | Differentially expressed (DE) genes in the anterior intestine of fish fed with the experimental diets when compared with those fed with the CTRL diet ($P < 0.05$). **(A)** Heatmap depicting the relative expression of the DEGs in the three dietary groups. Log_2 fold changes of differentially expressed genes (+SEM) in comparisons **(B)** non-PAP vs. CTRL and **(C)** PAP vs. CTRL are shown [asterisks represent statistically significant differences at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$].

Discriminant OTUs	AI DE Genes	HK DE Genes	L DE Genes
Nocardioides	*pcna, tlr9, tlr2*	*cd4-1*	
Psychrobacter piscatorii	*alpi*	*igt*	
Serratia nematodiphila	*il-10, lgals8*		*scd1b*
Anoxybacillus flavithermus	*muc13*		*cpt1a*
Chloroflexi	*muc13*		*cpt1a*
Nocardioides alpinus	*tlr9*		*cpt1a*
Nocardioides mesophilus	*igt*		*scd1a*
Pelosinus	*muc13*		*mn-sod/sod2*
Psychrosinus	*muc13*		*mn-sod/sod2*
Vibrio sagamiensis	*lgals1*		*elovl6*
Sphingomonas oligophenolica	*tlr9, pcna, igt, tlr2*		
Firmicutes	*tlr2, tlr9, igm*		
Clostridium aciditolerans	*tlr9, tlr2*		
Acinetobacter	*lgals8*		
Azotobacter salinestris	*tlr2*		
Catenococcus	*tlr5*		
Clostridiaceae	*ccr9*		
Clostridiales	*ccr9*		
Escherichia/Shigella	*igm*		
Mycobacterium	*lgals8*		
Phenylobacterium koreense	*tlr2*		
Pseudomonas fulva	*tlr2*		
Pseudomonas matsuisoli	*igm*		
Sphingomonadaceae	*igt*		
Sphingomonas	*igm*		
Staphylococcus petrasii	*igm*		
Streptococcus	*igt*		
Streptococcus cristatus	*igm*		
Psychrobacter		*cd8a, igt, cd3x*	
Bacilli		*il-8, tnf-a*	
Sphingomonas pituitosa		*cd8a, ccr3*	
Paracoccus koreensis		*igt*	
Paracoccus yeei		*tnf-a*	
Pleomorph. xiamenense		*igt*	
Rhodovulum phaeolacus		*ck8/ccl20*	
Brevinema andersonii			*scd1a, elovl6, scd1b*
Pseudomonas kilonensis			*hl, cpt1a*
Blastococcus aggregatus			*hl*
Celeribacter marinus			*ppara*
Haemophilus pittmaniae			*ppara*
Moraxellaceae			*ppara*
Skermanella aerolata			*igf-i*
Staphylococcus carnosus			*elovl6*

FIGURE 6 | (A) List of the 43 discriminant OTUs involved in significant ($P < 0.01$) correlations with intestinal (AI), head kidney (HK), and liver (L) differentially expressed genes (DEGs). Colors of genes in DE gene columns indicate if there is a direct (red) or inverse (green) OTU-gene correlation. **(B)** Dot plot depicting the normalized count number together with the proportion of correlated OTUs among the CTRL and experimental diets.

were strongly associated with seven head kidney DE genes (**Supplementary Figure 5B**).

DISCUSSION

Fish production continues to be strongly dependent on FM (Ytrestøyl et al., 2015; Hua et al., 2019), but there is now evidence that the use of a combination of plant proteins, poultry meal, and insect proteins is able to support optimal growth in a wide range of farmed marine fish, such as gilthead sea bream (Basto et al., 2021; Reis et al., 2021). Moreover, there is now evidence that the reshaping of gut microbiota by the replacement of FM with poultry meal and microbial biomass would serve to exert an adaptive and counter-regulatory inflammatory action at the local intestine level (Solé-Jiménez et al., 2021). Certainly, a number of factors (e.g., age, sex, genetics, and environment)

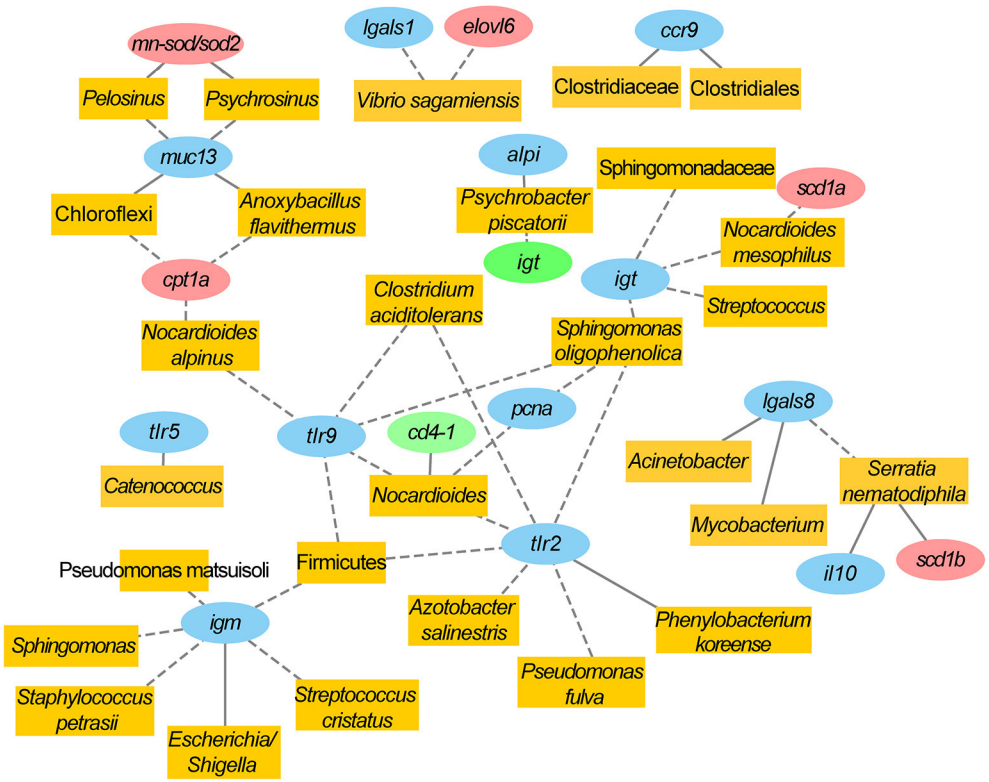

FIGURE 7 | Correlation network showing significant positive (straight lines) and negative (dotted lines) correlations (Spearman, $P < 0.01$) between discriminant OTUs (yellow) and differentially expressed genes (DEG) in the anterior intestine (blue). Differentially expressed genes in the head kidney (green) and liver (red) with common interaction with anterior intestine gene—OTU correlations are also shown.

regulate the composition of the microbial population, which could eventually influence growth performance and health (Nayak, 2010; Cordero et al., 2015). However, diet-associated factors are perhaps one of the most important (Silva et al., 2011; Ghanbari et al., 2015). For instance, in zebrafish, a gluten-formulated diet displayed heightened abundances of Legionellales, Rhizobiaceae, and *Rhodobacter*, as compared with the control diet (Koo et al., 2017). Regarding aquaculture species, in yellowtail kingfish (*Seriola lalandi*), diet- and diet-associated bacteria shaped gut microbiota through development (Wilkes Walburn et al., 2019). Legume-based diets increased the abundance of lactic acid bacteria in Atlantic salmon, *Salmo salar* (Gajardo et al., 2017). Likewise, long-term feeding trials with plant-based diets displayed a shift in the resident intestinal microbiota of gilthead sea bream, driven by a dramatic increase in the genus *Photobacterium* that was partially reversed by dietary butyrate supplementation (Piazzon et al., 2017). However, interactions among microbial community, fish metabolism, fish performance, and health are still largely unknown, and this study serves to highlight the different reshaping of gut microbiota with the PAP and non-PAP diets. Additionally, several correlations between microbial populations and host gene expression were disclosed, with a more pronounced local intestinal action, and with important interactions at the systemic level.

At a closer look, the non-PAP diet decreased ACE and diversity Shannon indices, resulting in reduced richness and alpha diversity, in line with the study of Solé-Jiménez et al. (2021) using poultry meal and microbial biomass as FM replacements. The general thinking is that the occurrence of less diverse bacterial populations is a bad sign, because it is prone to increasing the chance of opportunistic pathogenic bacteria to proliferate and cause host damage (Sekirov et al., 2010; Apper et al., 2016). However, a deeper and experiment-specific understanding of the changes in gut microbiota is needed before reaching such conclusion for a given fish species and experimental condition. For instance, the gut microbiota of fish selected for growth became more stable but at the same time was functionally more plastic against changes in diet compositions (Piazzon et al., 2020). In this study, the microbial composition at the phylum level also remained relatively stable (**Figure 3**), showing the typical predominant phyla of gilthead sea bream within the expected range of variations for Proteobacteria (38–50% vs. 40–60%), Firmicutes (20–38% vs. 15–40%), and Bacteroidetes (3% vs. 2–5%), and only some deviations for Actinobacteria (11–18% vs. 15–25%) (Parma et al., 2016; Piazzon et al., 2019; Rimoldi et al., 2020). Since this occurred under brackish water conditions (18 ppt), this would be indicative that the gut microbiota of an euryhaline fish, such as gilthead sea bream, has the capacity to remain almost unaltered at the

phylum level in the range of 18–37 ppt, which is in contrast with the dramatic changes observed with the sea water transfer in Atlantic salmon (Dehler et al., 2017). In this scenario, the PLS-DA disclosed 135 OTUs that drove (VIP $\geq$ 1) the separation among the groups (**Figure 4**). When this discriminant fraction of OTUs was considered more in-depth, the LEfSe analysis displayed a considerable number of taxa commonly related with opportunistic pathogenic bacteria in the gut of both the non-PAP- (Streptococcaceae, 3%; Flavobacteriaceae, 1%; *Sphingomonas,* 4.5%; *Staphylococcus,*2.5%) and PAP-fed fish (*Clostridium*_sensu_stricto, 5.5%) (Starliper, 2011; Sabry et al., 2016; Seghouani et al., 2017). However, assignments at high taxonomic levels, such as genus or families, do not necessarily reflect a pathogenic population, as these OTUs have been commonly detected in the microbial population of gilthead sea bream, and wide and diverse groups with several genus or species are implicated in diverse functions (Estruch et al., 2015; Parma et al., 2016; Nikouli et al., 2018; Piazzon et al., 2019).

Despite the increasing research on gut microbial composition, the understanding of how this population influences and is influenced by host gene expression remains relatively unexplored (Nichols and Davenport, 2021). Model organisms, such as zebrafish, *C. elegans*, *Drosophila melanogaster*, and mice are widely used to investigate these associations, with researchers having the control over the environment and complete control over diet, and importantly, the ability to study all tissues with advanced genomic tools (Borrelli et al., 2016; Wang et al., 2017). For aquaculture species, there is still a lack of information regarding the correlation between microbial populations and host gene expression, with some exceptions in black tiger shrimp (*Penaeus monodon*) (Uengwetwanit et al., 2020). Here, we take advantage of the genomic tools available for gilthead sea bream (Calduch-Giner et al., 2013; Pérez-Sánchez et al., 2019) to intersect the gene expression patterns of the intestine (from this study), liver, and head kidney (from Fernandes et al., 2021) with gut microbial community abundances (**Figures 6**, **7** and **Supplementary Figure 5**), putting together the paired samples from CTRL and fish fed with the PAP and non-PAP diets. It is worth noting that the high percentage of the OTUs (43 out of 135; ∼32%) driving the separation among the dietary groups in the PLS-DA was significantly correlated with DE genes at the local and systemic levels. This fact supports the usefulness of the PLS-DA filter strategy (VIP $\geq$ 1) for the detection of transcriptome-associated taxa, as reported in other research animal models correlating metagenomics with host transcriptomics and metabolomics (Yan et al., 2020; Liu and Zhang, 2021; Zhao et al., 2021). Nonetheless, there is an important gap in information on the long-range action of the microbiota, mainly due to the assumption that microorganisms establish their niches in the intestine and they and their response do not spread, and that when an asystemic response exists, it is not always of sufficient clinical relevance (Chiu et al., 2017). However, both assumptions are questionable, because several studies stated the ability of resident microbiota to exert important systemic effects (Brenner et al., 2015; Ho et al., 2015; Grigg and Sonnenberg, 2017). Thus, in this fish study, the number of discriminant OTUs correlating with DE genes decreased from 28 in the intestine to 16 in the liver, and 9 in the head kidney. Among them, 10 out of 48 were involved in local and systemic correlations at the same time. According to this, the host and microbial interactions were more pronounced at the local (gut) level, but the systemic action cannot be underestimated, as further discussed below. Besides, our correlation analysis was based on a targeted gene approach with different tissue-specific PCR arrays, but it remains elusive if the use of a massive gene expression approach (RNA-seq) could modify the established trend for the network of gut microbiota and host transcriptomics.

The homoeostasis of the constantly renewing intestinal epithelium relies on integrated control of proliferation, differentiation, and apoptosis (Macara et al., 2014). Thus, the down-regulation of *pcna* in gilthead sea bream fed with FM-based diets supplemented with olive oil bioactive compounds has been related to a lower epithelial turnover in association with a better gut health condition (Gisbert et al., 2017). The *pcna* gene was also down-regulated in fish fed with plant-based diets, and the restoration of control values by butyrate supplementation was considered part of the mechanisms driven by this gut health factor in gilthead sea bream (Estensoro et al., 2016; Piazzon et al., 2017). Accordingly, in this study, the PAP-based feed formulation, and, only to a lower extent (non-significantly), the non-PAP diet (with a higher content of plant proteins), triggered the up-regulation of *pcna* in the AI of the fish in these two groups. This feature will be prone to promoting cell proliferation rather than cell differentiation with the replacement of FM with non-conventional dietary protein sources in the PAP and non-PAP diets. Of note, we found that the *pcna* gene expression was inversely correlated to *Nocardioides* (CTRL.003%; non-PAP and PAP <0.001%) and *Sphingomonas oligophenolica* (CTRL 2%; ∼0.003% in the non-PAP and PAP fish), which suggests a role of these taxa in fish epithelial cell proliferation and regulation.

The integrity of the intestinal epithelium also relies on the maintenance of the mucus layer that is mainly composed of mucins, *O*-glycosylated glycoproteins that are present on the apex of all wet-surfaced epithelia, protecting epithelial cells from bacteria-, virus- or pH-derived damages and participating in cell signaling processes (Lang et al., 2007). Up to six mucins have been molecularly and transcriptionally characterized in gilthead sea bream, with *muc13* being extensively and constitutively expressed along the entire intestinal tract (Pérez-Sánchez et al., 2013). However, in this study, the expression of *muc13* was down-regulated in the non-PAP-fed fish, and to a lower extent (non-significantly), in the PAP fish. Besides, the correlation analysis highlighted a positive association of *muc13* with OTUs assigned to the phylum Chloroflexi (CTRL,0.58%; absent in PAP and non-PAP) and *Anoxybacillus flavithermus* (CTRL 0.01%; non-PAP and PAP < 0.001%). Since Chloroflexi and the majority of species of the *Anoxybacillus* genus are described as anaerobic bacteria (Pikuta et al., 2000; Speirs et al., 2019), the up-regulation of *muc13* gene expression will tend to produce a thicker layer with less available oxygen that will favor intestinal colonization by these specific taxa. Intriguingly, we also evidenced a negative association of *muc13*mRNA transcripts with OTUs assigned to *Pelosinus* and *Psychrosinus* genera (CTRL, <0.0001; NoPAP, 0.87%; PAP, 1.13%). To our knowledge, there

is no described correlation between these taxa and host gene expression. However, species belonging to these taxa are capable of altering metal speciation, being well-known iron reducers (Ray et al., 2018). Besides, the higher bioavailability of iron in the intestinal lumen of mice has been related to the action of these bacteria on the reduction of intestinal oxidative DNA damage (Li et al., 2015; Eteshola et al., 2020). In our case, the up-regulated expression of the hepatic superoxide dismutase [Mn] (*mn-sod/sod2*) positively correlated with the higher abundance of *Pelosinus* and *Psychrosinus* genera in the AI of the non-PAP- and PAP-fed fish, pointing out a possible systemic cross-talk between fish antioxidant defense and gut microbial community.

As for *pcna*, the intestinal expression of *alpi* increased from the CTRL fish to the PAP fish, with intermediate expression values in the fish fed with the non-PAP diet. ALPI is found in high concentrations in the brush border of intestinal epithelial cells of both fish and mammals (Estensoro et al., 2016), regulating the rate of lipid absorption (Mahmood et al., 2003). Additionally, ALPI has a role in the disassembly of lipopolysaccharides (LPS) of Gram-negative bacteria (Poelstra et al., 1997), reducing inflammatory response (Millán, 2006; Rader, 2017). Thus, as in other animal models, a decline in ALPI activity is generally associated with malnutrition in fish (Bakke-McKellep et al., 2000; Ducasse-Cabanot et al., 2007), and perhaps to a less effective response for preventing bacterial invasion across the gut mucosal barrier. In this regard, we found that the up-regulated expression of *alpi* was closely related with a high representation of *Psychrobacter piscatorii* (CTRL and non-PAP <0.005%; PAP1.6%) in the intestinal microbiota of gilthead sea bream. Overall, this can be viewed as a positive feature, because some species of the *Psychrobacter* genus are tolerant to alkaline phosphatase (Denner et al., 2001), and their use as a probiotic reduced the proportion of harmful and pro-inflammatory species in the gastrointestinal tract of cultured orange-spotted grouper (*Epinephelus coloides*) (Yang et al., 2011). However, the species of this genus also activate the pro-inflammatory NF-kB pathway (Chow et al., 1999; Yu et al., 2016), and a recent study on gilthead sea bream (Solé-Jiménez et al., 2021) associated the lower abundance of *Psychrobacter* with the reshaping of gut microbiota to preserve an anti-inflammatory gut profile in fish fed with an FM replacement with a high content of poultry meal.

The expression of intestinal *igm* was markedly up-regulated in the AI of fish fed with the PAP diet. Earlier studies on this fish species highlighted the up-regulation of the intestinal *igm* in response to changes in the nutritional background, and bacteria or parasite challenges (Estensoro et al., 2012; Piazzon et al., 2016; Simó-Mirabet et al., 2017). Therefore, it is not surprising that this gene correlated with up to six different discriminant OTUs (i.e., Firmicutes, *Sphingomonas*, *Escherichia/Shigella*, *Pseudomonas matsuisoli*, *Staphylococcus petrasii*, and *Streptococcus cristatus*). Among these, only the OTUs assigned to the *Escherichia/Shigella* genus were found in a considerable proportion (1.6%) in the gut of fish fed with the PAP diet, establishing a direct correlation. This genus is defined as an activator of the inflammasome system (Liu et al., 2020), suggesting the driving role of this bacteria in the host immune response. In the head kidney, the expression trend for *igm* in the PAP fish was also up-regulated. However, the opposite was found for the *igt* gene expression, which showed a more down-regulated pattern in the head kidney than in the AI, which confirms and extends the idea of differential regulation of these two types of immunoglobulins in fish, gilthead sea bream in particular (Reyes-Cerpa et al., 2014; Piazzon et al., 2016). Besides, both the systemic and local *igt* expressions were negatively correlated with the abundance of intestinal *Psychrobacter piscatorii*, reinforcing the aforementioned relationship of this bacteria with gut health in gilthead sea bream.

Another pro-inflammatory marker, *il8,* was up-regulated in the AI of fish fed with the PAP diet, although in this case, we failed to establish any significant correlation with the intestinal microbial population. However, at the systemic level, the expression of the head kidney *il8* was negatively correlated with Bacilli. This link is not surprising, as bacteria from this class are known to have anti-inflammatory and anti-oxidant properties (Giri et al., 2019), being used as probiotics to improve growth and intestinal health in mammals (Rhayat et al., 2019; Zhou et al., 2020) and livestock fish (Mingmongkolchai and Panbangred, 2018), such as gilthead sea bream (Simó-Mirabet et al., 2017; Moroni et al., 2021). In this regard, it must be noted that the alleviation of the intestinal pro-inflammatory pattern of non-PAP fish was concomitant with a higher abundance of the OTUs assigned to Bacilli class (non-PAP 1.63%; CTRL and PAP <0.001%). In the head kidney, this was extended to the expression pattern of *tnfα*, which achieved the highest expression level in fish fed with the PAP diet and with intermediate values in the non-PAP fish. TNFα has a central role in inflammation (Bradley, 2008), and microbiota have been addressed to block (Yang et al., 2020) or activate its gene expression (Mendes et al., 2019). Hence, in the study, *tnfα* expression was positively correlated with the abundance of *Paracoccus yeei*, not present in the gut of CTRL fish but increasing by up to 0.3 and 1.03% in the non-PAP and PAP fish, respectively. This might serve to exert a counter-regulatory inflammatory response that is supported by the inferred metagenomics pathway analysis, where *P. yeei* is the only contributor in the inferred over-representation of the TNF signaling pathway. Thus, although bacteria-host gene expression interactions can be sometimes difficult to interpret, the combination of different analyses provided support to the results.

Another sign of an anti-inflammatory profile in the non-PAP fish was the intestinal up-regulation of *il10,* a key anti-inflammatory cytokine used in both fish and mammals as an important marker for health status of the host (Levast et al., 2015; Piazzon et al., 2015). Some increase in the expression level of *il10* was also seen in the head kidney, although not significant, confirming the stronger modulation of this cytokine at the local level, as reported in earlier gilthead sea bream studies (Pérez-Cordón et al., 2014). Moreover, regarding microbiota and gene expression associations, the intestinal expression of *il10* positively correlated with the abundant *Serratia nematodiphila* in non-PAP fish (1.02%), while being poorly represented in the microbial population (<0.001%) of CTRL and PAP fish. In any case, the role of the *Serratia* genus in inflammatory processes remains controversial, because some species act as

pro-inflammatory agents through protease-activated receptors (Kida et al., 2007), whereas others are anti-inflammatory factors by means of enzymes with potent anti-oxidant power (Saeki et al., 1974; El-Abd and Ibrahim, 2020).

In a healthy gut, the recognition of microorganisms by PRRs has a primary role in the activation or repression of innate immunity (Fukata and Arditi, 2013), exerting diverse functions depending on the offending factor (Boltaña et al., 2011). In our experiment model, several PRRs, such as galectins and toll-like receptor genes, were monitored. Among them, it is worth noting that the pronounced down-regulation of intestinal *lgals8* in both PAP and non-PAP fish would contribute to the maintenance of the repressed immune system. This is in line with several pro-inflammatory molecules abundantly produced in mice by LGAL8-stimulated endothelial cells (Cattaneo et al., 2014). Otherwise, the intestinal expression of *lgals8* was significantly down-regulated in healthy gilthead sea bream juveniles fed with practical diets that are supplemented with sodium salt medium-chain fatty acids or *Bacillus*-based probiotic (Simó-Mirabet et al., 2017). Conversely, LGALs1 has a recognized role in the control of chronic inflammations, weakening cytokine synthesis and de-activating antigen presenting cells, causing an overall immune repression in mice (Seropian et al., 2018). Therefore, the up-regulated expression of intestinal *lgals1* in the non-PAP fish, but not in the PAP fish, reinforced the anti-inflammatory expression pattern of fish fed with the non-PAP diet. This was not the case for the *tlr* genes that showed an enhanced expression in the PAP and non-PAP fish (*tlr2*) or only in the NoPAP fish (*tlr9*). The functional significance of this finding remains unclear, but TLR signaling pathways play a key role in the regulation of the immune system, preventing autoimmune and inflammatory diseases in humans and rodents (Tang et al., 2012; Kawasaki and Kawai, 2014). Likewise, *tlr* genes are often up-regulated when fish deal with a bacterial infection (Reyes-Becerril et al., 2015), but there is now evidence that spirulina feed significantly enhanced the immune response of gibel carp (*Carassius auratus gibelio*) through the Tlr2 pathway (Cao et al., 2018). Since the non-PAP-based feed formulation also contained *Spirulina* as a microalgae meal, it is likely that the enhanced expression of *tlrs* in fish fed with the non-PAP diet was due, at least in part, to the use of this alternative dietary protein source. In any case, the number of OTUs (eight) associated with changes in the expression pattern of PPRs was relatively high. Besides, the correlated taxa belonged to different and diverse assignations (Firmicutes, *Clostridium*, *Nocardioides*, *Azotobacter*, *Pseudomonas*, and *Phenylobacterium*), showing the wide range of bacterial types that could be interacting with PRRs.

In a back-and-forth response, gut microbiota can exert a substantial influence on host lipid metabolism, and mechanistic links involving the microbial generation of SCFAs, microbial processing of bile acids, and bacterial-derived pro-inflammatory factors have been reported in mouse models (Schoeler and Caesar, 2019; Lamichhane et al., 2021). This highlights a gut microbiota-liver axis where SCFAs generated by the gut bacterial fermentation of dietary fiber fuel the SCD1 (Stearoyl CoA desaturase)-mediated lipogenesis in the liver of mice (Singh et al., 2015). Intriguing associations between host lipid metabolism and the composition of gut microbiota have also been reported in Atlantic salmon (Dvergedal et al., 2020) and rainbow trout (Yildirimer and Brown, 2018). However, in fish, these relationships remain in an infancy state, reinforcing the value of this study where, for the first time in a typical marine fish, a possible link between enzymes of lipid metabolism and gut microbiota was evidenced. Here, this can be exemplified by the lipogenic *scd1a* and *scd1b* enzymes, which becomes especially interesting in the case of *scd1a* given that a recent study revealed reliable epigenetic mechanisms (changes in DNA methylation rates of the *scd1a* promoter) by which the nutrition of parents can shape the s*cd1a* gene expression in the gilthead sea bream offspring (Perera et al., 2020). The extent to which this can be favored by changes in the gut microbiota remains unknown, but interestingly, the up-regulation of *scd1* genes in the fish fed with the non-PAP diet was associated with OTUs assigned to *Serratia nematodiphila* (NoPAP, 1.02%) and *Brevinema andersonii* (NoPAP 0.1%; CTRL and PAP <0.01%), giving these taxa a role as intestinal SCFA producers in humans (Parada Venegas et al., 2019) and enhancers of fatty acid metabolism in insects (Zhou et al., 2021). The case of *B. andersonii* merits further attention, as this species belongs to Spirochaetesphylum, which colonizes the gut gilthead sea bream microbiome to a large extent with advancing age (11% in 4-year-old individuals) (Piazzon et al., 2019). The physiological consequences of this finding merit further research, because studies on mice highlighted alterations of the hepatic lipid profile and fatty acid synthesis in response to gut colonization by aged microbiota (Albouery et al., 2020). Future studies should focus on enzymatic levels and activities to corroborate these results further.

Like hepatic SCD1, fatty acid elongase 6 (ELOVL6) is a rate-limiting enzyme of fatty acid synthesis and lipogenic activity, being triggered by the gut microbial production of SCFAs in mice (Kindt et al., 2018). This might also occur in our gilthead sea bream model, and the hepatic expression of *elovl6* was positively correlated with the non-pathogenic species *Staphylococcus carnosus* (non-PAP 0.03%; PAP <0.00001%), applied as a starter culture in industrialized processes (Janssens et al., 2012). Another type of association of lipid metabolism and gut microbiota was represented by the peroxisome proliferator-activated receptor α (*pparα*). This transcription factor of lipid metabolism is a well-known lipolytic factor (Mottillo et al., 2012; Pawlak et al., 2015), and its hepatic down-regulated expression in the fish fed with the non-PAP diet negatively correlated with OTUs assigned to Moraxellaceae, *Haemophillus pittmaniae*, and *Celeribacter marinus*. Therefore, the trade-off of host lipogenic and lipolytic pathways becomes related to the changes in the gut microbial population of a farmed marine fish.

In summary, even in the absence of major changes in growth performance, a number of processes related to epithelial cell turnover, immune response, and lipid metabolism were affected by dietary intervention. Both pro- and anti-inflammatory responses were triggered by the non-PAP- and PAP-based feed formulations, although the net effect would be prone to a slight pro-inflammatory status that appears mostly attenuated in the fish fed with the non-PAP diet. Thus, the formulation

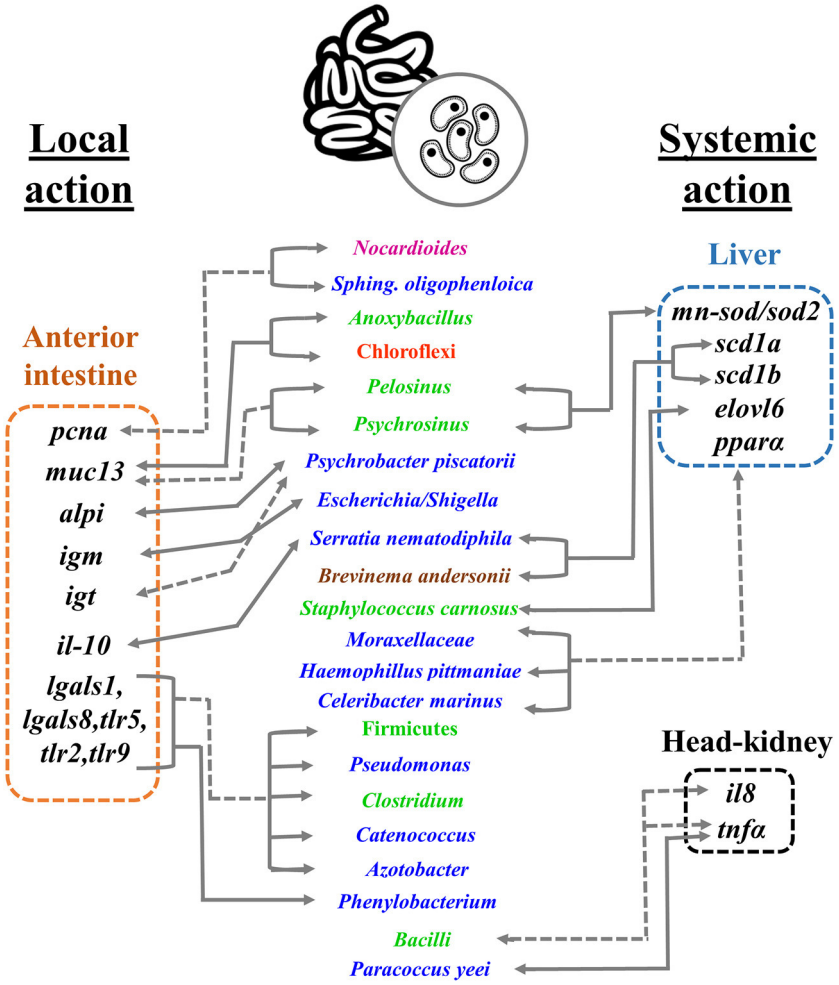

FIGURE 8 | Schematic representation of the main significant correlations between gut microbial community abundances and host gene expression at the local (AI) and systemic levels (liver, head-kidney). Gene symbols are boxed in dashed rectangles referring to each tissue: anterior intestine (orange), liver (blue), and head-kidney (black). Taxa names are in the middle column, with colors representing the phyla they belong: Proteobacteria (blue), Firmicutes (green), Actinobacteria (pink), Chloroflexi (red), and Spirochaetes (brown). Gray lines between taxa and gene symbol represent a positive (straight) or negative (dashed) correlation between gene and OTU.

of the non-PAP diet arises as an attractive formulation to be further studied for its use in gilthead sea bream aquaculture. Indeed, the fish fed with the non-PAP diet shared an enhanced lipogenic activity, which might suggest an enhanced microbial production of SCFAs. As summarized in **Figure 8**, remarkable correlations between changes in gut microbial populations and host gene expression were unveiled at the local and systemic levels. By correlating microbiome and host gene expression, we offer new insights into the physiological processes promoting both metabolic and gut homeostasis and, ultimately, the health of farmed fish. All this reinforces the action of the gut microbiome as a "second genome," being involved in—and/or being influenced by—the regulation of the transcriptomic response in fish fed with new feed formulations based on increased circularity and resource utilization. Future studies should focus on the effect of improved non-PAP-based formulations on fish growth and health, with a special focus on disease resistance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the Sequence Read Archive (SRA) under Bioproject accession number PRJNA745265 (BioSample accession numbers: SAMN20157689-709).

ETHICS STATEMENT

The animal study was reviewed and approved by Fernando Bernardo, Direçao Geral de Alimentaçao e Veterinaria, Lisboa, Portugal.

AUTHOR CONTRIBUTIONS

FN-C, GV, MP, AF, and JC-G: formal analysis. FN-C, GV, MP, and JP-S: writing—original manuscript. AS-B, LC, and JP-S:

conceptualization. All authors contributed to the experimental investigation, writing—review and editing, read, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.748265/full#supplementary-material

REFERENCES

Afgan, E., Baker, D., Batut, B., Van Den Beek, M., Bouvier, D., Ech, M., et al. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544. doi: 10.1093/nar/gky379

Albouery, M., Buteau, B., Grégoire, S., Cherbuy, C., Pais de Barros, J. –P., Martine, L., et al. (2020). Age-related changes in the gut microbiota modify brain lipid composition. *Front. Cell. Infect. Microbiol.* 9:444. doi: 10.3389/fcimb.2019.00444

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Apper, E., Weissman, D., Respondek, F., Guyonvarch, A., Baron, F., Boisot, P., et al. (2016). Hydrolysed wheat gluten as part of a diet based on animal and plant proteins supports good growth performance of Asian seabass (*Lates calcarifer*), without impairing intestinal morphology or microbiota. *Aquaculture* 453, 40–48. doi: 10.1016/j.aquaculture.2015.11.018

Aragão, C., Cabano, M., Colen, R., Fuentes, J., and Dias, J. (2020). Alternative formulations for gilthead seabream diets: towards a more sustainable production. *Aquac. Nutr.* 26, 444–455. doi: 10.1111/anu.13007

Bakke-McKellep, A., Nordrum, S., Krogdahl, Å., and Buddington, R. K. (2000). Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem.* 22, 33–44. doi: 10.1023/A:1007872929847

Basto, A., Calduch-Giner, J., Oliveira, B., Petit, L., Sá, T., Maia, M. R., et al. (2021). The use of defatted *Tenebrio molitor* larvae meal as a main protein source is supported in European sea bass (*Dicentrarchus labrax*) by data on growth performance, lipid metabolism and flesh quality. *Front. Physiol.* 12:659567. doi: 10.3389/fphys.2021.659567

Bledsoe, J. W., Peterson, B. C., Swanson, K. S., and Small, B. C. (2016). Ontogenetic characterization of the intestinal microbiota of channel catfish through 16S rRNA gene sequencing reveals insights on temporal shifts and the influence of environmental microbes. *PLoS ONE* 11:e0166379. doi: 10.1371/journal.pone.0166379

Boltaña, S., Roher, N., Goetz, F. W., and Mackenzie, S. A. (2011). PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish. *Dev. Comp. Immunol.* 35, 1195–1203. doi: 10.1016/j.dci.2011.02.010

Booman, M., Forster, I., Vederas, J. C., Groman, D. B., and Jones, S. R. M. (2018). Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*) and Chinook salmon (*Oncorhynchus tshawytscha*) but not in pink salmon (*O. gorbuscha). Aquaculture* 483, 238–243. doi: 10.1016/j.aquaculture.2017.10.025

Borrelli, L., Aceto, S., Agnisola, C., De Paolo, S., Dipineto, L., Stilling, R. M., et al. (2016). Probiotic modulation of the microbiota-gut-brain axis and behaviour in zebrafish. *Sci. Rep.* 6:30046. doi: 10.1038/srep30046

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *J. Pathol.* 214, 149–160. doi: 10.1002/path.2287

Brenner, D. A., Paik, Y. H., and Schnabl, B. (2015). Role of gut microbiota in liver disease. *J. Clin. Gastroenterol.* 49(Suppl. 1), S25–S27. doi: 10.1097/MCG.0000000000000391

Calduch-Giner, J. A., Bermejo-Nogales, A., Benedito-Palos, L., Estensoro, I., Ballester Lozano, G., Sitjà-Bobadilla, A., et al. (2013). Deep sequencing for *de novo* construction of a marine fish (*Sparus aurata*) transcriptome database with a large coverage of protein-coding transcripts. *BMC Genomics* 14:178. doi: 10.1186/1471-2164-14-178

Cao, S., Zhang, P., Zou, T., Fei, S., Han, D., Jin, J., et al. (2018). Replacement of fishmeal by *Spirulina Arthrospira platensis* affects growth, immune related-gene expression in gibel carp (*Carassius auratus gibelio* var. CAS III), and its challenge against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol.* 79, 265–273. doi: 10.1016/j.fsi.2018.05.022

Cattaneo, V., Tribulatti, M. V., Carabelli, J., Carestia, A., Schattner, M., and Campetella, O. (2014). Galectin-8 elicits pro-inflammatory activities in the endothelium. *Glycobiology* 24, 966–973. doi: 10.1093/glycob/cwu060

Chiu, L., Bazin, T., Truchetet, M. E., Schaeverbeke, T., Delhaes, L., and Pradeu, T. (2017). Protective microbiota: from localized to long-reaching co-immunity. *Front. Immunol.* 8:1678. doi: 10.3389/fimmu.2017.01678

Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274, 10689–10692. doi: 10.1074/jbc.274.16.10689

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. doi: 10.1093/nar/gkt1244

Conceição, L. E. C., Aragão, C., Dias, J., Costas, B., Terova, G., Martins, C., et al. (2012). Dietary nitrogen and fish welfare. *Fish Physiol. Biochem.* 38, 119–141. doi: 10.1007/s10695-011-9592-y

Cordero, H., Guardiola, F. A., Tapia-Paniagua, S. T., Cuesta, A., Meseguer, J., Balebona, M. C., et al. (2015). Modulation of immunity and gut microbiota after dietary administration of alginate encapsulated *Shewanella putrefaciens* Pdp11 to gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 45, 608–618. doi: 10.1016/j.fsi.2015.05.010

Dehler, C. E., Secombes, C. J., and Martin, S. A. M. (2017). Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Sci. Rep.* 7:13877. doi: 10.1038/s41598-017-13249-8

Delamare-Deboutteville, J., Batstone, D. J., Kawasaki, M., Stegman, S., Salini, M., Tabrett, S., et al. (2019). Mixed culture purple phototrophic bacteria is an effective fishmeal replacement in aquaculture. *Water Res. X* 4:100031. doi: 10.1016/j.wroa.2019.100031

Denner, E. B., Mark, B., Busse, H. J., Turkiewicz, M., and Lubitz, W. (2001). *Psychrobacter proteolyticus* sp. nov., a psychrotrophic, halotolerant

bacterium isolated from the Antarctic krill *Euphausia superba* Dana, excreting a cold-adapted metalloprotease. *Syst. Appl. Microbiol.* 24, 44–53. doi: 10.1078/0723-2020-00006

Ducasse-Cabanot, S., Zambonino-Infante, J., Richard, N., Medale, F., Corraze, G., Mambrini, M., et al. (2007). Reduced lipid intake leads to changes in digestive enzymes in the intestine but has minor effect on key enzymes of hepatic intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*). *Animal* 1, 1272–1282. doi: 10.1017/S1751731107000596

Dvergedal, H., Sandve, S. R., Angell, I. L., Klemetsdal, G., and Rudi, K. (2020). Association of gut microbiota with metabolism in juvenile Atlantic salmon. *Microbiome* 8:160. doi: 10.1186/s40168-020-00938-2

Egerton, S., Wan, A., Murphy, K., Collins, F., Ahern, G., Sugrue, I., et al. (2020). Replacing fishmeal with plant protein in Atlantic salmon (*Salmo salar*) diets by supplementation with fish protein hydrolysate. *Sci. Rep.* 10:4194. doi: 10.1038/s41598-020-60325-7

El-Abd, M. A., and Ibrahim, E. A. (2020). Production and one-step purification of serratiopeptidase enzyme from *Serratia marcescens* with potent anti-inflammatory and antioxidant power. *Egypt Pharmaceut. J.* 19, 238–243. doi: 10.4103/epj.epj_65_19

Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Grammes, F., Martos-Sitcha, J. A., Mydland, L. T., et al. (2016). Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. *PLoS ONE* 11:e0166564. doi: 10.1371/journal.pone.0166564

Estensoro, I., Calduch-Giner, J. A., Kaushik, S., Pérez-Sánchez, J., and Sitjà-Bobadilla, A. (2012). Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (*Myxozoa*). *Fish Shellfish Immunol.* 33, 401–410. doi: 10.1016/j.fsi.2012.05.029

Estruch, G., Collado, M. C., Peñaranda, D. S., Tomás Vidal, A., Jover Cerdá, M., Pérez Martínez, G., et al. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PLoS ONE* 10:e0136389. doi: 10.1371/journal.pone.0136389

Eteshola, E. O. U., Haupt, D. A., Koos, S. I., Siemer, L. A., and Morris Jr, D. L. (2020). The role of metal ion binding in the antioxidant mechanisms of reduced and oxidized glutathione in metal-mediated oxidative DNA damage. *Metallomics* 12:79. doi: 10.1039/c9mt00231f

Fernandes, A. M., Conceição, L. E. C., Calduch-Giner, J. A., Silva, B., Pereira, G. V., Costas, B., et al. (2021). "Evaluation of growth performance, oxidative stress and immune response response in gilthead seabream fed novel feed formulations," in *Proceedings of the Book of Abstracts of the Aquaculture Europe 2020 Congress,* 200–201.

Ferrer Llagostera, P., Kallas, Z., Reig, L., and Amores de Gea, D. (2019). The use of insect meal as a sustainable feeding alternative in aquaculture: current situation, Spanish consumers' perceptions and willingness to pay. *J. Clean. Prod.* 229, 10–21. doi: 10.1016/j.jclepro.2019.05.012

Firmino, J. P., Vallejos-Vidal, E., Balebona, M. C., Ramayo-Caldas, Y., Cerezo, I. M., Salomón, R., et al. (2021). Diet, immunity, and microbiota interactions: an integrative analysis of the intestine transcriptional response and microbiota modulation in gilthead seabream (*Sparus aurata*) fed an essential oils-based functional diet. *Front. Immunol.* 12:625297. doi: 10.3389/fimmu.2021.625297

Fukata, M., and Arditi, M. (2013). The role of pattern recognition receptors in intestinal inflammation. *Mucosal Immunol.* 6, 451–463. doi: 10.1038/mi.2013.13

Gajardo, K., Jaramillo-Torres, A., Kortner, T. M., Merrifield, D. L., Tinsley, J., Bakke, A. M., et al. (2017). Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*). *Appl. Environ. Microbiol.* 83, e02615–02616. doi: 10.1128/AEM.02615-16

Ghanbari, M., Kneifel, W., and Domig, K. J. (2015). A new view of the fish gut microbiome: advances from next-generation sequencing. *Aquaculture* 448, 464–475. doi: 10.1016/j.aquaculture.2015.06.033

Giri, S. S., Ryu, E. C., Sukumaran, V., and Park, S. C. (2019). Antioxidant, antibacterial, and antiadhesive activities of biosurfactants isolated from *Bacillus strains*. *Microb. Pathog.* 132, 66–72. doi: 10.1016/j.micpath.2019.04.035

Gisbert, E., Andree, K., Quintela, J., Calduch-Giner, J., Ipharraguerre, I., and Pérez-Sánchez, J. (2017). Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* 117, 351–363. doi: 10.1017/S0007114517000228

Grigg, J. B., and Sonnenberg, G. F. (2017). Host-microbiota interactions shape local and systemic inflammatory diseases. *J. Immunol.* 198, 564–571. doi: 10.4049/jimmunol.1601621

Hao, W. L., and Lee, Y. K. (2004). Microflora of the gastrointestinal tract: a review. *Methods Mol. Biol.* 268, 491–502. doi: 10.1385/1-59259-766-1-491

Ho, J. T., Chan, G. C., and Li, J. C. (2015). Systemic effects of gut microbiota and its relationship with disease and modulation. *BMC Immunol.* 16:21. doi: 10.1186/s12865-015-0083-2

Hua, K., Cobcroft, J. M., Cole, A., Condon, K., Jerry, D. R., Mangott, A., et al. (2019). The future of aquatic protein: implications for protein sources in aquaculture diets. *One Earth* 1, 316–329. doi: 10.1016/j.oneear.2019.10.018

Iwai, S., Weinmaier, T., Schmidt, B. L., Albertson, D. G., Poloso, N. J., Dabbagh, K., et al. (2016). Piphillin: improved prediction of metagenomic content by direct inference from human microbiomes. *PLoS ONE* 11:e0166104. doi: 10.1371/journal.pone.0166104

Janssens, M., Myter, N., De Vuyst, L., and Leroy, F. (2012). Species diversity and metabolic impact of the microbiota are low in spontaneously acidified Belgian sausages with an added starter culture of *Staphylococcus carnosus*. *Food Microbiol.* 29, 167–177. doi: 10.1016/j.fm.2011.07.005

Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. *Front. Immunol.* 5:461. doi: 10.3389/fimmu.2014.00461

Kida, Y., Inoue, H., Shimizu, T., and Kuwano, K. (2007). *Serratia marcescens* serralysin induces inflammatory responses through protease-activated receptor 2. *Infect. Immun.* 75, 164–174. doi: 10.1128/IAI.01239-06

Kieffer, D. A., Piccolo, B. D., Vaziri, N. D., Liu, S., Lau, W. L., Khazaeli, M., et al. (2016). Resistant starch alters gut microbiome and metabolomic profiles concurrent with amelioration of chronic kidney disease in rats. *Am. J. Physiol. Renal Physiol.* 310, F857–F871. doi: 10.1152/ajprenal.00513.2015

Kindt, A., Liebisch, G., Clavel, T., Haller, D., Hörmannsperger, G., Yoon, H., et al. (2018). The gut microbiota promotes hepatic fatty acid desaturation and elongation in mice. *Nat. Commun.* 9:3760. doi: 10.1038/s41467-018-05767-4

Koo, H., Hakim, J. A., Powell, M. L., Kumar, R., Eipers, P. G., Morrow, C. D., et al. (2017). Metagenomics approach to the study of the gut microbiome structure and function in zebrafish *Danio rerio* fed with gluten formulated diet. *J. Microbiol. Methods* 135, 69–76. doi: 10.1016/j.mimet.2017.01.016

Krogdahl, Å., Gajardo, K., Kortner, T. M., Penn, M., Gu, M., Berge, G. M., et al. (2015). Soya saponins induce enteritis in Atlantic salmon (*Salmo salar* L.). *J. Agric. Food Chem.* 63, 3887–3902. doi: 10.1021/jf506242t

Lamichhane, S., Sen, P., Alves, M. A., Ribeiro, H. C., Raunioniemi, P., Hyötyläinen, T., et al. (2021). Linking gut microbiome and lipid metabolism: moving beyond associations. *Metabolites* 11:55. doi: 10.3390/metabo11010055

Lang, T., Hansson, G. C., and Samuelsson, T. (2007). Gel-forming mucins appeared earlyin metazoan evolution. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16209–16214. doi: 10.1073/pnas.0705984104

Levast, B., Li, Z., and Madrenas, J. (2015). The role of IL-10 in microbiome-associated immune modulation and disease tolerance. *Cytokine* 75, 291–301. doi: 10.1016/j.cyto.2014.11.027

Li, H., Limenitakis, J., Fuhrer, T., Geuking, M. B., Lawson, M. A., Wyss, M., et al. (2015). The outer mucus layer hosts a distinct intestinal microbial niche. *Nat. Commun.* 6:8292. doi: 10.1038/ncomms9292

Li, H., Ma, M.-L., Luo, S., Zhang, R.-M., Han, P., and Hu, W. (2012). Metabolic responses to ethanol in *Saccharomyces cerevisiae* using a gas chromatography tandem mass spectrometry-based metabolomics approach. *Int. J. Biochem. Cell Biol.* 44, 1087–1096. doi: 10.1016/j.biocel.2012.03.017

Liu, M., Song, S., Hu, C., Tang, L., Lam, J. C. W., Lam, P. K. S., et al. (2020). Dietary administration of probiotic *Lactobacillus rhamnosus* modulates the neurological toxicities of perfluorobutanesulfonate in zebrafish. *Environ. Pollut.* 265:114832. doi: 10.1016/j.envpol.2020.114832

Liu, T., and Zhang, X. (2021). Transcriptome and metabolomic analyses reveal regulatory networks controlling maize stomatal development in response to blue light. *Int. J. Mol. Sci.* 22:5393. doi: 10.3390/ijms22105393

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. doi: 10.1186/s13059-014-0550-8

Macara, I. G., Guyer, R., Richardson, G., Huo, Y., and Ahmed, S. M. (2014). Epithelial homeostasis. *Curr. Biol.* 24, R815–R825. doi: 10.1016/j.cub.2014.06.068

Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., and Peres, H. (2017). Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture* 476, 79–85. doi: 10.1016/j.aquaculture.2017.04.021

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Gonçalves, J., Rocha, C., et al. (2017). Dietary supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (*Sparus aurata*). *Biol. Open* 6, 897–908. doi: 10.1242/bio.024299

Mahmood, A., Shao, J. S., and Alpers, D. H. (2003). Rat enterocytes secrete SLPs containing alkaline phosphatase and cubilin in response to corn oil feeding. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285, G433–441. doi: 10.1152/ajpgi.00466.2002

Maldonado-Othón, C. A., Pérez-Velázquez, M., Gatlin, D. M., and González-Félix, M. L. (2020). Replacement of fish oil by soybean oil and microalgal meals in diets for *Totoaba macdonaldi* (Gilbert, 1890) juveniles. *Aquaculture* 529, 735705. doi: 10.1016/j.aquaculture.2020.7357

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A., and Zenger, K. R. (2019). Methods for normalizing microbiome data: an ecological perspective. *Methods Ecol. Evol.* 10, 389–400. doi: 10.1111/2041-210X.13115

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Mekuchi, M., Asakura, T., Sakata, K., Yamaguchi, T., Teruya, K., and Kikuchi, J. (2018). Intestinal microbiota composition is altered according to nutritional biorhythms in the leopard coral grouper (*Plectropomus leopardus*). *PLoS ONE* 13:e0197256. doi: 10.1371/journal.pone.0197256

Mendes, V., Galvão, I., and Vieira, A. T. (2019). Mechanisms by which the gut microbiota influences cytokine production and modulates host inflammatory responses. *J. Interf. Cytok. Res.* 39, 393–409. doi: 10.1089/jir.2019.0011

Millán, J. L. (2006). Alkaline Phosphatases: structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signal.* 2, 335–341. doi: 10.1007/s11302-005-5435-6

Mingmongkolchai, S., and Panbangred, W. (2018). *Bacillus* probiotics: an alternative to antibiotics for livestock production. *J. Appl. Microbiol.* 124, 1334–1346. doi: 10.1111/jam.13690

Moroni, F., Naya-Català, F., Piazzon, M. C., Rimoldi, S., Calduch-Giner, J., Giardini, A., et al. (2021). Effects of nisin-producing *Lactococcus lactis* strain used as probiotic on gilthead sea bream (*Sparus aurata*) growth, gut microbiota, and transcriptional response. *Front. Mar. Sci.* 8:659519. doi: 10.3389/fmars.2021.659519

Mottillo, E. P., Bloch, A. E., Leff, T., and Granneman, J. G. (2012). Lipolytic products activate peroxisome proliferator-activated receptor (PPAR) α and δ in brown adipocytes to match fatty acid oxidation with supply. *J. Biol. Chem.* 287, 25038–25048. doi: 10.1074/jbc.M112.374041

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol.* 29, 2–14. doi: 10.1016/j.fsi.2010.02.017

Nebo, C., Overturf, K., Brezas, A., Dal-Pai-Silva, M., and Portella, M. C. (2017). Alteration in expression of atrogenes and IGF-1 induced by fasting in Nile tilapia *Oreochromis niloticus* juveniles. *Int. Aquat. Res.* 9, 361–372. doi: 10.1007/s40071-017-0182-1

Nichols, R. G., and Davenport, E. R. (2021). The relationship between the gut microbiome and host gene expression: a review. *Human Genet.* 140, 747–760. doi: 10.1007/s00439-020-02237-0

Nikouli, E., Meziti, A., Antonopoulou, E., Mente, E., and Kormas, K. A. (2018). Gut bacterial communities in geographically distant populations of farmed sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). *Microorganisms* 6, 92. doi: 10.3390/microorganisms6030092

Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10:277. doi: 10.3389/fimmu.2019.00277

Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., et al. (2016). Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (*Sparus aurata*, L.) fed low fishmeal based diets with increasing soybean meal levels. *Anim. Feed. Sci. Tech.* 222, 204–216. doi: 10.1016/j.anifeedsci.2016.10.022

Pawlak, M., Lefebvre, P., and Staels, B. (2015). Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J. Hepatol.* 62, 720–733. doi: 10.1016/j.jhep.2014.10.039

Peixoto, M. J., Ferraz, R., Magnoni, L. J., Pereira, R., Gonçalves, J. F., Calduch-Giner, J., et al. (2019). Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to bacterial infection. *Sci. Rep.* 9:16134. doi: 10.1038/s41598-019-52693-6

Pelusio, N. F., Scicchitano, D., Parma, L., Dondi, F., Brini, E., D'Amico, F., et al. (2021). Interaction between dietary lipid level and seasonal temperature changes in gilthead sea bream *Sparus aurata*: effects on growth, fat deposition, plasma biochemistry, digestive enzyme activity, and gut bacterial community. *Front. Mar. Sci.* 8:664701. doi: 10.3389/fmars.2021.664701

Pereira, H., Sardinha, M., Santos, T., Gouveia, L., Barreira, L., Dias, J., et al. (2020). Incorporation of defatted microalgal biomass (*Tetraselmis* sp. CTP4) at the expense of soybean meal as a feed ingredient for juvenile gilthead seabream (*Sparus aurata*). *Algal Res.* 47:101869. doi: 10.1016/j.algal.2020.101869

Perera, E., Turkmen, S., Simó-Mirabet, P., Zamorano, M. J., Xu, H., Naya-Català, F., et al. (2020). Stearoyl-CoA desaturase (*scd1a*) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). *Epigenetics* 15, 536–553. doi: 10.1080/15592294.2019.1699982

Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J. À., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2014). Interleukin gene expression is strongly modulated at the local levelin afisheparasite model. *Fish Shell. Immunol.* 37, 201–2018. doi: 10.1016/j.fsi.2014.01.022

Pérez-Sánchez, J., Estensoro, I., Redondo, M. J., Calduch-Giner, J. A., Kaushik, S., and Sitjà-Bobadilla, A. (2013). Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. *PLoS ONE* 8:e65457. doi: 10.1371/journal.pone.0065457

Pérez-Sánchez, J., Naya-Català, F., Soriano, B., Piazzon, M. C., Hafez, A., Gabaldón, T., et al. (2019). Genome sequencing and transcriptome analysis reveal recent species-specific gene duplications in the plastic gilthead sea bream (*Sparus aurata*). *Front. Mar. Sci.* 6:760. doi: 10.3389/fmars.2019.00760

Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha, J. A., Perera, E., Bermejo-Nogales, A., et al. (2018). Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Front. Endocrinol.* 9:687. doi: 10.3389/fendo.2018.00687

Piazzon, C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gómez-Casado, E., et al. (2016). Differential Modulation of IgT and IgM upon parasitic, bacterial, viral and dietary challenges in a perciform fish. *Front. Immunol.* 7:637. doi: 10.3389/fimmu.2016.00637

Piazzon, M. C., Calduch-Giner, J. A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., et al. (2017). Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* 5:164. doi: 10.1186/s40168-017-0390-3

Piazzon, M. C., Naya-Català, F., Perera, E., Palenzuela, O., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2020). Genetic selection for growth drives differences in intestinal microbiota composition and parasite disease resistance in gilthead sea bream. *Microbiome* 8:168. doi: 10.1186/s40168-020-00922-w

Piazzon, M. C., Naya-Català, F., Simó-Mirabet, P., Picard-Sánchez, A., Roig, F. J., Calduch-Giner, J. A., et al. (2019). Sex, age, and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite fish. *Front. Microbiol.* 10:2512. doi: 10.3389/fmicb.2019.02512

Piazzon, M. C., Savelkoul, H. S., Pietretti, D., Wiegertjes, G. F., and Forlenza, M. (2015). Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. *J. Immunol.* 194, 187–199. doi: 10.4049/jimmunol.1402093

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., et al. (2000). *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic,

alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavitherms* comb. nov. *Int. J. Syst. Evol. Microbiol.* 50, 2109–2117. doi: 10.1099/00207713-50-6-2109

Poelstra, K., Bakker, W. W., Klok, P. A., Kamps, J. A., Hardonk, M. J., and Meijer, D. K. (1997). Dephosphorylation of endotoxin by alkaline phosphatase *in vivo*. *Am. J. Clin. Pathol.*151, 1163–1169.

Rader, B. A. (2017). Alkaline phosphatase, an unconventional immune protein. *Front. Immunol.* 8:897. doi: 10.3389/fimmu.2017.00897

Ray, A. E., Connon, S. A., Neal, A. L., Fujita, Y., Cummings, D. E., Ingram, J. C., et al. (2018). Metal transformation by a novel *Pelosinus* isolate from a subsurface environment. *Front. Microbiol.* 9:1689. doi: 10.3389/fmicb.2018.01689

Reis, B., Ramos-Pinto, L., Martos-Sitcha, J. A., Machado, M., Azeredo, R., Fernández-Boo, S., et al. (2021). Health status in gilthead seabream (*Sparus aurata*) juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*. *J. App. Phycol.* 33, 979–996. doi: 10.1007/s10811-021-02377-4

Reyes-Becerril, M., Angulo, C., and Ascencio, F. (2015). Humoral immune response and TLR9 gene expression in Pacific red snapper (*Lutjanus peru*) experimentally exposed to *Aeromonas veronii*. *Fish Shellfish Immunol.* 42, 289–296. doi: 10.1016/j.fsi.2014.11.002

Reyes-Cerpa, S., Reyes-López, F., Toro-Ascuy, D., Montero, R., Maisey, K., Acuña-Castillo, C., et al. (2014). Induction of anti-inflammatory cytokine expression by IPNV in persistent infection. *Fish Shellfish Immunol.* 41, 172–182. doi: 10.1016/j.fsi.2014.08.029

Rhayat, L., Maresca, M., Nicoletti, C., Perrier, J., Brinch, K. S., Christian, S., et al. (2019). Effect of *Bacillus subtilis* strains on intestinal barrier function and inflammatory response. *Front. Immunol.* 10:564. doi: 10.3389/fimmu.2019.00564

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., and Terova, G. (2019). The effects of dietary insect meal from *Hermetia illucens prepupae* on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). *Animals* 9:143. doi: 10.3390/ani9040143

Rimoldi, S., Gini, E., Koch, J. F. A., Iannini, F., Brambilla, F., and Terova, G. (2020). Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (*Sparus aurata*) diet, on fish intestinal microbiome. *BMC Vet. Res.* 16:118. doi: 10.1186/s12917-020-02335-1

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Romarheim, O. H., Hetland, D. L., Skrede, A., Øverland, M., Mydland, L. T., and Landsverk, T. (2013). Prevention of soya-induced enteritis in Atlantic salmon (*Salmo salar*) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8α+ intraepithelial lymphocytes. *Br. J. Nutr.* 109, 1062–1070. doi: 10.1017/S0007114512002899

Sabry, M., Abd El-Moein, K., Hamza, E., and Abdel Kader, F. (2016). Occurrence of *Clostridium perfringens* types A, E, and C in fresh fish and its public health significance. *J. Food Prot.* 79, 994–1000. doi: 10.4315/0362-028X.JFP-15-569

Saeki, K., Endo, K., Tasaka, K., and Yamasaki, H. (1974). Anti-inflammatory polysaccharide produced by *Serratia piscatorum*. *Jpn. J. Pharmacol.* 24, 109–118. doi: 10.1254/jjp.24.109

Sánchez-Muros, M., de Haro, C., Sanz, A., Trenzado, C. E., Villareces, S., and Barroso, F. G. (2016). Nutritional evaluation of *Tenebrio molitor* meal as fishmeal substitute for tilapia (*Oreochromis niloticus*) diet. *Aquac. Nutr.* 22, 943–955. doi: 10.1111/anu.12313

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27, 863–864. doi: 10.1093/bioinformatics/btr026

Schoeler, M., and Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. *Rev. Endocr. Metab. Disord.* 20, 461–472. doi: 10.1007/s11154-019-09512-0

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Seghouani, H., Garcia-Rangel, C. E., Füller, J., Gauthier, J., and Derome, N. (2017). Walleye autochthonous bacteria as promising probiotic candidates against *Flavobacterium columnare*. *Front. Microbiol.* 8:1349. doi: 10.3389/fmicb.2017.01349

Sekirov, I., Russell, S. L., Antunes, L. C., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904. doi: 10.1152/physrev.00045.2009

Seropian, I. M., González, G. E., Maller, S. M., Berrocal, D. H., Abbate, A., and Rabinovich, G. A. (2018). Galectin-1 as an emerging mediator of cardiovascular inflammation: mechanisms and therapeutic opportunities. *Mediat. Inflamm.* 8696543, 1–11. doi: 10.1155/2018/8696543

Silva, F. C., de, P., Nicoli, J. R., Zambonino-Infante, J. L., Kaushik, S., and Gatesoupe, F. J. (2011). Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*). *FEMS Microbiol. Ecol.* 78, 285–296. doi: 10.1111/j.1574-6941.2011.01155.x

Simó-Mirabet, P., Piazzon, M. C., Calduch-Giner, J. A., Ortíz, A., Puyalto, M., Sitjà-Bobadilla, A., et al. (2017). Sodium salt medium-chain fatty acids and *Bacillus*-based probiotics strategies to improve growth and intestinal health of gilthead sea bream (*Sparus aurata*). *PeerJ* 5:e4001. doi: 10.7717/peerj.4001

Simon, C. J., Blyth, D., Ahmad Fatan, N., and Suri, S. (2019). Microbial biomass (NovacqTM) stimulates feeding and improves the growth performance on extruded low to zero-fishmeal diets in tilapia (GIFT strain). *Aquaculture* 501, 319–324. doi: 10.1016/j.aquaculture.2018.11.052

Simon, C. J., Truong, H. H., Noble, T. H., Osborne, S. A., Wynne, J. W., and Wade, N. M. (2020). Microbial biomass, marine invertebrate meals and feed restriction influence the biological and gut microbiota response of shrimp *Penaeus monodon*. *Aquaculture* 520:734679. doi: 10.1016/j.aquaculture.2019.734679

Singh, V., Chassaing, B., Zhang, L., San Yeoh, B., Xiao, X., Kumar, M., et al. (2015). Microbiota-dependent hepatic lipogenesis mediated by stearoyl CoA desaturase 1 (SCD1) promotes metabolic syndrome in TLR5-deficient mice. *Cell Metab.* 22, 983–996. doi: 10.1016/j.cmet.2015.09.028

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. L., and Ideker, T. (2011). Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* 27, 431–432. doi: 10.1093/bioinformatics/btq675

Solé-Jiménez, P., Naya-Català, F., Piazzon, M. C., Estensoro, I., Calduch-Giner, J. À., Sitjà-Bobadilla, A., et al. (2021). Reshaping of gut microbiota in gilthead sea bream fed microbial and processed animal proteins as the main dietary protein source. *Front. Mar. Sci.* 8:705041. doi: 10.3389/fmars.2021.705041

Speirs, L. B. M., Rice, D. T. F., Petrovski, S., and Seviour, R. J. (2019). The phylogeny, biodiversity, and ecology of the chloroflexi in activated sludge. *Front. Microbiol.* 10:2015. doi: 10.3389/fmicb.2019.02015

Starliper, C. E. (2011). Bacterial coldwater disease of fishes caused by*Flavobacterium psychrophilum*. *J. Adv. Res.* 2, 97–108. doi: 10.1016/j.jare.2010.04.001

Tacon, A., and Metian, M. (2015). Feed matters: satisfying the feed demand of aquaculture. *Trends Food Sci. Technol.* 23, 1–10. doi: 10.1080/23308249.2014.987209

Tang, D., Gao, Y., Wang, R., Sun, Y., and Xu, T. (2012). Characterization, genomic organization, and expression profiles of MyD88, a key adaptor molecule in the TLR signaling pathways in miiuy croaker (*Miichthys miiuy*). *Fish Physiol. Biochem.* 38, 1667–1677. doi: 10.1007/s10695-012-9663-8

Thévenot, E. A., Roux, A., Xu, Y., Ezan, E., and Junot, C. (2015). Analysis of the human adult urinary metabolome variations with age, body mass index, and gender by implementing a comprehensive workflow for univariate and OPLS statistical analyses. *J. Prot. Res.* 14, 3322–3335. doi: 10.1021/acs.jproteome.5b00354

Uengwetwanit, T., Uawisetwathana, U., Arayamethakorn, S., Khudet, J., Chaiyapechara, S., Karoonuthaisiri, N., et al. (2020). Multi-omics analysis to examine microbiota, host gene expression and metabolites in the intestine of black tiger shrimp (*Penaeus monodon*) with different growth performance. *PeerJ* 8:e9646. doi: 10.7717/peerj.9646

Urán, P. A., Schrama, J. W., Rombout, J. H., Taverne-Thiele, J. J., Obach, A., Koppe, W., et al. (2009). Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo salar* L., at two different soybean meal inclusion levels. *J. Fish. Dis.* 32, 733–744. doi: 10.1111/j.1365-2761.2009.01049.x

Wang, J., Thingholm, L., Skieceviciene, J., Rausch, P., Kummen, M., Hov, J., et al. (2017). Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat. Genet.* 546, 651–655. doi: 10.1038/nature22814.Trans-kingdom

Weiss, S., Van Treuren, W., Lozupone, C., Faust, K., Friedman, J., Deng, Y., et al. (2016). Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. *ISME J.* 10, 1669–1681. doi: 10.1038/ismej.2015.235

Welker, T. L., Lim, C., Yildirim-Aksoy, M., Shelby, R., and Klesius, P. H. (2007). Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents. *J. World Aquacult. Soc.* 38, 24–35. doi: 10.1111/j.1749-7345.2006.00070.x

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., et al. (2019). Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). *Microb. Biotechnol.* 12, 275–288. doi: 10.1111/1751-7915.13323

Wold, S., Sjöström, M., and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemom. Intell. Lab. Syst.* 58, 109–130. doi: 10.1016/S0169-7439(01)00155-1

Wu, Y. B., Ren, X., Chai, X. J., Li, P., and Wang, Y. (2018). Replacing fish meal with a blend of poultry by-product meal and feather meal in diets for giant croaker (*Nibea japonica*). *Aquacult. Nutr.* 24, 1085–1091. doi: 10.1111/anu.12647

Xia, J. H., Lin, G., Fu, G. H., Wan, Z. Y., Lee, M., Wang, L., et al. (2014). The intestinal microbiome of fish under starvation. *BMC Genomics* 15:266. doi: 10.1186/1471-2164-15-266

Yan, J., Qian, L., Zhu, W., Qiu, J., Lu, Q., Wang, X., et al. (2020). Integrated analysis of the transcriptome and metabolome of purple and green leaves of *Tetrastigma hemsleyanum* reveals gene expression patterns involved in anthocyanin biosynthesis. *PLoS ONE* 15:e0230154. doi: 10.1371/journal.pone.0230154

Yang, H. L., Sun, Y. Z., Ma, R. L., Li, J. S., and Huang, K. P. (2011). Probiotic *Psychrobacter* sp. improved the autochthonous microbial diversity along the gastrointestinal tract of grouper *Epinephelus coioides*. *J. Aquac. Res. Dev.* S1:001. doi: 10.4172/2155-9546.S1-001

Yang, Y., Gharaibeh, R. Z., Newsome, R. C., and Jobin, C. (2020). Amending microbiota by targeting intestinal inflammation with TNF blockade attenuates development of colorectal cancer. *Nat. Cancer* 1,723–734. doi: 10.1038/s43018-020-0078-7

Yildirimer, C. C., and Brown, K. H. (2018). Intestinal microbiota lipid metabolism varies across rainbow trout (*Oncorhynchus mykiss*) phylogeographic divide. *J. Appl. Microbiol.* 125, 1614–1625. doi: 10.1111/jam.14059

Ytrestøyl, T., Aas, T. S., and Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* 448, 365–374. doi: 10.1016/j.aquaculture.2015.06.023

Yu, L., Sun, G., Wei, J., Wang, Y., Du, C., and Li, J. (2016). Activation of macrophages by an exopolysaccharide isolated from Antarctic *Psychrobacter*sp. B-3. *Chin. J. Ocean Limnol.* 34, 1064–1071. doi: 10.1007/s00343-016-4393-x

Zhao, C., Su, W., Mu, Y., Mu, Y., and Jiang, L. (2021). Integrative metagenomics–metabolomics for analyzing the relationship between microorganisms and non-volatile profiles of traditional Xiaoqu. *Front. Microbiol.* 11:617030. doi: 10.3389/fmicb.2020.617030

Zhou, X., Ling, X., Guo, H., Zhu-Salzman, K., Ge, F., and Sun, Y. (2021). *Serratia symbiotica* enhances fatty acid metabolism of pea aphid to promote host development. *Int. J. Mol. Sci.* 22:5951. doi: 10.3390/ijms22115951

Zhou, Y., Zeng, Z., Xu, Y., Ying, J., Wang, B., Majeed, M., et al. (2020). Application of *Bacillus coagulans* in animal husbandry and its underlying mechanisms. *Animals* 10:454. doi: 10.3390/ani10030454

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Biofortified Diets Containing Algae and Selenised Yeast: Effects on Growth Performance, Nutrient Utilization, and Tissue Composition of Gilthead Seabream (*Sparus aurata*)

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Efforts have been made to find natural, highly nutritious alternatives to replace fish meal (FM) and fish oil (FO), which can simultaneously promote fish health and improve the nutritional quality of filets for human consumption. This study evaluated the impact of biofortified diets containing microalgae (as replacement for FM and FO), macroalgae (as natural source of iodine) and selenised yeast (organic source of selenium) on gilthead seabream growth, nutrient utilization, tissue composition and gene expression. A control diet (CTRL) with 15% FM and 5.5% FO was compared with three experimental diets (AD1, AD2, and AD3), where a microalgae blend (*Chlorella* sp., *Tetraselmis* sp., and DHA-rich *Schizochytrium* sp.) replaced 33% of FM. Diet AD1 contained 20% less FO. Diets were supplemented with *Laminaria digitata* (0.4% AD1 and AD2; 0.8% AD3) and selenised yeast (0.02% AD1 and AD2; 0.04% AD3). After feeding the experimental diets for 12 weeks, growth was similar in fish fed AD1, AD2, and CTRL, indicating that microalgae meal can partially replace both FM and FO in diets for seabream. But AD3 suppressed fish growth, suggesting that *L. digitata* and selenised yeast supplementation should be kept under 0.8 and 0.04%, respectively. Despite lower lipid intake and decreased PUFAs bioavailability in fish fed AD3, compared to CTRL, hepatic *elovl5* was upregulated resulting in a significant increase of muscle EPA + DHA. Indeed, filets of fish fed AD2 and AD3 provided the highest EPA + DHA contents (0.7 g 100 g^{-1}), that are well above the minimum recommended values for human consumption. Fish consuming the AD diets had a higher retention and gain of selenium, while iodine gain remained similar among diets. Upregulation of selenoproteins (*gpx1*, *selk,* and *dio2*) was observed in liver of fish fed AD1, but diets had limited impact on fish antioxidant status. Overall,

results indicate that the tested microalgae are good sources of protein and lipids, with their LC-PUFAs being effectively accumulated in seabream muscle. Selenised yeast is a good fortification vehicle to increase selenium levels in fish, but efforts should be placed to find new strategies to fortify fish in iodine.

Keywords: iodine, *Laminaria digitata*, microalgae, omega-3 fatty acids, selenium

INTRODUCTION

Fish are major dietary sources of high-quality proteins, vitamins (i.e., D, A and B12), n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), mainly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), iodine, selenium and iron for human consumption (EFSA, 2014), so farmed products must also provide adequate levels of such nutrients for consumers.

Gilthead seabream (*Sparus aurata*) is one of the main marine fish species for aquaculture production in the Mediterranean region (FAO, 2020). Plant-based products are now commonly used as substitutes for fish meal (FM) and fish oil (FO) in diets for several carnivorous farmed fish, but previous studies have shown that complete, or even partial, replacement of FM and FO with plant proteins (PP) and vegetable oils (VOs) in diets for *S. aurata* can compromise fish health and nutritional value of filets for human consumption (Izquierdo et al., 2005; Montero et al., 2008; Matos et al., 2017; Estruch et al., 2018). In this context, diets for farmed fish using natural products, such as selected microalgae (rich in nutrients including LC-PUFAs and bioactive compounds), seaweeds (rich in iodine) and selenised yeast (rich in selenium), with proven beneficial effects for both fish performance and filet quality, are of interest to both the scientific community and aquaculture sector (Vizcaíno et al., 2016; Ribeiro et al., 2017; Wang et al., 2018; Granby et al., 2020; Valente et al., 2021). However, fortification efficiency for a given nutrient is a complex process depending on many factors including origin, dose, form, and potential interactions with other dietary components (Ramos et al., 2008; Ribeiro et al., 2017; Saffari et al., 2017). Hence, new fortification strategies are required to maximize deposition of beneficial compounds contributing to the functional foods market in parallel with securing fish health.

The use of microalgae in fish diets has been explored previously, as promising sources of nutrients and bioactive compounds (Valente et al., 2021). Microalgae have been used successfully as partial replacements for FM and FO in diets for marine fish (Tibaldi et al., 2015; Cardinaletti et al., 2018; Batista et al., 2020). In European seabass (*Dicentrarchus labrax*), growth was not affected by the replacement of (45%) protein and (36%) lipids from FM with a blend of *Tisochrysis lutea* and *Tetraselmis suecica* (Cardinaletti et al., 2018). Seong et al. (2021) observed good results in terms of growth performance with the inclusion of mixed microalgae (*Schizochytrium* sp., *Nannochloropsis* sp. and *Chlorella* sp.) in non-FM and non-FO diets for red sea bream (*Pagrus major*), at an inclusion level of 45%. Thus, *Tetraselmis* sp. and *Chlorella* sp., due to their high protein content (35–60% dry weight), are potential candidates for inclusion in aquafeeds (Acquah et al., 2020). Certain microalgae, specifically *Schizochytrium* sp., are also rich sources of lipids and n-3 LC-PUFAs, such as docosahexaenoic acid (DHA) (Tocher, 2015), which together with eicosapentaenoic acid (EPA) have a crucial physiological role in both humans and fish (Swanson et al., 2012). In gilthead seabream larvae, dietary substitution of FO with *Schizochytrium* sp. resulted in whole-body DHA concentrations similar to those in fish fed FO-only diets (Ganuza et al., 2008). Like many other marine fish, the *S. aurata* capacity to synthesize highly unsaturated fatty acids (HUFAs) is limited, due to its low $\Delta 5$ desaturase activity (Tocher, 2010, 2015). However, several studies have shown that genes involved in synthesis of these fatty acids, namely desaturases and elongases, can be modulated nutritionally (Izquierdo et al., 2008; Benedito-Palos et al., 2014; Houston et al., 2017).

Macroalgae are also rich sources of bioactive compounds and their inclusion in aquafeeds has been associated with beneficial effects, including improved fish growth, welfare, and nutritional content of filets (Dantagnan et al., 2009; Wassef et al., 2013; Zeynali et al., 2020). Iodine-rich brown algae, such as *Saccharina latissima* and *Laminaria digitata*, have been shown to be promising vehicles for fortifying filets with iodine (Ribeiro et al., 2015, 2017; Granby et al., 2020). But previous reports have also evidenced impacts on lipid metabolism among several fish species, as a result of including macroalgae in diets (Xuan et al., 2013; Morshedi et al., 2018). In rainbow trout, for example, the dietary inclusion of *S. latissima* led to a downregulation of hepatic fatty acid synthase (*fas*) and a general reduction in fatty acid filet content. Nevertheless, EPA and DHA filet content did not decrease with inclusion of macroalgae, indicating a selective retention of these essential fatty acids in the muscle (Ferreira et al., 2020). Both micro- and macroalgae are also natural sources of compounds with bioactive properties, specifically carotenoids and polyphenols, capable of improving fish antioxidant status (Magnoni et al., 2017; Peixoto et al., 2019; Teimouri et al., 2019).

Selenium is an essential micronutrient involved in several biological processes in fish. Evidence suggests that organic Se has a higher bioavailability than inorganic forms (Khan et al., 2017; Sele et al., 2018) and is more easily incorporated into selenoproteins, involved in thyroid hormones metabolism (deiodinases) and oxidative stress pathways (glutathione peroxidase) (Mariotti et al., 2012). Dietary supplementation with selenised yeast was associated with increased growth of rainbow trout (Ribeiro et al., 2017; Wang et al., 2018) and *Megalobrama amblycephala* (Liu et al., 2017). Wang et al. (2018) also reported upregulation of several selenoproteins, both in liver and muscle, which were positively correlated with growth. In gilthead seabream, little is known about the impact of dietary selenised yeast or other organic forms of selenium on fish growth and metabolism (Mechlaoui et al., 2019).

This study aimed to evaluate the impact of biofortified diets containing a blend of microalgae (*Chlorella* sp., *Tetraselmis* sp., and DHA-rich *Schizochytrium* sp.), as partial replacement of FM alone or FM and FO. These diets were also supplemented with a seaweed, *L. digitata* (0.4 and 0.8%), and selenised yeast (0.02 and 0.04%), as natural sources of iodine and selenium, respectively. The effects of these biofortification strategies on growth performance, nutrient utilization and filet composition were assessed. Expression of genes involved in growth, selenium and iodine metabolism, oxidative stress, and lipid metabolism pathways were also evaluated to provide further insights into the potential of natural products (algae and yeast) to modulate fish growth and metabolism, nutrient utilization, and filet nutritional value for human consumption.

MATERIALS AND METHODS

Ethics Statement

Fish trials were conducted in compliance with the guidelines of the European Union (Directive 2010/63/EU) and approved by the Ethical Committee of the EPPO-IPMA (Estação Piloto de Piscicultura de Olhão, Av. Parque Natural da Ria Formosa N, 8700-194, Olhão), overseen by the National Competence Authority.

Experimental Diets

Gilthead seabream were fed with four isonitrogenous (50% dry matter, DM) and isoenergetic (24 kJ g^{-1} DM) diets, formulated and extruded by Sparos Lda., Olhão, Portugal. Feed ingredients and diet compositions are summarized in **Table 1**: a control diet (CTRL) with moderate FM (15%) and FO (5.5%) contents was compared with three experimental diets (AD1, AD2. and AD3) with 33% FM replaced with microalgae meal (*Chlorella* sp., *Tetraselmis* sp. and DHA-rich *Schizochytrium* sp.) and reduced vegetable oil contents (11% reduction in soybean, rapeseed, and linseed in AD1 and between 21–23% reduction in AD2 and AD3). AD1 also contained 20% less FO. The CTRL diet contained 1.34 mg kg^{-1} of iodine and 0.76 mg kg^{-1} of selenium. All AD diets were enriched with both iodine-rich macroalgae (*L. digitata*) and selenised yeast: AD1 and AD2 contained the same amount of *L. digitata* and selenised yeast (0.4 and 0.02%, respectively), providing iodine levels at 40% maximum legal limit of 20 mg kg^{-1} (8.10 and 8.97 mg kg^{-1} DM, respectively); and selenium levels at 150% of CTRL diet (1.15 and 1.07 mg kg^{-1} DM, respectively); whereas AD3 was supplemented with 0.8% *L. digitata* and 0.04% selenised yeast, providing iodine levels at 70% maximum legal limit (14.59 mg kg^{-1} DM) and selenium levels at 185% of CTRL diet (1.41 mg kg^{-1} DM). The adoption of selenium levels above the maximum legal limit is justified by the interest to assess any potential beneficial and/or detrimental effects of this trace mineral in the context of a biofortification feeding strategy.

All formulated diets were isonitrogeneous and isoenergetic (**Table 1**). Moreover, as expected AD1 and AD2 had similar concentrations of iodine and selenium, that were 6- and 1.5-times higher than the CTRL, respectively. AD3 had 86% more

TABLE 1 | Ingredients and proximate composition of the experimental diets.

Ingredients (%)	CTRL	AD1	AD2	AD3
Fishmeal LT70[1]	15.0	10.0	10.0	10.0
Fish protein concentrate[2]	2.5	2.5	2.5	2.5
Porcine blood meal[3]	2.5	2.5	2.5	2.5
Microalgae meal (*Chlorella* sp.)[4]		5.0	5.0	5.0
Microalgae meal (*Tetraselmis* sp.)[5]		0.5	0.5	0.5
Microalgae meal (*Schizochytrium* sp.)[6]		3.2	3.2	3.2
Macroalgae meal (*Laminaria digitata*)[7]		0.4	0.4	0.8
Soy protein concentrate[8]	17.0	17.0	17.0	17.0
Wheat gluten[9]	12.0	12.0	12.0	12.0
Corn gluten meal[10]	8.0	8.0	8.0	8.0
Soybean meal 48[11]	8.0	8.0	8.0	8.0
Wheat meal[12]	16.6	13.9	13.9	13.5
Fish oil[13]	5.5	4.4	5.5	5.5
Soybean oil[14]	2.8	2.5	2.2	2.12
Rapeseed oil[14]	5.6	5.0	4.3	4.3
Linseed oil[14]	0.9	0.8	0.7	0.7
Vitamins and minerals premix[15]	1.1	1.1	1.1	1.1
Binder[16]	1.0	1.0	1.0	1.0
Antioxidant[17]	0.2	0.2	0.2	0.2
Sodium propionate [18]	0.1	0.1	0.1	0.1
Monoammonium phosphate[19]	0.5	1.0	1.0	1.0
Selenised yeast[20]		0.02	0.02	0.04
L-Tryptophan [21]	0.1	0.1	0.1	0.1
D,L-Methionine[22]	0.2	0.3	0.3	0.3
L-Taurine[23]	0.4	0.5	0.5	0.5
Proximate composition				
Dry matter (DM,%)	92.1	91.1	90.9	91.2
Crude protein (% DM)	50.5	50.5	50.7	50.4
Crude fat (% DM)	16.7	16.2	15.7	14.8
Ash (% DM)	5.7	5.7	6.0	5.7
Energy (kJ g^{-1} DM)	23.8	24.0	23.9	23.8
Iodine (mg kg^{-1} DM)	1.34	8.10	8.97	14.59
Selenium (mg kg^{-1} DM)	0.76	1.15	1.07	1.41

[1]NORVIK LT: 70.6% crude protein (CP), 5.8% crude fat (CF), Sopropêche, France;
[2] CPSP90: 86% CP, 6% CF, Sopropêche, France;
[3]Porcine blood meal: 89% CP, 1% CF, SONAC BV, Netherlands;
[4]Chlorella sp. meal: 62% CP, 9% CF, ALLMICROALGAE, Portugal;
[5]Tetraselmis sp. meal: 23% CP, 6.2% CF, ALLMICROALGAE, Portugal;
[6]ALL-G RICH Schizochytrium sp.: 9.8% CP, 63.1% CF, Alltech Portugal;
[7]Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine kg^{-1}, Agrimer, France;
[8]Soycomil P: 6 3% CP, 0.8% CF, ADM, Netherlands;
[9]VITEN: 82% CP, 2.1% CF, Roquette, France;
[10]Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal;
[11]Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain;
[12]Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal;
[13]Sopropêche, France;
[14]Henry Lamotte Oils GmbH, Germany;
[15]INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's;
[16]Guar gum, SEAH International, France;
[17]VERDILOX, Kemin Europe NV, Belgium;
[18]PREMIX LDA., Portugal;
[19]Windmill AQUAPHOS, 26% P, ALIPHOS, Belgium;
[20]ALKOSEL R397: 2200 mg selenium kg^{-1}, Lallemand, France;
[21]TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany;
[22]DL-Methionine for Aquaculture 99%, EVONIK Nutrition & Care GmbH, Germany;
[23]L-Taurine 98%, ORFFA, Netherlands.

Se and an iodine concentration that was 11-times higher than the CTRL diet. The fatty acid composition (% total fatty acids) of the diets is presented in **Table 2**. The CTRL diet had the lowest concentrations of saturated fatty acids (SFAs), primarily due to the low palmitic acid (16:0) content; the highest concentrations of monounsaturated fatty acids (MUFAs), as a result of the highest oleic acid (OA, 18:1 n-9 c) content; and the highest concentrations of polyunsaturated fatty acids (PUFAs) due to the highest linoleic acid (LA, 18:2 n-6 c) and eicosapentaenoic acid (EPA, 20:5 n-3) contents. AD2 and AD3 had similar fatty acids contents with the highest concentrations of SFAs and lowest MUFAs. Compared to CTRL, all AD diets had higher docosahexaenoic acid (DHA, 22:6 n-3) contents (5–6 vs. 3% total fatty acids), resulting in higher DHA:EPA ratios (1.2–1.4 vs. 0.7). AD1, which had an additional 20% reduction in FO, also had the highest DHA:EPA ratio due to the lowest EPA content (3.8 vs. 4.5–4.7% total fatty acids in all other diets). Within the AD diets, AD1 had the lowest contents of SFAs and the lowest concentration of DHA (5.3 vs. 5.7–5.8% total fatty acids).

Experimental Trial and Fish Sampling

The trial was conducted in Olhão, Portugal, at the aquaculture research facility EPPO-IPMA between 9th July and 1st October 2018 (85 days). After quarantine (3 weeks), homogenous groups of fish (373.91 ± 10.16 g) were established and distributed into 12 identical circular fiberglass tanks (3 m^3 tanks; initial fish density of 6 kg m^{-3}) in an open seawater system. The system was supplied with continuous water flow (25.2 ± 1.4°C, 5.6 ± 0.9 mg L^{-1} dissolved oxygen). All the tanks were subjected to natural photoperiod summer conditions (14 light/10 dark). Experimental diets were tested in triplicate tanks (n = 50 fish per replicate/tank) and fish were hand-fed *ad libitum* four times a day, seven days a week. At the beginning and end of the trial, fish were bulk weighed and six fish per tank (18 fish per treatment) were anesthetized (phenoxyethanol, 500 ppm) before being weighed individually and their lengths recorded. These fish were euthanized by spinal cord section and stored at –20°C for proximate whole-body composition analysis. After 12 weeks, five fish per tank (15 fish per treatment) were anesthetized (phenoxyethanol, 500 ppm), measured and weighed, and euthanized for the collection of tissue samples. These fish were dissected, and viscera, liver and muscle weighed; livers and muscle were collected for further analysis. For muscle analysis, skinless fish fillets were collected and pooled (five fish fillets per pooled sample), resulting in three pooled samples per dietary treatment. For gene expression, small portions of liver tissue (five fish per tank) were collected from each fish and preserved immediately in RNAlater™ (Thermo Fisher Scientific, Waltham, United States). Then, the samples were transferred to 4°C for 24 h before being stored at –80°C until RNA extraction. For oxidative stress analysis, portions of liver from the same fish were stored at –80°C.

TABLE 2 | Fatty acid composition of the experimental diets (% total fatty acids).

Fatty acids	CTRL	AD1	AD2	AD3
14:0	2.52	2.65	3.23	3.26
16:0	12.25	16.94	18.53	18.54
18:0	2.99	2.75	3.05	3.07
[1] Σ SFA	19.42	24.41	27.06	27.11
16:1 n-7	2.76	2.35	2.70	2.70
18:1 n-7	2.52	2.26	2.24	2.22
18:1 n-9 c (OA)	29.95	26.62	24.06	24.04
20:1 n-9	1.39	1.12	1.13	1.13
[2] Σ MUFA	38.72	34.41	32.31	32.25
18:2 n-6 c (LA)	21.41	19.96	18.28	18.34
18:3 n-3 (ALA)	6.66	6.39	5.81	5.88
20:5 n-3 (EPA)	4.69	3.84	4.56	4.53
22:6 n-3 (DHA)	3.41	5.27	5.80	5.74
[a] EPA + DHA	8.11	9.11	10.35	10.28
DHA/EPA	0.73	1.37	1.27	1.27
[3] Σ PUFA	39.79	39.13	38.66	38.66

[1] Σ SFA is the sum of saturated fatty acids and also includes 15:0, 17:0, 20:0, 22:0 and 24:0;
[2] Σ MUFA is the sum of mono-unsaturated fatty acids and also includes 17:1 n-7, 22:1 n-9, 22:1 n-11 and 24:1 n-9;
[3] Σ PUFA is the sum of polyunsaturated fatty acids and also includes 18:3 n-6, 18:4 n-3, 20:2 n-6, 20:3 n-6, 20:4 n-6, 21:5 n-3, and 22:5 n-3;
[a] EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid. OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid.

Proximate Composition of Feed and Whole-Body

Fish collected from each tank (n = 6) were ground, pooled and freeze-dried for proximate composition analysis. Experimental diets were also homogenized prior to analysis. Proximate composition of whole-body samples and experimental diets were performed in duplicate, according to AOAC (2006) methods: dry matter (in an oven at 105°C to constant weight); ash (incinerated at 500°C for 5 h in a muffle furnace; Nabertherm L9/11/B170, Bremen, Germany); protein by quantitation of nitrogen (N) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, United States) and conversion (N × 6.25) to equivalent protein; gross energy using an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). Total lipids were extracted and quantified from whole-body and muscle samples according to Folch et al. (1957) and using Folch solution (dichloromethane:methanol 2:1 v/v with 0.01% butylated hydroxytoluene – BHT). Iodine and selenium contents of feed samples were determined according to the European standards EN 15111:2007 and EN 15763:2009, respectively. Inductively coupled plasma mass spectrometer (ICP-MS) (Thermo X series II, Thermo Fisher Scientific, Waltham, United States), after alkaline (iodine) or acid (selenium) digestion, was used for determination of iodine and selenium, as described by Barbosa et al. (2020).

Fatty Acid Analysis of Feed, Whole-Body, and Muscle

Fatty acid methyl esters (FAMEs) of muscle and whole-body lipid extracts and experimental diets were *trans*-esterified, according to Bondia-Pons et al. (2007). Samples were diluted in 1 mL of n-hexane and 100 μg L^{-1} of internal standard (13:0 or

21:0), and 5 mL of sodium methylate solution was added (0.5 M, NaOMe). After vigorous shaking, samples were heated to 100°C for 10 min and cooled for 5 min on ice. Samples were esterified with 5 mL of boron trifluoride-methanol at 100°C for 30 min and cooled to room temperature. FAMEs were isolated by adding 1 mL of *n*-hexane containing BHT (0.02%), the tubes were shaken, and 2 mL of sodium chloride (58 g L^{-1}) was added. Following centrifugation for 10 min at 2,000 × *g*, the top layer was retrieved and dried with anhydrous sodium sulfate. An aliquot (150 μL) was evaporated to dryness under nitrogen and re-constituted in *n*-hexane (1100 μL) before 100 μL of this sample was placed in a glass vial with an insert and subjected to analysis using flame ionization detection (GC-FID). A Shimadzu GC-2010 gas chromatograph (Kyoto, Japan) equipped with a FID and a Shimadzu AOC-20i autoinjector was used. Separation was carried out on an Agilent J&W CP-Sil 88 capillary column (60 m × 0.25 mm I.D., film thickness 0.20 μm). Operating conditions were as follows: split mode, with split ratio of 1:50 and an injection volume of 1.5 μL. The injector and detector temperatures were kept at 250 and 260°C, respectively. A flow rate of 30 mL min^{-1} of helium as carrier gas (Linde Sógas purity ≥ 99.999%), 40 mL min^{-1} of hydrogen, and 400 mL min^{-1} of air was provided. The column thermal gradient was as follows: 100°C for 5 min, then raise to 215°C at 1°C min^{-1} and held at this temperature for 12 min. FAMEs were identified by comparison with a known standard mixture (Sigma 47,885-U Supelco 37 Component FAME Mix, Darmstadt, Germany) and quantified using the software GCsolution for GC systems (Shimadzu, Kyoto, Japan). FAME contents in muscle tissue were expressed on the % of the total FAME basis. Quantification of each FAME as mg g^{-1} sample was estimated using tridecanoic acid (13:0) or heneicosanoic acid (21:0) as internal standards.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 15 mg of liver (n = 9 per treatment), using NZYol (Nyztech, Lisbon, Portugal) following the manufacturer's instructions, with some modifications, as previously described by Ferreira et al. (2020). RNA quantity and purity (A260/A280 ratios) were evaluated by spectrophotometry, using a Take 3 micro-volume plate on a Synergy™ HT Multi-Detection Reader and Gen5™ software (BioTek Instruments, Winooski, VT, United States). RNA integrity was assessed in 1% TAE (w/v) agarose electrophoresis gel stained with GelRed™ (Biotium, Hayward, United States). Subsequently, cDNA was synthesized from 1 μg of total RNA using a NZY First-Strand cDNA Synthesis Kit (Nyztech, Lisbon, Portugal), following the standard protocol.

Gene Expression Analysis

Reverse transcription (RT) q-PCR was performed to assess expression of 13 genes (**Table 3**) involved in (a) growth: *igf2*, insulin-like growth factor 2; (b) selenium metabolism: *selk*, selenoprotein K and *selp*, selenoprotein P; (c) iodine metabolism: *dio1*, iodothyronine deiodinase 1 and *dio2*, iodothyronine deiodinase 2; (d) oxidative stress: *gpx1a*, glutathione peroxidase 1a, *gpx1b*, glutathione peroxidase 1b, and *gpx4b*, glutathione peroxidase 4b; and (e) lipid metabolism: *cpt1a*, carnitine palmitoyl transferase 1a (lipolytic pathway related gene); *fas*, fatty acid synthase and *srebp1*, sterol regulatory element binding protein 1 (lipogenic pathway related genes); *fads2*, fatty acid desaturase 2 and *elovl5*, elongation of very long chain fatty acids protein 5 (desaturation and elongation pathway related genes). RT-qPCR was carried out using primers designed in previous studies (**Table 3**). For *selk*, *selp*, *dio1*, *dio2*, gene sequences were retrieved from NCBI GenBank and primers were designed with NCBI Primer Blast Tool (Ye et al., 2012), according to known qPCR restrictions: one of the primers was located at an exon-exon junction to avoid amplification of genomic DNA; primer length (18–22 bp); amplicon size (80–150 bp); Tm difference between primers; GC content (50–60%); and self-dimer or cross-dimer formation. PCR was performed in Eppendorf Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany) using NZYSpeedy qPCR Green Master Mix (2x) (Nyztech, Lisbon, Portugal), according to Ferreira et al. (2020). Specific annealing temperatures and PCR efficiencies for the target genes can be found in **Table 3**. Samples and standard curves were run in duplicate as well as a negative control without cDNA. Stabilities of three candidate reference genes (β-*actin*, *ef1α* and *rps18*) were determined using the geNorm algorithm (Vandesompele et al., 2002) implemented in qbase + software, version 3.2 (Biogazelle, Zwijnaarde, Belgium[1]). Based on these stabilities, geometric means for *ef1α*, *rps18* and β*actin* was used to calculate normalization factors for each liver sample. To evaluate transcript levels, $2^{-\Delta\ \Delta\ CT}$ was used (Livak and Schmittgen, 2001). Average CT for each sample was transformed into relative quantities, normalized with the respective factor, and standardized against CTRL diet.

Liver Preparation and Oxidative Stress Enzymatic Activity

Liver samples (n = 9 per treatment) were homogenized in phosphate-buffered saline (PBS, pH 7.4) using a tissue to PBS ratio of 1:15 (IKA T25 digital; Ultra Turrax). The homogenate was centrifuged at 13,500 × *g* for 15 min at 4°C (Z 383 K Hermle) and the resulting supernatant was aliquoted and stored at –80°C for determination of oxidative stress enzymatic activity. Analyses were performed in triplicate.

Tissue protein contents were determined according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976), and absorbances were measured at 595 nm. Superoxide dismutase (SOD) activity (EC 1.15.1.1) was carried out as described by Sun et al. (1988), using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), with 10 μL of liver extract and 250 μL of substrate (200 μL xanthine + 50 μL XOD), and a reaction time of 10 min. Samples absorbances were read at 550 nm and results are presented as units of SOD mg $protein^{-1}$, where 1 unit of enzyme equals 50% NBT inhibition (% inhibition = Δ control – Δ sample)/Δ control × 100; Δ = abs min^{-1}). Catalase (CAT) activity (EC 1.11.1.6) was determined based on consumption of hydrogen peroxide (H_2O_2, substrate; 250 μL), observed as the linear decrease in absorbance (240 nm) over time, according to the method of Beers and Sizer (1952),

[1]www.qbaseplus.com

TABLE 3 | Oligonucleotide primers used in the reverse transcription-qPCR analysis and respective annealing temperature (°C), PCR efficiency (%), accession number and reference.

Gene		Primer sequence 5′-3′	Annealing T (°C)	PCR efficiency (%)	Accession number	References
βactin	F	TGCGTGACATCAAGGAGAAG	58	91	X89920.1	Avella et al., 2010
	R	CAGGACTCCATACCGAGGAA				
ef1α	F	CTGTCAAGGAAATCCGTCGT	60	100	AF184170.1	Estruch et al., 2018
	R	TGACCTGAGCGTTGAAGTTG				
rps18	F	AGGGTGTTGGCAGACGTTAC	60	101	AM490061.1	Salmerón et al., 2016
	R	CTTCTGCCTGTTGAGGAACC				
Igf2	F	TGGGATCGTAGAGGAGTGTTGT	62	92	AY996778.1	Benedito-Palos et al., 2007
	R	CTGTAGAGAGGTGGCCGACA				
selk	F	TGTACGTGTCCAACGGTCAG	60	95	XM_030423889.1	–
	R	TGAATTCCACTGCTCCCCAG				
selp	F	CATTTATGACAGGTGTGGCCG	62	94	XM_030416800.1	–
	R	CGCTTGCAGTACGCATCTTT				
dio1	F	GACTCGTCAGGGACTTCAGC	60	97	DQ888894.1	–
	R	GAGCCTCTCAGGCATAGCAC				
dio2	F	GGCAGTTGGTTGAGGACTTC	60	91	DQ888895.1	–
	R	TCCATACAGTCAGCCACCAG				
gpx1a	F	TGGAGAAGGTGGATGTGAATG	58	92	KC201352.1	Malandrakis et al., 2014
	R	GCCATCAGGACCAACAAGG				
gpx1b	F	GCGTTACAGCAGGAGGTT	60	90	KC201353.1	Malandrakis et al., 2014
	R	TGAGCACATCTGGTCATTCA				
gpx4b	F	ACGGCTTGTCTGTAAACTC	60	99	KC201354.1	Malandrakis et al., 2014
	R	GCTGGTGAATGTCCTCTC				
cpt1a	F	GTGCCTTCGTTCGTTCCATGATC	62	99	JQ308822.1	Betancor et al., 2016
	R	TGATGCTTATCTGCTGCCTGTTTG				
Fas	F	TGCCATTGCCATAGCACTCA	58	92	JQ277708.1	Houston et al., 2017
	R	ACCTTTGCCCTTTGTGTGGA				
srebp1	F	AGGGCTGACCACAACGTCTCCTCTCC	60	93	JQ277709.1	Houston et al., 2017
	R	GCTGTACGTGGGATGTGATGGTTTGGG				
fads2	F	GCAGGCGGAGAGCGACGGTCTGTTCC	60	99	AY055749.1	Betancor et al., 2016
	R	AGCAGGATGTGACCCAGGTGGAGGCAGAAG				
elovl5	F	CCTCCTGGTGCTCTACAAT	60	92	AY660879.1	Betancor et al., 2016
	R	GTGAGTGTCCTGGCAGTA				

Reference genes: βactin, actin beta; ef1α, elongation factor 1 alpha and rps18, ribosomal protein S18. Target genes related to growth: igf2, insulin-like growth factor 2; selenoprotein genes: selk, selenoprotein K and selp, selenoprotein P; genes involved in iodine metabolism: dio1, iodothyronine deiodinase 1 and dio2, iodothyronine deiodinase 2; genes involved in oxidative stress: gpx1a, glutathione peroxidase 1a; gpx1b, glutathione peroxidase 1b and gpx4b, glutathione peroxidase 4b; and genes associated with lipid metabolism: cpt1a, carnitine palmitoyl transferase 1a; fas, fatty acid synthase; srebp1, sterol regulatory element binding protein 1; fads2, fatty acid desaturase 2; and elovl5, elongation of very long chain fatty acids protein 5.

adapted for microplates by Li and Schellhorn (2007). A sample volume of 10 μL was used, and H_2O_2 consumption was monitored every 30 s for 5 min at 240 nm and 25°C. Results are expressed in μmol min^{-1} mg^{-1} protein. Selenium-independent and dependent glutathione peroxidase (GPx and SeGPx) activities (EC 1.11.1.9) were evaluated spectrophotometrically using a coupled assay based on glutathione reductase (Flohe and Gunzler, 1984). Hydrogen peroxide and cumene hydroperoxide substrates (200 μL buffer + 50 μL substrate), with 10 μL liver extract, were used to analyze activities of SeGPx and GPx, respectively, following the decrease in NADPH consumed during re-formation of GSH from GSSG at 340 nm, for 5 min at 25°C. SeGPx activity was calculated as μmol oxidized NADPH min^{-1} mg^{-1} protein and GPx as nmol NADPH oxidized min^{-1} mg^{-1} protein, using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. Lipid peroxidation (LPO) concentrations were assessed by measuring malondialdehyde (MDA) using the method described by Erdelmeier et al. (1998), which is based on the reaction of a chromogenic compound, 1-methyl-2-phenylindole ($C_{15}H_{13}N$). Two moles of this reagent react with one mole of MDA at 45°C for 60 min to form a stable chromophore with a maximum absorbance at 586 nm.

Calculations

Growth performance calculations: Weight gain = final body weight (FBW) – initial body weight (IBW); Average body weight (ABW) = (FBW + IBW)/2; Condition factor (K) = 100 × (FBW/final body length^3); Specific growth rate (SGR) = (ln FBW – ln IBW)/days of the experiment × 100; Feed conversion ratio (FCR) = dry feed intake/(FBW – IBW); Protein efficiency ratio (PER) = (FBW – IBW)/crude protein

TABLE 4 | Growth performance and somatic indexes of gilthead seabream fed the experimental diets for 85 days.

	CTRL	AD1	AD2	AD3	*P*
Growth					
Initial body weight (g, IBW)	371.20 ± 14.50	378.95 ± 2.38	375.52 ± 13.39	369.97 ± 9.82	0.75
Final body weight (g, FBW)	625.76 ± 5.38^{a}	622.61 ± 6.77^{a}	623.11 ± 3.19^{a}	589.46 ± 5.49^{b}	<0.001
Weight gain (g, WG)	254.56 ± 11.18^{a}	243.66 ± 8.25^{ab}	247.59 ± 16.33^{ab}	219.49 ± 12.26^{b}	0.04
Final condition factor (K)	1.84 ± 0.05	1.89 ± 0.13	1.79 ± 0.03	1.78 ± 0.02	0.30
Specific growth rate (SGR)	0.73 ± 0.05	0.69 ± 0.02	0.70 ± 0.06	0.65 ± 0.04	0.23
Feed conversion ratio (FCR)	1.67 ± 0.02	1.72 ± 0.06	1.73 ± 0.12	1.91 ± 0.13	0.06
Protein efficiency ratio (PER)	1.20 ± 0.03^{a}	1.15 ± 0.04^{ab}	1.14 ± 0.07^{ab}	1.03 ± 0.07^{b}	0.03
Intake (g or kJ or mg kg^{-1} ABW day^{-1})					
Dry matter (g kg^{-1})	9.90 ± 0.39	9.85 ± 0.05	10.13 ± 0.19	10.40 ± 0.23	0.09
Protein (g kg^{-1})	5.00 ± 0.19	4.97 ± 0.03	5.14 ± 0.10	5.24 ± 0.12	0.10
Lipids (g kg^{-1})	1.65 ± 0.06^{a}	1.60 ± 0.01^{ab}	1.59 ± 0.04^{ab}	1.54 ± 0.03^{b}	0.04
Energy (kJ kg^{-1})	236.02 ± 9.19	235.89 ± 1.31	242.50 ± 4.61	247.23 ± 5.43	0.12
Iodine (mg kg^{-1})	13.31 ± 0.52^{d}	79.76 ± 0.44^{c}	90.88 ± 1.73^{b}	151.78 ± 3.34^{a}	<0.001
Selenium (mg kg^{-1})	7.50 ± 0.29^{c}	11.36 ± 0.06^{b}	10.82 ± 0.21^{b}	14.63 ± 0.32^{a}	<0.001
Somatic indexes					
Hepatosomatic index (HSI, %)	1.56 ± 0.12	1.31 ± 0.07	1.29 ± 0.05	1.34 ± 0.17	0.07
Viscerosomatic index (VSI, %)	5.70 ± 0.44	5.60 ± 0.32	5.75 ± 0.9	5.73 ± 0.09	0.97

Values are presented as mean ± standard deviation (SD). Growth performance and intake parameters were calculated with three pooled samples per treatment of six fish per tank. Somatic indexes were calculated with 15 fish per treatment (five fish per tank). Different superscript letters in a row denote significant differences among treatments.

TABLE 5 | Whole-body composition and nutrient retention and gain of gilthead seabream fed the experimental diets for 85 days.

	CTRL	AD1	AD2	AD3	*P*
Whole-body composition					
Moisture (% WW)	59.61 ± 0.66	60.14 ± 0.12	60.63 ± 0.95	59.87 ± 0.96	0.44
Ash (% WW)	3.79 ± 0.24	3.74 ± 0.18	3.76 ± 0.08	3.72 ± 0.15	0.97
Protein (% WW)	18.07 ± 0.46	18.35 ± 0.03	18.61 ± 0.30	18.18 ± 0.40	0.30
Lipids (% WW)	15.27 ± 0.43	15.69 ± 1.28	14.67 ± 1.23	14.52 ± 0.84	0.49
Energy (kJ g^{-1} WW)	10.94 ± 0.32	11.11 ± 0.43	10.7 ± 0.35	11.02 ± 0.37	0.60
Iodine (mg kg^{-1} WW)	0.19 ± 0.01	0.20 ± 0.03	0.20 ± 0.04	0.20 ± 0.01	0.96
Selenium (mg kg^{-1} WW)	0.24 ± 0.001^{c}	0.32 ± 0.01^{b}	0.33 ± 0.01^{b}	0.42 ± 0.02^{a}	<0.001
Retention (% of Intake)					
Dry matter	28.40 ± 1.40	26.68 ± 0.62	25.63 ± 0.50	24.43 ± 2.73	0.08
Protein	21.96 ± 1.24	21.85 ± 0.65	22.29 ± 2.05	19.12 ± 1.49	0.09
Lipids	68.81 ± 4.60	72.49 ± 10.47	64.25 ± 9.87	61.31 ± 10.90	0.51
Energy	33.52 ± 2.44	33.34 ± 1.72	30.45 ± 1.46	29.92 ± 3.75	0.24
Gain (g or kJ kg^{-1} ABW day^{-1})					
Dry matter	2.82 ± 0.24	2.63 ± 0.05	2.60 ± 0.10	2.54 ± 0.26	0.35
Protein	1.10 ± 0.04	1.09 ± 0.03	1.15 ± 0.12	1.00 ± 0.06	0.16
Lipids	1.14 ± 0.11	1.16 ± 0.17	1.02 ± 0.14	0.94 ± 0.15	0.30
Energy	0.79 ± 0.08	0.79 ± 0.05	0.74 ± 0.05	0.74 ± 0.09	0.66

Values are presented as mean ± SD, with three pooled samples per treatment of six fish per tank. Different superscript letters in a row denote significant differences among treatments.
ABW, average body weight; WW, wet weight.
Whole-body initial composition: 64.02 moisture (% WW); 35.98 DM (%); 4.32 ash (% WW); 17.94 protein (% WW); 12.78 lipids (% WW); 9.41 energy (kJ g^{-1}).

intake; Hepatosomatic index (HSI) = 100 × (liver weight/FBW); Viscerosomatic index (VSI) = 100 × (weight of viscera/FBW).

Nutrient intake, retention, and gain calculations: Protein (P), lipids (L), or energy (E) intake = DM intake × P, L, E (kJ g^{-1}) in the diet; DM retention/consumption = (FBW × final whole-body DM content – IBW × initial whole-body DM content)/dry feed consumption per fish; P, L, E or fatty acids (FAs) retention/consumption = 100 × (FBW × final whole-body P, L, E or FAs content in wet weight - IBW × initial whole-body P, L, E or FAs content in wet weight)/dry feed consumption per fish × P, L, FAs or E (kJ g^{-1}) in the diet in% DM; DM, P, L, E or FAs gain = (FBW × final whole-body DM, P, L, E or FAs content in wet weight x%) – (IBW × initial whole-body DM, P, L, E or FAs content in wet weight x%)/ABW (kg)/days of the experiment.

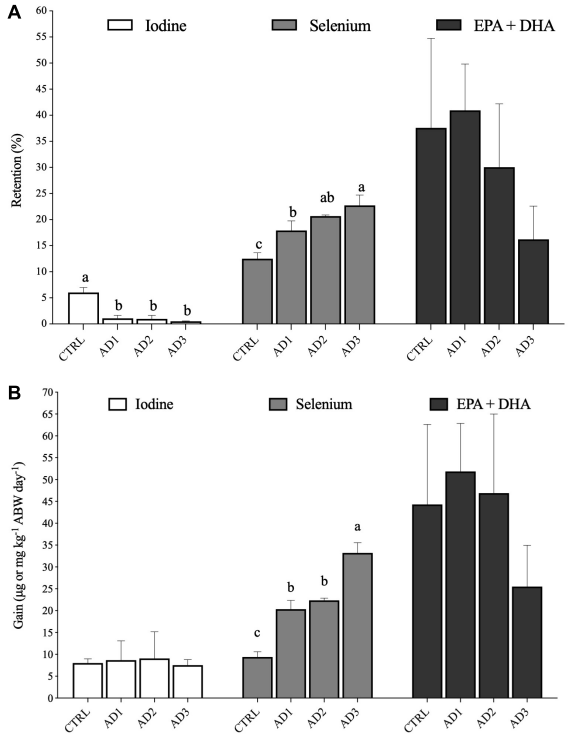

FIGURE 1 | (A) Retention (% feed intake) and **(B)** Gain of iodine and selenium (µg kg^{-1} ABW day^{-1}) and of EPA + DHA (mg kg^{-1} ABW day^{-1}). EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid. Values are presented as mean ± SD. Different letters denote significant differences among treatments.

Statistical Analysis

Statistical analyses were performed using IBM SPSS® Statistics 25.0 software (SPSS Inc., Chicago, IL, United States). All variables were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively, followed by one-way analysis of variance (ANOVA). A Tukey's multiple comparison test was applied to compare individual means. Data were log-transformed when normality and/or homogeneity of variances assumptions were not met and a non-parametric test (Kruskal–Wallis *H*-test) was used when required. Significant differences were considered for a $P < 0.05$. A Spearmen's rank correlation coefficient (*rs*) test was performed and significant correlations between different variables were considered at 0.05 (*) or 0.01 (**).

RESULTS

Growth Performance and Nutrient Utilization

The growth performance parameters are displayed in **Table 4**. All biofortified diets were well accepted by the gilthead seabream. At the end of 12 weeks of feeding, fish fed the CTRL diet reached a FBW of 625.8 ± 5.4 g, that was similar to that of fish fed with AD1 and AD2 (622.6 ± 6.8 g and 623.1 ± 3.2 g, respectively). But fish fed diet AD3 had a significantly lower FBW (589.5 ± 5.5 g) than fish consuming any other diet. Final condition factor (K), feed conversion ratio (FCR), and specific growth rate (SGR) remained unaffected by diet. Nonetheless, a tendency for increased FCR in fish fed diet AD3 was observed ($p = 0.06$). Likewise, hepatosomatic (HSI) and viscerosomatic (VSI) indices were not significantly different among treatments. Protein and energy intake did not differ between diets, but lipid intake was significantly lower in fish fed AD3 compared with the CTRL diet. Weight gain (WG) and protein efficiency ratio (PER) of fish fed AD3 were also significantly lower compared with those fed the CTRL diet. Iodine and selenium intake were significantly higher in fish fed the AD diets, with the highest values being observed in fish fed AD3.

Iodine intake was negatively correlated with FBW and PER, while selenium intake presented a negative correlation with FBW, WG and PER, and a positive correlation with FCR (**Supplementary Table 2**).

Whole-Body Composition, Fatty Acid Profile and Utilization

Whole-body composition, retention and gain in protein, lipids, energy were not significantly affected by diet (**Table 5**). Retention and gain in selenium, as well as whole-body composition (**Table 5** and **Figure 1**), were significantly higher in fish consuming the AD diets, with fish fed the AD3 diet presenting the highest values. For iodine, although retention was significantly decreased in fish fed the AD diets compared to CTRL, no significant differences could be observed on whole-body composition and gain among dietary treatments.

The whole-body fatty acid profile (% total fatty acids; **Table 6**) indicated that the most abundant fatty acids were OA (18:1 n-9 c, 34–35%) followed by palmitic acid (16:0, 21-26%) and LA (18:2 n-6 c, 13-17%), independently of the diet. Fish fed AD1 had significantly lower concentrations of myristic acid (14:0) compared to those fed AD3. Fish fed AD1 also had the highest PUFAs content, which was significantly higher than those fed AD3. Such differences were largely due to significantly higher concentrations of α-linolenic acid (ALA, 18:3 n-3) and EPA in the whole-body of fish fed AD1 in comparison with AD3. The whole-body DHA increased significantly in fish fed AD1 and AD2 compared to either CTRL or AD3 diets, reaching the highest values in fish fed AD1 diet.

Overall, fatty acid retention (**Table 6** and **Figure 1A**) and gain (**Supplementary Table 1** and **Figure 1B**) in gilthead seabream were not significantly affected by diet, except for 16:0, which was less retained in fish fed AD1 compared with those fed the CTRL diet. Moreover, fish fed AD1 had the lowest retention and gain of SFAs and MUFAs, while fish fed diet AD3 had the lowest PUFAs retention and gain, including EPA and DHA (although without statistical significance; **Figure 1**). The highest DHA retention and gain was observed in fish fed AD1, but again without statistical significance (**Table 6** and **Supplementary Table 1**).

Muscle Fatty Acid Profile

Muscle total lipids were not significantly affected by diet, while muscle fatty acid profiles differed significantly amongst the diets

(**Table 7**). SFAs, specifically myristic and palmitic acids (14:0 and 16:0), were significantly higher in fish fed AD2 compared with those fed CTRL. MUFAs were significantly reduced in fish fed AD2 and AD3 in comparison with CTRL, mainly due to significantly lower concentrations of OA (18:1 n-9) in those fish. Fish fed AD2 also had the lowest muscle concentrations of LA (18:2n-6 c), which was significantly lower compared with CTRL. Muscle from fish fed AD2 and AD3 had significantly higher DHA concentrations compared with CTRL, with the sum of EPA and DHA being highest in fish fed AD3 (% total fatty acids). Fish fed AD2 and AD3 diets had the highest net content of EPA + DHA (0.7 g 100 g^{-1} of filet), being significantly higher than in fish fed the CTRL (0.5 g 100 g^{-1} of filet).

Gene Expression in Liver

Expression of a panel of genes related to growth, selenoproteins involved in selenium and iodine metabolism and oxidative stress, and lipid metabolism were assessed in liver from gilthead seabream (**Figure 2**). Concerning genes associated with growth, the *igf2* expression (**Figure 2A**) was significantly downregulated in the liver of fish fed AD3 compared with those fed AD1. Moreover, hepatic expression of *igf2* was significantly and positively correlated with protein retention and gain (**Supplementary Table 2**).

In liver, the expression of several selenoprotein genes was affected by diet (**Figure 2B**). Transcript levels of *selk* were significantly upregulated in fish fed AD1 compared with fish fed the CTRL diet, but transcription of *selp* remained unaffected by diet. Expressions of liver *selk* and *dio2* were also significantly and positively correlated with hepatic *igf2* expression (**Supplementary Table 2**). AD1 was associated with increased hepatic expression of a selenoprotein gene related to iodine metabolism (*dio2*; **Figure 2C**) compared to AD2. The oxidative stress genes *gpx1a* and *gpx1b* were significantly upregulated in fish fed AD1 compared with those fed CTRL (**Figure 2D**).

For genes involved in hepatic lipid metabolism, increased expression of lipogenesis pathway related genes (*fas* and *srebp1*) was observed in fish consuming the AD1 diet compared with the other AD diets, but not in those fed the CTRL. The gene associated with the lipolytic process, *cpt1a*, was not affected by the dietary treatments. Concerning desaturation and elongation pathway related genes, no differences were observed on *fads2* expression while *elovl5* was up regulated in fish that consumed AD3 in comparison with the other diets (**Figure 2E**). A significant and negative correlation was observed between *elovl5* expression and several growth performance parameters (FBW, WG, DGI, SGR, and PER), lipid intake, and protein retention and gain (**Supplementary Table 2**).

Oxidative Stress Parameters in Liver

The oxidative stress parameters in liver are displayed in **Figure 3**. No significant differences in the activity of SOD, CAT, GPx, and SeGPx were observed between dietary treatments. Likewise, the LPO values were similar among fish fed the different experimental diets.

DISCUSSION

In recent years, efforts have been made to find natural, highly nutritious alternatives to replace FM and FO, which could simultaneously promote fish health and improve the nutritional quality of filets for human consumption. In the present study, fish fed diet AD1, with 10% FM and 4.4% FO, and with 20% less vegetable oils than the CTRL diet, grew equally well, indicating that a microalgae blend can be successfully used to partially replace FM and FO in diets for *S. aurata*. Moreover, a concomitant supplementation of seabream diets with 0.4% iodine-rich *L. digitata* and 0.02% selenised yeast can be achieved without negatively affecting fish growth. However, higher levels of *L. digitata* and selenised yeast resulted in lower seabream growth. The negative correlation observed between selenium and iodine intake and FBW and PER might be due to a decline in overall nutrient/energy bioavailability, which may have compromised nutrient absorption leading to growth impairment. Although digestibility of diets was not presently tested, previous studies with *S. latissima* associated growth impairment in rainbow trout with reduced nutrient digestibility and a reduction in tunica muscularis thickness, which could decrease intestine strength and motility (Granby et al., 2020). Very few studies evaluated the ability of fish to digest kelp, but according to Pereira et al. (2012), *Sargassum muticum* had comparatively lower nutrient and energy digestibility values compared to *Porphyra dioica*, *Ulva* spp., *Gracilaria vermiculophylla* in rainbow trout. The present results with gilthead seabream, although still preliminary, are encouraging, demonstrating the feasibility of using biofortified aquafeeds with algae and selenised yeast. But future studies should delve into the digestibility of these diets to have a more comprehensive perspective on the cost-benefit ratio of such feeds.

Somatotropic axis genes are reliable growth markers, and evidence suggests that both environmental and nutritional factors modulate expression and circulating concentrations of growth hormone receptors and insulin-like growth factors (*igfs*) (Pérez-Sánchez et al., 2018). Downregulation of hepatic *igf2* was observed in fish fed AD3, resulting in a significantly lower final body weight of these fish compared with CTRL. In a previous study evaluating the impact of FO replacement with VO in gilthead seabream, a significantly lower final body weight was observed in fish consuming the 100% VO diet compared to other diets (100% FO; 33% VO; and 66% VO), which was accompanied by a downregulation of hepatic *ig1* and *igf2* (Benedito-Palos et al., 2007). In the present study, *igf2* hepatic expression was positively correlated with protein retention and gain, which are major determinants of growth (Mommsen, 2001). Therefore, the downregulation of growth-related genes could have contributed to the growth impairment of fish fed AD3. But it would be necessary to measure the circulating concentrations of growth hormones and Igf1 to have a better understanding of nutritional modulation of the somatotropic axis.

The replacement of FO with VO in aquafeeds is known to reduce long-chain polyunsaturated fatty acids (LC-PUFAs), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)

TABLE 6 | Whole-body fatty acid composition (% total fatty acids) and retention (% feed intake).

	CTRL	AD1	AD2	AD3	*P*
Fatty acids					
14:0	3.08 ± 0.33^{ab}	2.71 ± 0.31^{b}	3.27 ± 0.17^{ab}	3.59 ± 0.20^{a}	0.02
16:0	22.57 ± 2.45	20.57 ± 2.57	23.38 ± 1.29	26.13 ± 2.06	0.07
18:0	5.54 ± 0.96	4.89 ± 0.65	5.50 ± 0.18	6.33 ± 0.76	0.17
[1] Σ SFA	32.36 ± 3.72	29.41 ± 3.58	33.46 ± 1.80	37.30 ± 2.91	0.07
16:1 n-7	4.04 ± 0.72	4.45 ± 0.45	4.36 ± 0.25	4.26 ± 0.61	0.81
18:1 n-9 c (OA)	35.42 ± 0.68	35.16 ± 0.81	34.17 ± 1.51	34.22 ± 0.51	0.33
20:1 n-9	1.60 ± 0.06	1.34 ± 0.16	1.53 ± 0.03	1.55 ± 0.06	0.11
[2] Σ MUFA	41.95 ± 1.04	41.91 ± 1.10	40.91 ± 1.63	40.84 ± 0.97	0.55
18:2 n-6 c (LA)	16.06 ± 2.05	16.79 ± 1.49	15.38 ± 0.30	13.43 ± 1.62	0.11
18:3 n-3 (ALA)	3.45 ± 0.52^{ab}	3.83 ± 0.43^{a}	3.24 ± 0.15^{ab}	2.68 ± 0.45^{b}	0.04
20:5 n-3 (EPA)	1.74 ± 0.13^{ab}	1.78 ± 0.17^{a}	1.74 ± 0.07^{ab}	1.36 ± 0.20^{b}	0.03
22:6 n-3 (DHA)	2.92 ± 0.28^{c}	4.36 ± 0.38^{a}	3.70 ± 0.14^{b}	2.85 ± 0.04^{c}	<0.001
[a]EPA + DHA	4.66 ± 0.33^{bc}	6.14 ± 0.54^{a}	5.44 ± 0.15^{ab}	4.21 ± 0.16^{c}	<0.001
[3] Σ PUFA	25.40 ± 2.66^{ab}	28.08 ± 2.52^{a}	25.22 ± 0.32^{ab}	21.46 ± 2.25^{b}	0.03
Retention (% of Intake)					
14:0	83.50 ± 14.23	48.73 ± 12.11	57.42 ± 14.73	62.89 ± 19.40	0.11
16:0	117.57 ± 27.58^{a}	52.11 ± 16.09^{b}	64.48 ± 17.31^{ab}	74.37 ± 27.02^{ab}	0.04
18:0	115.21 ± 35.27	68.88 ± 25.12	86.55 ± 33.97	105.96 ± 47.77	0.46
[1] Σ SFA	108.46 ± 24.88	52.84 ± 15.81	64.73 ± 18.17	74.22 ± 27.11	0.07
16:1 n-7	49.22 ± 39.50	40.99 ± 17.64	41.09 ± 38.66	32.04 ± 30.17	0.93
18:1 n-9 c (OA)	64.32 ± 25.10	47.18 ± 8.08	53.98 ± 31.26	49.46 ± 26.79	0.83
20:1 n-9	63.24 ± 16.75	29.95 ± 17.95	51.24 ± 24.26	50.51 ± 28.64	0.39
[2] Σ MUFA	57.52 ± 23.11	41.95 ± 7.44	47.06 ± 28.04	42.71 ± 24.11	0.81
18:2 n-6 c (LA)	44.95 ± 21.31	37.05 ± 10.12	35.39 ± 15.89	22.06 ± 11.75	0.39
18:3 n-3 (ALA)	42.64 ± 17.35	41.27 ± 9.09	35.91 ± 14.12	24.26 ± 7.38	0.34
20:5 n-3 (EPA)	30.47 ± 10.57	30.34 ± 5.87	25.22 ± 9.47	15.59 ± 4.38	0.16
22:6 n-3 (DHA)	47.62 ± 27.03	48.78 ± 11.15	33.94 ± 14.39	16.75 ± 8.83	0.15
[3] Σ PUFA	42.42 ± 18.81	37.89 ± 9.27	32.66 ± 13.69	20.29 ± 9.24	0.28

Values presented as mean ± SD, with three pooled samples per treatment of six fish per tank for final whole-body composition and two pooled samples of six fish per tank for initial whole-body composition. Different superscript letters in a row denote significant differences among treatments.
[1] Σ SFA is the sum of saturated fatty acids and also includes 15:0, 17:0, 20:0, 22:0 and 24:0;
[2] Σ MUFA is the sum of monounsaturated fatty acids and also includes 17:1 n-7, 22:1 n-9 and 24:1 n-9;
[3] Σ PUFA is the sum of polyunsaturated fatty acids and also includes 18:3 n-6, 20:2 n-6, 20:3 n-6 and 20:4 n-6;
[a]EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid. OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid.

(Nasopoulou and Zabetakis, 2012). This may negatively impact fish performance due to the reported limited capacity of a large variety of teleost fish lineages to synthesize LC-PUFAs (Tocher, 2015; Lopes-Marques et al., 2018). Yet, the replacement of 33% FM with microalgae meal and the inclusion of DHA-rich *Schizochytrium* sp. resulted in higher concentrations of DHA in biofortified diets, even in the case of AD1, which had an additional 20% reduction in FO. Moreover, none of the fortified diets significantly affected gilthead seabream whole-body composition, or nutrient retention and gain. Nevertheless, a trend for lower protein and lipid retention and gain in fish consuming AD3 may have contributed to their reduced growth.

In terms of essential fatty acids, the concomitant partial replacement of FM and FO either did not affect or have even increased whole-body and muscle EPA and DHA levels in fish. In fact, filets of fish consuming AD2 and AD3 diets presented the highest EPA + DHA contents, providing 2.6–2.7 times the recommended by EFSA 0.25 g per 100 g portion (EFSA, 2010). In general, muscle fatty acid profiles mirrored that of the diets, but whole-body trends were not the same. These results are indicative of selective retention of omega-3 fatty acids in muscle, which has been reported previously for farmed fish species (Mourente and Bell, 2006; Dantagnan et al., 2009). Together, our results indicate that DHA-rich *Schizochytrium* sp. is an effective substitute for FO in gilthead seabream diets since n-3 PUFAs were effectively retained in the muscle. In Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*), replacement of FO with *Schizochytrium* sp. meal resulted in increased DHA concentrations in filets (Miller et al., 2007; Sarker et al., 2016; Kousoulaki et al., 2020), further supporting the potential of LC-PUFA-rich microalgae as suitable candidates to replace FO in diets for farmed species. However, despite the slightly higher capacity of fish fed AD1 to retain DHA (49% *vs.* 48% in the CTRL) and EPA + DHA (41% *vs.* 38% in the CTRL) at whole-body level, differences were not significant among treatments. Ribeiro (2019) reported low retention values for EPA + DHA (17–24%)

TABLE 7 | Muscle total lipid content and fatty acid composition.

	CTRL	AD1	AD2	AD3	*P*
Total lipids (g 100 g^{-1} WW)	8.35 ± 0.34	8.27 ± 0.14	8.69 ± 0.76	8.97 ± 0.39	0.22
Fatty acids (% total fatty acids)					
14:0	2.47 ± 0.06^{b}	2.56 ± 0.09^{ab}	2.78 ± 0.17^{a}	2.69 ± 0.09^{ab}	0.04
16:0	18.15 ± 0.38^{b}	19.90 ± 1.23^{ab}	20.82 ± 0.84^{a}	20.14 ± 0.68^{ab}	0.02
18:0	4.48 ± 0.18	4.53 ± 0.24	4.64 ± 0.32	4.41 ± 0.23	0.74
[1] Σ SFA	26.09 ± 0.48^{b}	28.01 ± 1.36^{ab}	29.37 ± 1.20^{a}	28.32 ± 0.84^{ab}	0.03
16:1 n-7	4.42 ± 0.08	4.28 ± 0.07	4.44 ± 0.19	4.39 ± 0.20	0.56
18:1 n-9 c (OA)	35.93 ± 0.35^{a}	35.29 ± 0.91^{ab}	34.11 ± 0.34^{b}	33.91 ± 0.41^{b}	0.006
20:1 n-9	1.42 ± 0.02	1.44 ± 0.01	1.43 ± 0.04	1.43 ± 0.08	0.92
[2] Σ MUFA	42.82 ± 0.36^{a}	42.02 ± 1.04^{ab}	41.02 ± 0.31^{b}	40.73 ± 0.45^{b}	0.01
18:2 n-6 c (LA)	18.42 ± 0.39^{a}	17.56 ± 0.81^{ab}	16.64 ± 0.53^{b}	17.53 ± 0.39^{ab}	0.03
18:3 n-3 (ALA)	4.35 ± 0.37	3.87 ± 0.25	3.63 ± 0.22	3.95 ± 0.34	0.10
20:5 n-3 (EPA)	2.63 ± 0.18	2.18 ± 0.22	2.33 ± 0.16	2.46 ± 0.26	0.12
22:6 n-3 (DHA)	4.31 ± 0.24^{b}	5.03 ± 0.44^{ab}	5.60 ± 0.39^{a}	5.64 ± 0.34^{a}	0.006
[a]EPA + DHA	6.94 ± 0.12^{b}	7.21 ± 0.23^{ab}	7.92 ± 0.52^{ab}	8.10 ± 0.60^{a}	0.03
[3] Σ PUFA	30.90 ± 0.69	29.82 ± 1.17	29.40 ± 1.19	30.75 ± 1.04	0.30
Fatty acids (g 100 g^{-1} WW)					
EPA + DHA	0.53 ± 0.02^{b}	0.55 ± 0.02^{ab}	0.66 ± 0.08^{a}	0.67 ± 0.02^{a}	0.04

Values presented as mean ± SD, with three pooled muscle samples per treatment of six fish per tank. Different superscript letters in a row denote significant differences among treatments.

[1] Σ SFA is the sum of saturated fatty acids and also includes 15:0, 17:0, 20:0, 22:0 and 24:0;

[2] Σ MUFA is the sum of mono-unsaturated fatty acids and also includes 17:1 n-7, 22:1 n-9 and 24:1 n-9;

[3] Σ PUFA is the sum of polyunsaturated fatty acids and also includes 18:3 n-6, 20:2 n-6, 20:3 n-6 and 20:4 n-6;

[a]EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid.

OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; WW, wet weight.

in gilthead seabream filets fed diets with 15–30% FM and 12–15% FO, with DHA (24–37%) being retained better than EPA (9–15%). In the present study, and although the concomitant replacement of FM and FO (AD1 diet) led to the highest retention values of EPA + DHA (non-significant), new strategies must be developed to ensure higher retention of essential LC-PUFAs, since these values are still very low.

Previous studies have indicated that, like many marine fish, gilthead seabream cannot synthesize EPA and DHA efficiently (Tocher, 2015; Castro et al., 2016). Nevertheless, nutritional modulation of genes encoding LC-PUFAs biosynthetic enzymes has also been reported (Izquierdo et al., 2008). A previous study in black seabream (*Acanthopagrus schlegelii*) showed decreased expression of elongation of very long-chain fatty acids protein 5 (*elovl5*) in response to increased dietary n-3 LC-PUFAs (Jin et al., 2017a) or high DHA:EPA ratios (Jin et al., 2017b). Compared with CTRL, AD3 diet had higher concentrations of n-3 LC-PUFAs and a higher DHA:EPA ratio (1.2 for AD3 vs. 0.7 for CTRL). Therefore, the observed upregulation of hepatic *elovl5* in the present study might be a metabolic response to the lower bioavailability of LC-PUFAs in fish consuming AD3. The LC-PUFAs biosynthetic pathway is highly dependent on sequential steps of desaturation and elongation of C18 PUFAs (Tocher, 2010) and the increased hepatic expression of *elovl5* was accompanied by decreased whole-body C18 PUFAs and increased muscle n-3 LC-PUFAs concentrations. This suggests that fish fed AD3 elongated more C18 PUFAs to produce n-3 LC-PUFAs that were further retained by the muscle. Overall, our results support the hypothesis of a nutritional regulation of genes involved in the synthesis of LC-PUFAs. Additionally, a balance between the lipogenic and lipolytic processes is essential for lipid metabolism homeostasis. In the present study, the lipolytic pathway related gene (*cpt1a*) remained unaffected by the dietary treatments. In contrast, an upregulation of the lipogenic genes, *fas* and *srebp1*, was observed in response to the AD1 diet (with 20% reduction of FO), but only when compared with the other AD diets, not differing from the CTRL. Therefore, the concomitant replacement of FM and FO by microalgae is achievable without major impacts on both anabolic and catabolic processes associated with lipid metabolism.

Selenium and iodine are essential nutrients for human consumption and strategies to fortify fish in those elements are of particular relevance. In the present study, fish fed the diets supplemented with selenised yeast were able to increasingly retain the ingested selenium and accumulate it in their bodies. In a complementary study, with the same enriched feeds, it was demonstrated that the dietary supplementation with 0.02% selenised yeast was enough to achieve higher selenium contents in seabream filets compared with CTRL (0.23–0.36 μg g^{-1} in AD diets vs. 0.18 μg g^{-1} in CTRL), without increasing consumer's exposure to toxic elements (Barbosa et al., 2020). Together, these results indicate that organic selenium provided by selenised yeast is an effective vehicle to fortify gilthead seabream filets. Contrarily to selenium, iodine gain remained similar among diets, but its retention efficiency was comprised by the higher levels of iodine in diets supplemented with *L. digitata.* In fact, it was shown

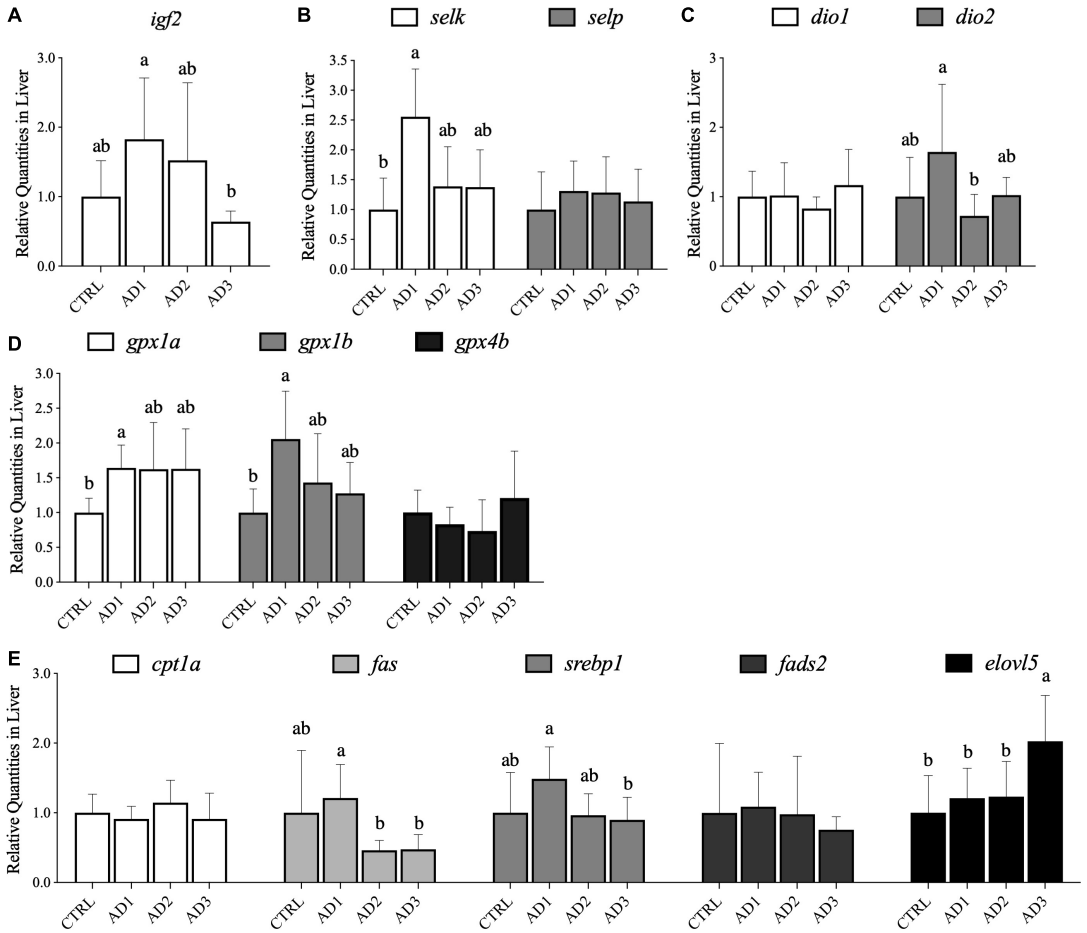

FIGURE 2 | Gene expression in the liver of gilthead seabream fed the experimental diets over 12 weeks. **(A)** Genes growth-related; **(B)** genes involved in selenium metabolism; **(C)** genes involved in iodine metabolism; **(D)** genes associated with oxidative stress; **(E)** genes involved in lipid metabolism (lipolytic, lipogenic, desaturation, and elongation pathway related gene). Relative abundance in liver is presented as mean ± SD (n = 9 fish per treatment). Different letters denote significant differences among treatments.

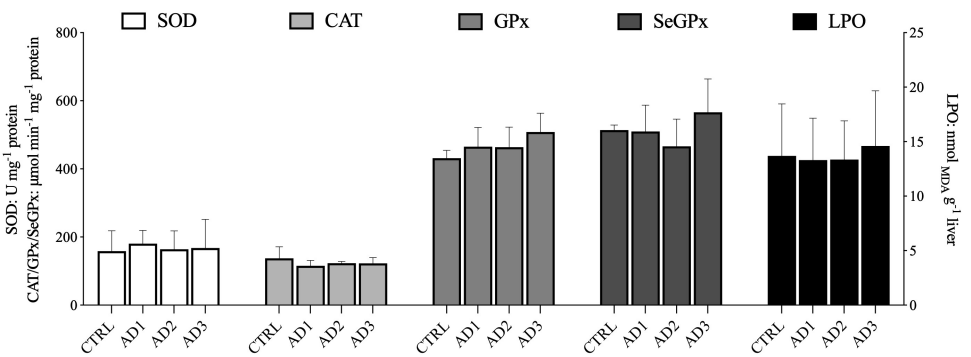

FIGURE 3 | Oxidative stress-related parameters in the liver of gilthead seabream fed the experimental diets over 12 weeks. No significant differences between dietary treatments were observed. Values are presented as mean ± SD.

that only the highest incorporation of *L. digitata* (0.8%) in AD3 fortified the filet with iodine (0.09 μg g^{-1} in AD3 vs. 0.07 μg g^{-1} in CTRL) (Barbosa et al., 2020), but impaired fish growth. Therefore, further studies are required to find sustainable feed formulations that can efficiently fortify the filets of gilthead seabream in iodine without compromising growth performance.

Dietary selenium is involved in several fish physiological processes, namely growth and antioxidant defense mechanism, through the action of selenoproteins such as deiodinases and glutathione peroxidase (Khan et al., 2017). In the present study, the hepatic expression of several selenoproteins (*selk*, *dio2*, *gpx1a,* and *gpx1b*) was evaluated and, generally, an upregulation was observed in fish consuming AD1, so the response of selenoproteins to dietary selenium level was not dose-dependent. Deiodinase enzymes have an important role in iodine and thyroid hormones metabolism, converting the inactive thyroxine (T4) to the active form triiodothyronine (T3) (Milanesi and Brent, 2017). The expression of the hepatic *dio2* followed the same trend observed for most selenoproteins rather than reflecting different iodine concentrations of diets supplemented with *L. digitata*. These results are in agreement with a previous study with rainbow trout, where diets containing high concentrations of iodine, through supplementation with the macroalga *S. latissima*, had limited impact on iodine-metabolism-related genes (Ferreira et al., 2020). In the present study, the expression of *dio2* was correlated positively with the expression of hepatic *igf2*, indicating that the effect of dietary selenium on growth might be partly exerted through the action of deidodinases. The determination of circulating concentrations of T3 and T4 could provide further insights into the impact of diets supplemented with organic selenium and iodine on thyroid hormone metabolism in fish.

As rich sources of bioactive compounds, the inclusion of algae in aquafeeds is often associated with protective effects against oxidative damage (Goiris et al., 2012; Wan et al., 2019). However, data from this study indicate a lack of differences in oxidative stress enzymes and LPO in the liver, which suggests that the experimental diets had limited impact on the antioxidant status, and that fish were able to maintain homeostasis. Antioxidant enzymes such as CAT, GPx and SeGPx are paramount to lessen the negative effects of hydroperoxides, increasing the body's ability to protect cells from oxidative damage, thus preventing lipid peroxidation. The expression of both *gpx1a* and *gpx1b*, which encode for glutathione peroxidase, increased in the liver of fish fed AD1. However, the differences at the phenotype level (activity of oxidative stress enzymes) did not reflect the genotypic differences observed in the liver. Since all fish were reared under optimal environmental conditions for the species, a pro-oxidant challenge should have been carried out to further clarify potential health benefits associated with the tested diets, especially if complemented with a quantification of liver glutathione. Additionally, the simultaneous upregulation of several genes encoding selenoproteins (*gpx1*, *selk,* and *dio2*) in fish consuming the AD1 diet may indicate that a prolonged exposure to this diet or a pro-oxidant challenge may modulate the levels of these proteins.

CONCLUSION

Our results demonstrated that biofortified diets with up to 0.4% *L. digitata* and 0.02% selenised yeast do not affect fish performance, but higher concentrations (0.8 and 0.04%, respectively) impair gilthead seabream growth. Fish consuming AD1 and AD2 grew equally well as fish fed the CTRL diet, indicating the potential of microalgae meal (*Chlorella* sp., *Tetraselmis* sp., and DHA-rich *Schizochytrium* sp.) to partially replace both FM and FO in diets for *S. aurata*. LC-PUFAs retention values were generally low, highlighting the need for new approaches to improve it in fish. Moreover, the high price and unpredictable supply of *Schizochytrium* sp. is still a limiting factor for global implementation in aquafeeds. Therefore, further efforts should be made to either reduce these costs or produce cost-effective feeds combining LC-PUFAs rich-microalgae supplementation with more affordable plant-based ingredients. A concomitant decreased bioavailability of LC-PUFAs and upregulation of hepatic *elovl5* in fish consuming AD3 resulted in higher EPA + DHA contents in the muscle. In fact, the filet of fish consuming this diet contained EPA and DHA levels that were 2.7 times above the minimum recommended levels for human consumption. Additionally, the experimental diets had limited effects on the lipogenic (*fas* and *srebp1*) and lipolytic (*cpt1a*) pathways related genes. Overall, omega-3 fatty acids from microalgae were effectively accumulated in *S. aurata* muscle, confirming their potential as a good source of dietary lipids for this fish species. A higher retention and gain of selenium were observed for fish consuming the AD diets, indicating the potential of selenised yeast to fortify fish in selenium. Supplementation with *L. digitata* led to similar iodine gain, but lower retention levels of iodine, evidencing the need for new approaches to fortify gilthead seabream in this essential element. Despite the absence of differences in antioxidant enzymes activities, hepatic expression of *gpx1a* and *gpx1b* was upregulated in fish fed AD1. Likewise, other selenoproteins genes (*selk*, *dio2*) were also upregulated in fish consuming this diet. Further studies seem to be required under challenging conditions to further evaluate the health benefits of the tested diets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of the EPPO-IPMA (Estação Piloto de Piscicultura de Olhão, Av. Parque Natural da Ria Formosa N, 8700-194, Olhão), overseen by the National Competence Authority.

AUTHOR CONTRIBUTIONS

MF: manuscript draft, investigation, formal analysis, and writing – review and editing. PR: investigation and formal analysis. LR: conceptualization, methodology, investigation, and writing – review and editing. MB: investigation. VD, SS, CS, and AlM: investigation and writing – review and editing. PP-F: funding acquisition and methodology. JD: conceptualization.

LC: investigation, methodology, and writing – review and editing. AnM and MN: funding acquisition, methodology, and writing – review and editing. LV: conceptualization, methodology, validation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.812884/full#supplementary-material

REFERENCES

Acquah, C., Tibbetts, S. M., Pan, S., and Udenigwe, C. (2020). "Chapter 19 - Nutritional quality and bioactive properties of proteins and peptides from microalgae," in *Handbook of Microalgae-Based Processes and Products*, eds E. Jacob-Lopes, M. M. Maroneze, M. I. Queiroz, and L. Q. Zepka (Cambridge, MA: Academic Press), 493–531. doi: 10.1016/B978-0-12-818536-0.00019-1

AOAC (2006). *Official Methods of Analysis*. Gaithersburg, MD: Association of Official Analytical Chemists.

Avella, M. A., Gioacchini, G., Decamp, O., Makridis, P., Bracciatelli, C., and Carnevali, O. (2010). Application of multi-species of *Bacillus* in sea bream larviculture. *Aquaculture* 305, 12–19. doi: 10.1016/j.aquaculture.2010.03.029

Barbosa, V., Maulvault, A. L., Anacleto, P., Santos, M., Mai, M., Oliveira, H., et al. (2020). Enriched feeds with iodine and selenium from natural and sustainable sources to modulate farmed gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*) fillets elemental nutritional value. *Food Chem. Toxicol.* 140:111330. doi: 10.1016/j.fct.2020.111330

Batista, S., Pereira, R., Oliveira, B., Baião, L. F., Jessen, F., Tulli, F., et al. (2020). Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. *J. Appl. Phycol.* 32, 2041–2059. doi: 10.1007/s10811-020-02118-z

Beers, R. F. Jr., and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 195, 133–140.

Benedito-Palos, L., Ballester-Lozano, G., and Pérez-Sánchez, J. (2014). Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene* 547, 34–42. doi: 10.1016/j.gene.2014.05.073

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.-A., Kaushik, S., and Pérez-Sánchez, J. (2007). Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. *Aquaculture* 267, 199–212. doi: 10.1016/j.aquaculture.2007.01.011

Betancor, M. B., Sprague, M., Montero, D., Usher, S., Sayanova, O., Campbell, P. J., et al. (2016). Replacement of marine fish oil with de novo omega-3 oils from transgenic camelina sativa in feeds for gilthead sea bream (*Sparus aurata* L.). *Lipids* 51, 1171–1191. doi: 10.1007/s11745-016-4191-4

Bondia-Pons, I., Molto-Puigmarti, C., Castellote, A. I., and Lopez-Sabater, M. C. (2007). Determination of conjugated linoleic acid in human plasma by fast gas chromatography. *J. Chromatogr.* 1157, 422–429. doi: 10.1016/j.chroma.2007.05.020

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Cardinaletti, G., Messina, M., Bruno, M., Tulli, F., Poli, B. M., Giorgi, G., et al. (2018). Effects of graded levels of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* dried biomass on growth and muscle tissue composition of European sea bass (*Dicentrarchus labrax*) fed diets low in fish meal and oil. *Aquaculture* 485, 173–182. doi: 10.1016/j.aquaculture.2017.11.049

Castro, L. F. C., Tocher, D. R., and Monroig, O. (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of *Fads* and *Elovl* gene repertoire. *Prog. Lipid Res.* 62, 25–40. doi: 10.1016/j.plipres.2016.01.001

Dantagnan, P., Hernández, A., Borquez, A., and Mansilla, A. (2009). Inclusion of macroalgae meal (*Macrocystis pyrifera*) as feed ingredient for rainbow trout (*Oncorhynchus mykiss*): effect on flesh fatty acid composition. *Aquac. Res.* 41, 87–94. doi: 10.1111/j.1365-2109.2009.02308.x

EFSA (2010). Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J.* 8:1461. doi: 10.2903/j.efsa.2010.1461

EFSA (2014). Scientific opinion on health benefits of seafood (fish and shellfish) consumption in relation to health risks associated with exposure to methylmercury. *EFSA J.* 12:3761.

Erdelmeier, I., Gérard-Monnier, D., Yadan, J.-C., and Chaudière, J. (1998). Reactions of N-Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem. Res. Toxicol.* 11, 1184–1194. doi: 10.1021/tx970180z

Estruch, G., Collado, M. C., Monge-Ortiz, R., Tomás-Vidal, A., Jover-Cerdá, M., Peñaranda, D. S., et al. (2018). Long-term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. *BMC Vet. Res.* 14:302. doi: 10.1186/s12917-018-1626-6

FAO (2020). *Fisheries And Aquaculture Software. FishStatJ-Software For Fishery Statistical Time Series*. Rome: FAO Fisheries and Aquaculture Department [online]. Available online at: http://www.fao.org/fishery/ (accessed March 2, 2021).

Ferreira, M., Larsen, B. K., Granby, K., Cunha, S. C., Monteiro, C., Fernandes, J. O., et al. (2020). Diets supplemented with *Saccharina latissima* influence the expression of genes related to lipid metabolism and oxidative stress modulating rainbow trout (*Oncorhynchus mykiss*) fillet composition. *Food Chem. Toxicol.* 140:111332. doi: 10.1016/j.fct.2020.111332

Flohe, L., and Gunzler, W. A. (1984). Assays of glutathione peroxidase. *Methods Enzymol.* 105, 114–121. doi: 10.1016/s0076-6879(84)05015-1

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226, 497–509.

Ganuza, E., Benítez-Santana, T., Atalah, E., Vega-Orellana, O., Ganga, R., and Izquierdo, M. S. (2008). *Crypthecodinium cohnii* and *Schizochytrium* sp. as potential substitutes to fisheries-derived oils from seabream (*Sparus aurata*) microdiets. *Aquaculture* 277, 109–116. doi: 10.1016/j.aquaculture.2008.02.005

Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., De Brabanter, J., and De Cooman, L. (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* 24, 1477–1486. doi: 10.1007/s10811-012-9804-6

Granby, K., Amlund, H., Valente, L. M. P., Dias, J., Adoff, G., Sousa, V., et al. (2020). Growth performance, bioavailability of toxic and essential elements and nutrients, and biofortification of iodine of rainbow trout (*Onchorynchus mykiss*) fed blends with sugar kelp (*Saccharina latissima*). *Food Chem. Toxicol.* 141:111387. doi: 10.1016/j.fct.2020.111387

Houston, S. J. S., Karalazos, V., Tinsley, J., Betancor, M. B., Martin, S. A. M., Tocher, D. R., et al. (2017). The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. *Br. J. Nutr.* 118, 1010–1022. doi: 10.1017/s0007114517002975

Izquierdo, M. S., Montero, D., Robaina, L., Caballero, M. J., Rosenlund, G., and Ginés, R. (2005). Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture* 250, 431–444. doi: 10.1016/j.aquaculture.2004.12.001

Izquierdo, M. S., Robaina, L., Juárez-Carrillo, E., Oliva, V., Hernández-Cruz, C. M., and Afonso, J. M. (2008). Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae (*Sparus aurata*). *Fish Physiol. Biochem.* 34, 117–127. doi: 10.1007/s10695-007-9152-7

Jin, M., Lu, Y., Yuan, Y., Li, Y., Qiu, H., Sun, P., et al. (2017a). Regulation of growth, antioxidant capacity, fatty acid profiles, hematological characteristics and expression of lipid related genes by different dietary n-3 highly unsaturated fatty acids in juvenile black seabream (*Acanthopagrus schlegelii*). *Aquaculture* 471, 55–65. doi: 10.1016/j.aquaculture.2017.01.004

Jin, M., Monroig, Ó, Lu, Y., Yuan, Y., Li, Y., Ding, L., et al. (2017b). Dietary DHA/EPA ratio affected tissue fatty acid profiles, antioxidant capacity, hematological characteristics and expression of lipid-related genes but not growth in juvenile black seabream (*Acanthopagrus schlegelii*). *PLoS One* 12:e0176216. doi: 10.1371/journal.pone.0176216

Khan, K. U., Zuberi, A., Fernandes, J. B. K., Ullah, I., and Sarwar, H. (2017). An overview of the ongoing insights in selenium research and its role in fish nutrition and fish health. *Fish Physiol. Biochem.* 43, 1689–1705. doi: 10.1007/s10695-017-0402-z

Kousoulaki, K., Gerd Marit, B., Mørkøre, T., Krasnov, A., Baeverfjord, G., Ytrestøyl, T., et al. (2020). Microalgal *Schizochytrium limacinum* biomass improves growth and filet quality when used long-term as a replacement for fish oil, in modern salmon diets. *Front. Mar. Sci* 7:57. doi: 10.3389/fmars.2020.00057

Li, Y., and Schellhorn, H. E. (2007). Rapid kinetic microassay for catalase activity. *J. Biomol. Tech.* 18, 185–187.

Liu, G.-X., Jiang, G.-Z., Lu, K.-L., Li, X.-F., Zhou, M., Zhang, D.-D., et al. (2017). Effects of dietary selenium on the growth, selenium status, antioxidant activities, muscle composition and meat quality of blunt snout bream, *Megalobrama amblycephala*. *Aquac. Nutr.* 23, 777–787. doi: 10.1111/anu.12444

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lopes-Marques, M., Kabeya, N., Qian, Y., Ruivo, R., Santos, M. M., Venkatesh, B., et al. (2018). Retention of fatty acyl desaturase 1 (*fads1*) in *Elopomorpha and Cyclostomata* provides novel insights into the evolution of long-chain polyunsaturated fatty acid biosynthesis in vertebrates. *BMC Evol. Biol.* 18:157. doi: 10.1186/s12862-018-1271-5

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Gonçalves, J. F. M., Rocha, C. M. R., et al. (2017). Dietary supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (*Sparus aurata*). *Biol. Open* 6, 897–908. doi: 10.1242/bio.024299

Malandrakis, E. E., Exadactylos, A., Dadali, O., Golomazou, E., Klaoudatos, S., and Panagiotaki, P. (2014). Molecular cloning of four glutathione peroxidase (GPx) homologs and expression analysis during stress exposure of the marine teleost *Sparus aurata*. *Comp. Biochem. Physiol. B* 168, 53–61. doi: 10.1016/j.cbpb.2013.11.005

Mariotti, M., Ridge, P. G., Zhang, Y., Lobanov, A. V., Pringle, T. H., Guigo, R., et al. (2012). Composition and evolution of the vertebrate and *mammalian selenoproteomes*. *PLoS One* 7:e33066. doi: 10.1371/journal.pone.0033066

Matos, E., Dias, J., Dinis, M. T., and Silva, T. S. (2017). Sustainability vs. Quality in gilthead seabream *(Sparus aurata L.)* farming: are trade-offs inevitable? *Rev. Aquac.* 9, 388–409. doi: 10.1111/raq.12144

Mechlaoui, M., Dominguez, D., Robaina, L., Geraert, P.-A., Kaushik, S., Saleh, R., et al. (2019). Effects of different dietary selenium sources on growth performance, liver and muscle composition, antioxidant status, stress response and expression of related genes in gilthead seabream (*Sparus aurata*). *Aquaculture* 507, 251–259. doi: 10.1016/j.aquaculture.2019.04.037

Milanesi, A., and Brent, G. A. (2017). "Chapter 12 - Iodine and thyroid hormone synthesis, metabolism, and action," in *Molecular, Genetic, and Nutritional Aspects of Major and Trace Minerals*, ed. J. F. Collins (Boston: Academic Press), 143–150. doi: 10.1016/B978-0-12-802168-2.00012-9

Miller, M. R., Nichols, P. D., and Carter, C. G. (2007). Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 148, 382–392. doi: 10.1016/j.cbpa.2007.05.018

Mommsen, T. P. (2001). Paradigms of growth in fish. *Comp. Biochem. Physiol. B* 129, 207–219. doi: 10.1016/S1096-4959(01)00312-8

Montero, D., Grasso, V., Izquierdo, M. S., Ganga, R., Real, F., Tort, L., et al. (2008). Total substitution of fish oil by vegetable oils in gilthead sea bream (*Sparus aurata*) diets: effects on hepatic Mx expression and some immune parameters. *Fish Shellfish Immunol.* 24, 147–155. doi: 10.1016/j.fsi.2007.08.002

Morshedi, V., Nafisi Bahabadi, M., Sotoudeh, E., Azodi, M., and Hafezieh, M. (2018). Nutritional evaluation of *Gracilaria pulvinata* as partial substitute with fish meal in practical diets of barramundi (*Lates calcarifer*). *J. Appl. Phycol.* 30, 619–628. doi: 10.1007/s10811-017-1199-y

Mourente, G., and Bell, J. G. (2006). Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax* L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp. Biochem. Physiol. B* 145, 389–399. doi: 10.1016/j.cbpb.2006.08.012

Nasopoulou, C., and Zabetakis, I. (2012). Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. *LWT* 47, 217–224. doi: 10.1016/j.lwt.2012.01.018

Peixoto, M. J., Ferraz, R., Magnoni, L. J., Pereira, R., Gonçalves, J. F., Calduch-Giner, J., et al. (2019). Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to bacterial infection. *Sci. Rep.* 9:16134. doi: 10.1038/s41598-019-52693-6

Pereira, R., Valente, L. M. P., Sousa-Pinto, I., and Rema, P. (2012). Apparent nutrient digestibility of seaweeds by rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). *Algal Res.* 1, 77–82. doi: 10.1016/j.algal.2012.04.002

Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha, J. A., Perera, E., Bermejo-Nogales, A., et al. (2018). Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Front. Endocrinol* 9:687. doi: 10.3389/fendo.2018.00687

Ramos, A., Bandarra, N. M., Rema, P., Vaz-Pires, P., Nunes, M. L., Andrade, A. M., et al. (2008). Time course deposition of conjugated linoleic acid in market size rainbow trout (*Oncorhynchus mykiss*) muscle. *Aquaculture* 274, 366–374. doi: 10.1016/j.aquaculture.2007.11.040

Ribeiro, A. R. (2019). *Farmed Fish As A Functional Food: Fortification Strategies With Health Valuable Compounds*. Doctoral Thesis, University of the Algarve, Portugal. Available online at: http://hdl.handle.net/10400.1/13604 (accessed September 20, 2021).

Ribeiro, A. R., Gonçalves, A., Bandarra, N., Nunes, M. L., Dinis, M. T., Dias, J., et al. (2017). Natural fortification of trout with dietary macroalgae and selenised-yeast increases the nutritional contribution in iodine and selenium. *Food Res. Int.* 99, 1103–1109. doi: 10.1016/j.foodres.2016.10.030

Ribeiro, A. R., Gonçalves, A., Colen, R., Nunes, M. L., Dinis, M. T., and Dias, J. (2015). Dietary macroalgae is a natural and effective tool to fortify gilthead seabream fillets with iodine: effects on growth, sensory quality and nutritional value. *Aquaculture* 437, 51–59. doi: 10.1016/j.aquaculture.2014.11.028

Saffari, S., Keyvanshokooh, S., Zakeri, M., Johari, S. A., and Pasha-Zanoosi, H. (2017). Effects of different dietary selenium sources (sodium selenite, selenomethionine and nanoselenium) on growth performance, muscle composition, blood enzymes and antioxidant status of common carp (*Cyprinus carpio*). *Aquac. Nutr.* 23, 611–617. doi: 10.1111/anu.12428

Salmerón, C., Riera-Heredia, N., Gutiérrez, J., Navarro, I., and Capilla, E. (2016). Adipogenic gene expression in gilthead sea bream mesenchymal stem cells from different origin. *Front. Endocrinol.* 7:113. doi: 10.3389/fendo.2016.00113

Sarker, P. K., Kapuscinski, A. R., Lanois, A. J., Livesey, E. D., Bernhard, K. P., and Coley, M. L. (2016). Towards sustainable aquafeeds: complete substitution of fish oil with marine microalga *Schizochytrium* sp. improves growth and fatty acid deposition in juvenile Nile tilapia (*Oreochromis niloticus*). *PLoS One* 11:e0156684. doi: 10.1371/journal.pone.0156684

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H. G., and Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *J. Trace Elem. Med. Biol.* 47, 124–133. doi: 10.1016/j.jtemb.2018.02.005

Seong, T., Uno, Y., Kitagima, R., Kabeya, N., Haga, Y., and Satoh, S. (2021). Microalgae as main ingredient for fish feed: non-fish meal and non-fish oil diet development for red sea bream, Pagrus major, by blending of microalgae *Nannochloropsis*, *Chlorella* and *Schizochytrium*. *Aquac. Res.* 52, 6025–6036. doi: 10.1111/are.15463

Sun, Y., Oberley, L. W., and Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. *Clin. Chem.* 34, 497–500.

Swanson, D., Block, R., and Mousa, S. A. (2012). Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Adv. Nutr.* 3, 1–7. doi: 10.3945/an.111.000893

Teimouri, M., Yeganeh, S., Mianji, G. R., Najafi, M., and Mahjoub, S. (2019). The effect of *Spirulina platensis* meal on antioxidant gene expression, total antioxidant capacity, and lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* 45, 977–986. doi: 10.1007/s10695-019-0608-3

Tibaldi, E., Chini Zittelli, G., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., et al. (2015). Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried Isochrysis sp. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. *Aquaculture* 440, 60–68. doi: 10.1016/j.aquaculture.2015.02.002

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac. Res.* 41, 717–732. doi: 10.1111/j.1365-2109.2008.02150.x

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* 449, 94–107. doi: 10.1016/j.aquaculture.2015.01.010

Valente, L. M. P., Cabrita, A. R. J., Maia, M. R. G., Valente, I. M., Engrola, S., Fonseca, A. J. M., et al. (2021). "Microalgae as feed ingredients for livestock production and aquaculture," in *Microalgae*, ed. C. M. Galanakis (Cambridge, MA: Academic Press), 239–312. doi: 10.1016/B978-0-12-821218-9.00009-8

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.0031. doi: 10.1186/gb-2002-3-7-research0034

Vizcaíno, A. J., Saéz, M. I., López, G., Arizcun, M., Abellán, E., Martínez, T. F., et al. (2016). *Tetraselmis suecia* and *Tisochrysis lutea* meal as dietary ingredients for gilthead sea bream (*Sparus aurata* L.) fry. *J. Appl. Phycol.* 28, 2843–2855. doi: 10.1007/s10811-016-0845-0

Wan, A. H. L., Davies, S. J., Soler-Vila, A., Fitzgerald, R., and Johnson, M. P. (2019). Macroalgae as a sustainable aquafeed ingredient. *Rev. Aquac.* 11, 458–492. doi: 10.1111/raq.12241

Wang, L., Zhang, X., Wu, L., Liu, Q., Zhang, D., and Yin, J. (2018). Expression of selenoprotein genes in muscle is crucial for the growth of rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with selenium yeast. *Aquaculture* 492, 82–90. doi: 10.1016/j.aquaculture.2018.03.054

Wassef, E. A., El-Sayed, A.-F. M., and Sakr, E. M. (2013). *Pterocladia* (Rhodophyta) and *Ulva* (Chlorophyta) as feed supplements for European seabass, *Dicentrarchus labrax L., fry. J. Appl. Phycol.* 25, 1369–1376. doi: 10.1007/s10811-013-9995-5

Xuan, X., Wen, X., Li, S., Zhu, D., and Li, Y. (2013). Potential use of macroalgae Gracilaria lemaneiformis in diets for the black sea bream. *Acanthopagrus schlegelii, juvenile*. *Aquaculture* 41, 167–172. doi: 10.1016/j.aquaculture.2013.07.022

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* 13:134. doi: 10.1186/1471-2105-13-134

Zeynali, M., Nafisi Bahabadi, M., Morshedi, V., Ghasemi, A., and Torfi Mozanzadeh, M. (2020). Replacement of dietary fishmeal with *Sargassum ilicifolium* meal on growth, innate immunity and immune gene mRNA transcript abundance in *Lates calcarifer* juveniles. *Aquac. Nutr.* 26, 1657–1668. doi: 10.1111/anu.13111

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Dietary Inclusion of Hydrolyzed *Debaryomyces hansenii* Yeasts Modulates Physiological Responses in Plasma and Immune Organs of Atlantic Salmon (*Salmo salar*) Parr Exposed to Acute Hypoxia Stress

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Stress related to salmon aquaculture practices (handling, sub-optimal nutrition, diseases, and environmental problems) may compromise fish welfare. This study describes the effects of two hydrolyzed *Debaryomyces hansenii* yeast-based products (LAN4 and LAN6) on physiological and immune responses of Atlantic salmon (*Salmo salar*) parr exposed to short hypoxia stress. A commercial-like diet (control diet: CD) and two experimental diets (CD supplemented with 0.1% of either component LAN4 or LAN6) were fed to fish for 8 weeks. At the end of the feeding experiment, fish were exposed to 1-min hypoxia and samples were collected at 0, 1, 3, 6, 12, and 24 h post-stress. Results showed that plasma cortisol reached a peak at 1 h post-stress in CD and LAN6 groups, whereas no significant increase in cortisol levels was detected in the LAN4 group. Moreover, the LAN6 group enhanced IL-10 responses to hypoxia, when compared to the control and LAN4 group. This suggests a regulation of immunosuppressive profiles in fish fed LAN4. Hypoxia stress increased TNFα in all groups, which indicates that fish may compensate for the short-term stress response, by modulating innate immune molecules. The apparent suppression of hypoxia responses in the LAN4 group coincided with the detection of differences in goblet cells size and Muc-like proteins production in DI; and upregulation (1 h post-stress) of pathways related to oxygen transport, hemoglobin complex, and glutathione transferase activity and the downregulation of fatty acid metabolism (6 h post-stress) in gills. To conclude, a 1-min hypoxia stress exposure affects the response to stress and immunity; and *D. hansenii*-based yeast products are promising components in functional aquafeeds for salmon due to their ability to counteract possible consequences of hypoxic stress.

Keywords: blood, distal intestine, gill, immunological markers, RNAseq, short-term stress, yeast-based products

INTRODUCTION

Aquaculture is a continuously expanding global industry. However, the rapid growth and the subsequent intensification in this sector causes increased stress incidences and increased risk of infectious diseases, which leads to large economic losses (Kiron, 2012; Food and Agriculture Organization of the United Nations, 2020).

Different processes related to salmonid farming such as transportation, grading, vaccination, and handling, where fish are taken out of the water or are exposed to low dissolved oxygen concentrations, can cause short-term stress, which deteriorates fish welfare (Braithwaite and Ebbesson, 2014; Peterson et al., 2019) due to negative synergistic effects on the physiological response of the fish (e.g., immune response) (Catalán et al., 2019; Reid et al., 2019).

Preventive measures and good husbandry practices are important for the sustainable growth of aquaculture. Nutrition and the use of functional feeds can help to alleviate stress. They can prevent immune suppression, as certain feed components can reduce the risk of diseases, and improve health and overall fish welfare. Modulation of the immune system by functional ingredients has previously been shown in several teleost fish such as Chinook salmon (*Onchoryhnchus tshawytscha*), rainbow trout (*Oncorhynchus mykiss*), African catfish (*Clarius gariepinus*), gilthead sea bream (*Sparus aurata*), European sea bass, (*Dicentrarchus labrax*), tilapia (*Oreochromis niloticus*), turbot (*Scophthalmus maximus*), and Indian carp (*Labeo rohita*). Moreover, in Atlantic salmon (*Salmo salar*), it has already been reported that functional ingredients or additives can beneficially impact health by modulating the respiratory burst of head kidney macrophage, controlling profiles associated with soybean meal-induced enteritis (SBMIE), and improving the robustness of the fish against various infectious agents (e.g., *Neoparamoeba perurans* and piscine myocarditis virus) (Torrecillas et al., 2012; Meena et al., 2013; Martinez-Rubio et al., 2014; Fuchs et al., 2017; Mullins et al., 2020; Agboola et al., 2021a; Rawling et al., 2021).

Among different functional ingredients, microbial ingredients (e.g., non-*Saccharomyces* yeast such as *Cyberlindnera jadinii*, *Blastobotrys adeninivorans,* and *Wickerhamomyces anomalus* or its cell components) are gaining increased interest in aquafeeds during recent years (Torrecillas et al., 2012; Ochangco et al., 2016; Øverland and Skrede, 2017; Glencross et al., 2020; Rawling et al., 2021). Yeast contains a wide range of microbe-associated molecular patterns (MAMPs) (e.g., α-glucan, β-glucan, α-mannan, and nucleic acids) (Navarrete and Tovar-Ramírez, 2014), which act on host pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), activating cells of the immune system (Whyte, 2007). Furthermore, these molecules can modulate the immune response of salmonids, which induces both local effects in the intestine (strengthening the intestinal barrier and increasing resistance to infectious pathogens) and systemic effects, by regulating pro- and anti-inflammatory cytokines, effector molecules, and coordinating antigen-presenting cells (Schmitt et al., 2015; Sahlmann et al., 2019; Morales-Lange et al., 2021).

A promising non-*Saccharomyces* yeast candidate as a functional ingredient for aquafeeds is *Debaryomyces hansenii.* This yeast has been isolated from different sources such as soil, seawater, foods, and clinical samples (Wrent et al., 2015). In different animal groups (from invertebrates to higher vertebrates), it has been described that *D. hansenii* possesses probiotic properties, which induces gut microbiota modulation [e.g., increasing operational taxonomic units (OTUs) of intestinal bacteria and promoting high colonization of the digestive tract], enhanced animal survival against pathogen challenges such as *Amyloodinium ocellatum* and *Vibrio anguillarum,* and stimulating the humoral immune response of the host (Reyes-Becerril et al., 2008; Caruffo et al., 2015; Angulo et al., 2018, 2020). Furthermore, selected inactivated fractions from this yeast by heat or hydrolysis (e.g., cell wall components and polyamines) have also shown immunomodulatory properties, increasing both phagocytic ability, and the production of nitric oxide in leukocytes, in addition to inducing the expression of immune-related genes such as NFκB, IL-1β, and TNFα (Angulo et al., 2018, 2020).

The main objective of this study was to evaluate the effect of two different hydrolyzed *D. hansenii* yeast-based products on plasma biomarkers and immune organs of Atlantic salmon after exposure to short-term hypoxia stress. This knowledge will facilitate the inclusion of bioactive components based on *D. hansenii* in functional feeds for salmonids, with the potential to modulate stress-related responses in fish exposed to challenging conditions.

MATERIALS AND METHODS

Experimental Design

The experiment was carried out at the Center for Sustainable Aquaculture at the Norwegian University of Life Sciences, Ås, Norway. All animals were treated according to the laws and regulations for experiments on live animals in the EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761).

A total of 540 Atlantic salmon pre-smolts (35 g initial body weight; GEN-innOva® GAIN from AquaGen AS; Trondheim, Norway) were randomly distributed in nine circular tanks (300-L fiberglass tanks with averaged 14.5°C recirculated fresh water at a flow rate keeping the oxygen level above 80% saturation). The experiment lasted 7 weeks, starting with 5 weeks of winter regime 8/16 h (L/D) and ending with 2 weeks of 24-h light. All units were controlled by OxyGuard water quality monitoring and control systems for aquaculture (OxyGuard International A/S, Denmark). No significant changes in water quality were detected.

Each tank was randomly allocated one of three experimental diets (in triplicate): a commercial-like diet (control diet: CD), and two experimental diets, where the control diet was supplemented with 0.1% of either compound LAN4 or LAN6 (hydrolyzed *D. hansenii*-based products from Lallemand SAS, France). The diets were produced by extrusion and subsequent vacuum coating with fish oil at the Centre for Feed Technology, Norwegian University of Life Sciences (Ås, Norway). The

TABLE 1 | Diets composition.

Ingredient (%)	CD	LAN4	LAN6
Fish meal[a]	39	39	39
Soy protein concentrate[b]	24.67	24.67	24.67
Wheat gluten[c]	3.34	3.34	3.34
Wheat[d]	11.8	11.8	11.8
Fish oil[e]	8.09	8.09	8.09
Rapeseed oil[f]	9	9	9
Premix[g]	2.85	2.85	2.85
Debaryomyces hansenii (LAN4)[h]	0	0.1	0
Debaryomyces hansenii (LAN6)[h]	0	0	0.1
Water correction	1.24	1.14	1.14
Yttrium oxide[i]	0.01	0.01	0.01

[a] *LT Fish meal, Norsildmel AS, Bergen, Norway.*
[b] *Soy protein concéntrate, Tradkon SPC HC-200, Sojaprotein, Becej, Serbia.*
[c] *Wheat gluten, Amilina AB, Panevezys, Lithuania.*
[d] *Wheat, Norgesmøllene, Bergen, Norway.*
[e] *Fish oil (28 % EPA + DHA), Nordsildmel AS, Bergen, Norway.*
[f] *Rapeseed oil, AAK, Karlshamn, Sweden.*
[g] *Premix (vitamineral-p-AA-kolin), BioMar AS, Norway.*
[h] *Debaryomyces hansenii, Lallemand, France.*
[i] *Yttrium oxide, Metal Rare Earth Limited, Shenzen, China.*

TABLE 2 | Characterization of hydrolyzed *D. hansenii*-based products.

	LAN4	LAN6
Cell size (μm)	3.2 ± 0.5	3.5 ± 0.6
Cell wall thickness (nm)	143.1 ± 35.7	119.2 ± 24.8
α-glucans (% w/w)	19.2	6.6
β–glucans (% w/w)	40.8	19.3
Mannans (% w/w)	7.4	14.8
Mannans length (nm)	83	105
Ratio βglucans/Mannans	5.5	1.3
Origin	Marine	Dairy

% w/w, expressed as % of dry matter.

composition of each diet is described in **Table 1**, and the morphological characterization, composition, and origin of both hydrolyzed *D. hansenii*-based products are shown in **Table 2**.

Fish were fed 10% excess for 7 weeks using automatic belt feeders (6 h a day) with a feeding level of 2% of body weight. After 7 weeks of feeding period, 42 Atlantic salmon per dietary group were distributed into seven tanks (6 fish/tank). Fish were acclimatized and continued to feed on their respective diets for 1 more week until the exposure to an acute hypoxia stress challenge.

The acute hypoxia stress challenge applied was described by Djordjevic et al. (2021a). Briefly, for each dietary treatment, a pre-stress group was sampled, and then, short-term hypoxia was inflicted by netting the fish out of the experimental tanks for 1 min and afterward placing them back into their respective tanks. After the stressful condition, six fish per diet and sampling time (0 h: immediately after stress, 1, 3, 6, 12, and 24 h post-stress exposure) were randomly netted out, sedated using 15 mg L^{-1} MS222, previously neutralized with sodium hydrogen carbonate (EMSURE® ACS, Reag.Ph Eur) and sampled for blood within 5 min from the moment they were placed into the sedation bath. Approximately 1 ml of blood was withdrawn from the caudal vein using 2-ml heparinized syringes. The blood samples were separated by centrifugation (3,000 × g; 5 min), and then, the plasma was aliquoted into 1.5-ml sterile Eppendorf tubes and stored at −80°C until analysis. Thereafter, each fish was killed by a quick blow to the head, measured for body weight, and dissected to obtain distal intestine (DI) and gill samples (from the left-hand-side second gill arch). For histology, DI samples were placed in 10% neutral buffered formalin for 48 h at room temperature and then processed according to the routine histological procedures. For molecular analysis, samples of DI and gill were immediately placed in RNA later, stored overnight at 4°C, and then kept at −80°C.

Plasma Parameters

Plasma levels of cortisol and glucose were assayed in duplicate per sample by a competitive enzyme-linked immunosorbent assay (ELISA) Cortisol assay kit (Ab108665, Abcam) and a Glucose assay kit (Ab65333, Abcam) using a SpectraMax microplate reader (Molecular Devices) and according to the manufacturer's instructions.

To detect immunological markers in plasma (TNFα; IL-10), an indirect ELISA was performed according to the study of Morales-Lange et al. (2021). Briefly, total proteins were quantified using the BCA Protein Assay Kit (Pierce) following the manufacturer's instructions. Then, each plasma sample was diluted in $NaHCO_3$ (60 mM pH 9.6) and seeded (by duplicate) in a 96-well plate (Nunc) at 50 ng μl^{-1} (100 μl) for overnight incubation at 4°C. After, 200 μl of Pierce Clear Milk Blocking Buffer 1x (BioRad) was incubated for 2 h at 37°C. Next, 100 μl of the primary antibody (**Table 3**) was added and incubated for 90 min at 37°C. After successive washes with PBST (PBS with 0.2% Tween 20), the secondary antibody was incubated for 60 min at 37°C (goat anti-mouse IgG-HRP diluted 1: 5,000). Finally, after several washes with PBST, chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (TMB, Thermo Fisher Scientific) was added (100 μl) and incubated for 20 min (in dark) at room temperature (RT). The reaction was stopped with 50 μl of 1 N sulfuric acid and read at 450 nm on a SpectraMax microplate reader (Molecular Devices).

Distal Intestine

Histological sections of DI were prepared following routine histology procedures. In brief, formalin-fixed DI samples were dehydrated, embedded in paraffin wax, sectioned at 5 μm thickness, dried in an oven overnight at 37°C, and stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS).

TABLE 3 | Primary antibodies for indirect enzyme-linked immunosorbent assay (ELISA).

Molecule	Source	Dilution	References
TNFα	Mouse	1:500	Morales-Lange et al., 2021
IL-10	Mouse	1:500	Morales-Lange et al., 2021
Muc-like proteins	Mouse	1:400	Djordjevic et al., 2021b

Sections were examined by light microscope (Zeiss Axio Lab.A1, Carl Zeiss, Germany) for the measurement of morphological parameters: villi simple fold length, goblet cells size, and goblet cell area according to the study of Djordjevic et al. (2021a). Furthermore, Muc-like proteins were detected in DI samples. For this, each DI sample was mechanically homogenized (with metal beads) in RIPA buffer (1:4) and then, total proteins were quantified through the BCA Protein Assay Kit. Thereafter, the indirect ELISA protocol was as follows: the samples were diluted in $NaHCO_3$ (60 mM pH 9.6) and seeded by duplicate in a 96-well plate (100 μl at 50 ng μl^{-1}) for overnight incubation (4°C). The next day, 200 μl of blocking buffer (1x) was incubated (2 h at 37°C), and then, the primary antibody against Muc-like proteins (**Table 3**) was added (100 μl) and incubated (90 min at 37°C). After successive washes with PBST, the secondary antibody (goat anti-mouse IgG-HRP diluted 1: 5,000) was incubated (60 min at 37°C) and washed (with PBST). TMB was added (100 μl) and incubated for 20 min (in dark) at RT. The reaction was stopped with 50 μl of 1 N sulfuric acid and read at 450 nm on a SpectraMax microplate reader.

RNA Sequencing

Gill samples from CD and LAN4 groups (3 fish per diet and sampling time: pre-stress, 1 h post-stress, and 6 h post-stress) were used to determine the gene expression by RNA sequencing (RNA-seq). Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the supplier's instructions. Each RNA sample was quantified using a NanoDrop^{TM} 8000 spectrophotometer (NanoDrop Technologies). RNA integrity was measured by Agilent Bioanalyzer 2100 (all samples showed an RNA integrity number $\geq$ 7). Thereafter, RNA-seq was performed by the Norwegian Sequencing Center (UiO, Norway) using Illumina NovaSeq 6000 System (150 bp paired-end RNA sequencing).

Data Analysis

Data analysis (means, standard deviation, and multiple comparisons test) and graphical presentation of the results were done using GraphPad Prism 8.0.2 (San Diego, CA, United States). One-way ANOVA with a Dunnett's multiple comparison *post-hoc* test was used to compare each post-stress time point with the pre-stress group (per diet). Furthermore, Tukey's multiple comparison test was used to compare the different diets within each time point. Differences were considered significant at $p < 0.05$.

Correlation coefficients were calculated using the corrplot package in R (Wei, 2021) among the different parameters by diet. The correlations were considered significant at $p < 0.01$ (degrees of freedom = 6).

RNA-seq raw data analysis was performed using nf-core rnaseq v3.3 (Ewels et al., 2020). Cleaned reads were aligned to *Salmo salar* genome SSAL_v3.1 (GenBank assembly accession: GCA_905237065.2). Fragment mapping was counted using featureCounts (subread v1.5.1), and differentially expressed genes (DEGs) were estimated using the SARTools R package (v1.7.3). Significant DEGs were determined when the adjusted *p*-value (padj) was < 0.05. ShinyGO v0.741 (Ge et al., 2020) was used to perform the enrichment analysis and functional classification of DEGs. Term categories (FDR < 0.05) were displayed and sorted by fold enrichment (minGSSize = 2).

RESULTS

Health Status and Growth Performance

During the trial, no mortality was recorded, and no significant differences in final body weight were observed between the dietary groups (CD: 103.6 g ± 15.8, LAN4: 108.7 g ± 17.7, LAN6: 104.4 g ± 19.2).

Plasma Biomarkers

Plasma cortisol results (**Figure 1A**) showed that the baseline levels (pre-stress groups) were as follows: 169.08 ± 77.38 ng ml^{-1} (CD), 152.38 ± 97.67 ng ml^{-1} (LAN4), and 57.04 ± 21.58 ng ml^{-1} (LAN6). Compared to their respective baseline levels, CD and LAN6 groups showed a significant increase in cortisol at 1 h post-stress (1,749.68 ± 806.99 ng ml^{-1} and 1,238.51 ± 419.41 ng ml^{-1}, respectively). In comparison, the LAN4 group did not show a significant increase in cortisol during the post-stress period; however, a significant decrease was measured in this dietary group at 0 (34.83 ± 9.54 ng ml^{-1}), 6 (60.36 ± 27.50 ng ml^{-1}), and 24 h (35.25 ± 24.97 ng ml^{-1}) post-stress (compared to the pre-stress group). In addition, by comparing the different diet groups within the sampling time, a significantly higher level of cortisol was detected in LAN4 in the pre-stress group (compared to LAN6). Nevertheless, LAN4 had a lower cortisol level (369.78 ± 29.04 ng ml^{-1}) compared to CD at 1 h post-stress.

Regarding plasma glucose levels (**Figure 1B**), at 24 h post-stress, both diets with the inclusion of *D. hansenii* showed lower glucose levels (LAN4: 195.59 ± 7.31 nmol μl^{-1} and LAN6: 204.19 ± 5.00 nmol μl^{-1}) than CD (219.71 ± 8.24 nmol μl^{-1}).

The analysis of cytokines in plasma samples (**Figure 2**) showed that TNFα secretion (**Figure 2A**) increased in all groups at 3 h post-stress (CD: 2.15-fold ± 0.38, LAN4: 2.00-fold ± 0.09, LAN6: 1.89-fold ± 0.23), 6 h post-stress (CD: 1.90-fold ± 0.26, LAN4: 1.39-fold ± 0.16, LAN6: 1.76-fold ± 0.12), and 24 h post-stress (CD: 1.79-fold ± 0.12, LAN4: 1.57-fold ± 0.15, LAN6: 1.86-fold ± 0.23) compared to their respective pre-stress group. Comparison between diets showed that LAN4 had significantly lower TNFα levels compared to CD and LAN6 at 6 h post-stress.

In the case of IL-10 (**Figure 2B**), the results showed that in the CD group, IL-10 levels increased at 6 h post-stress (1.14-fold ± 0.04) and decreases at 0 (0.54-fold ± 0.05), 1 (0.60-fold ± 0.04), 3 (0.82-fold ± 0.09), and 12 h (0.60-fold ± 0.06) post-stress compared to the pre-stress group (1.00-fold ± 0.07).

Whereas LAN4 showed a decrease of IL-10 at 0 (0.51-fold ± 0.04), 1 (0.51-fold ± 0.04), and 12 h (0.55-fold ± 0.07) post-stress (compared to pre-stress group), LAN6 showed increased levels at 3 (1.31-fold ± 0.06), 6 (1.68-fold ± 0.03), and 24 h (1.48-fold ± 0.06) post-stress (compared to the pre-stress group: 1.00-fold ± 0.25). Finally, the analysis by sampling time showed that LAN6 had higher levels of IL-10 than CD and LAN4

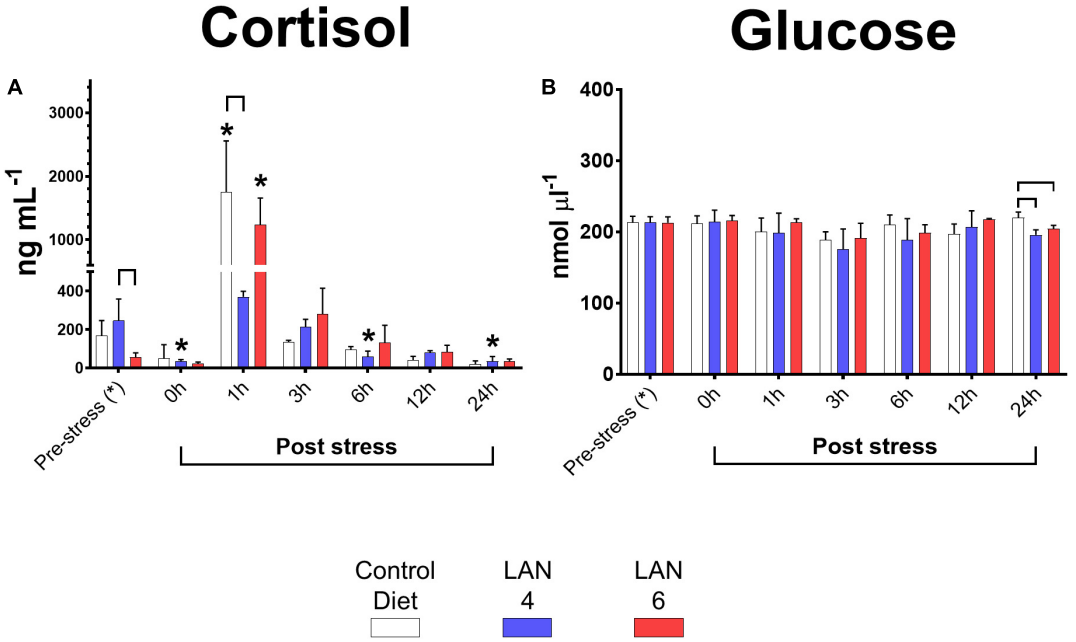

FIGURE 1 | Circulating cortisol and glucose in the plasma of Atlantic salmon exposed to acute hypoxia stress. **(A)** Cortisol (ng ml^{-1}). **(B)** Glucose level (nmol μl^{-1}). Sampling times: pre-stress, 0 h (immediately after stress), 1, 3, 6, 12, and 24 h post-stress. In white, CD. In blue, LAN4. In red, LAN6. Each bar: $n = 4$. *: Significant differences ($p < 0.05$) compared with pre-stress group (from each diet). Bracket: significant differences ($p < 0.05$) among dietary groups within sampling time (CD, LAN4, and LAN6, respectively).

(at 0, 1, 3, 12, and 24 h post-stress), and the CD group had higher levels than LAN4 at 6 h post-stress.

Biomarkers in the Distal Intestine

Morphometric data (**Figure 3**) from DI showed significant differences in villi simple fold length between diets at 1 h post-stress (LAN4: 1.14-fold ± 0.11 and LAN6: 0.90-fold ± 0.12) and 6 h post-stress (CD: 1.14-fold ± 0.11 and LAN4: 0.90-fold ± 0.12). Moreover, whereas in the goblet cell area, a significant difference was only detected at 6 h post-stress (increased value in LAN4: 2.18-fold ± 0.53, compared to CD: 0.60-fold ± 0.53 and LAN6: 0.74-fold ± 0.28). Several differences were detected for goblet cell size. In this parameter, an increase in the size of these cells was detected at 1 h post-stress in LAN4 (1.59-fold ± 0.29), compared to the pre-stress group (1.00-fold ± 0.17), and a decrease at 3 h post-stress in LAN6 (0.60-fold ± 0.15), compared to the pre-stress group (1.00-fold ± 0.10). Similarly, the analysis between the different diets showed that in the LAN6 group at 1 (0.95-fold ± 0.20), 3 (0.60-fold ± 0.15), and 6 h (0.78-fold ± 0.06), there was a smaller goblet cell, compared with LAN4 at the same post-stress sampling times (1.59-fold ± 0.29, 0.94-fold ± 0.19, 1.34-fold ± 0.32, respectively), and with CD at 3 h post-stress (1.26-fold ± 0.15). Representative histology images are shown in **Supplementary Data Sheet 2**.

The detection of Muc-like proteins showed differences in LAN4 at 1 (increase 1.35-fold ± 0.29), 3 (decrease 0.75-fold ± 0.05), 12 (increase: 1.45-fold ± 0.08), and 24 h (increase: 1.40-fold ± 0.07) post-stress compared to baseline levels (pre-stress). Comparison between diets within sampling time showed that CD group had a higher level of Muc-like proteins at 3 h post-stress (1.12-fold ± 0.21) compared to LAN4 (0.75-fold ± 0.05) and LAN6 (0.84-fold ± 0.08). In addition, the LAN4 group had higher levels of these proteins at 1, 12, and 24 h post-stress, compared with CD (at 12 h: 0.91-fold ± 0.09 and 24 h: 0.86-fold ± 0.15), and LAN6 (at 1 h: 0.88-fold ± 0.12, 12 h: 0.91-fold ± 0.06 and 24 h: 1.10-fold ± 0.03).

Correlations

The correlation among the different parameters (**Figure 4**) showed that cortisol levels in CD and LAN6 were significantly positively correlated (0.98). Moreover, glucose levels in LAN4 and LAN6 were also positively correlated (0.92). Regarding immunological markers, TNFα showed a significant positive correlation among all different diets (CD| L4 = 0.91, CD| L6 = 0.94, L4| L6 = 0.92).

In LAN6, TNFα was significantly negatively correlated with plasma glucose (−0.90) and goblet cell size (−0.90). In addition, goblet cell size was positively correlated with plasma glucose (0.92).

RNA-Seq in Gills

The comparison between LAN4 and CD showed that among the different sampling time points (pre-stress group, 1 h post-stress, and 6 h post-stress), DEGs were more upregulated than downregulated (**Table 4**): 238 upregulated DEGs and 125 downregulated DEGs were detected in the pre-stress group. At

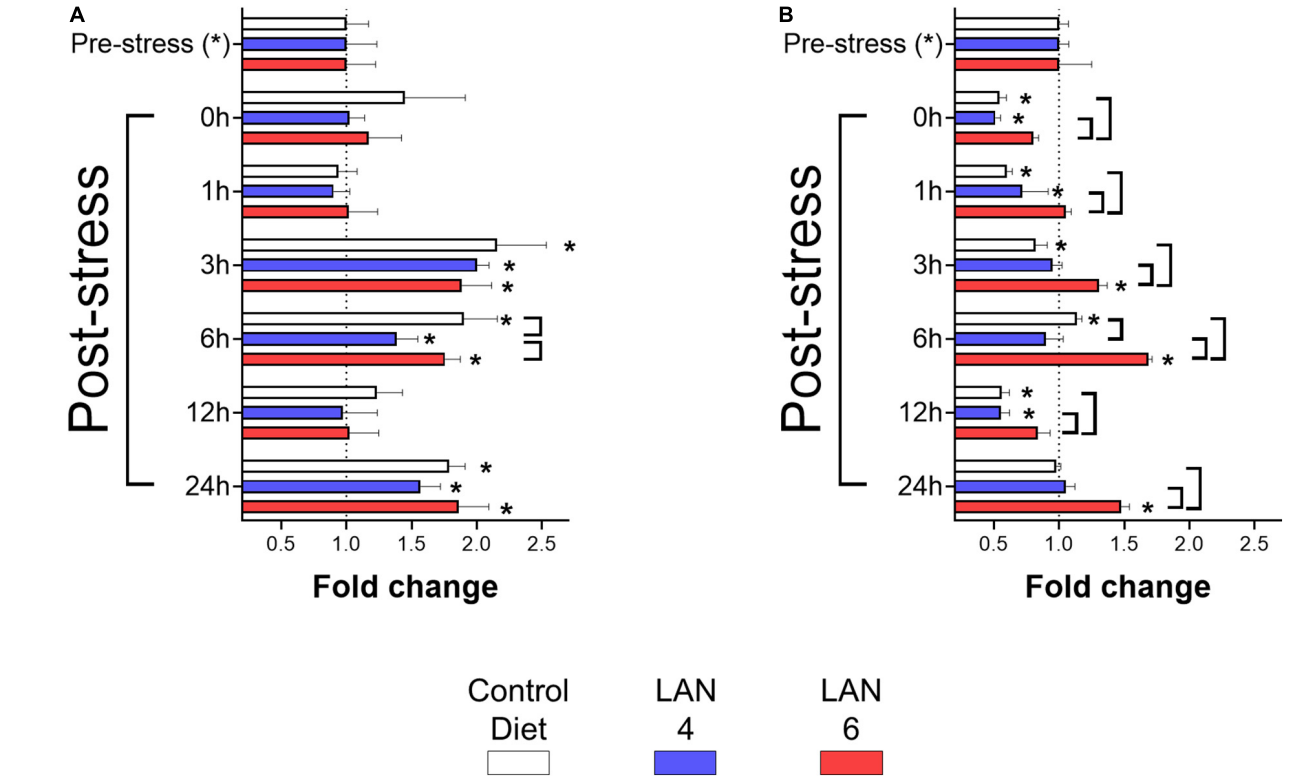

FIGURE 2 | Immunological biomarkers in plasma of Atlantic salmon exposed to acute hypoxia stress. Sampling times: pre-stress, 0 h (immediately after stress), 1, 3, 6, 12, and 24 h post-stress. In white: CD. In blue: LAN4. In red: LAN6. Each bar: $n = 4$. *: Significant differences ($p < 0.05$) compared with pre-stress group (from each diet). Bracket: significant differences ($p < 0.05$) between dietary groups at the same sampling time (CD, LAN4, and LAN6, respectively). **(A)** TNFα. **(B)** IL-10. **(A,B)** Data obtained from ELISA are expressed as fold change related to the pre-stress group of each diet.

1 h post-stress, 73 upregulated DEGs and 33 downregulated DEGs were detected, and at 6 h post-stress, the highest number of modulated DEGs was detected (686 upregulated and 421 downregulated).

The enrichment analysis and functional classification of DEGs in the gills (**Table 4**) between LAN4 and CD in the pre-stress group showed only upregulated terms (130) in LAN4. The same trend was observed at 1 h post-stress (30 upregulated terms). However, at 6 h post-stress, both upregulated and downregulated terms (208 and 144, respectively) were detected. The complete list of terms (enrichment FDR, number of genes, fold enrichment, and pathways) is shown in **Supplementary Data Sheet 1**. RNA-seq raw data are available in the Gene Expression Omnibus database (GEO-NCBI: GSE189236).

The top #30 terms per comparison showed that in the pre-stress group (**Figure 5A**), the upregulated terms (in LAN4) were mostly related to collagen fibril organization, phos/bisphosphoglycerate mutase activity, creatine kinase activity, glycolytic and gluconeogenesis process, metabolic and biosynthetic process, negative regulation of vasculature development, and extracellular matrix structural constituent. At 1 h post-stress (**Figure 5B**), the LAN4 group showed upregulated terms (compared to CD) associated with oxygen transport and carrier activity, hemoglobin complex, glutathione transferase activity, glutathione metabolic process, intracellular receptor signaling pathway, oxidation-reduction process, ligand-activated transcription factor activity, and sequence-specific DNA binding, among others.

The same analysis for 6 h post-stress samples (**Figure 5C**) showed that whereas the upregulation of terms was related to collagen, extracellular matrix assembly, termination of RNA polymerase I transcription, creatine kinase activity, metabolic or biosynthetic process, and dioxygenase activity, the downregulated terms were mainly associated with epimerase activity, galactose metabolic process, and fatty acyl-CoA metabolic or biosynthetic process.

DISCUSSION

Fish farming faces ongoing challenges during production related to multi-stressor conditions such as handling, sub-optimal nutrition, diseases, and environmental conditions. Hence, understanding the use of functional feeds in terms of stress

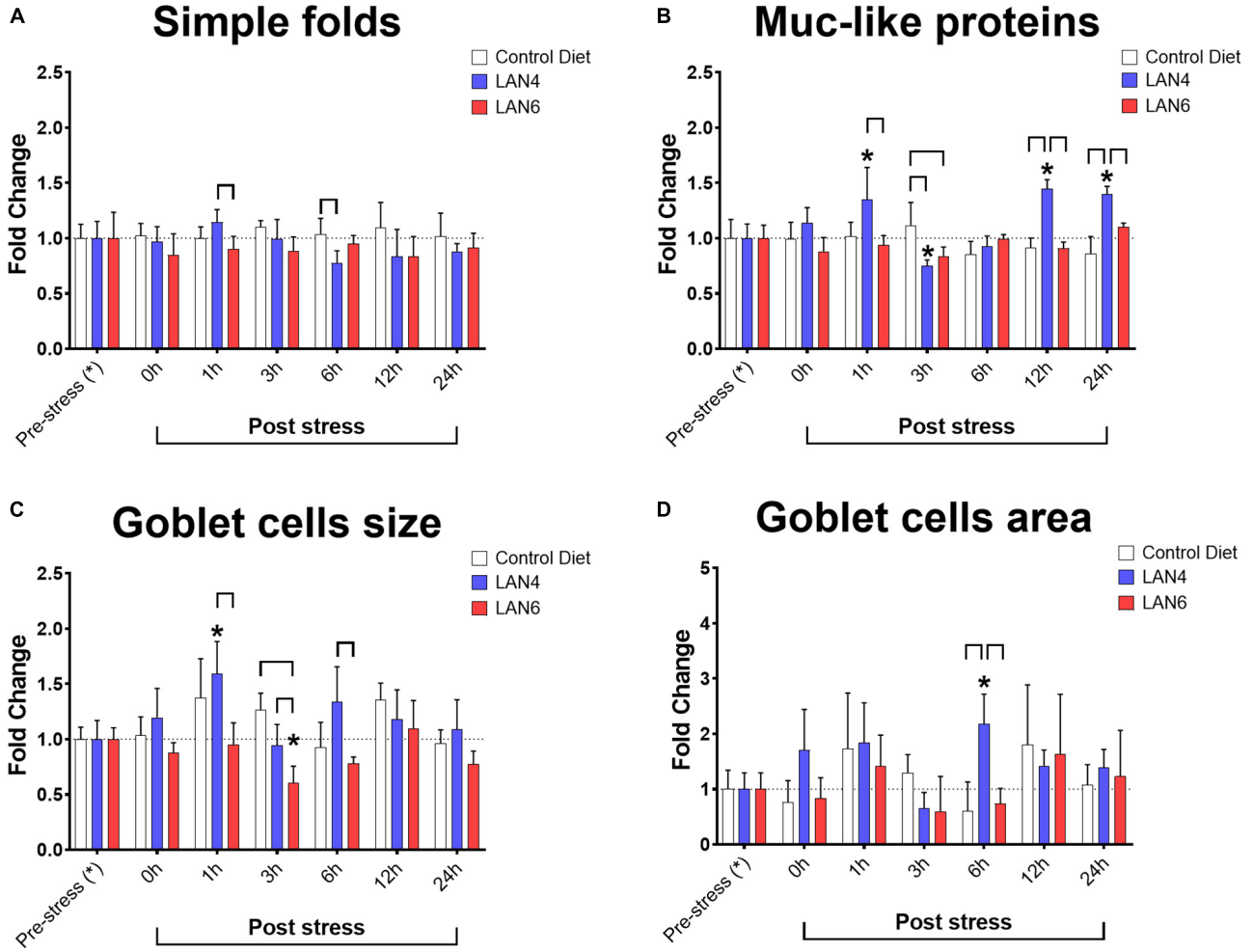

FIGURE 3 | Morphometric of PAS-stained tissue sections and detection of Muc-like proteins in the distal intestine from Atlantic salmon. Sampling times: pre-stress, 0 h (immediately after stress), 1, 3, 6, 12, and 24 h post-stress. In white: CD. In blue: LAN4. In red: LAN6. Each bar: $n = 4$. *: Significant differences ($p < 0.05$) compared with pre-stress group (from each diet). Bracket: significant differences ($p < 0.05$) between dietary groups at the same sampling time (CD, LAN4, and LAN6, respectively). **(A)** Simple folds. **(B)** Muc-like proteins. **(C)** Goblet cell size. **(D)** Goblet cell area. **(A–D)** Data are expressed as fold change related to the pre-stress group of each diet.

and disease prevention can add to the further development of sustainable aquaculture.

The main goal of this study was to assess the health beneficial effects of functional feeds containing two different hydrolyzed *D. hansenii* yeast-based products and to understand their modulating properties, associated with the regulation and control of stress-related responses during short-term hypoxia. Studies have already shown that microbial ingredients such as yeast cell wall components have the ability to modulate stress responses in fish (Forsatkar et al., 2017; Fuchs et al., 2017; Herrera et al., 2019). However, these effects can vary and are dependent on the yeast origin, processing, and inclusion rate in particular (Reveco-Urzua et al., 2019; Agboola et al., 2021b; Hansen et al., 2021). In this study, we found that adding 0.1% of two different strains (marine and dairy origin) of *D. hansenii* to diets induced distinctive physiological responses after exposure to acute hypoxia stress. Short-term hypoxia increased plasma cortisol levels 1 h post-stress in Atlantic salmon (with a positive correlation between CD and LAN6 dietary groups). This is in accordance with our previous study (Djordjevic et al., 2021a). However, whereas the LAN6 group seems to maintain a cortisol profile similar to CD, in the LAN4 group, we did not observe an increase in cortisol in the post-stress period, although we detected that in the pre-stress group, the cortisol levels in the LAN4 group were higher than in the LAN6 group. These differences raise new questions. For example, acute hypoxia could be a stress condition that stimulates a beneficial or compensatory response for the fish (as occurred in CD and LAN6), that could even benefit the fish against future similar conditions, or the regulation of the host response (as in LAN4 group) can be a way to avoid the energy expenditure that is associated with stress-related responses. In most vertebrates, cortisol is the main circulating glucocorticoid released during a primary stress response. This hormone is critical for mediating the stress response and can

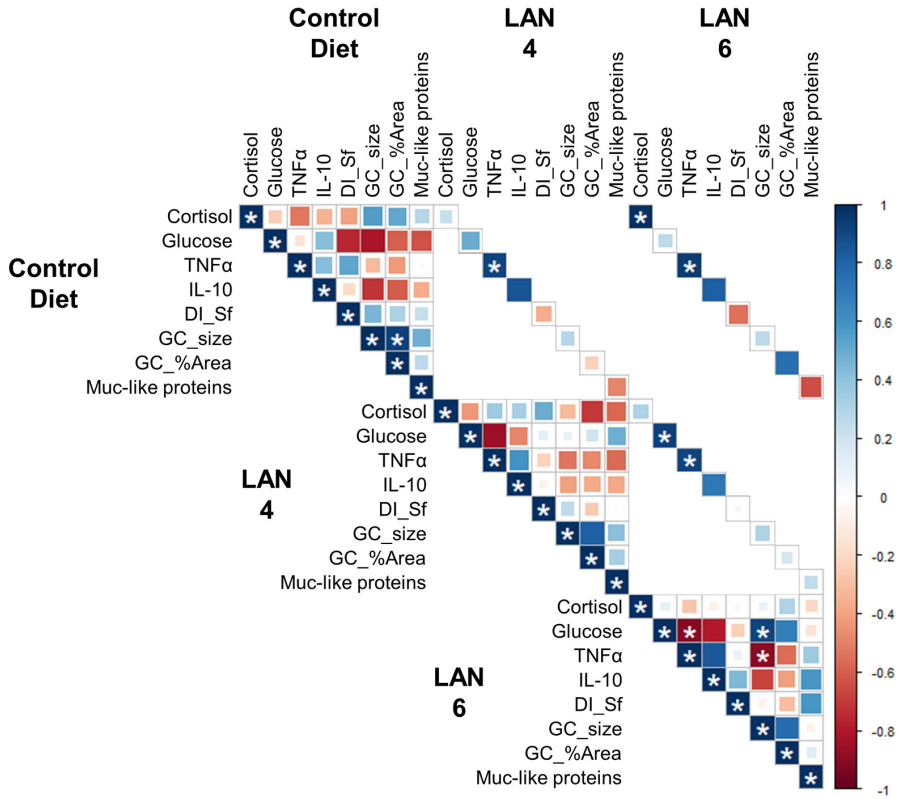

FIGURE 4 | Correlation analysis among the different parameters (cortisol, glucose, TNFα, IL-10, DI simple folds, goblet cells size, goblet cells area, and Muc-like proteins). All the parameters that are significantly correlated ($p < 0.01$) are denoted by *. Degrees of freedom = 6. In blue: positive correlation. In red: negative correlation.

induce both behavioral and metabolic pleiotropic effects (Ortega et al., 2021), and it is a classic marker for its evaluation. In Atlantic salmon, the previous studies have shown a typical pattern for this molecule. After acute stress exposure, the basal level of plasma cortisol (less than 100 ng ml^{-1}) increases 1–3 h and then decreases to basal levels (Djordjevic et al., 2012, 2021a; Soleng et al., 2019).

In recent years, there is the possibility of mitigating the negative effects of stress and disease susceptibility of fish through the use of feed additives such as functional amino acids (refers to amino acids beyond serving as the building blocks for proteins and peptides, Wu, 2010; Andersen et al., 2016), minerals, and fatty acids (Herrera et al., 2019). Moreover, it has also been observed that β-glucans and other yeast cell wall components can reduce the cortisol levels in zebrafish, turbot, and trout in response to different stressors (Kenari et al., 2013; Forsatkar et al., 2017; Fuchs et al., 2017). Although how these molecules modify and modulate stress response in fish is still unclear, there are indications that functional feed components may have different modes of action inducing multi-systematic responses. Herrera et al. (2019) described that amino acids such as arginine (critical for the production of reactive nitrogen species) can regulate the activation of cellular defense mechanisms in stimulated macrophages in fish.

Furthermore, leucine, isoleucine, and valine (branched-chain amino acids: BCAA) play an important role in regulating protein synthesis in skeletal muscle. Interestingly, it has been reported that fish under stressful situations have shown an increase in proteolytic activity and a decrease in BCAA plasma levels, which suggests that dietary supplementation appears to be a promising strategy to mitigate negative stress effects in fish (Herrera et al., 2019). Tryptophan also plays a role in the regulation of the stress response, since it can be converted to serotonin (5-HT). In the brain, 5-HT is involved in the control of the HPI axis, and in fish, a correlation between 5-HT activity and plasma cortisol levels has been observed. Related to hormone precursors and neurotransmitters (thyroxine, triiodothyronine, epinephrine, norepinephrine, dopamine, and melanin), tyrosine is a common precursor of these hormones and can influence pigmentation

TABLE 4 | Significant differentially expressed genes (DEGs) and term enrichment per comparison.

LAN4 | CD	DEGs		Term Enrichment	
	Upregulated	Downregulated	Upregulated	Downregulated
Pre-stress	238	125	130	0
1 h post-stress	73	33	29	0
6 h post-stress	686	421	208	144

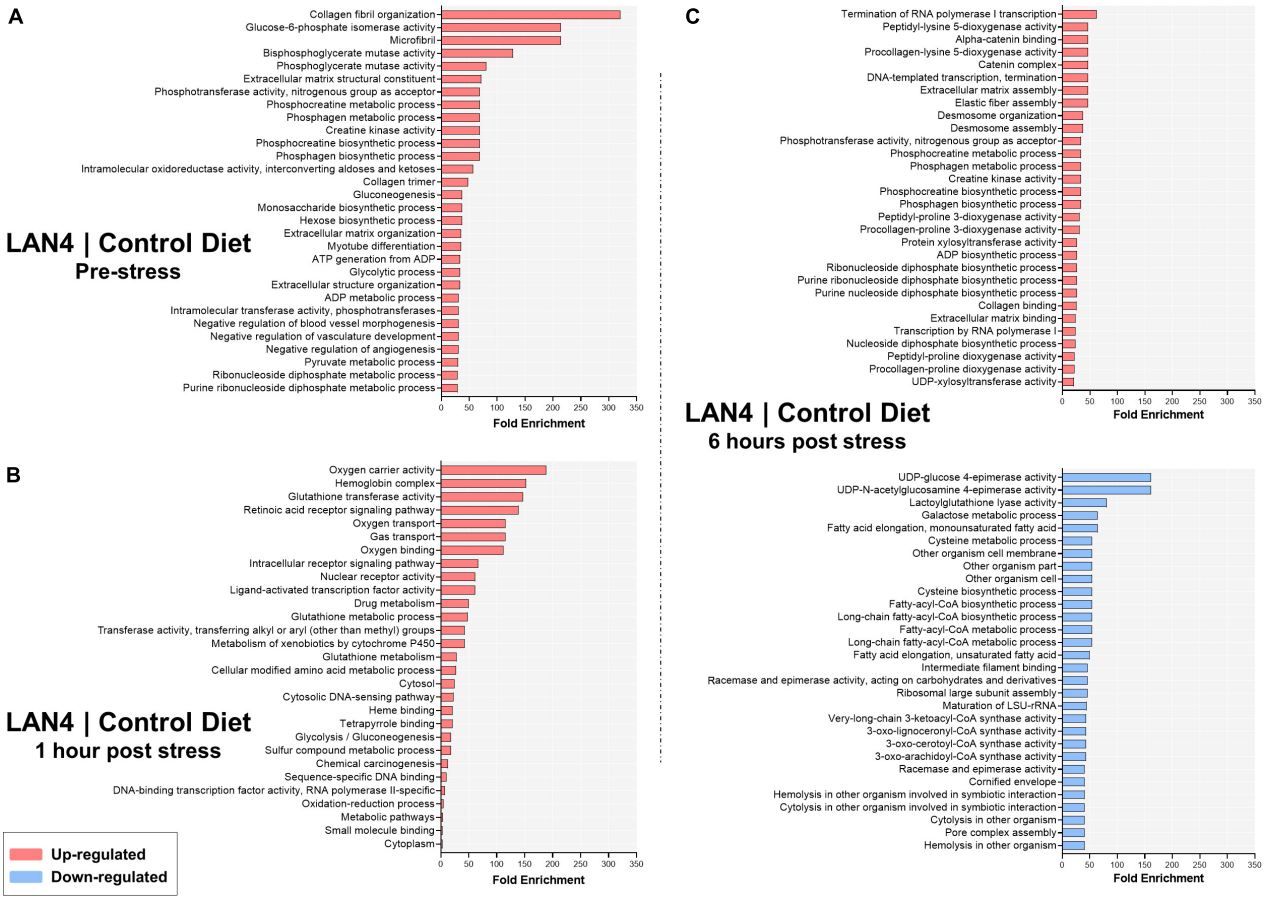

FIGURE 5 | Top #30 terms significantly enriched (minGSSize = 2) in gills from fish fed LAN4 diet compared to fish fed control diet (CD). The list is ordered by fold enrichment. **(A)** Terms in LAN4 compared to CD at the pre-stress group. **(B)** Terms in LAN4 compared to CD at 1 h post-stress. **(C)** Terms in LAN4 compared to CD at 6 h post-stress. In red: upregulated terms. In light blue: downregulated terms.

development, feed intake, growth performance, immunity, and survival of fish (Herrera et al., 2019). Methionine levels could be modulated by both acute and chronic stressful conditions. This amino acid also plays a role in the antioxidant and immune status of animals as the precursor of cysteine, which is required for the synthesis of glutathione. Glutathione is well known for its reactive species scavenging function, which plays a protective role during stress conditions (Cheng et al., 2015). Minerals (selenium, manganese, and zinc) also can induce stress attenuation, since they are cofactors for essential enzymes related to the control of oxidative stress. The importance of fatty acids during the stress response is associated with the formation of eicosanoids such as prostaglandins, which can modulate the HPI axis activity in fish (Herrera et al., 2019).

The two strains of *D. hansenii* tested differed in origin, chemical composition, and physical characteristics (**Table 2**). We speculate that some of these differences, such as the amount of β-glucan and/or difference in cell wall thickness or structure, might be the reason for different modulations of stress and immune responses in fish after short-term hypoxia. Stress and the subsequent increase in cortisol have also been found to be negatively correlated with the number of taxa of the gut microbiome (Uren Webster et al., 2020). A possible explanation could be that the addition of prebiotics, which enhances the growth of specific microbiota, prevented the increase in cortisol levels in the blood (Talpur et al., 2014). Although we did not perform this analysis in this study, further studies should investigate the correlation between cortisol and microbiota after different stress exposures in Atlantic salmon fed microbial ingredients. Furthermore, the higher IL-10 production after the hypoxia stress in LAN6 and CD group compared to LAN4 indicates that LAN4 prevents the secretion of IL-10 by an unknown mechanism. IL-10 is a cytokine that acts by modulating processes associated with immunosuppression (Zou and Secombes, 2016).

Usually, glucocorticoids are strong immunosuppressive agents, and immune suppression is observed during high cortisol secretion. In this study, the LAN4 group showed no increase in cortisol and no increase in IL-10 levels. This finding suggests that the LAN4 could be a beneficial functional ingredient during multiple stress events, for example, when fish are fighting against infectious agents or when they are trying

to respond efficiently to vaccination (Rodríguez et al., 2016). These situations make it interesting to evaluate *D. hansenii*-based products in future conditions such as chronic stress or after prolonged feeding times. Moreover, this would help to determine whether the regulation of cortisol secretion or the control of immunosuppressive profiles is beneficial or not for the fish in the different life stages of the animal or aquaculture production conditions.

Stress can enhance or suppress the immune response. An increase in TNFα and IL-10 levels in response to nano-encapsulated β-glucans has been reported in Atlantic salmon (Fredriksen et al., 2011). In this study, there was no difference in TNFα levels among diets. However, an increase of TNFα was detected at 3, 6, and 24 h post-stress in all diets (positively correlated), compared to the pre-stress group. This could be a conserved pattern of mobilization of the immune response during stressful events. In gilthead seabream, an *in vitro* study suggested that the adrenocorticotropin hormone (ACTH) can act independently of cortisol, which leads to increased TNFα mRNA levels in head kidney cells (Castillo et al., 2009). Nevertheless, more research is needed to further explain this phenomenon in Atlantic salmon.

In teleosts, mucosa-associated lymphoid tissues (MALTs) such as intestine (GALT) and gill (GIALT) are continually sensing information from the environment to guarantee the homeostasis necessary for fish survival. This important biological process can also be affected by stressor conditions, which impact health and animal welfare (Parra et al., 2015). In Atlantic salmon, it is known that short-term stress and cortisol can modulate the DI immune response (Niklasson et al., 2014; Djordjevic et al., 2021a) and the data from the present study reinforce this hypothesis. Morphometric changes related to goblet cells, which produce mucin proteins that act as a protective lubricant of the mucous layer against possible physical or chemical damage (Hur et al., 2016), were differentially modulated in CD and LAN diet groups. Whereas in the LAN4 group, fish displayed goblet cells with larger size (compared to those of the LAN6 group) and a higher level of DI Muc-like proteins (compared to the other dietary groups), LAN6 showed an opposite pattern, with the smallest goblet cells and a lower level of DI Muc-like proteins. We hypothesize that this response could be related to circulating cortisol since, in higher vertebrates, goblet cells secrete mucins stored in cytoplasmic granules, which results in small and thin goblet cells after stressful conditions (Johansson and Hansson, 2016). Moreover, in rainbow trout, Baumgarner et al. (2013) have described that short-term starvation stress is capable of inducing a decrease in the concentration of inhibitors that protect epithelia from enzymatic damage (e.g., serine proteases), compromising the ability of the mucosa to avoid bacterial infection in the intestine. Therefore, we propose that modulation of goblet cells and Muc-like proteins is a response of the fish to control or prevent intestinal damage after stress.

When analyzing the results, the LAN4 group showed different patterns of plasma biomarkers (no release of cortisol and IL-10) and DI parameters (goblet cells area and Muc-like proteins) compared to CD and LAN6. Stressors conditions can compromise the overall balance in the organism, increasing the demand for resources (e.g., a higher acquisition of food/energy) or inducing several physiological compensations to guarantee homeostasis. These processes can result in maladaptation (although this term is often associated with chronic stress), which indicates that the regulatory mechanisms in the animal have not been able to compensate for the effects of the stressor, which are very relevant in farmed animals, which includes fish subjected to artificial conditions (Balasch and Tort, 2019). To deepen our understanding of the molecular mechanisms behind these differences after hypoxia stress, we performed a transcriptomic analysis of gills using RNA-seq. Fish gills are important for many critical biological functions such as gas exchange, osmoregulation, excretion of nitrogenous waste, pH regulation, and hormone production (Herrero et al., 2018). Gills are also a relevant immune organ, which regulates interactions among the local microbiota and pathogens (Rességuier et al., 2020). In this organ, our data show that at 1 h post-stress, upregulation of genes related to biological pathways such as oxygen binding or transport, hemoglobin complex, oxidation-reduction process, metabolic pathways, and ligand-activated transcription factor activity occurred in fish fed LAN4. This suggests that LAN4 could modulate the response to short-term stress, seeking to guarantee fish homeostasis. In mammals, it has already been reported that during hypoxic conditions, cells activate adaptive responses to match oxygen supply with the metabolic, bio-energetic process and redox demands by hypoxia-inducible factors (Majmundar et al., 2010). The upregulation of glutathione transferase activity and glutathione metabolism in the LAN4 group could also be associated with oxidative stress-tolerance (Peña-Llopis et al., 2003; Srikanth et al., 2013). Furthermore, it is interesting that at 6 h post-stress, the LAN4 group displayed downregulation of fatty acyl-CoA biosynthetic or metabolic process pathways, which could be involved in the modulation of fatty acid oxidation (Kumari, 2018), which prevents oxidative stress by reactive species production (Ly et al., 2017). In fish, Atlantic salmon smolts exposed to an acute stressor (peracetic acid) were able to address oxidative stress (ROS imbalance), mobilizing systemic and mucosal defenses, by increasing the total antioxidant capacity in the plasma and modulating the gene expression of glutathione peroxidase, glutathione reductase, manganese superoxide dismutase, and copper or zinc superoxide dismutase in skin and gills (Soleng et al., 2019).

In summary, our study has shown that nutritional programming by the use of functional feed containing hydrolyzed *Debaryomyces hansenii* yeasts can be an interesting strategy to improve fish welfare in intensive aquaculture. Understanding mucosal responses to functional feeds in target organs such as gills and intestines during short-term stressors can open new avenues for monitoring fish health status. The results in this study showed that the *D. hansenii* yeast-based product (LAN4) was able to regulate the plasma secretion of cortisol and IL-10, in addition to modulating both the production of Muc-like proteins in DI and the gene expression of several metabolic pathways in Atlantic salmon. Based on these findings, we propose that inactivated D. hansenii yeast-based product (LAN4) could be a promising ingredient in functional feeds for salmon to mitigate stress-related responses.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189236.

ETHICS STATEMENT

All animals were treated according to the laws and regulations for experiments on live animals in the EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761).

AUTHOR CONTRIBUTIONS

BM-L and BD conceived the study with extensive inputs from LTM, JO, and MØ. AG, BD, BM-L, CP, and MI performed the experiments and data analysis. LM produced and validated the antibodies. MC produced and characterized the yeasts. LTM and MØ acquired the funds for this investigation. BM-L and BD drafted the manuscript with substantial contributions from all other authors. All authors contributed to the article and approved the submitted version.

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REFERENCES

Agboola, J. O., Øverland, M., Skrede, A., and Hansen, J. Ø (2021a). Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production. *Rev. Aquacult.* 13, 949–970. doi: 10.1111/raq.12507

Agboola, J. O., Schiavone, M., Øverland, M., Morales-Lange, B., Lagos, L., Arntzen, M. Ø, et al. (2021b). Impact of down-stream processing on functional properties of yeasts and the implications on gut health of Atlantic salmon (*Salmo salar*). *Sci. Rep.* 11:4496. doi: 10.1038/s41598-021-83764-2

Andersen, S. M., Waagbø, R., and Espe, M. (2016). Functional amino acids in fish nutrition, health and welfare. *Front. Biosci. (Elite Ed.)* 8:143–169. doi: 10.2741/757

Angulo, M., Reyes-Becerril, M., Medina-Córdova, N., Tovar-Ramírez, D., and Angulo, C. (2020). Probiotic and nutritional effects of *Debaryomyces hansenii* on animals. *Appl. Microbiol. Biotechnol.* 104, 7689–7699. doi: 10.1007/s00253-020-10780-z

Angulo, M., Reyes-Becerril, M., Tovar-Ramírez, D., Ascencio, F., and Angulo, C. (2018). Debaryomyces hansenii CBS 8339 β-glucan enhances immune responses and down-stream gene signaling pathways in goat peripheral blood leukocytes. *Dev. Comp. Immunol.* 88, 173–182. doi: 10.1016/j.dci.2018.07.017

Balasch, J. C., and Tort, L. (2019). Netting the stress responses in fish. *Front. Endocrinol.* 10:62. doi: 10.3389/fendo.2019.00062

Baumgarner, B. L., Bharadwaj, A. S., Inerowicz, D., Goodman, A. S., and Brown, P. B. (2013). Proteomic analysis of rainbow trout (*Oncorhynchus mykiss*) intestinal epithelia: physiological acclimation to short-term starvation. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 8, 58–64. doi: 10.1016/j.cbd.2012.11.001

Braithwaite, V. A., and Ebbesson, L. O. (2014). Pain and stress responses in farmed fish. *Rev. Sci. Tech. (Int. Office Epizoot.)* 33, 245–253. doi: 10.20506/rst.33.1.2285

Caruffo, M., Navarrete, N., Salgado, O., Díaz, A., López, P., García, K., et al. (2015). Potential probiotic yeasts isolated from the fish gut protect zebrafish (*Danio rerio*) from a *Vibrio anguillarum* challenge. *Front. Microbiol.* 6:1093. doi: 10.3389/fmicb.2015.01093

Castillo, J., Teles, M., Mackenzie, S., and Tort, L. (2009). Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immunol.* 27, 493–499. doi: 10.1016/j.fsi.2009.06.021

Catalán, I. A., Auch, D., Kamermans, P., Morales-Nin, B., Angelopoulos, N. V., Reglero, P., et al. (2019). Critically examining the knowledge base required to mechanistically project climate impacts: a case study of Europe's fish and shellfish. *Fish Fish.* 20, 501–517. doi: 10.1111/faf.12359

Cheng, M. C., Ko, K., Chang, W. L., Kuo, W. C., Chen, G. H., and Lin, T. P. (2015). Increased glutathione contributes to stress tolerance and global translational changes in *Arabidopsis*. *Plant J.* 83, 926–939. doi: 10.1111/tpj.12940

Djordjevic, B., Kristensen, T., Overli, O., Rosseland, B. O., and Kiessling, A. (2012). Effect of nutritional status and sampling intensity on recovery after dorsal aorta cannulation in free-swimming Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem.* 38, 259–272. doi: 10.1007/s10695-009-9362-2

Djordjevic, B., Morales-Lange, B., McLean Press, C., Olson, J., Lagos, L., Mercado, L., et al. (2021a). Comparison of circulating markers and mucosal immune parameters from skin and distal intestine of atlantic salmon in two models of acute stress. *Int. J. Mol. Sci.* 22:1028. doi: 10.3390/ijms22031028

Djordjevic, B., Morales-Lange, B., Øverland, M., Mercado, L., and Lagos, L. (2021b). Immune and proteomic responses to the soybean meal diet in skin and intestine mucus of Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr.* 27, 929–940. doi: 10.1111/anu.13248

Ewels, P. A., Peltzer, A., Fillinger, S., Patel, H., Alneberg, J., Wilm, A., et al. (2020). The nf-core framework for community-curated bioinformatics pipelines. *Nat. Biotechnol.* 38, 276–278. doi: 10.1038/s41587-020-0439-x

Food and Agriculture Organization of the United Nations (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome: Food and Agriculture Organization of the United Nations.

Forsatkar, M. N., Nematollahi, M. A., Rafiee, G., Farahmand, H., and Lawrence, C. (2017). Effects of the prebiotic mannan-oligosaccharide on the stress response of feed deprived zebrafish (*Danio rerio*). *Physiol. Behav.* 180, 70–77. doi: 10.1016/j.physbeh.2017.08.010

Fredriksen, B., Sævareid, K., McAuley, L., Lane, M., Bøgwald, J., and Dalmo, R. (2011). Early immune responses in Atlantic salmon (*Salmo salar* L.) after immunization with PLGA nanoparticles loaded with a model antigen and β-glucan. *Vaccine* 29, 8338–8349. doi: 10.1016/j.vaccine.2011.08.087

Fuchs, V. I., Schmidt, J., Slater, M. J., Buck, B. H., and Steinhagen, D. (2017). Influence of immunostimulant polysaccharides, nucleic acids, and

Bacillus strains on the innate immune and acute stress response in turbots (*Scophthalmus maximus*) fed soy bean- and wheat-based diets. *Fish Physiol. Biochem.* 43, 1501–1515. doi: 10.1007/s10695-017-0388-6

Ge, S. X., Jung, D., and Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 36, 2628–2629. doi: 10.1093/bioinformatics/btz931

Glencross, B. D., Baily, J., Berntssen, M. H., Hardy, R., MacKenzie, S., and Tocher, D. R. (2020). Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. *Rev. Aquacult.* 12, 703–758. doi: 10.1111/raq.12347

Hansen, J. Ø, Lagos, L., Lei, P., Reveco-Urzua, F. E., Morales-Lange, B., Hansen, L. D., et al. (2021). Down-stream processing of baker's yeast (*Saccharomyces Cerevisiae*): effect on nutrient digestibility and immune response in atlantic salmon (*Salmo Salar*). *Aquaculture* 530, 1–10. doi: 10.1016/j.aquaculture.2020.735707

Herrera, M., Mancera, J. M., and Costas, B. (2019). The use of dietary additives in fish stress mitigation: comparative endocrine and physiological responses. *Front. Endocrinol.* 10:447. doi: 10.3389/fendo.2019.00447

Herrero, A., Thompson, K. D., Ashby, A., Rodger, H. D., and Dagleish, M. P. (2018). Complex gill disease: an emerging syndrome in farmed atlantic salmon (*Salmo salar* L.). *J. Comp. Pathol.* 163, 23–28. doi: 10.1016/j.jcpa.2018.07.004

Hur, S. W., Kim, S. K., Kim, D. J., Lee, B. I., Park, S. J., Hwang, H. G., et al. (2016). Digestive physiological characteristics of the gobiidae: – characteristics of CCK-producing cells and mucus-secreting goblet cell of stomach fish and stomachless fish. *Dev. Reprod.* 20, 207–217. doi: 10.12717/DR.2016.20.3.207

Johansson, M. E., and Hansson, G. C. (2016). Immunological aspects of intestinal mucus and mucins. *Nat. Rev. Immunol.* 16, 639–649. doi: 10.1038/nri.2016.88

Kenari, A. A., Mahmoudi, N., Soltani, M., and Abediankenari, S. J. A. N. (2013). Dietary nucleotide supplements influence the growth, haemato-immunological parameters and stress responses in endangered Caspian brown trout (*Salmo trutta* caspius Kessler, 1877). *Aquacult. Nutr.* 19, 54–63. doi: 10.1111/j.1365-2095.2012.00938.x

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. *Anim. Feed Sci. Technol.* 173, 111–133. doi: 10.1016/j.anifeedsci.2011.12.015

Kumari, A. (2018). "Beta oxidation of fatty acids," in *Sweet Biochemistry*, ed. A. Kumari (Cambridge, MA: Academic Press), 17–19. doi: 10.1016/B978-0-12-814453-4.00004-2

Ly, L. D., Xu, S., Choi, S. K., Ha, C. M., Thoudam, T., Cha, S. K., et al. (2017). Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. *Exp. Mol. Med.* 49:e291. doi: 10.1038/emm.2016.157

Majmundar, A. J., Wong, W. J., and Simon, M. C. (2010). Hypoxia-inducible factors and the response to hypoxic stress. *Mol. Cell* 40, 294–309. doi: 10.1016/j.molcel.2010.09.022

Martinez-Rubio, L., Evensen, Ø, Krasnov, A., Jørgensen, S. M., Wadsworth, S., Ruohonen, K., et al. (2014). Effects of functional feeds on the lipid composition, transcriptomic responses and pathology in heart of Atlantic salmon (*Salmo salar* L.) before and after experimental challenge with Piscine Myocarditis Virus (PMCV). *BMC Genomics* 15:462. doi: 10.1186/1471-2164-15-462

Meena, D. K., Das, P., Kumar, S., Mandal, S. C., Prusty, A. K., Singh, S. K., et al. (2013). Beta-glucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiol. Biochem.* 39, 431–457. doi: 10.1007/s10695-012-9710-5

Morales-Lange, B., Agboola, J. O., Hansen, J. Ø, Lagos, L., Øyås, O., Mercado, L., et al. (2021). The spleen as a target to characterize immunomodulatory effects of down-stream processed cyberlindnera jadinii yeasts in atlantic salmon exposed to a dietary soybean meal challenge. *Front. Immunol.* 12:708747. doi: 10.3389/fimmu.2021.708747

Mullins, J., Nowak, B., Leef, M., Røn, Ø, Eriksen, T. B., and McGurk, C. (2020). Functional diets improve survival and physiological response of Atlantic salmon (*Salmo salar*) to amoebic gill disease. *J. World Aquacult. Soc.* 51, 634–648. doi: 10.1111/jwas.12692

Navarrete, P., and Tovar-Ramírez, D. (2014). "Use of yeasts as probiotics in fish aquaculture," in *Sustainable Aquaculture Techniques*, eds M. P. Hernandez-Vergara and C. I. Perez-Rostro (London: IntechOpen), doi: 10.5772/57196

Niklasson, L., Sundh, H., Olsen, R. E., Jutfelt, F., Skjødt, K., Nilsen, T. O., et al. (2014). Effects of cortisol on the intestinal mucosal immune response during cohabitant challenge with IPNV in Atlantic salmon (*Salmo salar*). *PLoS One* 9:e94288. doi: 10.1371/journal.pone.0094288

Ochangco, H. S., Gamero, A., Smith, I. M., Christensen, J. E., Jespersen, L., and Arneborg, N. (2016). In vitro investigation of *Debaryomyces hansenii* strains for potential probiotic properties. *World J. Microbiol. Biotechnol.* 32:141. doi: 10.1007/s11274-016-2109-1

Ortega, V. A., Mercer, E. M., Giesbrecht, G. F., and Arrieta, M. C. (2021). Evolutionary significance of the neuroendocrine stress axis on vertebrate immunity and the influence of the microbiome on early-life stress regulation and health outcomes. *Front. Microbiol.* 12:634539. doi: 10.3389/fmicb.2021.634539

Øverland, M., and Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *J. Sci. Food Agric.* 97, 733–742. doi: 10.1002/jsfa.8007

Parra, D., Reyes-Lopez, F. E., and Tort, L. (2015). Mucosal immunity and B cells in teleosts: effect of vaccination and stress. *Front. Immunol.* 6:354. doi: 10.3389/fimmu.2015.00354

Peña-Llopis, S., Ferrando, M. D., and Peña, J. B. (2003). Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by N-acetylcysteine. *Aquatic Toxicol.* 65, 337–360. doi: 10.1016/s0166-445x(03)00148-6

Peterson, B. C., Chatakondi, N. G., and Small, B. C. (2019). Ontogeny of the cortisol stress response and glucocorticoid receptor expression during early development in channel catfish, *Ictalurus punctatus*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 231, 119–123. doi: 10.1016/j.cbpa.2019.02.003

Rawling, M., Leclercq, E., Foey, A., Castex, M., and Merrifield, D. (2021). A novel dietary multi-strain yeast fraction modulates intestinal toll-like-receptor signalling and mucosal responses of rainbow trout (*Oncorhynchus mykiss*). *PLoS One* 16:e0245021. doi: 10.1371/journal.pone.0245021

Reid, G. K., Gurney-Smith, H. J., Flaherty, M., Garber, A. F., Forster, I., Brewer-Dalton, K., et al. (2019). Climate change and aquaculture: considering adaptation potential. *Aquacult. Environ. Interact.* 11, 603–624. doi: 10.3354/aei00333

Rességuier, J., Dalum, A. S., Du Pasquier, L., Zhang, Y., Koppang, E. O., Boudinot, P., et al. (2020). Lymphoid tissue in teleost gills: variations on a theme. *Biology* 9:127. doi: 10.3390/biology9060127

Reveco-Urzua, F. E., Hofossæter, M., Rao Kovi, M., Mydland, L. T., Ånestad, R., Sørby, R., et al. (2019). Candida utilis yeast as a functional protein source for Atlantic salmon (*Salmo salar* L.): local intestinal tissue and plasma proteome responses. *PLoS One* 14:e0218360. doi: 10.1371/journal.pone.0218360

Reyes-Becerril, M., Salinas, I., Cuesta, A., Meseguer, J., Tovar-Ramirez, D., Ascencio-Valle, F., et al. (2008). Oral delivery of live yeast *Debaryomyces hansenii* modulates the main innate immune parameters and the expression of immune-relevant genes in the gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 25, 731–739. doi: 10.1016/j.fsi.2008.02.010

Rodríguez, F. E., Valenzuela, B., Farías, A., Sandino, A. M., and Imarai, I. (2016). β-1,3/1,6-Glucan-supplemented diets antagonize immune inhibitory effects of hypoxia and enhance the immune response to a model vaccine. *Fish Shellfish Immunol.* 59, 36–45. doi: 10.1016/j.fsi.2016.10.020

Sahlmann, C., Djordjevic, B., Lagos, L., Mydland, L. T., Morales-Lange, B., Hansen, J. O., et al. (2019). Yeast as a protein source during smoltification of Atlantic salmon (*Salmo salar* L.), enhances performance and modulates health. *Aquaculture* 513:734396. doi: 10.1016/j.aquaculture.2019.734396

Schmitt, P., Wacyk, J., Morales-Lange, B., Rojas, V., Guzman, F., Dixon, B., et al. (2015). Immunomodulatory effect of cathelicidins in response to a beta-glucan in intestinal epithelial cells from rainbow trout. *Dev. Comp. Immunol.* 51, 160–169. doi: 10.1016/j.dci.2015.03.007

Soleng, M., Johansen, L.-H., Johnsen, H., Johansson, G. S., Breiland, M. W., Rørmark, L., et al. (2019). Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid. *Fish Shellfish Immunol.* 93, 895–903. doi: 10.1016/j.fsi.2019.08.048

Srikanth, K., Pereira, E., Duarte, A. C., and Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish–a review. *Environ. Sci. Pollut. Res. Int.* 20, 2133–2149. doi: 10.1007/s11356-012-1459-y

Talpur, A. D., Munir, M. B., Mary, A., and Hashim, R. J. A. (2014). Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against

Aeromonas hydrophila in snakehead (*Channa striata*) fingerlings. *Aquaculture* 426–427, 14–20. doi: 10.1016/j.aquaculture.2014.01.013

Torrecillas, S., Makol, A., Caballero, M. J., Montero, D., Dhanasiri, A. K. S., Sweetman, J., et al. (2012). Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. *J. Fish Dis.* 35, 591–602. doi: 10.1111/j.1365-2761.2012.01384.x

Uren Webster, T. M., Rodriguez-Barreto, D., Consuegra, S., and Garcia de Leaniz, C. (2020). Cortisol-related signatures of stress in the fish microbiome. *Front. Microbiol.* 11:1621. doi: 10.3389/fmicb.2020.01621

Wei, T. S. V. (2021). *R package 'Corrplot': Visualization Of A Correlation Matrix. (Version 0.90)*. Available online at: https://cran.r-project.org/web/packages/corrplot/corrplot.pdf [Accessed December 14, 2021].

Whyte, S. K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish Shellfish Immunol.* 23, 1127–1151. doi: 10.1016/j.fsi.2007.06.005

Wrent, P., Rivas, E. M., Gil de Prado, E., Peinado, J. M., and de Silóniz, M. I. (2015). Development of species-specific primers for rapid identification of *Debaryomyces hansenii*. *Int. J. Food Microbiol.* 193, 109–113. doi: 10.1016/j.ijfoodmicro.2014.10.011

Wu, G. (2010). Functional amino acids in growth, reproduction, and health. *Adv. Nutr.* 1, 31–37. doi: 10.3945/an.110.1008

Zou, J., and Secombes, C. J. (2016). The function of fish cytokines. *Biology* 5:23. doi: 10.3390/biology5020023

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Replacing Dietary Fish Meal with Defatted Black Soldier Fly (*Hermetia illucens*) Larvae Meal Affected Growth, Digestive Physiology and Muscle Quality of Tongue Sole (*Cynoglossus semilaevis*)

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For solving the global shortage of fish meal (FM) supplies from fisheries, the black soldier fly (*Hermetia illucens*) has become a new protein alternative in aquatic feeds. The present study investigated the effects of dietary inclusion of defatted *H. illucens* larvae meal (DBLM) on growth, serum biochemical parameters, digestive function, and muscle quality of tongue sole (*Cynoglossus semilaevis*). The feeding experiment consisted of five experimental diets: a control diet based on FM protein (H0) and four DBLM diets, substituting 25% (H25), 50% (H50), 75% (H75), and 100% (H100) of FM. *C. semilaevis* (initial weight 563.48 ± 22.81 g) were randomly allocated over five treatments in quadruplicate. After 65 days of feeding, the weight gain rate (WGR), specific growth rate (SGR), and protein efficiency ratio (PER) were significantly higher in H0 and H25 groups with less feed conversion ratio (FCR) and feed intake (FI). The concentrations of serum ALT, TG, T-CHO, ALB, and GLO and their ratio (i.e., A/G) in the H25 group were also significantly higher than those in the other DBLM diet-feeding groups. The digestive enzyme activities first increased (from 25% to 75%) and then decreased (from 75%) with the increased level of DBLM in diets. Meanwhile, there were significant improvements in the thickness of the intestinal longitudinal muscle (LM), circular muscle (CM), columnar epithelium (CE), and lamina propria (LP) in H25 *C. semilaevis* compared to the control group ($p < 0.05$). The fish from the other DBLM diets groups presented significant reductions in the thicknesses of LM, CM, CE, and LP, as well as the length of microvilli (ML) in a dose-dependent manner ($p < 0.05$). However, the substitution of FM increased up to 50% would result in intestinal structural damage. Moreover, the proximate compositions, antioxidant and water holding capacity, and muscular structures of *C. semilaevis* fillets were all significantly affected after substituting 25% FM with DBLM ($p < 0.05$). Except for the dry matter, moisture, ash, crude fat, and protein contents were significantly higher in H25 *C. semilaevis* muscles. The SOD activity in the H0 group was significantly lower than that in the H25 group. The CAT activity in *C. semilaevis* muscles

prominently reduced along with the increase in DBLM content in feeding diets ($p < 0.05$). The water holding capacity of *C. semilaevis* fillets was best in the H25 group. In summary, the optimum proportion of DBLM with FM for feeding *C. semilaevis* may be around 25%.

Keywords: fish meal substitution, *Hermetia illucens*, *Cynoglossus semilaevis*, growth performance, physiology and biochemistry

HIGHLIGHTS

(1) Replacing 25% of the fish meal with defatted black soldier fly (*Hermetia illucens*) larvae meal (DBLM) had positive effects on the weight gain rate, specific growth rate, condition factor, and survival rate of *C. semilaevis* while decreasing the feed conversion ratio and feed intake;
(2) Feeding *C. semilaevis* with DBLM diets brought the serum T-CHO and TG down and relieved the fat deposition in the fish body. Substituting fish meal with DBLM up to 50% resulted in intestinal structural damage and abnormal liver function;
(3) The replacement level of less than 75% could boost the antioxidant capacity of *C. semilaevis* muscles. The water holding capacity was the best in the H25 *C. semilaevis* fillets.

1 INTRODUCTION

It is well recognized that the increasing demand and unstable fish meal production have led to an increase in the cost of aquaculture production. The substitution of high-value and unsustainable fish meal (FM) by less expensive and more readily available alternatives in aquatic feeds is a priority for the sustainable development of the aquaculture sector (Jeong et al., 2021a). Therefore, it is of practical significance to replace FM with cheaper, protein-rich animal and plant ingredients (Tacon and Metian, 2008; Hardy, 2010). The growing empirical evidence has suggested that the insect meal has higher nutritive value, greater feed conversion efficiency, extremely smaller environmental footprint, and relatively lower price than FM and other alternative protein sources (Wang and Shelomi, 2017; Nogales-Mérida et al., 2019; Smets et al., 2020). Meanwhile, partially replacing FM with black soldier fly (*Hermetia illucens*) meal is highly feasible for most marine and freshwater fish species (Xu et al., 2020). Nevertheless, the extent to which FM can be spared by *H. illucens* meal without negative consequence depends on several factors, including fish species, life stages, and the differed processing technologies of *H. illucens*, as well as feeding strategies (Magalhães et al., 2017; Smets et al., 2020; Takakuwa et al., 2021).

Hermetia illucens can convert low-value organic waste into valuable fat- and protein-rich biomass (Raksasat et al., 2020). It has been testified that the extraction of lipid, protein, amino acid, and fatty acid profiles might differ among the developmental stages (larvae, prepupae, and pupae) of *H. illucens*. Especially, the most balanced and higher nutrients were measured in larvae (Smets et al., 2020). Specifically, their larvae are 38.9%~59.8% crude protein and 29%~41% fat, and the essential amino acid (EAA) profile is very similar to FM (Nogales-Mérida et al., 2019; Smets et al., 2020). When defatted, *H.* illucens larvae meal (DBLM) can have crude protein levels over 60% and more abundant nutrients (Spranghers et al., 2017; Wang and Shelomi, 2017). In addition, the total amount of EAA in DBLM was about 3 times that of FM; lauric acid (C12:0) is almost 2.6 times higher than that in FM; and the antimicrobial peptide was also identified in DBLM (Lee et al., 2020; Tippayadara et al., 2021). Therefore, DBLM has higher potential and more advantages to replace FM in aquatic feeds than either full-fat or partially defatted *H. illucens* larvae meal (Renna et al., 2017; Rawski et al., 2020).

The DBLM partial replacement of FM in diets has been successfully reported in several fish species. For example, the feed conversion ratio (FCR), total saturated, and monounsaturated fatty acids (SFA and MUFA) increased significantly at each increment level of DBLM, while polyunsaturated FA (particularly n-3 FA) decreased. Meanwhile, the DBLM diets resulted in similar apparent digestibility of nutrients, growth performances, proximate composition, and histomorphology of *Oncorhynchus mykiss* compared with the control group (Renna et al., 2017; Caimi et al., 2021). Li et al. (2017) confirmed that the DBLM diets had no significant effects on *Cyprinus carpio* growth, nutrient utilization, and digestive enzyme activity. However, the DBLM diets boosted the antioxidant capacity of *C. carpio* by strengthening the CAT activity and reduced cholesterol content and lipid deposits in tissues. Thus, it is suitable to replace up to 50% of FM with DBLM for *C. carpio*; the dietary stress and intestinal histopathological damages were observed if the replacement levels exceeded 75% (Li et al., 2017). In *Perca fluviatilis* research, reduced fatty acid content and n-3/n-6 ratio were found with increasing DBLM inclusion, whereas the higher specific growth rate was tested in the control and 20% and 40% DBLM groups. Therefore, 40% inclusion of DBLM can be used successfully in standard diets for *P. fluviatilis* (Stejskal et al., 2020). The abovementioned similar findings were confirmed once again in *Danio rerio*, which also revealed the positive impacts of DBLM on fish at the molecular level (Lanes et al., 2021). In general, the DBLM not only has high nutritive value but also represents good palatability, characteristics that can be better absorbed and utilized, and some beneficial effects on farmed fish. However, it is still blank for the application of DBLM in tongue sole (*Cynoglossus semilaevis* Günther) diets and its improving effect on fish flesh quality.

Cynoglossus semilaevis is distributed in Chinese coastal waters, particularly abundant in the northern region, such as off Tianjin and Shandong peninsula (Lin et al., 2021). This kind of fish is a promising species for aquaculture and a popular commercial flatfish in China due to its high nutritive value and pleasant taste. Intensive farming based on the utilization of artificial feeds has contributed to the continuous growth in the production of *C.*

TABLE 1 | Ingredients and proximate composition of the experimental diets.

Ingredients (g/kg)	Experiment diets				
	H0[a]	H25	H50	H75	H100
Super steam fish meal	50.00	37.50	25.00	12.50	0.00
Defatted *H. illucens* larvae meal	0.00	14.41	28.81	43.22	57.63
Fermented soybean meal	13.50	13.50	13.50	13.50	13.50
Broken yeast	4.00	4.00	4.00	4.00	4.00
Shrimp meal	6.00	6.00	6.00	6.00	6.00
Gluten	4.47	4.20	3.90	3.67	3.37
Fish oil	5.90	7.00	8.10	9.20	10.30
Microcrystalline cellulose	11.77	8.83	5.93	2.98	0.07
Calcium dihydrogen phosphate	1.40	1.40	1.40	1.40	1.40
Multivitamins[b]	1.50	1.50	1.50	1.50	1.50
Complex minerals[c]	0.10	0.10	0.10	0.10	0.10
50% choline chloride	1.00	1.00	1.00	1.00	1.00
Lysine	0.00	0.15	0.30	0.42	0.55
Methionine	0.00	0.05	0.10	0.15	0.22
Sodium polyacrylate	0.30	0.30	0.30	0.30	0.30
Clomerin	0.03	0.03	0.03	0.03	0.03
Ethoxyquinoline dry powder	0.03	0.03	0.03	0.03	0.03
Total	100.00	100.00	100.00	100.00	100.00
Proximate composition (%)					
Crude fat	10.10	10.50	10.20	10.40	10.90
Crude protein	56.55	56.62	53.91	53.93	52.46
Ash	5.64	5.85	5.93	5.12	4.82
Moisture	10.77	12.92	13.50	16.30	15.88

[a]*H0 = 100% super steam fish meal; H25 = 25% DBLM; H50 = 50% DBLM; H75 = 75% DBLM; H100 = 100% DBLM.*

[b]*Mineral premix (g/kg of mixture): $Ca(H_2PO_4)_2{\cdot}H_2O$, 14; $ZnSO_4{\cdot}7H_2O$, 0.065; $CuSO_4{\cdot}5H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.109; $MnSO_4{\cdot}H_2O$, 0.024; KI, 0.032; $CoCl_2{\cdot}6H_2O$, 0.0002; Na_2SeO_3, 0.0003; zeolite powder, 5.76.*

[c]*Vitamin premix (g/kg of mixture): V_A 0.03; V_{D3} 0.01; nicotinamide 0.25; inositol 1.00; calcium pantothenate 0.06; V_{B1} 0.01; V_{B2} 0.05; V_{B6} 0.02; V_{B12} 0.012; folic acid 0.015; V_E 0.40; V_C 0.60; V_{K3} 0.005; biotin 0.06; wheat middling 5.133.*

semilaevis. Unfortunately, the fish flesh quality declined over time through the course of artificial aquaculture based on artificial feeds (rich in FM protein) feeding (Valente et al., 2013). Searching for alternative proteins for FM and obtaining high-quality fish products are the key targets for a successful aquaculture industry in the future. To fulfill this aim, we conducted a comprehensive study to compare the effects of DBLM diets and artificial feeds on the growth, digestion, muscle characteristics, and quality of *C. semilaevis*. The results would provide a theoretical basis and reference for further research on the wide application of DBLM in aquatic feeds and improving the flesh quality of aquatic products fed with DBLM diets.

2 METHODS AND MATERIALS

2.1 Experimental Diets and Proximate Composition

The primary dietary ingredients of conventional *C. semilaevis* feeds are as follows: super steam fish meal (FM), defatted *H. illucens* larvae meal (DBLM), fermented soybean meal, shrimp meal, and fish oil. The five isonitrogenous and isolipidic experimental diets were formulated by substituting 0%, 25%, 50%, 75%, and 100% FM protein with DBLM (H0, H25, H50, H75, and H100, respectively). The composition and proportion of other components in experimental diets were summarized in **Table 1** (fish oil, high gluten flour, and microcrystalline cellulose contents were different in order to regulate the lipid and protein contents in each group's feeding diet).

Chemical analyses of the experimental diets were analyzed according to AOAC methods (AOAC, 2012). The samples were dried to a constant weight at 105°C for 24 h to determine the dry matter content. Then, the crude protein was determined by the Kjeldahl method after acid digestion. The percentage of chitin and its nitrogen content is not considered. Crude fat was carried out according to Folch et al.'s method (Folch et al., 1957). The ash content was determined using incineration in a muffle furnace at 550°C for 12 h (ISO 5984-2002). The proximate compositions of the five experimental diets are given in **Table 1**.

2.2 Experimental Fish and Feeding Trial

Cynoglossus semilaevis (mean initial weight 563.48 ± 22.81 g) was provided by Haisheng Aquaculture Co., Ltd. (Tianjin, China). The experimental fish were randomly distributed into 20 tanks (80 L) with n = 5 fish per tank and acclimatized to the feeding experimental conditions for 7 days. The feeding trials were conducted in an indoor recirculating system. The fish were fed with an experimental diet of the corresponding experimental group prior to the formal feeding experiment, twice a day at 9:00 and 19:00. Each tank was outfitted with a continuous aeration device. During the whole feeding period, the water temperature was maintained at 17~20°C. The dissolved oxygen in the water

was controlled above 7 mg/L, ammonia-N less than 0.01 mg/L. For 65 days, *C. semilaevis* were fed to apparent satiation, at 9:00 and 19:00, with a daily feeding rate of 0.5% of body weight. The dead fish were weighed and their amount in each group was recorded, respectively.

2.3 Sample Collection and Analysis

After 24 h starvation, five fish from each tank were anesthetized by 100 mg L^{-1} of MS-222 (Sigma, St. Louis, MO, United States). The growth performances (including final body weight—FBW, body length, and body height) were measured and calculated from a total of 20 tails of fish per group before slaughter. In each tank, three individuals collected blood samples from the caudal vein using 2 ml syringes, the separated serum was removed by centrifuging (3,500 r/min, 10 min) after keeping it at 4°C for 12 h. Then, the separated serum was stored at −80°C until the serum biochemical analysis. After blood sampling, all of the experimental fish were immediately sampled liver, intestine, and muscle tissues, then frozen in liquid nitrogen. All these samples were transferred and preserved at −80°C until subsequent detection.

Afterward, the instantaneous growth rate (IGR), specific growth rate (SGR), weight gain rate (WGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (ConF), feed intake (FI), survival rate (SR) were calculated via the following formulas (Crane et al., 2020):

$$\mathrm{IGR}\ (\%) = \frac{\mathrm{Ln}\ (W_f) - \mathrm{Ln}\ (W_i)}{T}; \quad (1)$$

$$\mathrm{SGR}\ (\%) = 100\left(e^{IGR} - 1\right);$$

$$\mathrm{WGR}\ (\%) = \frac{W_f - W_i}{W_i} \times 100\%; \quad (2)$$

$$\mathrm{FCR} = \frac{W_0}{W_f - W_i}; \quad (3)$$

$$\mathrm{FI}\ (\%/\mathrm{tail}) = \frac{W_F}{(W_f + W)_i/2} \times 100\%; \quad (4)$$

$$\mathrm{PER} = \frac{W_f + W' - W_i}{\mathrm{FI} \times P} \times 100\%; \quad (5)$$

$$\mathrm{ConF} = \frac{W_f}{L^3} \times 100; \quad (6)$$

$$\mathrm{SR}\ (\%) = \frac{N_1}{N_2} \times 100\%. \quad (7)$$

Here, W_0, amount of feeds given (g); W_f, final body weight (g); W_i, initial Weight (g); W', weight of dead fish during the feeding period (g); W_F, the total amount of the feed consumed (g); T, feeding days (65 d); P, protein content in the specific experimental feeds (%); L, body length (cm); N_1, the final tails of fish in the specific experimental group; N_2, the initial number of fish in the corresponding group.

This study complied with the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines and the "Guidelines for Experimental Animals" from the Ministry of Science and Technology (Beijing, China). Furthermore, the Institutional Animal Care and Use Ethics Committee of Tianjin Agricultural University had approved our study. All efforts were made to minimize the suffering of sampled fish individuals.

2.4 Determination of Serum and Intestine Biochemical Parameters

The serum samples were prepared according to the previous method (Shi et al., 2006). The total protein (TP), albumin (ALB), globulin (GLO), aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), and total cholesterol (T-CHO) were tested using an automatic biochemistry analyzer (Hitachi 7020, Hitachi High Technologies, Inc., Ibaraki, Japan). The test kits were purchased from Nanjing Jiancheng Biochemical Corporation (Nanjing Jiancheng Biochemical Corporation, Nanjing, China).

In addition, the activities of trypsin and lipase in the intestines were determined using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The procedures followed were in accordance with the instructions of the kit.

2.5 Histological Analysis

The midgut (i.e., mid-intestine) was dissected and stored in 10% buffered formalin. The samples were processed according to the standard histological method in place at the Institute of Aquaculture (Stirling, United Kingdom). Briefly, the samples were dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin. Transverse cuts of approximately 8 μm were stained with hematoxylin & eosin (H&E staining) (Rasmussen and Ostenfeld, 2000). Each slide was observed under a microscope. ImageJ was used to quantify the effects of DBLM diets on intestinal morphology (for example, lumen, columnar epithelium, lamina propria, goblet cells, stratum granulosum, circular muscle, longitudinal muscle, and serosa) of *C. semilaevis* (Urán et al., 2008; Cavrois-Rogacki et al., 2022).

Serial transverse 8 μm-thick sections were stained with H&E according to the procedures (Rasmussen and Ostenfeld, 2000). A total of 200–400 fibers of white muscle per fish were studied using a Leica MZ 6 microscope for their cross-sectional area (CSA) and the diameter ($d = 2r$) of each fiber was calculated from the fiber area (A) [$A = \pi \times r^2$, thus, $d = 2\sqrt{(A \times \pi^{-1})}$]. A size limit for identifying the fibers was set at fiber diameters ≥10 μm since the optical resolution below this limit did not allow for sufficient identification and accuracy in the analyses (Luther et al., 1995). The circularity of each fiber ($4\pi \times A \times \mathrm{circumference}^{-2}$) was also determined. A circularity of 1.0 indicated a complete circle. The free software ImageJ (http://rsb.info.nih.gov/ij/) was also used for quantitative statistics and analyses.

2.6 Antioxidant Capacity and Oxidative Stress

For the analyses of antioxidant enzymes activity and oxidative stress, muscle samples were collected (three fish per tank) and used for determination. All the samples were flash-frozen in liquid nitrogen and stored at −80°C until follow-up analysis. Quality muscle samples were taken, the surface blood was washed with normal saline, and then, nine times the volume of 0.85%

normal saline was added, homogenized in an ice water bath, and centrifuged at 4°C (2,500 r/min, 15 min). The supernatant was used for the determination of antioxidant enzymes, including superoxide dismutase (SOD), total antioxidant capacity (T-AOC), malondialdehyde (MDA), catalase (CAT), and lipid peroxide (LPO) contents with test kits (Nanjing Jiancheng Bioengineering Institute, China).

2.7 Measurement of Muscle Drip Loss, Frozen Exudation Rate, and Cooking Loss

Each fish took three parts of about 3.00 g peeled white muscle and weighed (W_1, W_3, and W_5), which were stored at 4°C for 24 h, −20°C for 24 h, and put into a 5 ml centrifuge tube, respectively. After removing the muscle, wipe off the moisture from the surface, weigh (W_2 and W_4), and calculate the drip loss (DL) and the frozen exudation rate (FET). The centrifuge tube cover is cutoff, sealed with a sealing film, and a vent tied on the sealing film, which is put into a 75°C water bath for 15 min. Then, the moisture on the surface of muscle samples is absorbed, and subsequently weighed (W_6). Afterward the DL, FET, and cooking loss (CL) were calculated based on the measured data.

$$\mathbf{DL}\,(\%) = (W_1 - W_2)/W1 \times 100\%; \quad (8)$$

$$\mathbf{FET}\,(\%) = (W_3 - W_4)/W_3 \times 100\%; \quad (9)$$

$$\mathbf{CL}\,(\%) = (W_5 - W_6)/W_5 \times 100\%. \quad (10)$$

2.8 Statistical Analysis

The white muscle fiber circularity and intestinal morphology data were ln-transformed using software ImageJ. The fiber diameter was additionally fitted as a covariate to fiber circularity. For comparing the distributions of muscle fiber sizes, the non-parametric Kolmogorov-Smirnov two-sample test was applied. This test is very sensitive in detecting the differences in dispersions and skewness compared to other statistical tests (Zar, 1996). The ImageJ software was also used for intestinal morphometric analysis. One-way ANOVA (multiple comparisons) was used for analyzing the differences in the muscle characteristics and intestinal structures among groups. The differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

The data analyses of all test indicators (growth performances, biochemical indexes, and muscle drip loss, frozen exudation rate, and cooking loss) were presented as the "mean ± SE." One-way analysis of variance was used to test the effect of diets. Duncan's multiple range test was used for multiple comparisons. The 0.05 probability level was used to denote statistically significant data, while the 0.01 probability level was used to denote highly significant data.

3 RESULTS

3.1 Growth Performances and Feeding Situations

The effects of experimental diets formulated by substituting different levels of FM protein with DBLM on survival, growth performance, and feed utilization of *C. semilaevis* were illustrated in **Table 2**. No significant differences were observed in the survival rate (SR) among the five groups, and the SR of *C. semilaevis* fed with DBLM diets remained higher (90%~100%). The weight gain rate (WGR), specific growth rate (SGR), and protein efficiency ratio (PER) were significantly lower in H50, H75, and H100 groups than those in the control group ($p < 0.05$). However, the WGR, IGR, SGR, and PER in the H25 group were all significantly greater than those in H0 ($p < 0.05$). The less feed conversion ratio (FCR) was tested in the H0 and H25 groups; which were significantly different from that of H50, H75, and H100 groups ($p < 0.05$). Meanwhile, the greatest FCR was found in the H75 group. When replacing the FM with DBLM up to 50%, it had no significant effect on the feed intake (FI) of fish, and then, it significantly became enhanced in the H75 and H100 groups. Moreover, there was no significant difference in the condition factor (ConF) among different treatments ($p > 0.05$). Overall, most of the indices investigated the largest and optimal values in the H25 group, namely, the 25% DBLM could better promote the growth and feed utilization of *C. semilaevis*.

3.2 Serum Biochemical Indices

As shown in **Figure 1**, no significant difference was found in the serum TP content for *C. semilaevis* among all the groups ($p > 0.05$). In the H25 group, the serum ALB, GLO and their ratio (i.e., A/G), as well as ALT, TG, and T-CHO contents were significantly higher than those of the other DBLM diet-feeding groups (including H50, H75, and H100 groups). The highest ALB, ALT, and TG levels were also detected in the same group. However, the comparison for each index between H25 and H0 groups showed complexity and index specificity. For instance, the levels of ALB, GLO, ALT, and TG in serum from the H25 group were all significantly higher compared to the H0 group, but the greater level of A/G and T-CHO were found in the control group ($p < 0.05$). For the AST activity, it was significantly inhibited in the H25 group, whilst exhibiting strengthened activity with the increase of FM replacement level. Accordingly, the greatest activity of AST was detected in the H100 group ($p < 0.05$) (**Figure 1**).

3.3 Intestinal Biochemical Indices

Cynoglossus semilaevis fed with the DBLM diets (namely, from H25, H50, H75, and H100 groups) presented significantly lower activities of intestinal digestive enzymes than those in the control group ($p < 0.05$) (**Figure 2**). The highest levels of amylase and trypsin were measured in the H0 group. However, the greatest activity of lipase was observed in the H75 group, it showed no significant changes relative to the H0 group ($p > 0.05$). The comparisons between DBLM diet-feeding groups illustrated that the intestinal amylase, lipase, and trypsin activities had a similar altered trend with the increased substitution level of DBLM for FM in feeds. To be specific, the activity of digestive enzymes firstly increased (from 25% to 75%) and then decreased (from 75%) with the increased level of DBLM in diets. The lowest values of amylase, lipase, and trypsin were detected in the H25 and H50 groups, respectively (**Figure 2**).

3.4 Morphology of the *Cynoglossus semilaevis* Intestine

The midgut construction of *C. semilaevis* was assayed in **Figure 3**; meanwhile, the statistical information of the data quantified from the

TABLE 2 | Growth performances of *C. semilaevis* fed with different levels of DBLM (n = 20).

	H0	H25	H50	H75	H100
IW (g)	520.98 ± 10.39	522.79 ± 21.57	563.48 ± 22.81	588.47 ± 22.76	585.05 ± 21.37
FW (g)	686.75 ± 10.42	709.14 ± 21.06	699.67 ± 21.66	690.74 ± 22.23	722.22 ± 20.69
WGR (%)	31.86 ± 0.76^{c}	35.84 ± 1.61^{d}	24.31 ± 1.16^{b}	17.46 ± 0.76^{a}	23.55 ± 0.95^{b}
FCR	1.26 ± 0.04^{a}	1.14 ± 0.05^{a}	1.61 ± 0.09^{b}	2.34 ± 0.09^{c}	1.75 ± 0.05^{b}
IGR (%/d)	0.43 ± 0.01^{c}	0.47 ± 0.02^{d}	0.33 ± 0.01^{b}	0.25 ± 0.01^{a}	0.33 ± 0.01^{b}
SGR (%)	1.66 ± 0.05^{c}	1.97 ± 0.12^{d}	1.16 ± 0.07^{b}	0.77 ± 0.04^{a}	1.12 ± 0.06^{b}
PER	30.32 ± 0.16^{c}	31.22 ± 0.12^{d}	28.04 ± 0.11^{b}	25.56 ± 0.19^{a}	25.63 ± 0.17^{a}
FI (%/d)	2.12 ± 0.04^{a}	2.12 ± 0.01^{a}	2.13 ± 0.06^{a}	2.30 ± 0.01^{b}	2.27 ± 0.02^{b}
ConF (g/cm^{3})	0.77 ± 0.02	0.81 ± 0.03	0.79 ± 0.03	0.75 ± 0.04	0.75 ± 0.02
SR (%)	95.00 ± 5.00	100.00 ± 0.00	90.00 ± 5.77	100.00 ± 0.00	95.00 ± 5.00

Initial weight, IW; final body weight, FW; weight gain rate, WGR; feed conversion ratio, FCR; instantaneous growth rate, IGR; specific growth rate, SGR; protein efficiency ratio, PER; feed intake, FI; condition factor, ConF; survival rate, SR. The data measured traits of growth performances were presented as the "Mean ± SE." Different lowercase letters mean the significant differences (p < 0.05) from each other.

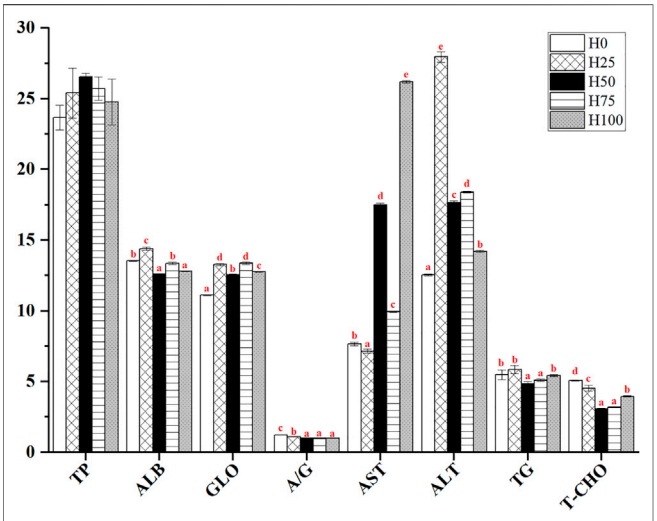

FIGURE 1 | Serum biochemical parameters of *C. semilaevis* were fed with different levels of DBLM diets (n = 4). Total protein (TP, g/L), albumin (ALB, g/L), globulin (GLO, g/L), albumin/globulin ratio (A/G), aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L), triglyceride (TG, mmol/L), and total cholesterol (T-CHO, mmol/L). The data were expressed by "mean ± SE." Different lowercase letters were used for presenting the significant differences among the five experimental groups, and significant at the 0.05 level.

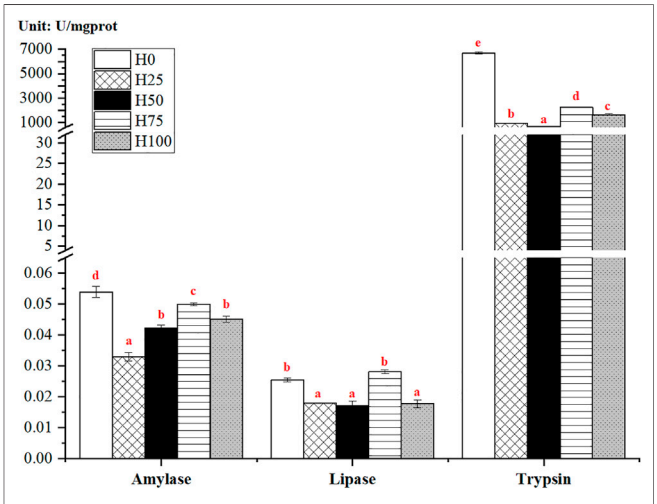

FIGURE 2 | Activity of the intestinal digestive enzymes in *C. semilaevis* fed with experimental feeds substituting FM with different levels of DBLM (n = 4). The activities of intestinal amylase (U/mgprot), lipase (U/mgprot), and trypsin (U/mgprot) in *C. semilaevis* from five experimental groups, as well as their pairwise comparisons between groups. The significant differences between the groups were indicated by various red lowercase letters (p < 0.05).

tissue sections was supplied in **Table 3**. In the H25 group *C. semilaevis*, there were significant improvements in the thickness of intestinal longitudinal muscle (LM), circular muscle (CM), the columnar epithelium (CE), and lamina propria (LP) in comparison to the control group (p < 0.05). Whereas the fish from the other DBLM diets-feeding groups (i.e., H50, H75, and H100) presented significant reductions in thicknesses of LM, CM, CE, and LP, as well as the length of microvilli (ML) compared to the H0 and H25 groups, in a dose-dependent manner (p < 0.05). Thereby, the smallest CE, LP, and ML were observed in the H100 group. However, the LM and CM were thinnest in the H75 group, then they showed slight recoveries in the H100 group (**Table 3**). Furthermore, the DBLM diets also had significant effects on the goblet cell (GC). The numbers of GC increased with the increasing replacement level of FM (**Figure 3**). Moreover, the substitution level of the FM increased up to approximately 50%, accompanied by intestinal structural damage, such as splits in serosa and intestinal villus (**Figure 3**).

3.5 The Comparison of the Muscle Characteristics of *Cynoglossus semilaevis* Between Groups

3.5.1 Proximate Compositions of *Cynoglossus semilaevis* Flesh

Compared to the H0 group, moisture, ash, crude fat (CF), and protein (CP) contents of *C. semilaevis* flesh all significantly enhanced after substituting 25% FM with DBLM (p < 0.05). There were no significant alterations in moisture, DM, and ash contents along with the changes in substitute rate of DBLM

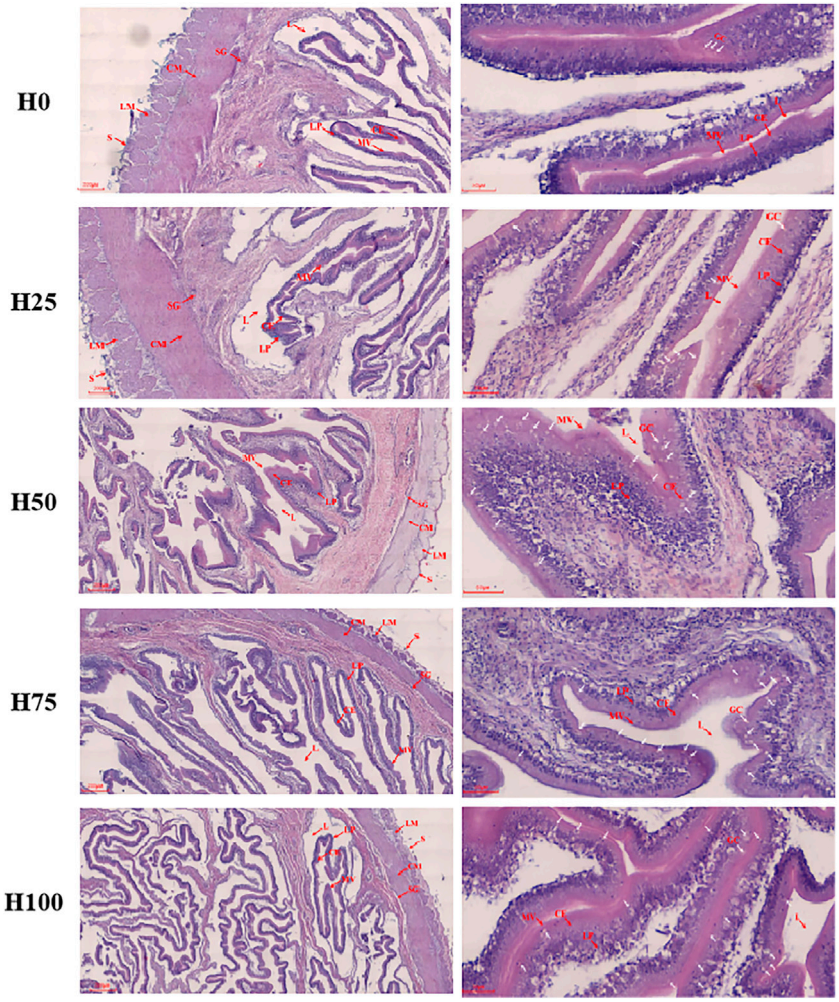

FIGURE 3 | Midgut construction of *C. semilaevis* fed with the five different experimental diets. Transverse section of *C. semilaevis* mid-intestine (left column: ×40, right column: ×200) with structural organization. L, lumen; CE, columnar epithelium; LP, lamina propria; GC, goblet cells (multiple white arrow marked); MV, microvilli; SG, stratum granulosum; CM, circular muscle; LM, longitudinal muscle; and S, serosa.

TABLE 3 | Jejunum morphology of *C. semilaevis* in each experimental group.

	H0	H25	H50	H75	H100
Longitudinal muscle (μm)	125.86 ± 4.00^{c}	188.82 ± 3.63^{d}	60.59 ± 1.64^{b}	43.90 ± 1.19^{a}	57.35 ± 1.33^{b}
Circular muscle (μm)	211.91 ± 4.76^{d}	281.30 ± 3.06^{e}	76.33 ± 2.96 ^{ab}	70.26 ± 1.55^{a}	83.54 ± 1.13 ^{bc}
Columnar epithelium (μm)	27.57 ± 0.48^{b}	32.38 ± 0.59^{d}	37.55 ± 0.34^{e}	29.74 ± 0.21^{c}	24.91 ± 0.19^{a}
Lamina propria (μm)	51.54 ± 0.46^{c}	60.98 ± 0.81^{e}	54.57 ± 0.37^{d}	38.69 ± 0.24^{b}	26.31 ± 0.21^{a}
Microvilli length (μm)	31.00 ± 0.58^{e}	19.34 ± 0.34^{c}	22.41 ± 0.31^{d}	8.19 ± 0.19^{b}	6.99 ± 0.13^{a}

(namely, from 25% to 100%). As for the CP, it significantly decreased with the increase of replacement level of FM with DBLM in feeds ($p < 0.05$). However, the CF in *C. semilaevis* muscles demonstrated complex changes among different feeding groups. It rose dramatically in the H25 group, decreased and then increased slightly in the H50 and H75 groups, declined sharply in the H100 group, respectively ($p < 0.05$). It is noteworthy that the higher levels of muscular moisture, ash, CF, and CP were found in the H25 group, while their lowest values were mostly detected in the either H0 group or the H100 group (**Table 4**).

3.5.2 Antioxidant Capacity in *Cynoglossus semilaevis* Muscles

Data on the anti-oxidative responses in the muscles of *C. semilaevis* under distinct diets-feeding are shown in **Table 5**. Significant differences were detected in SOD, T-AOC, CAT, MDA, and

TABLE 4 | Proximate compositions of *C. semilaevis* muscles from the five experimental groups (% dry weight).

	H0	H25	H50	H75	H100
Moisture	79.57 ± 0.55^{a}	80.92 ± 0.66^{b}	80.08 ± 0.21 ^{ab}	80.02 ± 0.17 ^{ab}	80.21 ± 0.25 ^{ab}
Dry matter	20.43 ± 0.23^{b}	19.08 ± 0.28^{a}	19.92 ± 0.21^{b}	19.98 ± 0.17^{b}	19.79 ± 0.25^{b}
Ash	5.23 ± 0.01^{a}	5.43 ± 0.02^{b}	5.60 ± 0.02^{c}	5.37 ± 0.01^{b}	5.42 ± 0.04^{b}
Crude fat	2.29 ± 0.03^{b}	2.61 ± 0.04^{d}	2.25 ± 0.02^{b}	2.39 ± 0.02^{c}	2.01 ± 0.02^{a}
Crude protein	89.47 ± 0.25^{c}	90.38 ± 0.15^{d}	89.73 ± 0.38^{cd}	88.63 ± 0.18^{b}	86.32 ± 0.16^{a}

The different lowercase letters, e.g., a, b, c, and d, have the meaning of significant differences (p < 0.05) from each other. The analyzed data are presented as the "Means ± SE."

TABLE 5 | Antioxidation of *C. semilaevis* fed with different diets formulated with levels of DBLM (n = 4).

	H0	H25	H50	H75	H100
SOD	51.60 ± 0.10^{b}	55.93 ± 0.09^{c}	45.72 ± 0.23^{a}	56.84 ± 0.06^{d}	51.53 ± 0.11^{b}
T-AOC	0.42 ± 0.00^{c}	0.40 ± 0.00^{a}	0.44 ± 0.00^{d}	0.41 ± 0.00^{b}	0.41 ± 0.00 ^{ab}
CAT	6.64 ± 0.07^{d}	3.77 ± 0.01^{c}	3.62 ± 0.02^{c}	3.19 ± 0.00^{b}	2.83 ± 0.09^{a}
MDA	2.78 ± 0.02^{d}	2.67 ± 0.02^{c}	2.47 ± 0.00^{a}	2.61 ± 0.01^{b}	5.64 ± 0.01^{e}
LPO	0.23 ± 0.01^{b}	0.22 ± 0.00^{b}	0.08 ± 0.00^{a}	0.28 ± 0.00^{c}	0.28 ± 0.00^{c}

Superoxide dismutase (SOD, U/mgprot), total antioxidant capacity (T-AOC, U/mL), malondialdehyde (MDA, mmol/L), catalase activity (CAT, U/mgprot), and lipid peroxide (LPO, mmol/L). The data were expressed by "mean ± SE." Different lowercase letters mean the significant differences among the five experimental groups (p < 0.05).

LPO activities among the treatments (p < 0.05). Concretely, no significant difference was detected in SOD between H0 and H100 groups, their SOD activities were greatly lower than that in the H25 and H75 groups. Meanwhile, the lowest level of SOD was measured in the H50 group. For the T-AOC, the smallest activity in the H25 *C. semilaevis* muscles was detected, their higher levels were analyzed in the H75, H100, and H0 groups, and yet less than that in the H50 group. The CAT activity in *C. semilaevis* muscles prominently reduced along with the rise in the DBLM content in feeding diets (p < 0.05). The MDA levels showed a markedly downward trend to the minimal activity (in the H50 group) at first, and then significantly increased to the greatest activity in the H100 group (p < 0.05). No significant differences in the LPO were shown between the H0 and H25 groups or between the H75 and H100 groups, but their activities were all significantly greater than that in the H50 group (**Table 5**).

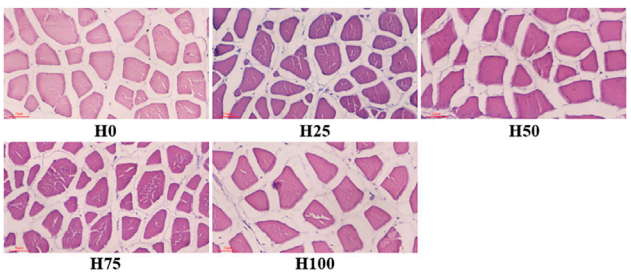

FIGURE 4 | Cross sections of *C. semilaevis* muscles from the five experimental groups. Cross sections of *C. semilaevis* muscle tissue (×200 times) with structural organization. The red scale bar in the lower-left represents 50 μm and applies to all panels. The muscle fibers were stained with H&E.

3.5.3 Histological Observation of *Cynoglossus semilaevis* Muscles

The histological changes were recorded using diameter and density of muscle fibers, interstitium between myofibers through the observation of cross sections of *C. semilaevis* muscles (**Figures 4**, **5**). First, the distances between myofibers significantly reduced in the H25, H50, and H75 groups compared to the H0, but the maximum gap was detected in the H100 group (p < 0.05). There was an obvious downward trend in the density of muscle fibers as the increasing levels of DBLM in feeding diets. There were about 4, 5, 3, 2, and 2 fibers per unit area (r = 250 μm). It was also found that the percentage of muscle fiber area in the H75 group was significantly larger than that in other groups, whilst lowest in the H100 group (p < 0.05). Furthermore, no significant difference was found in the muscle fiber diameter among the H0, H50, and H100 groups, whose values were significantly larger than the H25 group's, and smaller than that in the H75 *C. semilaevis* muscles.

3.5.4 Water Holding Capacity of *Cynoglossus semilaevis* Muscles From Different Groups

The effect of levels of dietary DBLM on muscular water holding capacity was explained by the indexes, including the cooking loss rate (CLR), drip loss rate (DLR), and freeze exudation rate (FER). The CLR, DLR, and FER of *C. semilaevis* muscles were significantly influenced by the 5 dietary treatments (**Figure 6**). Meanwhile, these three indicators showed a similar change rule among the groups. The CLR, DLR, and FER of H0 *C. semilaevis* muscles were all significantly stronger than those of the H25 fish samples (p < 0.05), and almost the same or less than those in the H50 group. The three indexes were markedly reduced in the H75 group, but their levels were still higher than those in the H25 group (p < 0.05). The highest and lowest levels of CLR, DLR, and FER were detected in H0 and H100 groups, H100 and H25 groups, H25 and H50 groups, respectively.

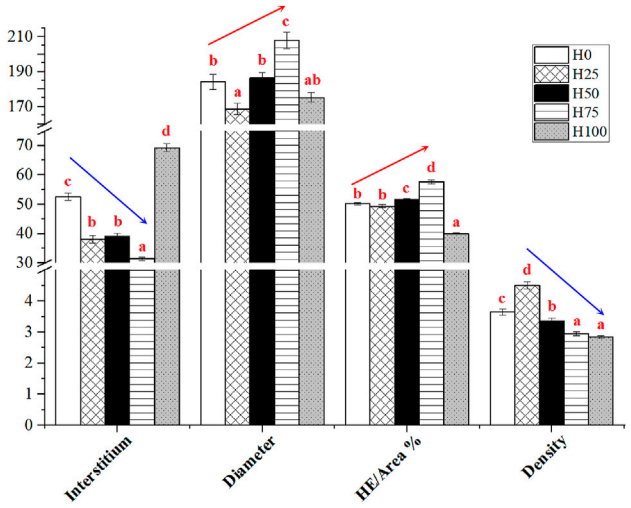

FIGURE 5 | Quantitative results of the effect of DBLM diets on muscle fiber characteristics of *C. semilaevis*. Interstitium (Unit: μm), the distance between muscle fibers; diameter (Unit: μm), myofiber diameter; HE/area (%), the area of muscle fibers stained with H&E as the percentages of the area of the selected views; density, the numbers of myofibers within the area (r = 250 μm). The different lowercase letters indicate the significant differences among the five experimental groups ($p < 0.05$). The arrows represent the trends of indicators among the treatments. Data are presented as the "Mean ± SE."

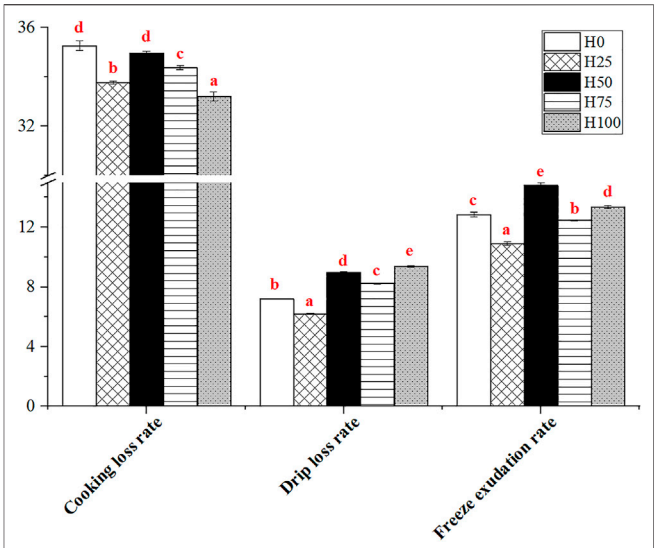

FIGURE 6 | Effects of levels of DBLM diets on water holding capacity of *C. semilaevis* muscles (n = 12). The different lowercase letters mean the significant differences among groups ($p < 0.05$). Data are presented as the "Mean ± SE."

4 DISCUSSION

4.1 Differences in the Growth of *Cynoglossus semilaevis* Induced by DBLM Diet Feeding

In the present study, *C. semilaevis* fed with diets replaced 25% FM with DBLM showed significant improvements in weight gain rate (WGR), specific growth rate (SGR), condition factor (ConF), and survival rate (SR) compared to the control group, demonstrating the positive influence of supplemented lower level of DBLM on the growth of *C. semilaevis*. This conclusion has been confirmed in the study of the other species. For example, dietary replacement of FM by 25% insect powder, such as black soldier fly larvae meal (BLM), defatted BLM (DBLM), or *Tenebrio molitor* larvae meal, had no side effect on the growth performances of *O. mykiss* (Renna et al., 2017), *Litopenaeus vannamei* (Wang et al., 2021), *Clarias gariepinus* (Adeoye et al., 2020), and *Sparus aurata* (Piccolo et al., 2017). However, the level of insect meal replacing FM exceeded 50% and commonly resulted in a significant decline in the growth of fed fish. The dietary replacement of FM by 80% DBLM reduced the growth of *L. vannamei* (Wang et al., 2021), 100% BLM significantly restrained the SGR of *C. gariepinus* (Adeoye et al., 2020). In current work, the significantly reduced WGR, SGR, and ConF were found in the H50 group, and their lowest results were all detected in the H75 group, accompanied by the increases in feed conversion ratio (FCR) and feed intake (FI). It is the same as the results in *Ictalurus punctatus* (Yildirim-Aksoy et al., 2020). More specifically, the final weight gain of *I. punctatus* significantly increased when the feeding diets contained BLM at levels from 100 to 300 g/kg. *I. punctatus* fed diets without BLM, and with 300 g/kg BLM, showed the lowest and highest FI, respectively. The protein efficiency ratio (PER), however, was significantly lower in *I. punctatus* fed BLM at levels of 200 g/kg and higher compared to the FM diet (Yildirim-Aksoy et al., 2020). However, the research on *Dicentrarchus labrax* found that the dietary FM partially replaced by BLM showed no influences not only in growth performances of *D. labrax* but also in the feed utilization (Magalhães et al., 2017). Basto's study found that it is feasible to substitute FM by defatted *T. molitor* larvae meal up to 80% without any significant effect on the growth of *D. labrax* (Basto et al., 2021). In *S. aurata* study testified that the more the replacement of FM by *T. molitor* larvae meal in diets, the less the FCR (Piccolo et al., 2017). The above diverse results could be due to the differences in fish or insect species, the different developmental stages of insects, as well as the varied processing methods for insects (Tschirner and Simon, 2015; Renna et al., 2017; English et al., 2021). In this study, the growth performances and feed utilization indices were observed as the largest or optimal values in the H25 group. Hence, the DBLM could replace 25% FM without any negative effects on the growth and feed utilization of *C. semilaevis*.

4.2 Effects of the Levels of Dietary DBLM on the Serum Biochemical Parameters of *Cynoglossus semilaevis*

Serum biochemical parameters are often used as a sensitive biomarker for confirming the aquatic product quality and for monitoring any changes in aquaculture in many fish species (Polakof et al., 2012). Currently, feeding *C. semilaevis* with

DBLM diets brought the concentrations of serum T-CHO and TG down relative to the H0 group, which indicate that the more dietary DBLM supplied in diets could cut down the lipid level and fat accumulation in farmed fish. It is similar to the results in *D. labrax* (Magalhães et al., 2017), *C. carpio* (Li et al., 2017), *Lateolabrax japonicus* (Wang et al., 2019). These studies tend to claim that using DBLM instead of FM in feeding diets could effectively decrease the T-CHO and TG concentrations, meanwhile, relieve the fat deposition in the fish body that is exposed in the process of long-term feeding with FM feeds (Zhao et al., 2018). The higher content of chitosan in insects might be one of the important reasons which lead to the benefits abovementioned (Xia et al., 2011; Mohan et al., 2020). Another study demonstrates that the dietary inclusion of medium-chain fatty acid (MCFA) also resulted in decreased fat deposition in the tissues with improved growth of fish, and the MCFA is particularly high in black soldier fly larvae (Li S. et al., 2016).

The ALB, GLO, ALT, and AST are the available and sensitive indicators for the identification of hepatocyte injury and liver necrosis (Sheikhzadeh et al., 2012). Accordingly, the activities of serum GLO, AST, and ALT in DBLM diets-feeding groups (including H25, H50, H75, and H100 groups) were significantly higher than those in the H0 group, as well as the lower levels of ALB in DBLM diets-feeding groups. They all mean that abnormal liver function is likely to occur when *C. semilaevis* fed with DBLM diets (Lu et al., 2020). Whereas, no inflammation was found in the *D. rerio* liver fed with a diet supplemented with 50% BLM (Zarantoniello et al., 2018). Therefore, we boldly assume that the varied processing methods for insects also have quite different effects on the physiological and biochemical parameters of fed fish.

4.3 Differences in Digestive Enzyme Activity and Intestinal Microstructure Among Groups

The activity of intestinal digestive enzymes adapts to the changes in diets, also reflects the dietary influence on the feed utilization and growth performance of fish (Li et al., 2017; Zhang H. et al., 2020). The FCR and FI, as well as the activities of amylase, lipase, and trypsin were, examined higher even highest levels in the H75 *C. semilaevis*, with the smallest PER, WGR, SGR, and SR. Correspondingly, the H25 *C. semilaevis* exhibited the greatest PER, WGR, SGR, ConF, as well as the lowest FCR, FI and digestive enzyme activities. However, the negative correlation between growth performances and digestive enzyme activity is rarely reported in the investigation on the applications of BLM or DBLM in other species. Most studies suggested that the DBLM or BLM as an alternative protein ingredient had no significant effects on the activity of digestive enzymes, e.g., in *C. carpio* (Li et al., 2017), *L. vannamei* (Wang et al., 2021), *L. japonicus* (Wang et al., 2019) and *Micropterus salmoides* (Xu et al., 2021). The decreased activities of intestinal trypsin and lipase in *C. semilaevis* firstl, then enhanced with the increase in the addition level of DBLM. A similar result has been confirmed in *D. labrax* that increasing levels of DBLM in diets lowered the activities of intestinal trypsin and lipase (Lu et al., 2020). Because the different experimental diets in our study were isonitrogenous and isolipid, the alterations in digestive enzyme activities of *C. semilaevis* were not caused by the differences in the nutritional composition of feeding diets. The specific reason needs to be further verified.

The intestinal morphology (such as LM, CM, CE, ML, and LP) is valuable for the assessment of the gut health of fish (Yu et al., 2020). Accordingly, the significant improvements in LM, CM, CE, ML, and LP were found in the H25 group, whereas they were evidently reduced in the H50, H75, and H100 groups by a DBLM level-dependent manner. Ordinarily, the thinning of the intestinal lining is beneficial in increasing the absorption of nutrients which leads to optimize utilization and resulting in better growth (Cetingul et al., 2015; Wang et al., 2019). Therefore, the decreased thickness of CE, LP, CM, LM, and the length of microvilli, indicated that replacing FM with DBLM is helpful in improving the digestion and absorption of nutrients by *C. semilaevis*. However, it is worth noting that the substitution of FM increased up to 50%, accompanied by intestinal structural damage, such as splits of serosa and intestinal villus. In addition, the observations in intestinal morphology attributed to the varied contents of chitin in our five different feeding diets (Li Q. P. et al., 2016). Chitin was reported to decrease the thickness of intestinal wall and the length of microvilli (Li Q. P. et al., 2016).

4.4 Effects of Different Levels of DBLM on Meat Quality Characteristics of *Cynoglossus semilaevis*

The antioxidant system is the most important defense line of the body against oxidative damage (Xu et al., 2022). We found that superoxide dismutase (SOD) and catalase (CAT) declined significantly in the muscles of *C. semilaevis*, as the proportion of DBLM in the diets increased up to 75%. The data means that replaced level of less than 75% could boost the antioxidant capacity of *C. semilaevis* muscles, while the capacity would be inhibited if the level of replacing FM with DBLM was further increased. This situation was in accordance with Wang et al. (2019) who found that SOD activities in *L. japonicus* serum produced a similar trend when using DBLM as an alternative protein ingredient in diets. As mentioned previously, the more insect powder used, the more chitin contained, which is reported to have antioxidants, immune enhancement and other biological functions (Benhabiles et al., 2012; Mengíbar et al., 2013; Zou et al., 2016; Naveed et al., 2019). However, we need to pay attention to the negative effect of the excessive use of DBLM. For instance, substituting the FM with DBLM over 75% resulted in an oxidative damage in *C. semilaevis* muscles, characterized by increasing the products of lipid peroxidation, such as MDA and LPO. This conclusion was supported by the previous findings in *D. rerio* (Zarantoniello et al., 2018) and *L. japonicus* (Wang et al., 2019).

In addition, the contents of crude fat (CF), crude protein (CP), and ash in *C. semilaevis* fillets were not affected by dietary DBLM levels, without changes in moisture and dry matter (DM). It is similar to the results reported in *Paralichthys olivaceus*, which

revealed that no changes were evident in moisture and ash, but the CF and CP contents showed a significant decreasing trend with the rise in dietary insect powder (Jeong et al., 2021b). Belforti et al. (2016) found that the CP in fillets rose significantly with the increased DBLM in diets. Water holding capacity, an important index to evaluate the meat quality, is reflected by DLR, CLR, and FER (Zhang L. et al., 2020). There was research illustrated that water holding capacity poses positive correlations with the density of myofibers and the CP in fillets (Periago et al., 2005; Cheng et al., 2014). Accordingly, the largest density of myofibers, most abundant CP, and the lowest DLR, CLR, FER were all detected in the H25 *C. semilaevis* fillets. Consequently, the water holding capacity of H25 *C. semilaevis* muscles was the best among the groups. The study on *O. mykiss* came to a same conclusion (Caimi et al., 2021).

5 CONCLUSION

We conducted a comprehensive study to investigate the effects of different levels of DBLM on the growth, digestive function, and flesh quality of *C. semilaevis*. The alterations in all testing indices are deeply intertwined and eventually give rise to the differences in the muscle growth, digestion, and muscle quality among groups. Concretely, *C. semilaevis* fed with diets replaced by 25% FM with DBLM showed significant improvements in WGR, SGR, ConF, and SR compared to the H0 group, demonstrating the positive influence of supplemented lower level of DBLM on the growth of *C. semilaevis*, accompanied with the decline in feed conversion ratio (FCR) and feed intake (FI). Feeding *C. semilaevis* with DBLM diets could cut down the lipid level and relieve the fat deposition in the fish body by downregulating the concentrations of serum T-CHO and TG. Furthermore, the thinning of the intestinal lining, found in the H50, H75, and H100 groups, is beneficial in enhancing the absorption of nutrients leading to optimize the utilization and resulting in better growth. However, the proportion of DBLM replacing FM exceeded 50% also caused intestinal histopathological damage. In addition, the negative effects (e.g., splits of serosa and intestinal villus, increasing the products of lipid peroxidation, and abnormal liver function) of excessive use of DBLM (>75%) were also proved in our study, though the significantly improved antioxidant capacity was detected in the H75 *C. semilaevis* muscles. In addition, the largest density of myofibers, most abundant of CP, and lowest DLR, CLR, FER were all detected in the H25 *C. semilaevis* fillets. By inference, the water holding capacity was the best in the H25 group.

In conclusion, the application of *H. illucens* in aquatic feeds to replace a fish meal with a lower level for culturing *C. semilaevis* could improve feed utilization, promote growth performances, enhance the digestive ability, and obtain better quality of farmed fish. This research consists of an important step toward looking for an efficiently alternative protein source of fish meal, meeting consumers' demand for high-quality, safe, and healthy aquatic products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ministry of Science and Technology (Beijing, China) and the Institutional Animal Care and Use Ethics Committee of Tianjin Agricultural University.

AUTHOR CONTRIBUTIONS

The author XL had roles in culturing fish, collecting samples, data collection, and analysis. The manuscript was written through contributions of HZ and QW. CQ and HZ provided valuable assistance in data analysis. All authors have given approval to the final version of the manuscript and decided to submit the work for publication.

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REFERENCES

Adeoye, A. A., Akegbejo-Samsons, Y., Fawole, F. J., and Davies, S. J. (2020). Preliminary Assessment of Blackoldier Fly (Hermetia Illucens) Larval Meal in the Diet of African Catfish (*Clarias gariepinus*): Impact on Growth, Body index, and Hematological Parameters. *J. World Aquacult Soc.* 51, 1024–1033. doi:10.1111/jwas.12691

AOAC (2012). *Official Methods of Analysis (19th Edition), Chapter 4, Association of Official Analytical Chemists*. Gaithersburgh, USA: AOAC International, 9–13.

Basto, A., Calduch-Giner, J., Oliveira, B., Petit, L., Sá, T., Maia, M. R. G., et al. (2021). The Use of Defatted *Tenebrio molitor* Larvae Meal as a Main Protein

Source Is Supported in European Sea Bass (*Dicentrarchus labrax*) by Data on Growth Performance, Lipid Metabolism, and Flesh Quality. *Front. Physiol.* 12 (12), 659567. doi:10.3389/fphys.2021.659567

Belforti, M., Gai, F., Lussiana, C., Renna, M., Malfatto, V., Rotolo, L., et al. (2016). Tenebrio MolitorMeal in Rainbow Trout (Oncorhynchus Mykiss) Diets: Effects on Animal Performance, Nutrient Digestibility and Chemical Composition of Fillets. *Ital. J. Anim. Sci.* 14 (4), 4170. doi:10.4081/ijas.2015.4170

Benhabiles, M. S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., and Mameri, N. (2012). Antibacterial Activity of Chitin, Chitosan and its Oligomers Prepared from Shrimp Shell Waste. *Food Hydrocolloids* 29 (1), 48–56. doi:10.1016/j.foodhyd.2012.02.013

Caimi, C., Biasato, I., Chemello, G., Oddon, S. B., Lussiana, C., Malfatto, V. M., et al. (2021). Dietary Inclusion of a Partially Defatted Black Soldier Fly (*Hermetia Illucens*) Larva Meal in Low Fishmeal-Based Diets for Rainbow trout (*Oncorhynchus mykiss*). *J. Anim. Sci Biotechnol* 12 (1), 50. doi:10.1186/s40104-021-00575-1

Cavrois-Rogacki, T., Leeming, D., Lopez, P. M., Davie, A., and Migaud, H. (2022). Plant-based Protein Ingredients Can Successfully Replace Fish Meal in the Diet of Ballan Wrasse (*Labrus Bergylta*) Juveniles. *Aquaculture* 546, 737419. doi:10.1016/j.aquaculture.2021.737419

Cetingul, I. S., Rahman, A., Ulucan, A., Keles, H., Bayram, I., Uyarlar, C., et al. (2015). Effect of Mentha Piperita on Some Morphological Characteristics of Intestine in Japanese Quails (*Coturnix coturnix Japonica*). *Archiva Zootechnica* 18 (2), 53–60.

Cheng, J.-H., Sun, D.-W., Han, Z., and Zeng, X.-A. (2014). Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review. *Compr. Rev. Food Sci. Food Saf.* 13 (1), 52–61. doi:10.1111/1541-4337.12043

Crane, D. P., Ogle, D. H., and Shoup, D. E. (2020). Use and Misuse of a Common Growth Metric: Guidance for Appropriately Calculating and Reporting Specific Growth Rate. *Rev. Aquac.* 12 (3), 1542–1547. doi:10.1111/raq.12396

English, G., Wanger, G., and Colombo, S. M. (2021). A Review of Advancements in Black Soldier Fly (*Hermetia Illucens*) Production for Dietary Inclusion in Salmonid Feeds. *J. Agric. Food Res.* 5, 100164. doi:10.1016/j.jafr.2021.100164

Folch, J., Lees, M., and Stanley, G. H. S. (1957). A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *J. Biol. Chem.* 226 (1), 497–509. doi:10.1016/S0021-9258(18)64849-5

Hardy, R. W. (2010). Utilization of Plant Proteins in Fish Diets: Effects of Global Demand and Supplies of Fishmeal. *Aquac. Res.* 41 (5), 770–776. doi:10.1111/j.1365-2109.2009.02349.x

Jeong, S.-M., Khosravi, S., Yoon, K.-Y., Kim, K.-W., Lee, B.-J., Hur, S.-W., et al. (2021b). Mealworm, *Tenebrio molitor*, as a Feed Ingredient for Juvenile Olive Flounder, *Paralichthys olivaceus*. *Aquac. Rep.* 20, 100747. doi:10.1016/j.aqrep.2021.100747

Jeong, S. M., Khosravi, S., Mauliasari, I. R., Lee, B. J., You, S. G., and Lee, S. M. (2021a). Nutritional Evaluation of Cricket, Gryllus Bimaculatus , Meal as Fish Meal Substitute for Olive Flounder, *Paralichthys olivaceus* , Juveniles. *J. World Aquac. Soc.* 52, 859–880. doi:10.1111/jwas.12790

Lanes, C. F. C., Pedron, F. A., Bergamin, G. T., Bitencourt, A. L., Dorneles, B. E. R., Villanova, J. C. V., et al. (2021). Black Soldier Fly (*Hermetia Illucens*) Larvae and Prepupae Defatted Meals in Diets for Zebrafish (*Danio rerio*). *Animals* 11 (3), 720. doi:10.3390/ani11030720

Lee, D.-H., Chu, K.-B., Kang, H.-J., Lee, S.-H., and Quan, F.-S. (2020). Peptides in the Hemolymph of *Hermetia Illucens* Larvae Completely Inhibit the Growth of *Klebsiella Pneumonia In Vitro* and *In Vivo*. *J. Asia-Pacific Entomol.* 23, 36–43. doi:10.1016/j.aspen.2019.10.004

Li, Q. P., Gooneratne, S. R., Wang, R. L., Zhang, R., An, L. L., Chen, J. J., et al. (2016a). Effect of Different Molecular Weight of Chitosans on Performance and Lipid Metabolism in Chicken. *Anim. Feed Sci. Tech.* 211, 174–180. doi:10.1016/j.anifeedsci.2015.11.013

Li, S., Ji, H., Zhang, B., Tian, J., Zhou, J., and Yu, H. (2016b). Influence of Black Soldier Fly (Hermetia Illucens) Larvae Oil on Growth Performance, Body Composition, Tissue Fatty Acid Composition and Lipid Deposition in Juvenile Jian Carp (*Cyprinus carpio* Var. Jian). *Aquaculture* 465, 43–52. doi:10.1016/j.aquaculture.2016.08.020

Li, S., Ji, H., Zhang, B., Zhou, J., and Yu, H. (2017). Defatted Black Soldier Fly (Hermetia Illucens) Larvae Meal in Diets for Juvenile Jian Carp (*Cyprinus carpio* Var. Jian): Growth Performance, Antioxidant Enzyme Activities, Digestive Enzyme Activities, Intestine and Hepatopancreas Histological Structure. *Aquaculture* 477, 62–70. doi:10.1016/j.aquaculture.2017.04.015

Lin, G., Gao, D., Lu, J., and Sun, X. (2021). Transcriptome Profiling Reveals the Sexual Dimorphism of Gene Expression Patterns during Gonad Differentiation in the Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Mar. Biotechnol.* 23 (1), 18–30. doi:10.1007/s10126-020-09996-x

Lu, R., Chen, Y., Yu, W., Lin, M., Yang, G., Qin, C., et al. (2020). Defatted Black Soldier Fly (*Hermetia Illucens*) Larvae Meal Can Replace Soybean Meal in Juvenile Grass Carp (*Ctenopharyngodon idellus*) Diets. *Aquac. Rep.* 18, 100520. doi:10.1016/j.aqrep.2020.100520

Luther, P. K., Munro, P. M. G., and Squire, J. M. (1995). Muscle Ultrastructure in the Teleost Fish. *Micron* 26, 431–459. doi:10.1016/0968-4328(95)00015-1

Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., and Peres, H. (2017). Black Soldier Fly (*Hermetia Illucens*) Pre-pupae Meal as a Fish Meal Replacement in Diets for European Seabass (*Dicentrarchus labrax*). *Aquaculture* 476, 79–85. doi:10.1016/j.aquaculture.2017.04.021

Mengíbar, M., Mateos-Aparicio, I., Miralles, B., and Heras, A. (2013). Influence of the Physico-Chemical Characteristics of Chito-Oligosaccharides (COS) on Antioxidant Activity. *Carbohydr. Polym.* 97 (2), 776–782. doi:10.1016/j.carbpol.2013.05.035

Mohan, K., Ganesan, A. R., Muralisankar, T., Jayakumar, R., Sathishkumar, P., Uthayakumar, V., et al. (2020). Recent Insights into the Extraction, Characterization, and Bioactivities of Chitin and Chitosan from Insects. *Trends Food Sci. Tech.* 105, 17–42. doi:10.1016/j.tifs.2020.08.016

Naveed, M., Phil, L., Sohail, M., Hasnat, M., Baig, M. M. F. A., Ihsan, A. U., et al. (2019). Chitosan Oligosaccharide (COS): An Overview. *Int. J. Biol. Macromolecules* 129, 827–843. doi:10.1016/j.ijbiomac.2019.01.192

Nogales-Mérida, S., Gobbi, P., Jozefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., et al. (2019). Insect Meals in Fish Nutrition. *Rev. Aquac.* 11, 1080–1103.

Periago, M. J., Ayala, M. D., López-Albors, O., Abdel, I., Martínez, C., García-Alcázar, A., et al. (2005). Muscle Cellularity and Flesh Quality of Wild and Farmed Sea Bass. *Dicentrarchus labrax L. Aquac.* 249 (1-4), 175–188. doi:10.1016/j.aquaculture.2005.02.047

Piccolo, G., Iaconisi, V., Marono, S., Gasco, L., Loponte, R., Nizza, S., et al. (2017). Effect of *Tenebrio molitor* Larvae Meal on Growth Performance, *In Vivo* Nutrients Digestibility, Somatic and Marketable Indexes of Gilthead Sea Bream (*Sparus Aurata*). *Anim. Feed Sci. Tech.* 226, 12–20. doi:10.1016/j.anifeedsci.2017.02.007

Polakof, S., Panserat, S., Soengas, J. L., and Moon, T. W. (2012). Glucose Metabolism in Fish: a Review. *J. Comp. Physiol. B* 182, 1015–1045. doi:10.1007/s00360-012-0658-7

Raksasat, R., Lim, J. W., Kiatkittipong, W., Kiatkittipong, K., Ho, Y. C., Lam, M. K., et al. (2020). A Review of Organic Waste Enrichment for Inducing Palatability of Black Soldier Fly Larvae: Wastes to Valuable Resources. *Environ. Pollut.* 267, 115488. doi:10.1016/j.envpol.2020.115488

Rasmussen, R. S., and Ostenfeld, T. H. (2000). Influence of Growth Rate on white Muscle Dynamics in Rainbow trout and brook trout. *J. Fish Biol.* 56, 1548–1552. doi:10.1111/j.1095-8649.2000.tb02164.x

Rawski, M., Mazurkiewicz, J., Kierończyk, B., and Józefiak, D. (2020). Black Soldier Fly Full-Fat Larvae Meal as an Alternative to Fish Meal and Fish Oil in Siberian sturgeon Nutrition: The Effects on Physical Properties of the Feed, Animal Growth Performance, and Feed Acceptance and Utilization. *Animals* 10, 2119. doi:10.3390/ani10112119

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., et al. (2017). Evaluation of the Suitability of a Partially Defatted Black Soldier Fly (*Hermetia Illucens* L.) Larvae Meal as Ingredient for Rainbow trout (*Oncorhynchus mykiss* Walbaum) Diets. *J. Anim. Sci Biotechnol* 8, 57. doi:10.1186/s40104-017-0191-3

Sheikhzadeh, N., Tayefi-Nasrabadi, H., Khani Oushani, A., and Najafi Enferadi, M. H. (2012). Effects of *Haematococcus pluvialis* Supplementation on Antioxidant System and Metabolism in Rainbow trout (*Oncorhynchus mykiss*). *Fish. Physiol. Biochem.* 38 (2), 413–419. doi:10.1007/s10695-011-9519-7

Shi, X., Li, D., Zhuang, P., Nie, F., and Long, L. (2006). Comparative Blood Biochemistry of Amur sturgeon, Acipenser Schrenckii, and Chinese Surgeon, Acipenser Sinensis. *Fish. Physiol. Biochem.* 32, 63–66. doi:10.1007/s10695-006-7134-9

Smets, R., Verbinnen, B., Van De Voorde, I., Aerts, G., Claes, J., and Van Der Borght, M. (2020). Sequential Extraction and Characterisation of Lipids,

Proteins, and Chitin from Black Soldier Fly (*Hermetia Illucens*) Larvae, Prepupae, and Pupae. *Waste Biomass Valor.* 11, 6455–6466. doi:10.1007/s12649-019-00924-2

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., et al. (2017). Nutritional Composition of Black Soldier Fly (*Hermetia Illucens*) Prepupae Reared on Different Organic Waste Substrates. *J. Sci. Food Agric.* 97, 2594–2600. doi:10.1002/jsfa.8081

Stejskal, V., Tran, H. Q., Prokesova, M., Gebauer, T., Giang, P. T., Gai, F., et al. (2020). Partially Defatted *Hermetia Illucens* Larva Meal in Diet of Eurasian Perch (*Perca fluviatilis*) Juveniles. *Animals* 10 (10), 1876. doi:10.3390/ani10101876

Tacon, A. G. J., and Metian, M. (2008). Global Overview on the Use of Fish Meal and Fish Oil in Industrially Compounded Aquafeeds: Trends and Future Prospects. *Aquaculture* 285 (1-4), 146–158. doi:10.1016/j.aquaculture.2008.08.015

Takakuwa, F., Tanabe, R., Nomura, S., Inui, T., Yamada, S., Biswas, A., et al. (2021). Availability of Black Soldier Fly Meal as an Alternative Protein Source to Fish Meal in Red Sea Bream (*Pagrus Major*, Temminck & Schlegel) Fingerling Diets. *Aquac. Res.* 00, 1–14. doi:10.1111/are.15550

Tippayadara, N., Dawood, M. A. O., Krutmuang, P., Hoseinifar, S. H., Doan, H. V., and Paolucci, M. (2021). Replacement of Fish Meal by Black Soldier Fly (*Hermetia Illucens*) Larvae Meal: Effects on Growth, Haematology, and Skin Mucus Immunity of Nile tilapia, *Oreochromis niloticus*. *Animals* 11, 193. doi:10.3390/ani11010193

Tschirner, M., and Simon, A. (2015). Influence of Different Growing Substrates and Processing on the Nutrient Composition of Black Soldier Fly Larvae Destined for Animal Feed. *J. Insects as Food Feed* 1 (4), 249–259. doi:10.3920/jiff2014.0008

Urán, P. A., Schrama, J. W., Rombout, J. H. W. M., Obach, A., Jensen, L., Koppe, W., et al. (2008). Soybean Meal-Induced Enteritis in Atlantic salmon (*Salmo salar* L.) at Different Temperatures. *Aquac. Nutr.* 14, 324–330. doi:10.1111/j.1365-2095.2007.00534.x

Valente, L. M. P., Moutou, K. A., Conceição, L. E. C., Engrola, S., Fernandes, J. M. O., and Johnston, I. A. (2013). What Determines Growth Potential and Juvenile Quality of Farmed Fish Species? *Rev. Aquacult.* 5, 168–193. doi:10.1111/raq.12020

Wang, G., Peng, K., Hu, J., Mo, W., Wei, Z., and Huang, Y. (2021). Evaluation of Defatted Hermetia Illucens Larvae Meal for Litopenaeus Vannamei : Effects on Growth Performance, Nutrition Retention, Antioxidant and Immune Response, Digestive Enzyme Activity and Hepatic Morphology. *Aquacult Nutr.* 27 (4), 986–997. doi:10.1111/anu.13240

Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., et al. (2019). Evaluation of Defatted Black Soldier Fly (*Hermetia Illucens* L.) Larvae Meal as an Alternative Protein Ingredient for Juvenile Japanese Seabass (*Lateolabrax Japonicus*) Diets. *Aquaculture* 507, 144–154. doi:10.1016/j.aquaculture.2019.04.023

Wang, Y.-S., and Shelomi, M. (2017). Review of Black Soldier Fly (*Hermetia Illucens*) as Animal Feed and Human Food. *Foods* 6, 91. doi:10.3390/foods6100091

Xia, W., Liu, P., Zhang, J., and Chen, J. (2011). Biological Activities of Chitosan and Chitooligosaccharides. *Food Hydrocolloids* 25 (2), 170–179. doi:10.1016/j.foodhyd.2010.03.003

Xu, F.-M., Hou, S.-W., Wang, G.-X., Gong, J.-Y., Zhou, L., Huang, Y.-H., et al. (2021). Effects of Zymolytic Black Soldier Fly (*Hermetia Illucens*) Pulp as Dietary Supplementation in Largemouth Bass (*Micropterus salmoides*). *Aquac. Rep.* 21, 100823. doi:10.1016/j.aqrep.2021.100823

Xu, M., Chen, X., Huang, Z., Chen, D., Li, M., He, J., et al. (2022). Effects of Dietary Grape Seed Proanthocyanidin Extract Supplementation on Meat Quality, Muscle Fiber Characteristics and Antioxidant Capacity of Finishing Pigs. *Food Chem.* 367, 130781. doi:10.1016/j.foodchem.2021.130781

Xu, X. X., Ji, H., Yu, H. B., and Zhou, J. S. (2020). Influence of Dietary Black Soldier Fly (Hermetia Illucens Linnaeus) Pulp on Growth Performance, Antioxidant Capacity and Intestinal Health of Juvenile Mirror Carp (*Cyprinus carpio* Var. Specularis). *Aquac. Nutr.* 00, 1–12. doi:10.1111/anu.13005

Yildirim-Aksoy, M., Eljack, R., and Beck, B. H. (2020). Nutritional Value of Frass from Black Soldier Fly Larvae, *Hermetia Illucens*, in a Channel Catfish, *Ictalurus punctatus*, Diet. *Aquac. Nutr.* 26 (3), 812–819. doi:10.1111/anu.13040

Yu, L., Wen, H., Jiang, M., Wu, F., Tian, J., Lu, X., et al. (2020). Effects of Ferulic Acid on Intestinal Enzyme Activities, Morphology, Microbiome Composition of Genetically Improved Farmed tilapia (*Oreochromis niloticus*) Fed Oxidized Fish Oil. *Aquaculture* 528, 735543. doi:10.1016/j.aquaculture.2020.735543

Zar, J. H. (1996). *Biostatistical Analysis*. 3rd Edn. Prentice-Hall International. Hoboken, New Jersey.

Zarantoniello, M., Bruni, L., Randazzo, B., Vargas, A., Gioacchini, G., Truzzi, C., et al. (2018). Partial Dietary Inclusion of *Hermetia Illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications. *Zebrafish* 15 (5), 519–532. doi:10.1089/zeb.2018.1596

Zhang, H., Ding, Q., Wang, A., Liu, Y., Teame, T., Ran, C., et al. (2020a). Effects of Dietary Sodium Acetate on Food Intake, Weight Gain, Intestinal Digestive Enzyme Activities, Energy Metabolism and Gut Microbiota in Cultured Fish: Zebrafish as a Model. *Aquaculture* 523, 735188. doi:10.1016/j.aquaculture.2020.735188

Zhang, L., Huang, H., Wang, P., Xing, T., and Xu, X. (2020b). Water-spraying Forced Ventilation during Holding Improves the Water Holding Capacity, Impedance, and Microstructure of Breast Meat from Summer-Transported Broiler Chickens. *Poult. Sci.* 99 (3), 1744–1749. doi:10.1016/j.psj.2019.10.077

Zhao, H., Xia, J., Zhang, X., He, X., Li, L., Tang, R., et al. (2018). Diet Affects Muscle Quality and Growth Traits of Grass Carp (*Ctenopharyngodon idellus*): A Comparison between Grass and Artificial Feed. *Front. Physiol.* 9, 283. doi:10.3389/fphys.2018.00283

Zou, P., Yang, X., Wang, J., Li, Y., Yu, H., Zhang, Y., et al. (2016). Advances in Characterisation and Biological Activities of Chitosan and Chitosan Oligosaccharides. *Food Chem.* 190, 1174–1181. doi:10.1016/j.foodchem.2015.06.076

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On the Utilization of Dietary Glycerol in Carnivorous Fish—Part II: Insights Into Lipid Metabolism of Rainbow Trout (*Oncorhynchus mykiss*) and European Seabass (*Dicentrarchus labrax*)

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Glycerol is the by-product of biodiesel production and its utilisation in fish feed has recently gained relevance. As an important metabolic intermediate and structural component of triacylglycerol (TAG), it is still unclear whether its supplementation affects lipid utilisation and/or deposition in different tissues. Accordingly, a set of studies was aimed to evaluate how increasing levels of dietary glycerol (0, 2.5 and 5%) affect lipid synthesis in the liver, muscle and adipose tissue. After a growth trial with rainbow trout (8 weeks) and European seabass (6 weeks) fish were sampled at 6 and 24 h to assess mRNA levels of lipid metabolism-related enzymes. The remaining fish were subjected to a metabolic trial consisting of a 6-day residence in deuterated water (^2H_2O) for metabolic flux calculations. This study stands as the second part of a broader experiment that also aimed at evaluating the carbohydrate metabolism (Viegas et al., 2022). Dietary supplementation at 5% glycerol significantly increased plasma TAG levels in both species and liver TAG levels in seabass, with no repercussions on the perivisceral fat index. Despite responding as expected in a postprandial setting, only *fas* and Δ*6-fad* in trout and Δ*6-fad* in seabass responded significantly by increasing with the dietary supplementation of glycerol. In trout, the observed differences in the regulation of these enzymes were not reflected in the *de novo* lipogenic fluxes. The fractional synthetic rates (FSR) were overall low in all tissues with an average of 0.04, 0.02 and 0.01% d^{-1}, for liver, muscle and perivisceral fat, respectively. In seabass on the other hand, and despite increased mRNA levels in Δ*6-fad*, the overall lipid profile in the liver muscle and perivisceral fat was higher in MUFA at the expense of lower

PUFA. Moreover, glycerol supplementation altered the lipogenic capacity of seabass with hepatic fractional synthetic rates for TAG-bound FA increasing with increasing glycerol levels (0.32 ± 0.18, 0.57 ± 0.18, and 0.82 ± 0.24 for 0%, 2.5% and 5% glycerol supplementation, respectively). The findings of the present study suggest that supplementation up to 2.5% of glycerol did not severely impact the lipid metabolism nor increased lipogenic potential in liver, muscle and perivisceral fat accumulation.

Keywords: ^{2}H NMR, aquaculture, circular economy, deuterated water, glycerol, muscle, perivisceral fat, triacylglycerol (TAG)

INTRODUCTION

Glycerol (1,2,3-propanetriol or glycerine; ChEBI: 17754) is a water-soluble, almost colorless, odorless, viscous, hygroscopic 3-carbon sugar alcohol. Glycerol is an important structural component of triglycerides and phospholipids, as well as an important metabolic intermediate at the crossroad of several pathways. Its sites of activation to glycerol-3-phosphate, transportation between cells, as well as regulatory roles of related enzymes or genes on lipid metabolism have been extensively studied and reviewed (Xue et al., 2017). However, several aspects of the complex interplay between glycerol and lipid metabolism are yet to be unraveled. Early on, studies in mice hypothesised that dietary glycerol supplementation could cause problems associated with hepatic lipid mobilisation by increasing the liver ability to sequester and re-esterify fatty acids (FA) from tissue (Lin et al., 1976). Glycerol could also stimulate hepatic lipogenesis and speed up the induction of fatty acid desaturases resulting in changes in liver FA composition and in altered membrane permeability (Narayan and Mcmullen, 1979). The metabolisation of glycerol in liver has been clearly associated with the pentose phosphate pathway, from which NADPH, the reducing agent for lipogenesis, is produced (Jin et al., 2014). Besides, the versatility of this 3-carbon substrate puts it at the centre of lipid metabolism also in extrahepatic tissues (Coppack et al., 1999; Nye et al., 2008). This is particularly evident in scenarios of energy storage in adipose tissue (Rotondo et al., 2017; Ho-Palma et al., 2019) and energy mobilisation (e.g., exercise) in muscle tissue (Montell et al., 2002; van Hall et al., 2002).

With the rampant increase in biodiesel production in the early 2000s, where 1 kg of crude glycerol can be generated for every 10 kg of biodiesel (Ayoub and Abdullah, 2012), soon the potential of glycerol as ingredient for livestock feed was identified and evaluated (Food and Agriculture Organization [FAO], 2012). In beef cattle, diets at levels up to 15% glycerol did not impair performance or carcass characteristics (Del Bianco Benedeti et al., 2016). In feedlot young bulls, the fat content in the *longissimus dorsi* muscle, particularly oleic acid (18:1n-9) increased, while the saturated fatty acids (SFA) content decreased linearly as function of glycerol addition (Carvalho et al., 2014). In dairy cows glycerol supplementation did not affect overall intake but impacted feeding patterns including particle sorting (Carvalho E.R. et al., 2012). Glycerol utilisation in swine was even more appealing due to the metabolisable-to-digestible energy ratio similar to corn or soybean oil (Lammers et al., 2008b). Supplementations up to ∼10% did not impair performance, carcass characteristics, and meat quality (Lammers et al., 2008a; Schieck et al., 2010; Ordoñez-Gomez et al., 2017) as well as plasmatic parameters such as glucose, triglycerides, cholesterol, and urea nitrogen (Carvalho P.L.D.O. et al., 2012). Similar to what has been observed in cattle, adding 5% glycerol also increased significantly the proportion of oleic acid in pig backfat but at the expense of linoleic (18:2n-6) and linolenic acids (18:3n-3; 18:3n-6), consequently decreasing the unsaturation index of fat (Mourot et al., 1994). In broilers diets containing 10% glycerol influenced performance thus 5% is considered the appropriate supplementation level (Cerrate et al., 2006; Freitas et al., 2017). However, while these studies reported no alterations in abdominal fat content, others reported increased abdominal fat pad weight when compared to control diets (Lessard et al., 1993).

The use of glycerol in fish feed was recently reviewed (Mauerwerk et al., 2021a) but studies and insight into lipid metabolism are scarce and its putative lipogenic potential is yet to be evaluated thoroughly. Early studies with rainbow trout (*Oncorhynchus mykiss*) reported that glycerol was not an effective precursor for lipogenesis (Menton et al., 1986) but since then evidence has been presented that this may not be the case in other species and/or dosages. In juvenile Mozambique tilapia (*Oreochromis mossambicus*), the incorporation of dietary glycerol into liver and muscle lipids was demonstrated using pellets labeled with ^{14}C-glycerol. This incorporation into muscle was statistically lower in glycerol-supplemented fish while no difference in incorporation was observed into hepatic lipids (Costa et al., 2017). In another study with the same species, glycerol supplementation > 10% increased triacylglycerol (TAG) levels in liver and white muscle (Costa et al., 2015). On the contrary, in channel catfish (*Ictalurus punctatus*), glycerol supplementation > 15% decreased liver lipids (Li et al., 2010). Fish plasma TAG seems to be irresponsive to glycerol supplementation (Neu et al., 2013; Costa et al., 2015; Mauerwerk et al., 2021b) with the exception of curimbatá (*Prochilodus lineatus*) (Balen et al., 2017) which presented significantly increased TAG levels

Abbreviations: ^{2}H, deuterium; $^{2}H_2O$, deuterated water; BW, body water; *cpt1*, carnitine palmitoyltransferase I (EC: 2.3.1.21); DNL, *de novo* lipogenesis; *elovl*, elongation of very long-chain fatty acid protein (EC 2.3.1.199); FA, fatty acids; *fas*, fatty acid synthase (EC 2.3.1.85); FSR, fractional synthetic rate; *dgat*, diglyceride acyltransferase (EC 2.3.1.20); *hoad*, 3-hydroxyacyl-CoA dehydrogenase EC 1.1.1.35; HSI, hepatosomatic index; MUFA, monounsaturated fatty acid; n-3, omega-3; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; RAS, recirculated aquatic system; SFA, saturated fatty acid; TAG, triacylglycerol; TW, tank water; UFA, unsaturated fatty acid; Δ*6-fad,* acyl-CoA 6-desaturase (EC:1.14.19.3).

at 12% supplementation. Although these studies provide valuable insight, and in the absence of enzymatic studies, plasma TAG or liver lipids are merely indicative of the actual status of lipid metabolism. In order to evaluate how dietary stimuli affects lipid utilisation and deposition in several tissues, namely liver, muscle and adipose tissue, deuterated water (^2H_2O) has been employed successfully in fish (Viegas et al., 2016, 2019). Deuterium (^2H) is differentially incorporated into different positions of the molecules and, if using ^2H NMR, it is possible to discriminate such positional ^2H-enrichment, an advantage over other types of instrumentation able to detect overall ^2H-enrichments. This means that in this case, within a TAG molecule, ^2H NMR is able to separately quantify the enrichment in the glycerol backbone and on its three esterified FA. Differential positional labeling should stem, amongst others, from pathways where unlabeled glycerol (in this case, dietary glycerol) is involved. Accordingly, the present study aimed to describe how dietary glycerol affects lipid synthesis and accumulation in different tissues. ^2H_2O-based metabolic flux calculations were paired with the mRNA levels of important lipid metabolism-related enzymes using as model two representative carnivorous species of European aquaculture: freshwater rainbow trout (*Oncorhynchus mykiss*) and marine European seabass (*Dicentrarchus labrax*).

MATERIALS AND METHODS

This work is the second of two parts focussing on the utilisation of dietary glycerol in carnivorous fish. The first part, providing insights into hepatic carbohydrate metabolism (Viegas et al., 2022), and the present manuscript are based on the same set of experiments. Viegas et al. (2022) will be often referred to as "Part I" whenever necessary and describes in detail all the experimental procedures that led to the production of the data presented hereafter. The "*Ethics statement*," "*Diet Formulation*," and "*Growth trials – fish handling and sampling*" sections of Part I cover in its entirety the information needed for replicating the experiment. Further details about diet formulations can be found in Part I as Supplementary Information (Viegas et al., 2022 – Supplementary Table 1). The formulation was the same for both species (D0, D2.5 and D5), but for the sake of clarity and context they will hereafter be referred to T0, T2.5, T5 or S0, S2.5, S5, if when addressing the rainbow trout or European seabass experiment, respectively. All remaining details specific to replicating the present experiment are provided in the following sections. Briefly, the experiment consisted of a 6- to 8-week feeding trial (Viegas et al., 2022 – Table 1), after which samples taken 6 h and 24 h after last meal were used to assess the molecular regulation of hepatic enzymes (two to three fish per tank for a total n = 8 for trout and n = 7 for seabass, per diet, per sampling time). The remaining fish were transferred to ^2H_2O (4%) for 6 days for a metabolic trial (total n = 24 per diet for both species). Here, ^2H gets incorporated into metabolites and molecules following well-defined metabolic steps with consequent analysis of the positional ^2H-enrichment by ^2H NMR. Further details on the growth trials can be found in Palma et al. (2019) and Magnoni et al. (2021) for rainbow trout and in Palma et al. (2019) for European seabass.

Molecular Analysis of Intermediary Metabolism

Sampling, sample treatment and consequent relative gene mRNA level determination by real-time quantitative polymerase chain reaction (qPCR) are extensively described in Part I, including the PCR protocol and amplification cycles. Plasma and liver TAG were quantified using a commercial kit (TRG050; Sobioda). The primer sequences used in real-time quantitative qPCR assays for lipid metabolism gene and other information for the analysis are presented in **Supplementary Table 1** for trout and **Supplementary Table 2** for seabass. mRNA level for key enzymes of lipogenesis (*fas*, fatty acid synthase EC 2.3.1.85); *dgat* (diglyceride acyltransferase EC 2.3.1.20), lipolysis (*hoad*, 3-hydroxyacyl-CoA dehydrogenase EC 1.1.1.35; *cpt1a and cpt1b* carnitine palmitoyltransferase I isoforms a and b EC: 2.3.1.21) and enzymes involved in long chain polyunsaturated fatty acids (PUFA) biosynthesis (Δ*6-fad,* acyl-CoA 6-desaturase EC:1.14.19.3; *elovl2* and *elovl5,* elongation of very long-chain fatty acid protein 2 and 5 EC 2.3.1.199). Elongation factor-1 alpha (*ef1α*) was used as reference gene.

Metabolic Trial – Labeling of Triacylglycerol With ^2H_2O

As described in Part I, the fish remaining from the growth trials were transferred to a closed recirculated system consisting of 3 × 150 L tanks for the metabolic trials. Tank water (TW) contained ∼4% ^2H-enriched water, achieved by the addition of 99%-enriched ^2H_2O (CortecNet, France) to water from the system where the respective growth trial took place as previously described (Viegas et al., 2011). Fish remained in ^2H-enriched water for 6 days, being fed once a day (*ad libitum*) and euthanized 24 h after last meal on day 5. Fish were anesthetised (MS-222; 3-aminobenzoic acid ethyl ester, 0.1 g L^{-1}), euthanized and sampled for blood, liver, muscle and perivisceral fat as described in Part I. Plasma TAG was quantified with commercial kit (I.S.E. S.r.l.; A-R0100000901). In order to ensure samples that yielded ^2H NMR spectra with high signal-to-noise ratios, tissue from 2 to 4 fish were pooled (for a final n = 6 per diet).

TAG were extracted from N_2-pulverised (liver and muscle) or recently thawed (perivisceral fat) tissue according to Matyash et al. (2008) using methyl tert-butyl ether, methanol and water mixture (MTBE:MeOH:H_2O, 10:3:2.5; 20 mL g^{-1} of tissue), after 1 h in agitation at room temperature. The organic phase was then transferred to glass amber vials, dried under N_2 stream and stored at −20°C. TAG were purified by solid phase extraction with pre-packed 2 g cartridges (Discovery® DSC-NH2 52.641-U, Supelco) and TAG identity was confirmed by spotting onto thin by thin-layer chromatography (TLC) plates alongside TAG standards. The plate was developed with petroleum ether/diethyl ether/acetic acid (7.0/1.0/0.1; v/v/v). After drying, lipid spots were visualised under iodine vapour and correspondent fractions dried under N_2 stream and stored at −20°C until NMR analysis (Viegas et al., 2016).

^{2}H NMR Acquisition and Metabolic Flux Calculations

Proton-decoupled ^{2}H NMR spectra were obtained with a Bruker Avance III HD 500 spectrometer using a ^{2}H-selective 5 mm probe incorporating a ^{19}F-lock channel. Body water (BW) and tank water (TW) ^{2}H-enrichments were quantified as previously described in Part I according to Jones et al. (2001). Triacylglycerol samples were reconstituted in 400 μL of chloroform (Sigma-Aldrich 528730; HPLC grade, ≥99.9%) and transferred to a 5-mm NMR-grade tube with a pyrazine standard and 50 μL of hexafluorobenzene. TAG spectra were obtained at 25°C: ^{1}H NMR spectra were acquired with a 90° pulse, 3 s of acquisition time and 5 s of pulse delay, for 16 scans; ^{2}H NMR spectra were acquired with a 90° pulse, 0.37 s of acquisition time and 0.1 s of pulse delay, for 10,000 to 20,000 scans. As control for the TAG extraction, a FA/glycerol ratio (should be ∼3) was calculated from the area of all α protons times 2, divided by TAG-bound glycerol *sn*1,3 protons (Duarte et al., 2014; Viegas et al., 2016). The FA profile (in percentage) for saturated (SFA) and unsaturated fatty acids (UFA), both poly- (PUFA) and monounsaturated fatty acids (MUFA) were estimated from ^{1}H NMR spectra according to Viegas et al. (2016). ^{2}H-enrichments were quantified from the ^{1}H and ^{2}H NMR spectra by measuring the ^{1}H and ^{2}H intensities of selected signals relative to the ^{1}H and ^{2}H intensities of a pyrazine standard, as described previously (Duarte et al., 2014; Viegas et al., 2016). Briefly, TAG-bound FA synthetised *de novo* were estimated by determining the ^{2}H-enrichment in the FA terminal methyl site and newly synthesized and/or turned-over TAG-bound glycerol was estimated by determining the ^{2}H-enrichment in the *sn*-1,3 glycerol site. Estimates on desaturation rates were made by determining the ^{2}H-enrichment in the MUFAs' allylic protons. Estimating elongation rates are based on the principle that during the residence in $^{2}H_2O$, the terminal methyl site is enriched during the first round of FA synthesis, while the α protons incorporate ^{2}H in the last round of elongation. Therefore, if elongation occurs on pre-existing (unlabeled) FA, the α- and methyl-protons will be differentially labeled and will inform of the fractional contribution of elongation to lipid synthesis. Excess TAG positional ^{2}H-enrichments were calculated after systematic subtraction of the values with 0.0156%, taken as natural abundance ^{2}H-enrichment. Fractional synthetic rates (FSR; in% day^{-1}) were estimated by dividing these above described positional TAG enrichments by that of body water. Spectra were processed by applying exponential multiplication to the free-induction decay (^{1}H: 0.1 Hz; ^{2}H: 1.0 Hz), and analyzed using the curve-fitting routine supplied with ACD/NMR Processor Academic Edition from ACD/Labs 12.0 software (Advanced Chemistry Development, Inc.).

Statistics

Data are presented as mean ± SD. Normality (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test) were verified prior to the analysis. For the plasma and hepatic parameters and mRNA levels of lipid-related enzymes, the effects of sampling time and diet were analyzed by two-way ANOVA, with time (6 h and 24 h) and glycerol level (D0, D2.5 and D5) as variables. In the case of significant interactions, one-way ANOVA was performed followed by Tukey's HSD for multiple comparison. For the parameters evaluated following the metabolic trial, such as FSR for *de novo* lipogenesis, glycerol turnover, elongation and desaturation rates, were analyzed by one-way ANOVA. In the case of detecting significant differences through the one-way ANOVA, a Tukey multiple range test was performed to discriminate differences between diets. Statistical analysis was performed in GraphPad Prism software (GraphPad Software, La Jolla, CA, United States) and differences were considered statistically significant at $p < 0.05$.

RESULTS

Tank water (TW) ^{2}H-enrichment was maintained between 3.5 and 4.0%. Body water ^{2}H-enrichment ranged from 2.7 to 4.1% (average of 3.6 ± 0.3%) with no statistical differences between dietary treatments for both species. After the growth trial no differences in the final body weight for both diet (D0, D2.5, D5) and sampling time (6 h vs. 24 h after last meal) were observed for trout or seabass (Viegas et al., 2022).

Rainbow Trout

Plasma and liver TAG levels were not significantly affected by diet and sampling time. However, it is worth noting that for TAG levels in plasma p-value for diet was 0.065, which may indicate that increased glycerol dietary levels may lead to hypertriglyceridaemia. This was corroborated by the plasma TAG levels from the fish at the end of the metabolic trial, which were sampled only 24 h after last meal but that were significantly increased in T5. As explained in Part I for liver glycogen analysis the liver tissue assigned for the TAG assay was poorly maintained and this parameter was not measured (Viegas et al., 2022). The perivisceral fat index did not reveal any statistical differences. The results from both trials in trout are summarised in **Table 1**.

In order to assess the impact of glycerol-supplemented diets on hepatic metabolism, mRNA levels of key enzymes involved in the regulation of lipid metabolism were assessed after the growth trial at two different sampling times after last meal (6 and 24 h) by real-time qPCR (**Figure 1**). At 6 h after last meal, as expected in a postprandial setting, and as already observed in Part I with the enzymes related to the carbohydrate metabolism (Viegas et al., 2022), most enzymes related with lipid metabolism significantly differed between sampling times. In addition, as expected in a postprandial setting, the mRNA levels of lipogenic enzymes were up-regulated and the lipolytic enzymes were down-regulated at 6 h after last meal. But contrary to that observed in the enzymes related to carbohydrate metabolism where none of them responded to dietary treatment, *fas* and *Δ6-fad* (involved in the *de novo* synthesis of FA and long chain PUFA biosynthesis, respectively), involved in the *de novo* synthesis of FA and long chain PUFA biosynthesis, respectively, were significantly increased with up-regulated levels of dietary glycerol. The differences observed in the regulation of enzymes involved in lipid metabolism were not reflected in the overall lipid profile of TAG-bound FA in liver (**Table 2**). However, in muscle and perivisceral TAG there seemed to be an increase in MUFA at

TABLE 1 | Plasma and liver parameters for rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks sampled at the end of the growth trial at 6 h and 24 h after last meal and the metabolic trial (6-day residence in ^{2}H-enriched water) 24 h after last meal.

Growth trial									
	6 h			24 h			2-way ANOVA p-value		
Rainbow trout	**T0**	**T2.5**	**T5**	**T0**	**T2.5**	**T5**	**time**	**glycerol**	**interaction**
Plasma TAG (mM)	3.67 ± 0.97	3.89 ± 0.20	5.75 ± 1.90	3.36 ± 1.55	3.54 ± 1.95	3.92 ± 1.49	0.091	0.065	0.349
Hepatic TAG (mg/g)	20.66 ± 10.60	18.68 ± 11.75	18.63 ± 12.77	16.56 ± 3.27	19.25 ± 15.93	15.05 ± 11.75	0.432	0.824	0.783
Metabolic trial – $^{2}H_2O$				**T0**	**T2.5**	**T5**	**1-way ANOVA p-value**		
Plasma TAG (mM)				2.19 ± 0.65a	2.19 ± 0.51a	2.78 ± 0.52b		**0.022**	
PVFI*				2.52 ± 0.80	2.50 ± 0.78	2.42 ± 0.79		0.892	

**Perivisceral Fat Index as PVFI = (perivisceral fat weight/body weight) *100.*
Values are mean ± SD. Significant differences in the growth trial (n = 8) were evaluated by two-way ANOVA (p < 0.05, values in bold). Significant differences between dietary treatments in the metabolic trial (n = 12) are indicated by different letters (one-way ANOVA, p < 0.05; followed by Tukey's test).

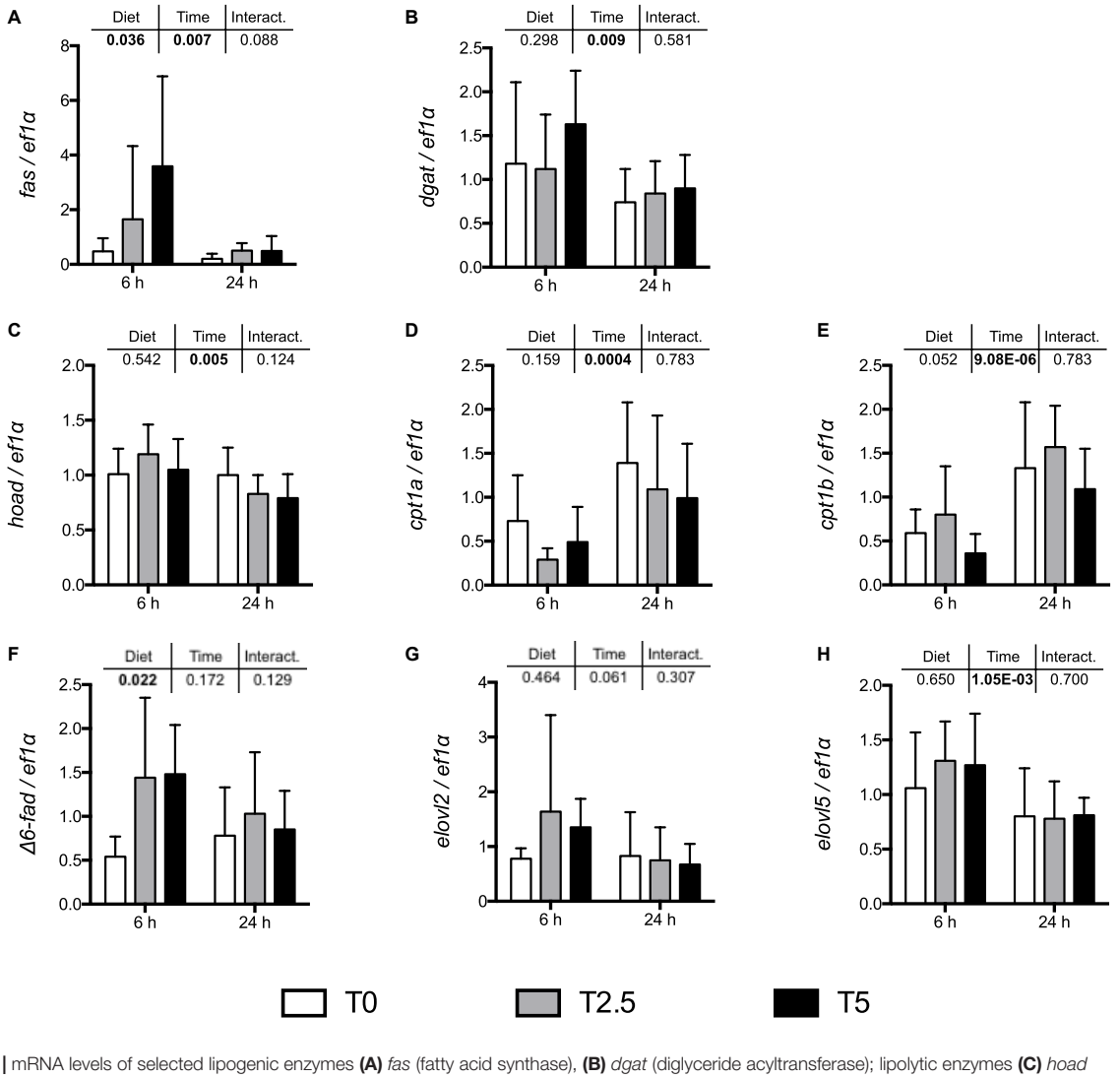

FIGURE 1 | mRNA levels of selected lipogenic enzymes **(A)** *fas* (fatty acid synthase), **(B)** *dgat* (diglyceride acyltransferase); lipolytic enzymes **(C)** *hoad* (3-hydroxyacyl-CoA dehydrogenase), **(D)** *cpt1a* and **(E)** *cpt1b* (carnitine palmitoyltransferase I a and b, respectively); and enzymes involved in long chain PUFA biosynthesis: **(F)** Δ*6-fad* (acyl-CoA 6-desaturase), **(G)** *elovl2* and **(H)** *elovl5* (elongation of very long-chain fatty acid protein 2 and 5, respectively), in the liver of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks. Values are mean ± SD (n = 8); nd: not detected. Significant differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

TABLE 2 | Percentage of fatty acid classes as determined from ^{1}H NMR spectra of TAG from liver, muscle and perivisceral fat of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%).

	Liver			Muscle			Perivisceral fat		
Rainbow trout	**T0**	**T2.5**	**T5**	**T0**	**T2.5**	**T5**	**T0**	**T2.5**	**T5**
n-3 FA	7.3 ± 2.2	7.4 ± 1.0	7.2 ± 2.4	24.5 ± 2.0	26.3 ± 3.9	23.4 ± 1.0	23.4 ± 1.4	23.0 ± 0.9	23.8 ± 1.6
non n-3 FA	92.7 ± 2.2	92.6 ± 1.0	92.8 ± 2.4	75.5 ± 2.0	73.7 ± 3.9	76.6 ± 1.0	76.6 ± 1.4	77.0 ± 0.9	76.2 ± 1.6
SFA	21.4 ± 6.1	24.3 ± 2.5	21.1 ± 3.1	22.0 ± 2.0	20.9 ± 3.9	22.4 ± 2.6	22.0 ± 1.5	23.1 ± 1.8	21.0 ± 1.9
UFA	78.7 ± 6.1	75.7 ± 2.5	78.9 ± 3.1	78.0 ± 2.0	79.2 ± 3.9	77.6 ± 2.6	78.0 ± 1.5	76.9 ± 1.8	79.0 ± 1.9
PUFA	35.5 ± 4.7	33.3 ± 3.2	34.1 ± 5.9	43.2 ± 3.6 ab	46.2 ± 7.2 b	38.3 ± 1.3 a	41.2 ± 0.7	41.8 ± 1.2	40.2 ± 2.8
MUFA	43.2 ± 3.1	42.5 ± 2.3	44.8 ± 3.0	34.8 ± 1.7 a	32.9 ± 3.6 a	39.3 ± 2.2 b	36.9 ± 2.1 ab	35.1 ± 1.0 a	38.8 ± 2.7 b

Values are mean ± SD (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test). MUFA, monounsaturated fatty acid; n-3 FA, omega-3 fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid UFA, unsaturated fatty acid.

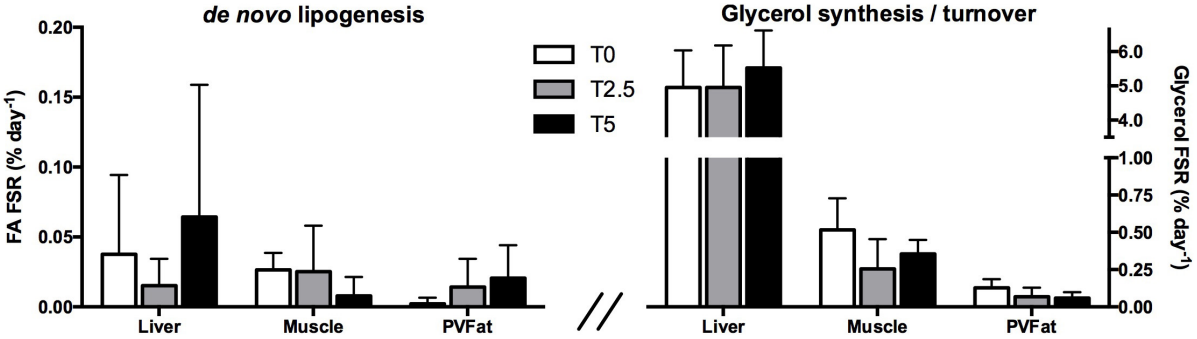

FIGURE 2 | TAG-bound fatty acid fractional synthetic rate (FA FSR; *de novo lipogenesis*) expressed as percent of newly synthesized fatty acids per day (left) and TAG-bound glycerol fractional synthetic rate (Glycerol FSR; glycerol synthesis/turnover) expressed as percent of newly synthesized/turned over glycerol per day in hepatic, muscular and perivisceral TAG of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) after a 6-day residence in a tank with ∼4% ^{2}H-enriched water. Mean values ± SD are presented ($n = 6$). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test).

the highest glycerol inclusion (T5) when compared with T2.5 but not when compared with T0.

A deeper insight into the impact of glycerol-supplemented diets on lipid metabolism was gained through the analysis of the positional ^{2}H-labeling of TAG with $^{2}H_2O$ (**Figure 2**). Glycerol supplementation did not alter the lipogenic capacity of rainbow trout as evaluated by the ^{2}H-enrichment of TAG-bound FA (**Figure 2**). These were overall low in all tissues with average fractional synthetic rates at 0.04, 0.02 and 0.01% d^{-1}, for liver, muscle and perivisceral fat, respectively. It is worth noting that the variability was very high amongst fish from the same dietary treatment. TAG-bound glycerol revealed much higher ^{2}H-enrichment relative to TAG-bound FA without significant differences between levels of glycerol supplementation for all tissues. The hepatic glycerol pool was completely turned over with fractional synthetic rates similar to TW ^{2}H-enrichment. Glycerol fractional synthetic rates for muscle and perivisceral fat (0.38 and 0.09% d^{-1}, respectively) were lower compared to the ones observed in the liver.

European Seabass

After the growth trial significant differences were found in plasma TAG levels between diets (S0, S2.5, S5) and sampling time (6 h, 24 h after last meal). Significant differences were also found in hepatic TAG but only between sampling time. In the metabolic trial, no alterations were found in plasma TAG levels or in the perivisceral fat index. The results from both trials in seabass are summarised in **Table 3**.

Similar to that observed in trout, most mRNA levels of key enzymes involved in the regulation of lipid metabolism did not respond significantly to the different levels of dietary glycerol with the exception was Δ*6-fad* (**Figure 3**). However, the differences observed in the regulation of this enzyme were not reflected in the overall lipid profile of TAG-bound FA in liver muscle and perivisceral fat TAG. In fact, an increase in MUFA was observed in every tissue while PUFA decrease regardless of increased Δ*6-fad* expression observed in the liver (**Table 4**).

Contrary to observed in rainbow trout, glycerol supplementation altered the lipogenic capacity of seabass with hepatic fractional synthetic rates for TAG-bound FA increasing with increasing glycerol levels (**Figure 4**) (0.32 ± 0.18, 0.57 ± 0.18, and 0.82 ± 0.24 for 0%, 2.5% and 5% glycerol supplementation, respectively). No ^{2}H-enrichment was detected in muscle TAG while the enrichments found in perivisceral fat were extremely low (0.01% d^{-1}) and not influenced by dietary treatment. TAG-bound glycerol revealed much higher ^{2}H-enrichment relative to TAG-bound FA. Also as observed in rainbow trout, the hepatic glycerol pool was completely

TABLE 3 | Plasma and liver parameters for European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 6 weeks sampled at the end of the growth trial at 6 h and 24 h after last meal and the metabolic trial (6-day residence in ^{2}H-enriched water) 24 h after last meal.

Growth trial									
	6 h			**24 h**			**2-way ANOVA p-value**		
European Seabass	**S0**	**S2.5**	**S5**	**S0**	**S2.5**	**S5**	**time**	**glycerol**	**interaction**
Plasma TAG (mM)	11.72 ± 3.47	18.22 ± 4.34	27.06 ± 7.13	9.28 ± 3.63	10.19 ± 2.63	19.37 ± 4.17	**2.59E-4**	**6.63E-8**	0.243
Hepatic TAG (mg/g)	120.51 ± 22.0	110.71 ± 26.6	133.44 ± 10.6	122.04 ± 20.0	129.53 ± 6.9	137.10 ± 11.3	0.127	**0.033**	0.334
Metabolic trial – $^{2}H_2O$				**S0**	**S2.5**	**S5**	**1-way ANOVA p-value**		
Plasma TAG (mM)				8.45 ± 5.45	10.31 ± 6.25	11.43 ± 7.42	0.524		
PVFI*				1.96 ± 1,29	2.05 ± 1.26	2.23 ± 1.54	0.773		

**Perivisceral Fat Index as PVFI = (perivisceral fat weight/body weight) *100.*
Values are mean ± SD. Significant differences in the growth trial (n = 7) were evaluated by two-way ANOVA ($p < 0.05$, values in bold). Significant differences between dietary treatments in the metabolic trial (n = 12) are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test).

turned over with fractional synthetic rates similar to TW ^{2}H-enrichment, without significant differences between levels of glycerol supplementation for all tissues.

DISCUSSION

Our understanding of fish nutrition and metabolism has advanced substantially and the use of isotopes has greatly assisted to follow the metabolic fate of various metabolites, glycerol included. Delivery strategies may be methodologically challenging in aquatic organisms but are valid both *in vitro*, by using labeling incubation medium for fish hepatocytes (Lech, 1970) or muscle slices (Kam and Milligan, 2006), and *in vivo*. Amongst the latter, the feeding of labeling pellets (Costa et al., 2017) raises the issue of leaching of costly compounds into the water. This can be circumvented by forced feeding or gavage which then require handling, anaesthesia and more invasive protocols. The same applies to intraperitoneal (IP) injection (Savina and Wojtczak, 1977; Rito et al., 2019). This methodology assures the delivery of a controlled amount of label, in spite of doing so in a somehow unrealistic transfer (in terms of rate and dosage) of the labeling metabolite into circulation. On the other hand, dorsal aorta cannulation in combination with a continuous infusion of a radiolabeled metabolite requires a surgical procedure, which is highly dependent on fish size for access through the mouth and on the skills of highly trained personnel (Haman and Weber, 1996). This is an invasive method that, if properly setup, can allow for accurate estimation of substrate fluxes (Weber et al., 2016), including glycerol (Bernard et al., 1999; Magnoni et al., 2008), in fully recovered fish under minimal stress. If using radioactive isotopes like [^{3}H] or [^{14}C] glycerol, additional laboratory security procedures and safe discard of leftover solutions and biomass must be in place, amounting to experimental costs. Despite all the technicalities behind these approaches, the aforementioned studies were able to clearly demonstrate that glycerol is incorporated into lipids. Li et al. (2016) using [^{14}C]glycerol on Atlantic cod (*Gadus morhua*) larvae, were even able to determine that the predominant destination lipid classes were TAG and phosphatidyl-choline (PC), followed by phosphatidylethanolamine (PE). A significant increase in [^{14}C]PC and a decreasing percentage of [^{14}C]TAG over time were also reported. On the other hand, deuterium ^{2}H is stable (non-radioactive) isotope, and if delivered on the form of $^{2}H_2O$ is ideally suited for metabolic studies in fish. It can be incorporated into tank water safely for an indefinite period, if periodically adjusted for evaporation, ^{2}H-enrichment and water quality. ^{2}H-enrichment incorporation allows for a non-invasive measurement of glycerol utilisation on naturally feeding fish, and in the case of TAG, separately quantify the enrichment of the glycerol backbone and its esterified FA.

Small 3-carbon glucose precursors like lactate, pyruvate, alanine and glycerol are metabolically versatile and display inter-organ moiety with several described metabolic shuttles. However, if considered as aquafeed ingredients, glycerol has the advantage of being a gluconeogenic substrate that does not generate nitrogenous disposal. This is particularly relevant in fish farmed at high densities or using a recirculated aquatic system (RAS), and even more relevant to carnivorous fish species that do not tolerate high levels of dietary carbohydrate and depend highly on gluconeogenesis to regulate glycaemia and glycogen reserves (Viegas et al., 2013, 2015, 2019). This was confirmed and thoroughly discussed in Part I of the present study for hepatic glycogen synthesis in rainbow trout and blood glucose regulation in European seabass (Viegas et al., 2022). Moreover, high glycerol turnover allows for incredibly high rate of re-esterification (or TAG/FA cycling) in rainbow trout at rest (Magnoni et al., 2008; Turenne and Weber, 2018) which surprisingly remains unaltered when in exercise. This is a very different paradigm when compared with mammals that need to boost their lipolytic rates (measured as R_a glycerol) by 2–5 fold (Turenne and Weber, 2018). This mechanism in fish has also been suggested as a means to achieve rapid membrane remodeling in variable environments.

Liver

Alteration to lipid metabolism may be revealed by a spectrum of parameters amongst which the most common are plasma and liver TAG/lipid levels. In the present study both species provided evidence that dietary glycerol may in fact increase plasma TAG levels. In Nile tilapia and silver catfish (*Rhamdia*

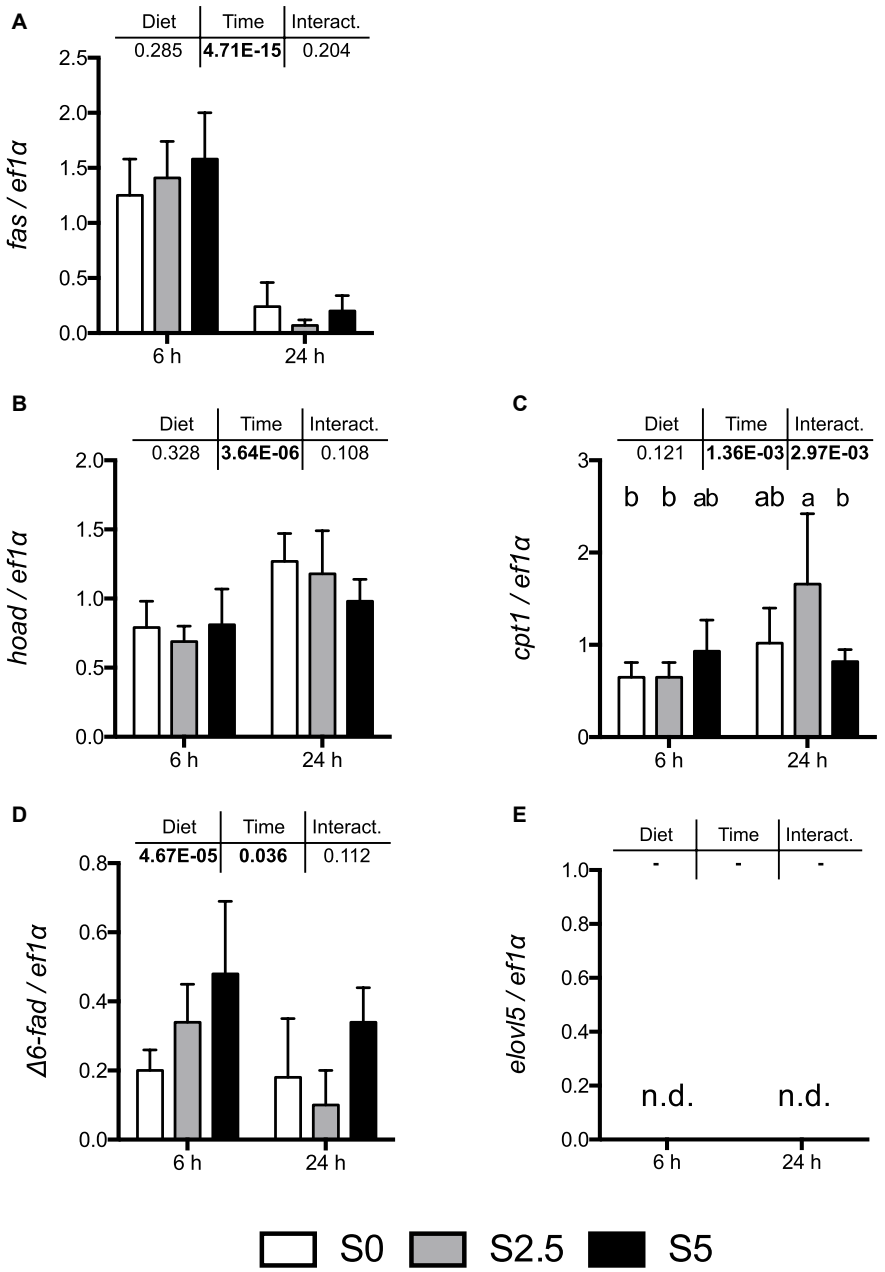

FIGURE 3 | mRNA levels of selected lipogenic enzymes **(A)** *fas* (fatty acid synthase); lipolytic enzymes **(B)** *hoad* (3-hydroxyacyl-CoA dehydrogenase) and **(C)** *cpt1* (carnitine palmitoyltransferase I); and enzymes involved in long chain PUFA biosynthesis: **(D)** Δ*6-fad* (acyl-CoA 6-desaturase) and **(E)** *elovl5* (elongation of very long-chain fatty acid protein 5) in the liver of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks. Values are mean ± SD ($n = 7$); n.d.: not detected. Significant differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold). In the case of significant interactions, significant differences are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test).

quelen) dietary glycerol did not influence plasma TAG (Costa et al., 2015; Mauerwerk et al., 2021b) even with diets with up to 15% and 7.5% glycerol, respectively. Moreover, dietary glycerol appeared to result in an increase of liver TAG levels of seabass, as previously observed in Nile tilapia but at supplementation levels of 15% glycerol (Santos et al., 2019). However, in tilapia, indirect measurements of adiposity in liver through the assessment of histological parameters, suggested that there was no strong relationship between diet and the accumulation of lipids in the hepatocytes (Mewes et al., 2016) or alteration in hepatocyte area (Santos et al., 2019). In channel catfish, diets containing 5 and 10% glycerol provoked no significant differences in liver lipid level while fish fed diets containing 15 and 20% glycerol reduced liver lipid content (Li et al., 2010). In seabass, the increase in

TABLE 4 | Percentage of fatty acid classes as determined from ^{1}H NMR spectra of TAG from liver, muscle and perivisceral fat of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%).

	Liver			Muscle			Perivisceral fat		
European seabass	**S0**	**S2.5**	**S5**	**S0**	**S2.5**	**S5**	**S0**	**S2.5**	**S5**
n-3	17.8 ± 2.6	17.1 ± 2.2	16.3 ± 2.5	20.2 ± 0.7b	17.2 ± 0.6a	17.7 ± 0.7a	18.3 ± 3.4	17.6 ± 0.9	15.2 ± 3.7
non n-3	82.2 ± 2.6	82.9 ± 2.2	83.7 ± 2.5	79.9 ± 0.7 a	82.8 ± 0.6 b	82.3 ± 0.7b	81.7 ± 3.4	82.4 ± 0.9	84.8 ± 3.7
SFA	29.3 ± 5.1	25.5 ± 2.6	26.3 ± 2.4	22.7 ± 1.0	23.7 ± 1.0	24.9 ± 1.4	22.3 ± 3.3	25.0 ± 2.1	25.5 ± 2.0
UFA	70.7 ± 5.1	74.5 ± 2.6	73.7 ± 2.4	77.4 ± 1.0	76.3 ± 1.0	75.1 ± 1.4	77.8 ± 3.3	75.0 ± 2.1	74.4 ± 2.0
PUFA	32.1 ± 3.7 b	31.2 ± 2.7 ab	26.7 ± 3.6 a	39.0 ± 1.7 b	34.6 ± 0.7 a	32.6 ± 2.6 a	34.7 ± 3.7 b	31.7 ± 1.3 ab	28.2 ± 3.6 a
MUFA	38.6 ± 3.1 a	43.3 ± 2.6 b	47.0 ± 2.3 b	38.3 ± 1.4 a	41.8 ± 1.3 b	42.5 ± 1.3 b	43.1 ± 4.4	43.3 ± 1.0	46.2 ± 4.9

Values are mean ± SD (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test). MUFA, monounsaturated fatty acid; n-3 FA, omega-3 fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid UFA, unsaturated fatty acid.

FIGURE 4 | Triacylglycerol-bound fatty acid fractional synthetic rate (FA FSR; *de novo lipogenesis*) expressed as percent of newly synthesized fatty acids per day (left) and triacylglycerol-bound glycerol fractional synthetic rate (Glycerol FSR; glycerol synthesis/turnover) expressed as percent of newly synthesized/turned over glycerol per day in hepatic, muscular and perivisceral TAG of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) after a 6-day residence in a tank with ∼4% ^{2}H-enriched water. Mean values ± SD are presented ($n = 6$). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$; followed by Tukey's test). n.d.: not detected.

liver TAG levels was accompanied by a change in its composition in S5 provoking a significant decrease in PUFA at the expense of an increase in MUFA. This pattern in FA composition was observed also in muscle and perivisceral fat. No such alterations in lipid composition were observed in trout liver that was apparently able to compensate by significantly up-regulating Δ*6-fad*. Despite not displaying any significant alterations to liver TAG and its composition, trout liver responded to dietary glycerol with a significant up-regulation of *fas*. Excess FA may have been exported to the muscle where the same pattern of decrease in PUFA at the expense of an increase in MUFA verified in seabass tissue was observed. As mentioned before, glycerol supplementation may alter the unsaturation index of fat in cattle and pigs (Mourot et al., 1994; Carvalho et al., 2014) and the mechanisms behind this pattern should be addressed in future studies. Nonetheless, the up-regulation of *fas* was not corroborated by the metabolic flux data, where no significant increase in *de novo* lipogenesis was observed in trout. In seabass, the increase in liver TAG levels was corroborated not by not by changes in *fas* mRNA levels but by a sustained increased in *de novo* lipogenesis as revealed by ^{2}H-enriched TAG-bound FA terminal CH_3. In fact, the FA fractional synthetic rates in seabass were almost 10-fold of those found in trout. This is revealing of the capacity of trout to efficiently retain dietary lipid as previously described in gilthead seabream fed with 5% glycerol (Silva et al., 2012), or in trout where fat is being replaced by starch (Figueiredo-Silva et al., 2012). During the 6-day residence in $^{2}H_2O$ the glycerol turnover in liver was almost complete and not influenced by dietary glycerol. This is asserted by the fact that TAG-bound glycerol ^{2}H-enrichment equalled that of the tank water (average 5.1 ± 0.3 vs. 3.9% TW in trout; average 4.1 ± 0.1 vs. 3.5% TW in seabass). Other than *fas* in trout and Δ*6-fad* in trout and seabass, the mRNA levels of the other lipid metabolism-related enzymes did not respond to the dietary treatment. This is in agreement with the results obtained from the glycerol metabolism-related enzymes evaluated in trout from the present study (Panserat et al., 2020). Even if poorly regulated by glycerol, the mRNA levels of these enzymes in liver were overall responsive to time after last meal (6 vs. 24 h), as previously described for both species (Panserat et al., 2009; Kamalam et al., 2013; Castro et al., 2015a,b).

Muscle and Perivisceral Fat

One of the first reports on the effects of dietary glycerol in fish (rainbow trout) revealed that the addition of 1% free glycerol (approximately the amount of bound glycerol found in a 10%

addition of lipid to the control diets) resulted in no effect on the final carcass lipid levels (either whole or eviscerated fish) (Menton et al., 1986). This supplementation, even if at a relatively low dosage, was made to low and high-energy diets, and the lack of significant alteration appeared to indicate that dietary free glycerol had no significant effect on lipogenic activity in trout. Other studies in other fish species reported that, in general, fillet protein and fat generally decrease, while moisture increases as dietary glycerol level increase (Li et al., 2010; Moesch et al., 2016; Santos et al., 2019). In humans, glycerol has been associated with increased fluid retention via osmotic effect on the kidneys, being independent of hormonal response (Goulet et al., 2007; Nelson and Robergs, 2007). It is, however, speculative to assert the same mechanisms for aquatic organisms, especially marine species that have to finely osmoregulate, mechanisms to which glycerol has not been clearly associated yet. In spite of the potential impact of glycerol on water retention, whole body moisture in trout reared in the present study was not affected by diet as reported elsewhere (Magnoni et al., 2021).

The association between increasing levels of glycerol and muscle lipids are complex to interpret since most studies test a wide range of supplementations. In Nile tilapia, carcass lipids showed the highest values at 5% and the lowest at 10% (Neu et al., 2013) which may represent not a linear metabolic response but a severe metabolic deregulation at higher dosages. In gilthead seabream, the individual muscle FA analyzed did not differ between diets (control vs. 5% glycerol), and there were no significant differences between major lipid classes (Silva et al., 2012). In the present study there was a decrease in muscle PUFA at the expense of an increase in MUFA in both species, even if apparent digestibility coefficient for crude lipid was not impacted by diet in trout as previously reported elsewhere for the same fish from the present study (Magnoni et al., 2021). Choline is an essential component of cell membranes in the form of PC preventing excessive lipid accumulation. In trout, muscle choline was decreased in the T5 group compared to the T0 group as previously reported for in a metabolomic analysis performed in the same fish from this study (Palma et al., 2019). As stated before, PC is the major fate lipid class to glycerol after TAG (Li et al., 2016), and as most diets shift toward plant-based ingredients an exogenous supply of choline may be essential for normal metabolism. This is particularly true since choline is essential for transport and export of lipids across the mucosa of the pyloric caeca and for prevention of lipid malabsorption syndrome (LMS) (Hansen et al., 2020). At the time of death, gilthead seabream fed with glycerol had a significantly higher ATP content in the muscle (Silva et al., 2012); however, fish from the present study (both species) revealed no effects on the overall energetic state of the muscle as calculated by adenylate energy charge (Palma et al., 2019). From a consumer's perspective, glycerol increased the hardness of raw gilthead seabream fillets, but this effect dissipated in the cooked fillets (Silva et al., 2012).

From a metabolic point of view, TAG in extrahepatic tissues TAG presented much lower enrichments compared to the liver, since in the muscle of seabass no ^{2}H-enrichment was detected (neither in the TAG-bound FA nor glycerol) as previously described (Viegas et al., 2019). Relatively lower TAG-bound glycerol ^{2}H-enrichments were also observed in extrahepatic tissues of trout (muscle: 0.38 $\pm$ 0.13% d^{-1}; perivisceral fat; 0.09 $\pm$ 0.04% d^{-1}) and seabass (muscle: not detected; perivisceral fat; 0.12 $\pm$ 0.03% d^{-1}). As the liver is the main metabolically active organ, this may stem from the limited capacity of the liver to export labeled metabolites, or of the peripheral tissues to incorporate circulating dietary (unlabeled) glycerol. The latter seems to be the case for seabass since Viegas et al. (2019) reported glycerol ^{2}H-enrichments in muscle between 1 and 2.5% for the same period of residence in $^{2}H_2O$. Overall, no differences were found due to the dietary treatment in extrahepatic tissues for TAG-bound FA and glycerol. As in the present study, liposomatic index, visceral fat weight or perivisceral fat/viscerosomatic index were not correlated (Silva et al., 2012; Neu et al., 2013; Gonçalves et al., 2015) or were even negatively correlated with glycerol supplementation (Santos et al., 2019) in several other species. Visceral fat composition mimicked what was in generally observed in liver and muscle with a significant decrease in PUFA in seabass and a significant increase in MUFA in trout.

CONCLUSION

In conclusion, we present evidence that dietary glycerol supplementation at 5% significantly increased plasma TAG levels in both species and liver TAG levels in seabass. A supplementation of 2.5% glycerol is recommended since it did not severely impact the lipid metabolism nor increased lipogenic potential in liver, muscle and perivisceral fat accumulation in both species. Moreover, the TAG-bound FA composition was in general higher in MUFA at the expense of lower PUFA in all tissues. This means that increasing the PUFA profile in the diet formulation should be considered to compensate this effect of glycerol supplementation. Overall, endogenous turnover rates of TAG-bound glycerol were not impacted by dietary glycerol but extrapolations to other species should be cautious. Specific differences were evident since seabass synthesized FA *de novo* at much higher rates than trout and in proportion to increasing dietary glycerol.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Portuguese Veterinary Authority (1005/92), DGAV-Portugal, according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

AUTHOR CONTRIBUTIONS

IV, LJM, and SP: conceptualization, supervision, data treatment, and writing of the original draft. LJM, RO, ES, JR, LFH, LCT, MP, and IV: growth and metabolic trials. SP and EP-J: molecular analysis of enzymes. JR, ES, LFH, LCT, MP, and IV: NMR spectral acquisition and processing. IV and LJM: funding acquisition, project administration, and resources. All authors reviewed, edited, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.836612/full#supplementary-material

REFERENCES

Ayoub, M., and Abdullah, A. Z. (2012). Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. *Renew. Sustain. Energy Rev.* 16, 2671–2686. doi: 10.1016/j.rser.2012.01.054

Balen, R. E., Junior, G. B., Colpini, L. M. S., Bombardelli, R. A., Silva, L. C. R., and Meurer, F. (2017). Energia digestível e inclusão da glicerina bruta em dietas para juvenis de curimbatá concentrations of ammonia. *Bol. Inst. Pesca* 43, 347–357. doi: 10.20950/1678-2305.2017v43n3p347

Bernard, S. F., Reidy, S. P., Zwingelstein, G., and Weber, J. (1999). Glycerol and fatty acid kinetics in rainbow trout: effects of endurance swimming. *J. Exp. Biol.* 202, 279–288. doi: 10.1242/jeb.202.3.279

Carvalho, E. R., Schmelz-Roberts, N. S., White, H. M., Wilcox, C. S., Eicher, S. D., and Donkin, S. S. (2012). Feeding behaviors of transition dairy cows fed glycerol as a replacement for corn. *J. Dairy Sci.* 95, 7214–7224. doi: 10.3168/jds.2010-3584

Carvalho, P. L. D. O., Moreira, I., Martins, E. N., Piano, L. M., Toledo, J. B., and Costa Filho, C. D. L. (2012). Crude glycerine in diets for piglets. *Rev. Bras. Zootec.* 41, 1654–1661. doi: 10.1590/S1516-35982012000700014

Carvalho, J. R. R., Chizzotti, M. L., Ramos, E. M., Machado Neto, O. R., Lanna, D. P. D., Lopes, L. S., et al. (2014). Qualitative characteristics of meat from young bulls fed different levels of crude glycerin. *Meat Sci.* 96, 977–983. doi: 10.1016/j.meatsci.2013.10.020

Castro, C., Corraze, G., Panserat, S., and Oliva-Teles, A. (2015a). Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquac. Nutr.* 21, 592–603. doi: 10.1111/anu.12184

Castro, C., Corraze, G., Pérez-Jiménez, A., Larroquet, L., Cluzeaud, M., Panserat, S., et al. (2015b). Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Br. J. Nutr.* 114, 1143–1156. doi: 10.1017/S0007114515002731

Cerrate, S., Yan, F., Wang, Z., Coto, C., Sacakli, P., and Waldroup, P. W. (2006). Evaluation of Glycerine from Biodiesel Production as a Feed Ingredient for Broilers. *Int. J. Poult. Sci.* 5, 1001–1007. doi: 10.3923/ijps.2006.1001.1007

Coppack, S. W., Persson, M., Judd, R. L., and Miles, J. M. (1999). Glycerol and nonesterified fatty acid metabolism in human muscle and adipose tissue in vivo. *Am. J. Physiol. Endocrinol. Metab.* 276, E233–E240. doi: 10.1152/ajpendo.1999.276.2.E233

Costa, D. V. D., Dias, J., Colen, R., Rosa, P. V., and Engrola, S. (2017). Partition and metabolic fate of dietary glycerol in muscles and liver of juvenile tilapia. *Arch. Anim. Nutr.* 71, 165–174. doi: 10.1080/1745039X.2017.1281579

Costa, D. V. D., Paulino, R. R., Okamura, D., Oliveira, M. M. D., and Rosa, P. V. E. (2015). Growth and energy metabolism of Nile tilapia juveniles fed glycerol. *Pesqui. Agropecu. Bras.* 50, 347–354. doi: 10.1590/S0100-204X2015000500001

Del Bianco Benedeti, P., Paulino, P. V. R., Marcondes, M. I., Maciel, I. F. S., Da Silva, M. C., and Faciola, A. P. (2016). Partial Replacement of Ground Corn with Glycerol in Beef Cattle Diets: intake, Digestibility, Performance, and Carcass Characteristics. *PLoS One* 11:e0148224. doi: 10.1371/journal.pone.0148224

Duarte, J. A. G., Carvalho, F., Pearson, M., Horton, J. D., Browning, J. D., Jones, J. G., et al. (2014). A high-fat diet suppresses de novo lipogenesis and desaturation but not elongation and triglyceride synthesis in mice. *J. Lipid Res.* 55, 2541–2553. doi: 10.1194/jlr.M052308

Figueiredo-Silva, A. C., Saravanan, S., Schrama, J. W., Panserat, S., Kaushik, S., and Geurden, I. (2012). A comparative study of the metabolic response in rainbow trout and Nile tilapia to changes in dietary macronutrient composition. *Br. J. Nutr.* 109, 816–826. doi: 10.1017/S000711451200205X

Food and Agriculture Organization [FAO] (2012). *Biofuel co-products as livestock feed – Opportunities and challenges*. Rome: FAO. Available online at: http://www.fao.org/3/i3009e/i3009e.pdf

Freitas, L., Menten, J., Zavarize, K., Pereira, R., Romano, G., Lima, M., et al. (2017). Evaluation of Dietary Glycerin Inclusion During Different Broiler Rearing Phases. *Braz. J. Poult. Sci.* 19, 91–96. doi: 10.1590/1806-9061-2016-0226

Gonçalves, L. U., Cerozi, B. D. S., Silva, T. S. C., Zanon, R. B., and Cyrino, J. E. P. (2015). Crude glycerin as dietary energy source for Nile tilapia. *Aquaculture* 437, 230–234. doi: 10.1016/j.aquaculture.2014.12.004

Goulet, E. D. B., Aubertin-Leheudre, M., Plante, G. E., and Dionne, I. J. (2007). A Meta-Analysis of the Effects of Glycerol-Induced Hyperhydration on Fluid Retention and Endurance Performance. *Int. J. Sport Nutr. Exerc. Metab.* 17, 391–410. doi: 10.1123/ijsnem.17.4.391

Haman, F., and Weber, J. M. (1996). Continuous tracer infusion to measure in vivo metabolite turnover rates in trout. *J. Exp. Biol.* 199, 1157–1162. doi: 10.1242/jeb.199.5.1157

Hansen, A. K. G., Kortner, T. M., Denstadli, V., Måsøval, K., Björkhem, I., Grav, H. J., et al. (2020). Dose–response relationship between dietary choline and

lipid accumulation in pyloric enterocytes of Atlantic salmon (Salmo salar L.) in seawater. *Br. J. Nutr.* 123, 1081–1093. doi: 10.1017/S0007114520000434

Ho-Palma, A. C., Toro, P., Rotondo, F., Romero, M. D. M., Alemany, M., Remesar, X., et al. (2019). Insulin Controls Triacylglycerol Synthesis through Control of Glycerol Metabolism and Despite Increased Lipogenesis. *Nutrients* 11:513. doi: 10.3390/nu11030513

Jin, E. S., Sherry, A. D., and Malloy, C. R. (2014). Interaction between the Pentose Phosphate Pathway and Gluconeogenesis from Glycerol in the Liver. *J. Biol. Chem.* 289, 32593–32603. doi: 10.1074/jbc.M114.577692

Jones, J. G., Merritt, M., and Malloy, C. (2001). Quantifying tracer levels of 2H2O enrichment from microliter amounts of plasma and urine by 2H NMR. *Magn. Reson. Med.* 45, 156–158. doi: 10.1002/1522-2594(200101)45:1<156::aid-mrm1020>3.0.co;2-z

Kam, J. C., and Milligan, C. L. (2006). Fuel use during glycogenesis in rainbow trout (*Oncorhynchus mykiss* Walbaum) white muscle studied *in vitro*. *J. Exp. Biol.* 209, 871–880. doi: 10.1242/jeb.02071

Kamalam, B. S., Médale, F., Larroquet, L., Corraze, G., and Panserat, S. (2013). Metabolism and Fatty Acid Profile in Fat and Lean Rainbow Trout Lines Fed with Vegetable Oil: effect of Carbohydrates. *PLoS One* 8:e76570. doi: 10.1371/journal.pone.0076570

Lammers, P. J., Kerr, B. J., Weber, T. E., Dozier, W. A. III, Kidd, M. T., Bregendahl, K., et al. (2008b). Digestible and metabolizable energy of crude glycerol for growing pigs. *J. Anim. Sci.* 86, 602–608. doi: 10.2527/jas.2007-0453

Lammers, P. J., Kerr, B. J., Weber, T. E., Bregendahl, K., Lonergan, S. M., Prusa, K. J., et al. (2008a). Growth performance, carcass characteristics, meat quality, and tissue histology of growing pigs fed crude glycerin-supplemented diets1. *J. Anim. Sci.* 86, 2962–2970. doi: 10.2527/jas.2008-0972

Lech, J. J. (1970). Glycerol kinase and glycerol utilization in trout (*Salmo gairdneri*) liver. *Comp. Biochem. Physiol.* 34, 117–124. doi: 10.1016/0010-406X(70)90058-7

Lessard, P., Lefrançois, M. R., and Bernier, J. F. (1993). Dietary Addition of Cellular Metabolic Intermediates and Carcass Fat Deposition in Broilers. *Poult. Sci.* 72, 535–545. doi: 10.3382/ps.0720535

Li, K., Olsen, R. E., Østensen, M.-A., Altin, D., Kjørsvik, E., and Olsen, Y. (2016). Atlantic cod (*Gadus morhua*) larvae can biosynthesis phospholipid de novo from 2-oleoyl-glycerol and glycerol precursors. *Fish Physiol. Biochem.* 42, 137–147. doi: 10.1007/s10695-015-0125-y

Li, M. H., Minchew, C. D., Oberle, D. F., and Robinson, E. H. (2010). Evaluation of Glycerol from Biodiesel Production as a Feed Ingredient for Channel Catfish, *Ictalurus punctatus*. *J. World Aquac. Soc.* 41, 130–136. doi: 10.1111/j.1749-7345.2009.00320.x

Lin, M. H., Romsos, D. R., and Leveille, G. A. (1976). Effect of Glycerol on Lipogenic Enzyme Activities and on Fatty Acid Synthesis in the Rat and Chicken. *J. Nutr.* 106, 1668–1677. doi: 10.1093/jn/106.11.1668

Magnoni, L., Rema, P., Silva-Brito, F., Rito, J., Palma, M., Ozorio, R., et al. (2021). Dietary glycerol inclusion decreases growth performance and nitrogen retention efficiency in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 535:736383. doi: 10.1016/j.aquaculture.2021.736383

Magnoni, L., Vaillancourt, E., and Weber, J.-M. (2008). In vivo regulation of rainbow trout lipolysis by catecholamines. *J. Exp. Biol.* 211, 2460–2466. doi: 10.1242/jeb.018143

Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., and Schwudke, D. (2008). Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res.* 49, 1137–1146. doi: 10.1194/jlr.D700041-JLR200

Mauerwerk, M. T., Zadinelo, I. V., and Meurer, F. (2021a). Use of glycerol in fish nutrition: a review. *Rev. Aquac.* 13, 853–861. doi: 10.1111/raq.12502

Mauerwerk, M. T., Zadinelo, I. W., Zanella Júnior, M. C., Balen, R. E., Bombardelli, R. A., Da Silva, L. C. R., et al. (2021b). Glycerol effects on silver catfish (Rhamdia quelen) fingerling feeding: morphometric, zootechnical and blood parameters. *Aquaculture* 535:736361. doi: 10.1016/j.aquaculture.2021.736361

Menton, D. J., Slinger, S. J., and Hilton, J. W. (1986). Utilization of free glycerol as a source of dietary energy in rainbow trout (*Salmo gairdneri*). *Aquaculture* 56, 215–227. doi: 10.1016/0044-8486(86)90337-6

Mewes, J. K., Meurer, F., Tessaro, L., Buzzi, A. H., Syperreck, M. A., and Bombardelli, R. A. (2016). Diets containing crude glycerin damage the sperm characteristics and modify the testis histology of Nile tilapia broodstock. *Aquaculture* 465, 164–171. doi: 10.1016/j.aquaculture.2016.08.035

Moesch, A., Meurer, F., Zadinelo, I. V., Carneiro, W. F., Da Silva, L. C. R., and Dos Santos, L. D. (2016). Growth, body composition and hepatopancreas morphology of Nile tilapia fingerlings fed crude glycerol as a replacement for maize in diets. *Anim. Feed Sci. Technol.* 219, 122–131. doi: 10.1016/j.anifeedsci.2016.05.009

Montell, E., LeríN, C., Newgard, C. B., and Gómez-Foix, A. M. (2002). Effects of Modulation of Glycerol Kinase Expression on Lipid and Carbohydrate Metabolism in Human Muscle Cells. *J. Biol. Chem.* 277, 2682–2686. doi: 10.1074/jbc.M107227200

Mourot, J., Aumaitre, A., Mounier, A., Peiniau, P., and François, A. C. (1994). Nutritional and physiological effects of dietary glycerol in the growing pig. Consequences on fatty tissues and post mortem muscular parameters. *Livest. Prod. Sci.* 38, 237–244. doi: 10.1016/0301-6226(94)90175-9

Narayan, K. A., and Mcmullen, J. J. (1979). The Interactive Effect of Dietary Glycerol and Corn Oil on Rat Liver Lipids, Serum Lipids and Serum Lipoproteins. *J. Nutr.* 109, 1836–1846. doi: 10.1093/jn/109.11.1836

Nelson, J. L., and Robergs, R. A. (2007). Exploring the Potential Ergogenic Effects of Glycerol Hyperhydration. *Sports Med.* 37, 981–1000. doi: 10.2165/00007256-200737110-00005

Neu, D. H., Furuya, W. M., Boscolo, W. R., Potrich, F. R., Lui, T. A., and Feiden, A. (2013). Glycerol inclusion in the diet of Nile tilapia (*Oreochromis niloticus*) juveniles. *Aquac. Nutr.* 19, 211–217. doi: 10.1111/j.1365-2095.2012.00968.x

Nye, C. K., Hanson, R. W., and Kalhan, S. C. (2008). Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis in vivo in the rat. *J. Biol. Chem.* 283, 27565–27574. doi: 10.1074/jbc.M804393200

Ordoñez-Gomez, C., Afanador-Tellez, G., Castañeda, S., Florez, H., and Ariza-Nieto, C. (2017). Growth performance, carcass characteristics, meat quality of growing pigs fed diets supplemented with crude glycerin derived from palm oil. *Ciênc. Anim. Bras.* 18:e40769. doi: 10.1590/1089-6891v18e-40769

Palma, M., Tavares, L. C., Rito, J., Henriques, L. F., Silva, J. G., Ozório, R., et al. (2019). Metabolic Effects of Dietary Glycerol Supplementation in Muscle and Liver of European Seabass and Rainbow Trout by 1H NMR Metabolomics. *Metabolites* 9:202. doi: 10.3390/metabo9100202

Panserat, S., Plagnes-Juan, E., Gazzola, E., Palma, M., Magnoni, L. J., Marandel, L., et al. (2020). Hepatic Glycerol Metabolism-Related Genes in Carnivorous Rainbow Trout (*Oncorhynchus mykiss*): insights Into Molecular Characteristics, Ontogenesis, and Nutritional Regulation. *Front. Physiol.* 11:882. doi: 10.3389/fphys.2020.00882

Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C., et al. (2009). Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities?. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297, R707–R715. doi: 10.1152/ajpregu.00120.2009

Rito, J., Viegas, I., Pardal, M. Â, Metón, I., Baanante, I. V., and Jones, J. G. (2019). Utilization of glycerol for endogenous glucose and glycogen synthesis in seabass (*Dicentrarchus labrax*): a potential mechanism for sparing amino acid catabolism in carnivorous fish. *Aquaculture* 498, 488–495. doi: 10.1016/j.aquaculture.2018.08.066

Rotondo, F., Ho-Palma, A. C., Remesar, X., Fernández-López, J. A., Romero, M. D. M., and Alemany, M. (2017). Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover. *Sci. Rep.* 7:8983. doi: 10.1038/s41598-017-09450-4

Santos, L. D. D., Zadinelo, I. V., Moesch, A., Bombardelli, R. A., and Meurer, F. (2019). Crude glycerol in diets for Nile tilapia in the fattening stage. *Pesqui. Agropecu. Bras.* 54:e00460. doi: 10.1590/S1678-3921.pab2019.v54.00460

Savina, M. V., and Wojtczak, A. B. (1977). Enzymes of gluconeogenesis and the synthesis of glycogen from glycerol in various organs of the lamprey (*Lampetra fluviatilis*). *Comp. Biochem. Physiol. B Comp. Biochem.* 57, 185–190. doi: 10.1016/0305-0491(77)90141-9

Schieck, S. J., Shurson, G. C., Kerr, B. J., and Johnston, L. J. (2010). Evaluation of glycerol, a biodiesel coproduct, in grow-finish pig diets to support growth and pork quality. *J. Anim. Sci.* 88, 3927–3935. doi: 10.2527/jas.2010-2858

Silva, T. S., Matos, E., Cordeiro, O. D., Colen, R., Wulff, T., Sampaio, E., et al. (2012). Dietary Tools To Modulate Glycogen Storage in Gilthead Seabream Muscle: glycerol Supplementation. *J. Agric. Food Chem.* 60, 10613–10624. doi: 10.1021/jf3023244

Turenne, E. D., and Weber, J.-M. (2018). Lean, mean, lipolytic machines: lipid mobilization in rainbow trout during graded swimming. *J. Exp. Biol.* 221:jeb171553. doi: 10.1242/jeb.171553

van Hall, G., Sacchetti, M., Rådegran, G., and Saltin, B. (2002). Human Skeletal Muscle Fatty Acid and Glycerol Metabolism During Rest, Exercise and Recovery. *J. Physiol.* 543, 1047–1058. doi: 10.1113/jphysiol.2002.023796

Viegas, I., Jarak, I., Rito, J., Carvalho, R. A., Metón, I., Pardal, M. A., et al. (2016). Effects of dietary carbohydrate on hepatic *de novo* lipogenesis in European seabass (*Dicentrarchus labrax* L.). *J. Lipid Res.* 57, 1264–1272. doi: 10.1194/jlr.M067850

Viegas, I., Mendes, V. M., Leston, S., Jarak, I., Carvalho, R. A., Pardal, M. Â, et al. (2011). Analysis of glucose metabolism in farmed European sea bass (*Dicentrarchus labrax* L.) using deuterated water. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 160, 341–347. doi: 10.1016/j.cbpa.2011.06.029

Viegas, I., Rito, J., González, J. D., Jarak, I., Carvalho, R. A., Metón, I., et al. (2013). Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 166, 399–405. doi: 10.1016/j.cbpa.2013.07.013

Viegas, I., Rito, J., Jarak, I., Leston, S., Caballero-Solares, A., Metón, I., et al. (2015). Contribution of dietary starch to hepatic and systemic carbohydrate fluxes in European seabass (*Dicentrarchus labrax* L.). *Br. J. Nutr.* 113, 1345–1354. doi: 10.1017/S0007114515000574

Viegas, I., Trenkner, L. H., Rito, J., Palma, M., Tavares, L. C., Jones, J. G., et al. (2019). Impact of dietary starch on extrahepatic tissue lipid metabolism in farmed European (*Dicentrarchus labrax*) and Asian seabass (*Lates calcarifer*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 231, 170–176. doi: 10.1016/j.cbpa.2019.02.025

Viegas, I., Tavares, L. C., Plagnes-Juan, E., Silva, E., Rito, J., Marandel, L., et al. (2022). On the utilization of dietary glycerol in carnivorous fish – Part I: insights into hepatic carbohydrate metabolism of juvenile rainbow trout (*Oncorhynchus mykiss*) and European seabass (*Dicentrarchus labrax*). *Front. Mar. Sci.* 9:836610. doi: 10.3389/fmars.2022.836610

Weber, J.-M., Choi, K., Gonzalez, A., and Omlin, T. (2016). Metabolic fuel kinetics in fish: swimming, hypoxia and muscle membranes. *J. Exp. Biol.* 219, 250–258. doi: 10.1242/jeb.125294

Xue, L.-L., Chen, H.-H., and Jiang, J.-G. (2017). Implications of glycerol metabolism for lipid production. *Prog. Lipid Res.* 68, 12–25. doi: 10.1016/j.plipres.2017.07.002

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A Newly Isolated Strain of *Haematococcus pluvialis* GXU-A23 Improves the Growth Performance, Antioxidant and Anti-Inflammatory Status, Metabolic Capacity and Mid-intestine Morphology of Juvenile *Litopenaeus vannamei*

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Haematococcus pluvialis can be used as a green additive in aquafeeds due to it contains rich astaxanthin and polyunsaturated fatty acid. In the present study, a newly strain of *H. pluvialis* GXU-A23 with high concentration of astaxanthin was firstly isolated by a newly culture strategy in our laboratory. In addition, *H. pluvialis* GXU-A23 was applied in the *Litopenaeus vannamei* feed for determining whether it has positive effects on the growth performance, antioxidant and anti-inflammatory status, metabolic capacity and mid-intestine morphology of juvenile *L. vannamei*. Shrimp with 0.63 g approximately initial body weight were fed diets supplemented with/without 50 g/kg *H. pluvialis* GXU-A23. After 8 weeks feeding intervention, significantly higher growth performance of *L. vannamei* was obtained in the *H. pluvialis* GXU-A23 treatment group compared to the control group ($p < 0.05$). At the same time, *L. vannamei* fed with *H. pluvialis* GXU-A23 acquired significantly better antioxidant and anti-inflammatory status than the control group ($p < 0.05$). In addition, higher RNA expression level of hepatopancreas digestive enzyme, hepatopancreas lipid and glucose metabolic enzymes as well as better mid-intestine morphology were found in the *H. pluvialis* GXU-A23 treatment group than the control group ($p < 0.05$). These results indicated that 50 g/kg *H. pluvialis* GXU-A23 was suitable for the *L. vannamei* feed, which could improve the growth performance, antioxidant and anti-inflammatory status, metabolic capacity and mid-intestine morphology of juvenile *L. vannamei.*

Keywords: *Haematococcus pluvialis* GXU-A23, *Litopenaeus vannamei*, growth performance, hepatopancreas health, metabolism, intestine morphology

INTRODUCTION

The speedy development of aquaculture provided considerable high-quality protein for human (Costello et al., 2020; Cottrell et al., 2021). In fact, high production is attributed to high density farming (Bostock et al., 2010). On the other hand, many aquaculture environments were polluted due to the improvement of human activities (Zhang et al., 2019). However, these two factors might cause the growth of pathogen microorganisms, such as white spot syndrome virus (WSSV) (Verbruggen et al., 2016) and *Vibrio* parahaemolyticus (Soto-Rodriguez et al., 2015), in water and thus inducing the low survival rate of *Litopenaeus vannamei*, which severely limited the development of the shrimp industry. In order to reduce adverse effects of shrimp as caused by pathogen microorganisms, antibiotics were widely used in recent 20 years (Romero et al., 2012). However, limitations of antibiotic used in aquaculture are antibiotic resistance and drug residues (Sørum, 2005; Santos and Ramos, 2016). Therefore, to promote the development of aquaculture industry, proper green additives must be exploited for substituting the antibiotic used during the farming.

The flesh pigment is one of the essential factors which might influence the shrimp price since customers generally regard the optimal pigment as high quality (Diler and Gokoglu, 2004). However, crustaceans are unable to biosynthesize carotenoids *de novo*, while they can obtain and convert pigment from the feed into carotenoids and then deposit in the flesh (Niu et al., 2009). Therefore, optimization of the flesh pigment could be taken into consideration when it comes to exploiting a shrimp additive.

Astaxanthin, one of the keto carotenoids, is mainly existing in algae (like *Haematococcus pluvialis*, *Chlorella zofingiensis*), bacteria (like *Phaffia rhodozyma*) and crustaceans (Johnson and Lewis, 1979; Ip and Chen, 2005). The antioxidant property of astaxanthin was demonstrated more 100–500 folds than vitamin E to inhibit the lipid peroxidation *in vitro* (Ni et al., 2015). Dietary supplementation of astaxanthin bring many benefits to aquatic animals. For example, improving the growth performance (Wang et al., 2018), reducing the interval of molt cycle (Petit et al., 1997), enhancing the antioxidant and anti-inflammatory capacity (Xie J. et al., 2020), optimization of shrimp pigment (Ju et al., 2011).

Newly strain of *H. pluvialis* GXU-A23 with high concentration astaxanthin (33 g/kg) was isolated and cultured by a newly two-step batch culture strategy in our laboratory (Wang et al., 2019). In this method, modified Bold's Basal medium (mBBM) (Wang et al., 2019) with 9.0 mM urea was provided to culture the *H. pluvialis* GXU-A23. Compared to the modified BG-11 medium (mBG-11) (Gao et al., 2016), the *H. pluvialis* could obtain remarkably higher astaxanthin content in the mBBM (Domınguez-Bocanegra et al., 2004; Nahidian et al., 2018). Apart from that, the *H. pluvialis* showed the better astaxanthin accumulation property in the urea as nitrogen source than $NaNO_3$ and NH_4HCO_3 (Wang et al., 2019). In our previous study, the two-step batch culture strategy was used to successfully culture *H. pluvialis* JNU35, which contained 31.70 g/kg astaxanthin (Zhao et al., 2021). Since astaxanthin was mostly produced by nature *H. pluvialis*, the newly strain of *H. pluvialis* GXU-A23 could remarkably improve the production industry of astaxanthin as well as reduce the product budget. In addition, this microalga also could be used as a green additive in aquafeeds and beneficial for sustainable development of aquaculture.

The present study aims to evaluate whether there are beneficial effects of the newly isolated strain of *H. pluvialis* GXU-A23 on *L. vannamei*. Therefore, an 8 weeks feeding experiment was conducted to investigate effects of *H. pluvialis* GXU-A23 on the growth performance, antioxidant and anti-inflammatory status, metabolic capacity and mid-intestine morphology of juvenile *L. vannamei*. These results might provide a reference for feed formulation of *L. vannamei*.

TABLE 1 | Ingredients and proximate compositions of two experimental diets (g/kg).

Ingredients	D1	D2
Fish meal	250	250
Soybean meal	270	270
Peanut meal	120	120
Wheat flour	234	184
Beer yeast	30	30
Shrimp bran powder	30	30
Fish oil	10	10
Soybean lecithin	10	10
Soybean oil	10	10
Choline chloride (50%)	5	5
Vitamin C phosphate	1	1
Vitamin and mineral premix[a]	20	20
Monocalcium phosphate	10	10
Haematococcus pluvialis GXU-A23 [b]	0	50
Sum	1000	1000
Nutrient levels[c](%)		
Moisture	7.45	7.63
Crude lipid	7.12	7.01
Crude protein	40.52	40.74
Ash	9.46	9.59
Astaxanthin	–	0.16

[a]*Composition of vitamin and mineral mixture (kg^{-1} of mixture): vitamin A, 250,000 IU; riboflavin, 750 mg; pyridoxine HCL, 500 mg; cyanocobalamin, 1 mg; thiamin, 500 mg; menadione, 250 mg; folic acid, 125 mg; biotin, 10 mg; a-tocopherol, 3750 mg; myo-inositol, 2500 mg; calcium pantothenate, 1250 mg; nicotinic acid, 2000 mg; vitamin D_3, 45,000 IU; vitamin C, 7000 mg, Zn, 4000 mg; K, 22.500 mg; I, 200 mg; NaCl, 2.6 g; Cu, 500 mg; Co., 50 mg; $FeSO_4$, 200 mg; Mg, 3000 mg; Se, 10 mg.*
[b]Haematococcus pluvialis *GXU-A23: 33 g/kg astaxanthin (Dry matter).*
[c]*Measured values (Dry matter).*

MATERIALS AND METHODS

Haematococcus pluvialis GXU-A23 Culture

H. pluvialis GXU-A23 was obtained from Zixi Mountain of Chuxiong (YunNan, China), and these microalgae were bacteria-free cultured in our laboratory. The culture method of *H. pluvialis* GXU-A23 was following the manuscript by Wang et al. (2019). Briefly, *H. pluvialis* GXU-A23 was scale-up cultured in a flat glass photobioreactor (length: 240 cm; height: 120 cm) with 6 cm light paths. mBBM (9.0 mM urea), bubbled gas of 1% CO2 (v/v) as well as 100 μmol/m^2 s^{-1} continuous unilateral lighting were provided to culture the *H. pluvialis* GXU-A23

for 15 days. Afterward, *H. pluvialis* GXU-A23 was transferred into the same sized photobioreactor with nitrogen-free medium, 3 cm light paths and continuous bilateral illumination of 400 μmol m^{-2} s^{-1} for 15 days to accumulate astaxanthin. Afterward, red cells of *H. pluvialis* GXU-A23 were harvested by auto-precipitation and freeze-dried using freezing dryer. The freeze-drying biomass of *H. pluvialis* GXU-A23 contained 33 g/kg astaxanthin.

Diet Preparation

As shown in **Table 1**, two isonitrogen and isolipids experimental diets were formulated with/without *H. pluvialis* GXU-A23 (D1: 0; D2: 50 g/kg) respectively. Dietary ingredients were purchased from Guangzhou Chengyi Company Ltd. (Guangzhou, China). The level of *H. pluvialis* GXU-A23 used in the present study was referred to our previous study (Zhao, et al., 2020), which was normally the highest concentration of additive used in the aquafeed.

Measured nutrition values of diets were 7% crude lipid and 40% protein approximately (**Table 1**). The method of diet preparation was following the reported by Yu et al. (2016). Briefly, all dried ingredients and oils were weighted following **table 1** and then completely homogeneous in the Hobart-type mixer (A-200T Mixer, Canada). Then, deionized water (250 ml/kg dried ingredients mixture) was added into the above ingredient to thoroughly mix for 15 min. Then, diets (1.2 mm diameters) were extruded using the pelletizer (South China University of Technology, China). Then, diets were heated in the 50°C ventilated oven for 120 min. Then, diets were stored at −20°C and kept away from the light until the feeding trial.

Feeding Experiment

Juvenile *L. vannamei* were obtained and cultured at the experimental station of the Chinese Academy of Fishery Science (Lingshui, China). Before the trial, shrimp were acclimated to the experimental environment by feeding with D1 diet for 30 days 320 lively shrimp with an initial body weight of 0.63 g approximately were distributed randomly into the recirculating water system with eight cylindrical fiber tanks (300 L). Each of diets was randomly allocated to quadruplicate tanks. The feeding frequency was three times daily at 06:00, 12:00, and 18:00 with 8% of total shrimp weight and lasted for 8 weeks. During the period of feeding, environmental conditions were maintained as follows: water temperature: 26.8–28.1°C; pH: 7.5–7.7; salinity: 29—32‰; dissolved oxygen: > 7.0 mg/L; total ammonia nitrogen: < 0.1 mg/L; sulfide: < 0.05 mg/L. Natural light-dark (12–12 h) cycle was used during the feeding trial.

Sample Collection

After 8 weeks feeding, *L. vannamei* were starved for 24 h. Then, all shrimp from each tank were weighed, counted and then recorded. Then, eight individuals from each tank were randomly collected and anesthetized (MS-222, 98%, Sigma, United States) for obtaining the blood sample. Then, hepatopancreas samples were removed for analysis of antioxidant parameters and mRNA expression; same sections of mid-intestine were removed and fixed in 4% paraformaldehyde (Beyotime, China) for intestinal histological examination. Blood samples were stored at the fridge (4°C, 12 h) and then centrifuged (7,100 g, 10 min, at 4°C) to obtain hemolymph for antioxidant parameters analysis. All hepatopancreas and hemolymph samples were separated rapidly and then maintained at −80°C until examination.

Astaxanthin Analysis of *Haematococcus pluvialis* GXU-A23 and Feeds

Astaxanthin contents of *H. pluvialis* GXU-A23 and feeds were determined by spectrophotometrically as the description by Li, et al. (2012).

Chemical Analysis of Feeds

Chemical compositions (moisture, crude lipid, crude protein and ash) of feeds were determined according to standard methods of AOAC (Horwitz, 2010). Briefly, moisture was analyzed by drying in the ventilated oven at 105°C until constant weight; crude lipid examination was performed following the Soxhlet extractor method (Soxtec System HT6, Tecator, Sweden); crude protein (N × 6.25) was measured following the Kjeldahl method (1030—Autoanalyzer; Tecator, Höganäs, Sweden); ash was analyzed using muffle furnace at 550°C until constant weight.

Quantification of Hepatopancreas and Hemolymph Parameters Related to Antioxidant Status

Hepatopancreas were homogenized and centrifuged according to the description of Fang et al. (2021). Briefly, hepatopancreas were homogenized (1:9) in phosphate buffer. Afterward, above homogenates were centrifuged (10 min, 4°C, 1200 g) and then supernatants were collected.

Enzyme activities of total superoxide dismutase (T-SOD) (A001-1), total antioxidant capacity (T-AOC) (A015–2), glutathione peroxidase (GSH-PX) (A005-1) as well as the content of malondialdehyde (MDA) (A003-1) were measured according to instructions of reagent (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Instructions of reagent were shown in additional files).

Examination of Mid-intestine Histology

Mid-intestine sections were obtained and stained following the manuscript of Zhao et al. (2020). Briefly, tissue sections were stained using the hematoxylin and eosin (Beyotime, China), and mid-intestine histology were observed using the microscope (Olympus CKX41 microscope, Tokyo, Japan). The villus height and the mucosal layer thickness are equating to the average value of randomly selected eight villi and eight mucosal per slide respectively (Chen et al., 2020).

mRNA Isolation and Expression Quantification

Hepatopancreas total RNA isolation and mRNA expression examination were performed following our previous manuscript (Fang et al., 2019). Briefly, the total RNA was

TABLE 2 | Sequences of primers used for real-time quantitative PCR.

Gene	Primer Sequence (5′-3′)	References
ef1a-F	TGGCTGTGAACAAGATGGAC	Xie et al. (2018)
ef1a-R	AGATGGGGATGATTGGGACC	
sod-F	CCGTGCAGATTACGTGAAGG	Duan et al. (2018)
sod-R	GTCGCCACGAGAAGTCAATG	
gsh-px F	GGCACCAGGAGAACACTAC	Xie et al. (2018)
gsh-px R	CGACTTTGCCGAACATAAC	
cat-F	TACTGCAAGTTCCATTACAAGACG	Xie et al. (2019)
cat-R	GTAATTCTTTGGATTGCGGTCA	
relish-F	CTACATTCTGCCCTTGACTCTGG	Xie et al. (2018)
relish-R	GGCTGGCAAGTCGTTCTCG	
rho-F	GTGATGGTGCCTGTGGTAAA	Xie et al. (2018)
rho-R	GCCTCAATCTGTCATAGTCCTC	
chymotrypsin-F	GGCTCTCTTCATCGACG	Xie J. et al. (2020)
chymotrypsin-R	CGTGAGTGAAGAAGTCGG	
trypsin-F	TCCAAGATCATCCAACACGA	Xie S. et al. (2020)
trypsin-R	GACCCTGAGCGGGAATATC	
hk-F	AGTCGCAGCAACAGGAAGTT	Yang et al. (2021)
hk-R	CGCTCTTCTGGCACATGATA	
fas-F	GCGTGATAACTGGGTGTCCT	Yang et al. (2021)
fas-R	ACGTGTGGGTTATGGTGGAT	

TABLE 3 | Growth performance and feed utilization of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days.

	D1	D2
IBW	0.64 ± 0.01	0.63 ± 0.01
FBW	5.98 ± 0.03	6.25 ± 0.01
WGR	828.31 ± 15.07	925.12 ± 14.84
SGR	3.98 ± 0.03	4.16 ± 0.03
FCR	1.24 ± 0.03	1.17 ± 0.01
SR	96.25 ± 1.25	96.88 ± 0.63

IBW (g per shrimp): initial body weight.
FBW (g per shrimp): final body weight.
*Weight gain rate (WGR, %) = 100 * (final body weight—initial body weight)/initial body weight.*
Specific growth rate (SGR, % day^{-1}): 100× (Ln $_{final\ shrimp\ weight}$ - Ln $_{initial\ shrimp\ weight}$)/the experimental duration in days.
Feed conversion ratio (FCR) = dry diet fed/wet weight gain.
*Survival rate (SR) (%) = 100 * (final number of shrimp)/(initial number of shrimp).*
Values are mean ± SE (n = 4). Means in the same row with different superscripts are significantly different (p < 0.05).

isolated using Trizol® reagent (Invitrogen, United States) following the manufacturer's instruction. 1% agarose gel electrophoresis and spectrophotometer (NanoDrop 2000; Thermo Fisher, United States) were used to ascertain RNA quality and quantity, respectively. Afterward, cDNA was synthesized using the PrimeScript TM RT Reagent kit (Takara, Japan), following the manufacturer's instruction. Real-time PCR for the target genes were performed using SYBR® Premix Ex TaqTM II (Takara, Japan) and quantified on the LightCycler 480 (Roche Applied Science, Basel, Switzerland).

Primers related to the present study were listed in **table 2**. The elongation factor a (*ef1a*) was used as a housekeeping gene for RNA expression analysis (Guzmán-Villanueva et al., 2020). The relative mRNA expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

TABLE 4 | Hepatopancreas and hemolymph antioxidant status parameters of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days.

	D1	D2
Hepatopancreas		
T-SOD (U/mgprot)	10.4 ± 0.88^{a}	5.61 ± 1.00^{b}
T-AOC (U/mgprot)	0.27 ± 0.01	0.18 ± 0.03
GSH-PX (U/mg prot)	624.12 ± 49.36^{a}	233.92 ± 56.78^{b}
MDA (nmol/mgprot)	1.26 ± 0.03	1.1 ± 0.08
Hemolymph		
T-SOD (U/mL)	273.75 ± 6.08^{a}	239.53 ± 9.52^{b}
T-AOC (U/mL)	3.7 ± 0.12	3.66 ± 0.23
GSH-PX (U/mL)	419.35 ± 54.11	380.65 ± 19.36
MDA (mmol/ml)	8.27 ± 1.04^{a}	3.84 ± 0.21^{b}

Values are mean ± SE (n = 4).
Means in the same row with different superscripts are significantly different (p < 0.05).

Statistical Analysis

Experimental data in the present study are shown as means ± standard error (SE). Data were checked for normality and homogeneity of variance in the software of SPSS 22.0 (Chicago, United States) and then analyzed by independent-sample t-test. $p < 0.05$ was regarded as the significant difference between groups.

RESULT

Growth Performance and Feed Utilization

As shown in **Table 3**, dietary *H. pluvialis* GXU-A23 supplementation significantly altered the growth performance of *L. vannamei*. Significantly higher final body weight (FBW), weight gain rate (WGR) and specific growth rate (SGR) of *L. vannamei* were found in the D2 group than that of the D1 group ($p < 0.05$). However, dietary *H. pluvialis* GXU-A23 supplementation was unable to change the feed conversion ratio (FCR) of *L. vannamei* ($p > 0.05$). After 8 weeks feeding, survival rate (SR) of *L. vannamei* fed with/without *H. pluvialis* GXU-A23 were 96% approximately ($p > 0.05$).

Oxidative Status Parameters

Antioxidant parameters of *L. vannamei* under different dietary intervention were shown in **Table 4**. Results showed that enzyme activities of hepatopancreas T-SOD, hepatopancreas GSH-PX as well as hemolymph T-SOD were significantly decreased in the D2 group than that in the D1 group ($p < 0.05$). Meanwhile, relatively lower hepatopancreas MDA content ($p > 0.05$) and remarkably lower hemolymph MDA content ($p < 0.05$) were found in the dietary *H. pluvialis* GXU-A23 supplementation group than the control group. No statistical differences of hepatopancreas T-AOC, hemolymph T-AOC and hemolymph GSH-PX were obtained between two experimental groups ($p > 0.05$).

Hepatopancreas mRNA Expression Related to Immunity

mRNA expression levels of genes related to antioxidation of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 were shown in **Figure 1**. Compared to the control group, the

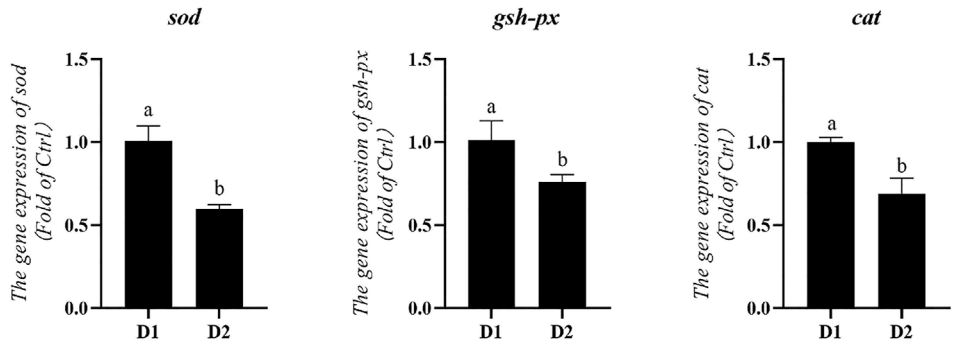

FIGURE 1 | Hepatopancreas mRNA levels of antioxidative genes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days. Values are mean ± SE (n = 4). The small letters indicated significant differences at $p < 0.05$.

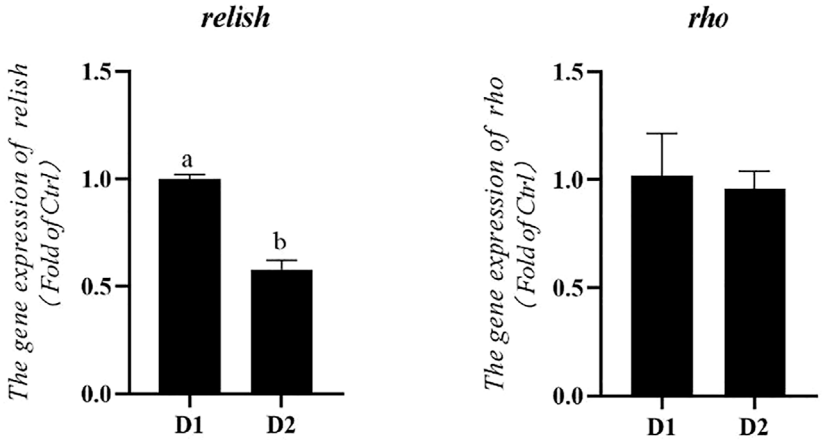

FIGURE 2 | Hepatopancreas mRNA levels of anti-inflammatory genes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days. Values are mean ± SE (n = 4). The small letters indicated significant differences at $p < 0.05$.

dietary *H. pluvialis* GXU-A23 supplementation group obtained significantly lower mRNA expression levels of *sod*, *gsh-px* and *cat* ($p < 0.05$).

mRNA expression levels of anti-inflammatory genes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 were shown in **Figure 2**. Remarkably lower mRNA expression level of relish was obtained in the D2 group compared to the control group ($p < 0.05$). No statistical difference of the *rho* mRNA expression level was observed between two groups ($p > 0.05$).

Hepatopancreas mRNA Expression Related to Digestive and Metabolic Enzymes

mRNA expression levels of digestive enzymes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 were shown in **Figure 3**. The mRNA expression level of *chymotrypsin* of *L. vannamei* was significantly increased after dietary *H. pluvialis* GXU-A23 intervention ($p < 0.05$). However, no statistical difference of *trypsin* mRNA expression level was observed between two groups ($p > 0.05$).

Dietary *H. pluvialis* GXU-A23 supplementation significantly altered the mRNA expression level of metabolic enzymes of *L. vannamei* (**Figure 4**). mRNA expression levels of *hexokinase* (*hk*) and *fatty acid synthase* (*fas*) were significantly higher in the *H. pluvialis* GXU-A23 treatment group compared to the control group ($p < 0.05$).

Light Microscopy Observation of Mid-intestine Morphology

Light microscopy of mid-intestine morphology of *L. vannamei* exposed to different dietary treatment for 56 days was shown in **Figure 5**. Results showed that the intestinal mucosal layer thickness and villa height of *L. vannamei* fed with *H. pluvialis* GXU-A23 was significantly higher than that of the control group ($p < 0.05$).

DISCUSSION

In recent years, microalgae had been gained widely attention in aquafeeds due to it is the green additive with high nutrition (Roy

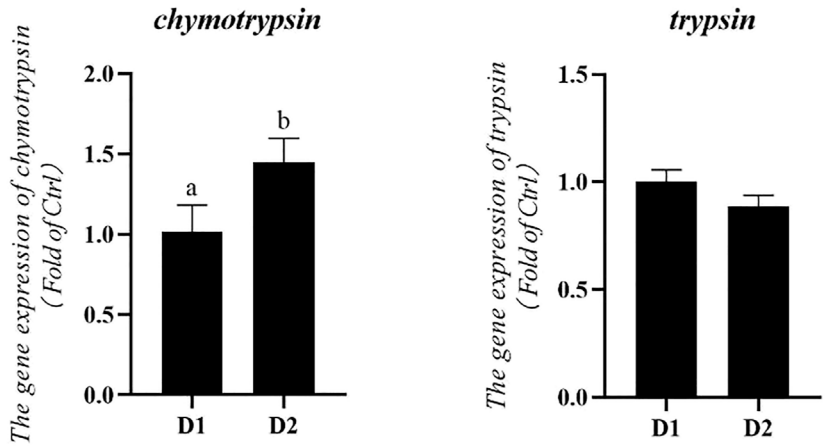

FIGURE 3 | Hepatopancreas mRNA levels of digestive enzyme genes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days. Values are mean ± SE (n = 4). The small letters indicated significant differences at $p < 0.05$.

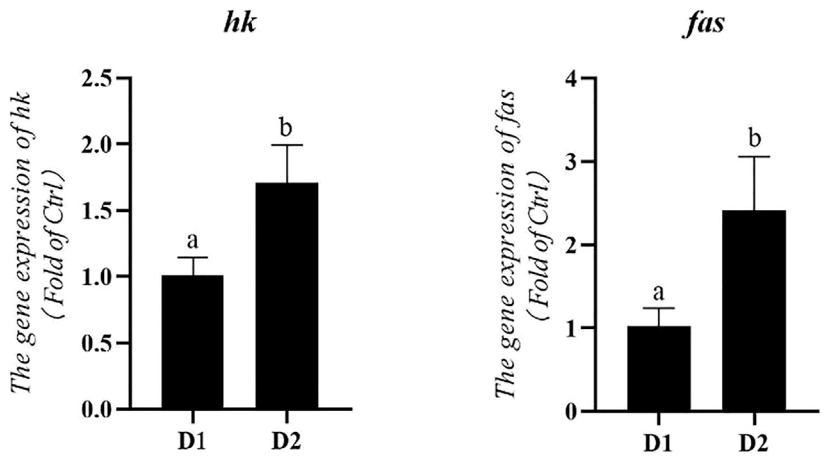

FIGURE 4 | Hepatopancreas mRNA levels of metabolic enzymes genes of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days. Values are mean ± SE (n = 4). The small letters indicated significant differences at $p < 0.05$.

and Pal, 2015). Different microalgae might contain different nutrients, such as high lipid and protein (Aaronson et al., 1980; Webb, 1983), proper amino acid pattern (Becker, 2004), polysaccharide (Chu et al., 1982; Lama et al., 1996), pigments (Metting, 1996) and vitamins (Brown and Farmer, 1994). Supplementation of microalgae in aquafeed can partly substitute for minerals (Fabregas and Herrero, 1986), fishmeal and fish oil (Shah et al., 2018). Microalgae as an aquafeed additive for improving the growth performance and immunity of animals was also widely reported (Cerezuela et al., 2012; Reyes-Becerril et al., 2013, 2014).

In the present study, *L. vannamei* fed with the *H. pluvialis* GXU-A23 diet obtained the better growth performance (WG and SGR) compared to that of the control group. Similar results also reported in *Pseudosciaena crocea* (Li et al., 2014), *Trachinotus ovatus* (Zhao et al., 2021), *L. vannamei* (initial weight: ~ 1.0 g) (Ju et al., 2012). *H. pluvialis* GXU-A23 contains astaxanthin with 3S-3′S type which is the same structure in *Salmo salar* and other aquatic animals (Higuera-Ciapara et al., 2006). The main reason for astaxanthin could improve the growth performance of aquatic animals is that this pigment could mediate intermediate metabolism, resulting in enhancing nutrients utilization and thus optimization of the growth performance of *L. vannamei* (Han et al., 2018). However, *H. pluvialis* was unable to alter the growth performance in post-larval *L. vannamei* (5 days after metamorphosing of mysis stage) (Xie et al., 2018), *L. vannamei* (initial weight: 0.94–0.99 g) (Ju et al., 2011) and *Cichlasoma citrinellum* (Pan and Chien, 2009). These different results might be attributed to the source and dose used of dietary *H. pluvialis,* the growth stage of animals as well as the experimental environment. Besides, the hepatopancreas mRNA expression level of *chymotrypsin* was upregulated in the *H.*

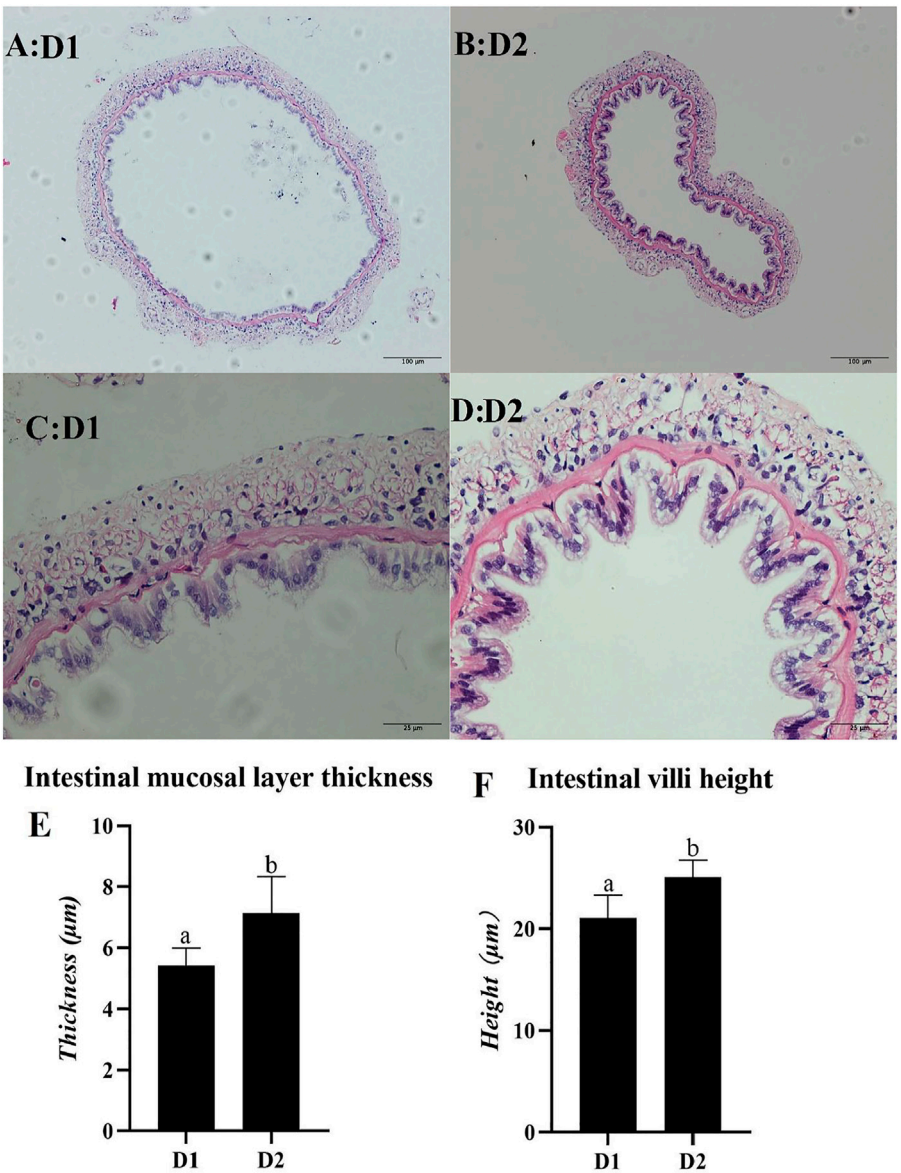

FIGURE 5 | Light microscopy of mid-intestine morphology of *L. vannamei* fed diet supplemented with/without *H. pluvialis* GXU-A23 for 56 days. The scale bars of picture **(A,B)** were 100 μm, while the scale bars of picture **(C,D)** were 25 μm respectively. Picture **(E,F)** represents the intestinal mucosal layer thickness and intestinal villi height of *L. vannamei* respectively. Values are mean ± SE (n = 4). The small letters indicated significant differences at $p < 0.05$.

pluvialis GXU-A23 feeding group than that of the control group. High expression of protease could improve the digestion and absorption of protein, and thus enhancing growth (Zokaeifar et al., 2012). Apart from digestive enzyme, the intestine morphology was strongly contributed to the growth of shrimp. Higher intestinal villi height represented the larger contact surface area between the intestine and nutrients (Emami et al., 2012), and the increasing of intestinal mucosal layer thickness meaning the improvement digestion and absorption ability of shrimp (Chen et al., 2020). In the present study, remarkably higher intestinal villi height and intestinal mucosal layer thickness were found in the dietary *H. pluvialis* GXU-A23 treatment group compared to the control group, indicating that *H. pluvialis* GXU-A23 has protective effect on mid-intestine morphology of *L. vannamei* and thus improves the growth performance of shrimp, which is consistent with the present result.

Generally, aquatic animals have the poor glucose utilization capacity because of the low level of insulin released (Chen et al., 2020). However, glycolysis is the only pathway of glucose metabolism in animals (Li et al., 2018). Among them, hepatic HK was a fundamental limitation enzyme in the glycolysis process (Lu et al., 2018). In the present study, the *H. pluvialis* GXU-A23 feeding *L. vannamei* group obtained higher

hepatopancreas mRNA expression level of *hk* than the control group, indicating that dietary *H. pluvialis* GXU-A23 supplementation could improve the utilization capacity of blood glucose for satisfying higher energy requirement. Apart from glucose metabolism, lipid metabolism also plays a major role in health of aquatic animals. In particular, FAS plays an essential role in lipogenesis by catalyzing the *de novo* biosynthesis of fatty acids (Lu et al., 2018). In the present study, higher mRNA expression level of *fas* in the *L. vannamei* fed with *H. pluvialis* GXU-A23 group than that in the control group, indicating dietary *H. pluvialis* GXU-A23 supplementation was beneficial for the synthesis of hepatopancreas fatty acids.

When shrimp was subjected to environmental pressures, the breathing burst would be occurred to produce reactive oxygen species (ROS) for attacking invading microorganisms (Zhao et al., 2020). However, overproduction ROS might attack normal cells and then cause oxidative damages to shrimp. To avoid the riskiness of ROS, cells have developed an antioxidant system which involve various antioxidant enzymes, like SOD, GSH-PX, CAT (Zhao et al., 2017). In the present study, significantly lower antioxidant enzyme activities (hepatopancreas T-SOD, hepatopancreas GSH-PX and hemolymph T-SOD) as well as hepatopancreas mRNA expression levels (*sod*, *gsh-px* and *cat*) were obtained in dietary *H. pluvialis* GXU-A23 treatment group compared to the control group. Lower antioxidant parameters in the D2 group was attributed to the astaxanthin in *H. pluvialis* GXU-A23, which contains the ionone ring with hydroxyl and keto and thus it could scavenge ROS in crustaceans (Ambati et al., 2014). As a result, *L. vannamei* was unnecessary to produce more antioxidant enzymes. MDA is a lipid peroxidation product which is generally regarded as an essential parameter to evaluate the oxidative damage of animals (Larbi Ayisi et al., 2018). In the present study, *L. vannamei* fed with *H. pluvialis* GXU-A23 diet obtained the remarkably lower hemolymph MDA compared to the control group, indicating *H. pluvialis* GXU-A23 could prohibit the lipid peroxidation of cells and enhance the antioxidant capacity of *L. vannamei*.

Except for the antioxidant system, aquatic animals also responses to environmental stresses by regulating inflammatory responses (Fazelan et al., 2020). If subjected to stress, inflammatory mediators (like cytokines or prostaglandins) would be produced in cells for mediating the inflammatory system to remove detrimental irritations (Boltana et al., 2018). However, excessive inflammation response might lead to various pathological diseases, such as fever (Evans et al., 2015), loss of tissue function (Takeuchi and Akira, 2010). NF-κB signal pathway is closely correlated with the pathogenesis of inflammatory diseases (Yu et al., 2020). Among them, relish was a key NF-κB family protein in *L. vannamei* (Qiu et al., 2014). In the present study, the mRNA expression level of relish in the dietary *H. pluvialis* GXU-A23 supplementation group was significantly higher than that of the control group, indicating *H. pluvialis* GXU-A23 have a positive effect on inhibiting the NF-κB singal pathway. The prohibition of NF-κB pathway might narrow the production of pro-inflammatory cytokines, resulting in mitigating inflammatory responses (Xie et al., 2011). Therefore, *H. pluvialis* GXU-A23 plays an important role in alleviating inflammatory responses of *L. vannamei.*

CONCLUSION

Overall, our present study demonstrated that dietary *H. pluvialis* GXU-A23 supplementation enhanced the growth performance of *L. vannamei* by improving antioxidant and anti-inflammatory status, metabolic metabolism and mid-intestine morphology. Therefore, 50 g/kg *H. pluvialis* GXU-A23 was recommended for the *L. vannamei* feed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Experimental Animal Ethics Committee of Sun-Yat San University.

AUTHOR CONTRIBUTIONS

HF, JN, and WZ designed the study, LH culture the *Haematococcus pluvialis* GXU-A23, HF and ZZ analyzed data, HF carried out the experiment and wrote this paper, ZW modified the language.

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REFERENCES

Aaronson, S., Berner, T., and Dubinsky, Z. (1980). "Microalgae as a Source of Chemicals and Natural Products," in *Algae Biomass Prod.* Amsterdam, Netherlands: Elsevier Press. Editors G. Shelef and C. J. Soeder.

Ambati, R., Phang, S.-M., Ravi, S., and Aswathanarayana, R. (2014). Astaxanthin: Sources, Extraction, Stability, Biological Activities and its Commercial Applications-A Review. *Mar. Drugs* 12, 128–152. doi:10.3390/md12010128

Becker, W., (2004). "18 Microalgae in Human and Animal Nutrition," in *Handbook of Microalgal Culture: Biotechnology and Applied Phycology* (United States: Wiley Online Library).

Boltana, S., Sanhueza, N., Donoso, A., Aguilar, A., Crespo, D., Vergara, D., et al. (2018). The Expression of TRPV Channels, Prostaglandin E2 and Pro-inflammatory Cytokines during Behavioural Fever in Fish. *Brain Behav. Immun.* 71, 169–181. doi:10.1016/j.bbi.2018.03.023

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., et al. (2010). Aquaculture: Global Status and Trends. *Phil. Trans. R. Soc. B* 365, 2897–2912. doi:10.1098/rstb.2010.0170

Brown, M. R., and Farmer, C. L. (1994). Riboflavin Content of Six Species of Microalgae Used in Mariculture. *J. Appl. Phycol.* 6, 61–65. doi:10.1007/bf02185905

Cerezuela, R., Guardiola, F. A., Meseguer, J., and Esteban, M. Á. (2012). Enrichment of Gilthead Seabream (*Sparus aurata* L.) Diet with Microalgae: Effects on the Immune System. *Fish. Physiol. Biochem.* 38, 1729–1739. doi:10.1007/s10695-012-9670-9

Chen, M., Chen, X.-Q., Tian, L.-X., Liu, Y.-J., and Niu, J. (2020). Beneficial Impacts on Growth, Intestinal Health, Immune Responses and Ammonia Resistance of pacific white Shrimp (Litopenaeus Vannamei) Fed Dietary Synbiotic (Mannan Oligosaccharide and Bacillus Licheniformis). *Aquacult. Rep.* 17, 100408. doi:10.1016/j.aqrep.2020.100408

Chu, F.-L. E., Dupuy, J. L., and Webb, K. L. (1982). Polysaccharide Composition of Five Algal Species Used as Food for Larvae of the American Oyster, *Crassostrea virginica*. *Aquacul.* 29, 241–252. doi:10.1016/0044-8486(82)90138-7

Costello, C., Cao, L., Gelcich, S., Cisneros-Mata, M. Á., Free, C. M., Froehlich, H. E., et al. (2020). The Future of Food from the Sea. *Nature* 588, 95–100. doi:10.1038/s41586-020-2616-y

Cottrell, R. S., Metian, M., Froehlich, H. E., Blanchard, J. L., Sand Jacobsen, N., McIntyre, P. B., et al. (2021). Time to Rethink Trophic Levels in Aquaculture Policy. *Rev. Aquac.* 13, 1583–1593. doi:10.1111/raq.12535

Diler, I., and Gokoglu, N. (2004). Investigation of the Sensory Properties of the Flesh of Rainbow trout (*Oncorhynchus mykiss*) Fed Diets with Astaxanthin, Shrimp Waste Meal and Red Pepper Meal. *Eur. Food Res. Technol.* 219, 217–222. doi:10.1007/s00217-004-0923-4

Domınguez-Bocanegra, A. R., Legarreta, I. G., Jeronimo, F. M., and Campocosio, A. T. (2004). Influence of Environmental and Nutritional Factors in the Production of Astaxanthin from Haematococcus pluvialis. *Bioresour. Technol.* 92, 209–214. doi:10.1016/j.biortech.2003.04.001

Duan, Y., Wang, Y., Zhang, J., Liu, Q., and Ding, X. (2018). Morphologic, Digestive Enzymes and Immunological Responses of Intestine from Litopenaeus Vannamei after Lipopolysaccharide Injection. *J. Invertebr. Pathol.* 153, 186–194. doi:10.1016/j.jip.2018.03.003

Evans, S. S., Repasky, E. A., and Fisher, D. T. (2015). Fever and the thermal Regulation of Immunity: the Immune System Feels the Heat. *Nat. Rev. Immunol.* 15, 335–349. doi:10.1038/nri3843

Fabregas, J., and Herrero, C. (1986). Marine Microalgae as a Potential Source of Minerals in Fish Diets. *Aquaculture* 51, 237–243. doi:10.1016/0044-8486(86)90315-7

Fang, H., Xie, J., Liao, S., Guo, T., Xie, S., Liu, Y., et al. (2019). Effects of Dietary Inclusion of Shrimp Paste on Growth Performance, Digestive Enzymes Activities, Antioxidant and Immunological Status and Intestinal Morphology of Hybrid Snakehead (Channa Maculata ♀ × *Channa argus* ♂). *Front. Physiol.* 10, 1027. doi:10.3389/fphys.2019.01027

Fang, H., Zhao, W., Xie, J., Yin, P., Zhuang, Z., Liu, Y., et al. (2021). Effects of Dietary Lipid Levels on Growth Performance, Hepatic Health, Lipid Metabolism and Intestinal Microbiota on Trachinotus Ovatus. *Aquac. Nutr.* 27, 1554–1568. doi:10.1111/anu.13296

Fazelan, Z., Hoseini, S. M., Yousefi, M., Khalili, M., Hoseinifar, S. H., and Van Doan, H. (2020). Effects of Dietary Eucalyptol Administration on Antioxidant and Inflammatory Genes in Common Carp (*Cyprinus carpio*) Exposed to Ambient Copper. *Aquaculture* 520, 734988. doi:10.1016/j.aquaculture.2020.734988

Gao, B., Yang, J., Lei, X., Xia, S., Li, A., and Zhang, C. (2016). Characterization of Cell Structural Change, Growth, Lipid Accumulation, and Pigment Profile of a Novel Oleaginous Microalga, Vischeria Stellata (Eustigmatophyceae), Cultured with Different Initial Nitrate Supplies. *J. Appl. Phycol.* 28, 821–830. doi:10.1007/s10811-015-0626-1

Guzmán-Villanueva, L. T., Escobedo-Fregoso, C., Barajas-Sandoval, D. R., Gomez-Gil, B., Peña-Rodríguez, A., Martínez-Diaz, S. F., et al. (2020). Assessment of Microbial Dynamics and Antioxidant Enzyme Gene Expression Following Probiotic Administration in Farmed Pacific white Shrimp (Litopenaeus Vannamei). *Aquaculture* 519, 734907. doi:10.1016/j.aquaculture.2019.734907

Han, T., Li, X., Wang, J., Wang, C., Yang, M., and Zheng, P. (2018). Effects of Dietary Astaxanthin (AX) Supplementation on Pigmentation, Antioxidant Capacity and Nutritional Value of Swimming Crab, Portunus Trituberculatus. *Aquac.* 490, 169–177. doi:10.1016/j.aquaculture.2018.02.030

Higuera-Ciapara, I., Félix-Valenzuela, L., and Goycoolea, F. M. (2006). Astaxanthin: a Review of its Chemistry and Applications. *Crit. Rev. Food Sci. Nutr.* 46, 185–196. doi:10.1080/10408690590957188

Horwitz, W. (20101997). "Official Methods of Analysis of AOAC International," in *Volume I, Agricultural Chemicals, Contaminants, Drugs*. Editor W. Horwitz (Gaithersburg (Maryland): AOAC International).

Ip, P.-F., and Chen, F. (2005). Production of Astaxanthin by the green Microalga Chlorella Zofingiensis in the Dark. *Process Biochem.* 40, 733–738. doi:10.1016/j.procbio.2004.01.039

Johnson, E. A., and Lewis, M. J. (1979). Astaxanthin Formation by the Yeast Phaffia Rhodozyma. *J. Gen. Microbiol.* 115, 173–183. doi:10.1099/00221287-115-1-173

Ju, Z. Y., Deng, D.-F., and Dominy, W. (2012). A Defatted Microalgae (Haematococcus pluvialis) Meal as a Protein Ingredient to Partially Replace Fishmeal in Diets of Pacific white Shrimp (Litopenaeus Vannamei, Boone, 1931). *Aquaculture* 354-355 (355), 50–55. doi:10.1016/j.aquaculture.2012.04.028

Ju, Z. Y., Deng, D.-F., Dominy, W. G., and Forster, I. P. (2011). Pigmentation of Pacific white Shrimp, Litopenaeus Vannamei, by Dietary Astaxanthin Extracted from Haematococcus pluvialis. *J. World Aquac. Soc.* 42, 633–644. doi:10.1111/j.1749-7345.2011.00511.x

Khodambashi Emami, N., Samie, A., Rahmani, H. R., and Ruiz-Feria, C. A. (2012). The Effect of Peppermint Essential Oil and Fructooligosaccharides, as Alternatives to Virginiamycin, on Growth Performance, Digestibility, Gut Morphology and Immune Response of Male Broilers. *Anim. Feed Sci. Tech.* 175, 57–64. doi:10.1016/j.anifeedsci.2012.04.001

Lama, L., Nicolaus, B., Calandrelli, V., Manca, M. C., Romano, I., and Gambacorta, A. (1996). Effect of Growth Conditions on Endo- and Exopolymer Biosynthesis in Anabaena Cylindrica 10 C. *Phytochemistry* 42, 655–659. doi:10.1016/0031-9422(95)00985-x

Larbi Ayisi, C., Zhao, J., and Wu, J.-W. (2018). Replacement of Fish Oil with palm Oil: Effects on Growth Performance, Innate Immune Response, Antioxidant Capacity and Disease Resistance in Nile tilapia (*Oreochromis niloticus*). *PLoS One* 13, e0196100. doi:10.1371/journal.pone.0196100

Li, M., Wu, W., Zhou, P., Xie, F., Zhou, Q., and Mai, K. (2014). Comparison Effect of Dietary Astaxanthin and Haematococcus pluvialis on Growth Performance, Antioxidant Status and Immune Response of Large Yellow Croaker Pseudosciaena Crocea. *Aquaculture* 434, 227–232. doi:10.1016/j.aquaculture.2014.08.022

Li, R., Liu, H., Dong, X., Chi, S., Yang, Q., Zhang, S., et al. (2018). Molecular Characterization and Expression Analysis of Glucose Transporter 1 and Hepatic Glycolytic Enzymes Activities from Herbivorous Fish *Ctenopharyngodon idellus* in Respond to a Glucose Load after the Adaptation to Dietary Carbohydrate Levels. *Aquaculture* 492, 290–299. doi:10.1016/j.aquaculture.2018.04.028

Li, Y., Miao, F., Geng, Y., Lu, D., Zhang, C., and Zeng, M. (2012). Accurate Quantification of Astaxanthin from Haematococcus Crude Extract Spectrophotometrically. *Chin. J. Ocean. Limnol.* 30, 627–637. doi:10.1007/s00343-012-1217-5

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *methods* 25, 402–408. doi:10.1006/meth.2001.1262

Lu, S., Wu, X., Gao, Y., Gatlin, D. M., III, Wu, M., Yao, W., et al. (2018). Effects of Dietary Carbohydrate Sources on Growth, Digestive Enzyme Activity, Gene Expression of Hepatic GLUTs and Key Enzymes Involved in Glycolysis-Gluconeogenesis of Giant Grouper Epinephelus Lanceolatus Larvae. *Aquaculture* 484, 343–350. doi:10.1016/j.aquaculture.2017.07.033

Metting, F. B. (1996). Biodiversity and Application of Microalgae. *J. Ind. Microbiol. Biotechnol.* 17, 477–489. doi:10.1007/bf01574779

Nahidian, B., Ghanati, F., Shahbazi, M., and Soltani, N. (2018). Effect of Nutrients on the Growth and Physiological Features of Newly Isolated Haematococcus pluvialis TMU1. *Bioresour. Tech.* 255, 229–237. doi:10.1016/j.biortech.2018.01.130

Ni, Y., Nagashimada, M., Zhuge, F., Zhan, L., Nagata, N., Tsutsui, A., et al. (2015). Astaxanthin Prevents and Reverses Diet-Induced Insulin Resistance and Steatohepatitis in Mice: A Comparison with Vitamin E. *Sci. Rep.* 5, 1–15. doi:10.1038/srep17192

Niu, J., Tian, L.-X., Liu, Y.-J., Yang, H.-J., Ye, C.-X., Gao, W., et al. (2009). Effect of Dietary Astaxanthin on Growth, Survival, and Stress Tolerance of Postlarval Shrimp, Litopenaeus Vannamei. *J. World Aquac. Soc.* 40, 795–802. doi:10.1111/j.1749-7345.2009.00300.x

Pan, C.-H., and Chien, Y.-H. (2009). Effects of Dietary Supplementation of algaHaematococcus pluvialis(Flotow), Synthetic Astaxanthin and β-carotene on Survival, Growth, and Pigment Distribution of Red Devil,Cichlasoma citrinellum(Günther). *Aquac. Res.* 40, 871–879. doi:10.1111/j.1365-2109.2008.02153.x

Petit, H., Nègre-Sadargues, G., Castillo, R., and Trilles, J.-P. (1997). The Effects of Dietary Astaxanthin on Growth and Moulting Cycle of Postlarval Stages of the Prawn, Penaeus Japonicus (Crustacea, Decapoda). *Comp. Biochem. Physiol. A: Physiol.* 117, 539–544. doi:10.1016/s0300-9629(96)00431-8

Qiu, W., Zhang, S., Chen, Y.-G., Wang, P.-H., Xu, X.-P., Li, C.-z., et al. (2014). Litopenaeus Vannamei NF-Kb Is Required for WSSV Replication. *Dev. Comp. Immunol.* 45, 156–162. doi:10.1016/j.dci.2014.02.016

Reyes-Becerril, M., Angulo, C., Estrada, N., Murillo, Y., and Ascencio-Valle, F. (2014). Dietary Administration of Microalgae Alone or Supplemented with Lactobacillus Sakei Affects Immune Response and Intestinal Morphology of Pacific Red Snapper (Lutjanus peru). *Fish Shellfish Immunol.* 40, 208–216. doi:10.1016/j.fsi.2014.06.032

Reyes-Becerril, M., Guardiola, F., Rojas, M., Ascencio-Valle, F., and Esteban, M. Á. (2013). Dietary Administration of Microalgae Navicula Sp. Affects Immune Status and Gene Expression of Gilthead Seabream (*Sparus aurata*). *Fish Shellfish Immunol.* 35, 883–889. doi:10.1016/j.fsi.2013.06.026

Romero, J., Feijoó, C. G., and Navarrete, P. (2012). Antibiotics in Aquaculture–Use, Abuse and Alternatives. *Heal. Environ. Aquac.* 159, 159–198.

Roy, S. S., and Pal, R. (2015). Microalgae in Aquaculture: a Review with Special References to Nutritional Value and Fish Dietetics, *Proc. Zool Soc.* 68. 1–8. doi:10.1007/s12595-013-0089-9

Santos, L., and Ramos, F. (2016). Analytical Strategies for the Detection and Quantification of Antibiotic Residues in Aquaculture Fishes: A Review. *Trends Food Sci. Tech.* 52, 16–30. doi:10.1016/j.tifs.2016.03.015

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaeimehr, A., et al. (2018). Microalgae in Aquafeeds for a Sustainable Aquaculture Industry. *J. Appl. Phycol.* 30, 197–213. doi:10.1007/s10811-017-1234-z

Sørum, H. (2005). "Antimicrobial Drug Resistance in Fish Pathogens". in *Antimicrob. Resist. Bact. Anim. Orig.*, 213–238. Hoboken, NJ, United States: Wiley Press

Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., and Morales-Covarrubias, M. S. (2015). Field and Experimental Evidence of Vibrio Parahaemolyticus as the Causative Agent of Acute Hepatopancreatic Necrosis Disease of Cultured Shrimp (Litopenaeus Vannamei) in Northwestern Mexico. *Appl. Environ. Microbiol.* 81, 1689–1699. doi:10.1128/aem.03610-14

Takeuchi, O., and Akira, S. (2010). Pattern Recognition Receptors and Inflammation. *Cell* 140, 805–820. doi:10.1016/j.cell.2010.01.022

Verbruggen, B., Bickley, L., Van Aerle, R., Bateman, K., Stentiford, G., Santos, E., et al. (2016). Molecular Mechanisms of white Spot Syndrome Virus Infection and Perspectives on Treatments. *Viruses* 8, 23. doi:10.3390/v8010023

Wang, F., Gao, B., Wu, M., Huang, L., and Zhang, C. (2019). A Novel Strategy for the Hyper-Production of Astaxanthin from the Newly Isolated Microalga Haematococcus pluvialis JNU35. *Algal Res.* 39, 101466. doi:10.1016/j.algal.2019.101466

Wang, W., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M. A., and Zhang, Y. (2018). Effects of Dietary Astaxanthin Supplementation on Survival, Growth and Stress Resistance in Larval and post-larval Kuruma Shrimp, *Marsupenaeus japonicus*. *Aquac. Res.* 49, 2225–2232. doi:10.1111/are.13679

Webb, K. L. (1983). "Phytoplankton as a Food Source for Bivalve Larvae," in Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfis, Baton Rouge: Lousiana State University Press, 272–291.

Xie, C., Kang, J., Ferguson, M. E., Nagarajan, S., Badger, T. M., and Wu, X. (2011). Blueberries Reduce Pro-inflammatory Cytokine TNF-α and IL-6 Production in Mouse Macrophages by Inhibiting NF-Kb Activation and the MAPK Pathway. *Mol. Nutr. Food Res.* 55, 1587–1591. doi:10.1002/mnfr.201100344

Xie, J., Fang, H., He, X., Liao, S., Liu, Y., Tian, L., et al. (2020). Study on Mechanism of Synthetic Astaxanthin and Haematococcus pluvialis Improving the Growth Performance and Antioxidant Capacity under Acute Hypoxia Stress of golden Pompano (Trachinotus Ovatus) and Enhancing Anti-inflammatory by Activating Nrf2-ARE Pathway to Antagonize the NF-Kb Pathway. *Aquaculture* 518, 734657. doi:10.1016/j.aquaculture.2019.734657

Xie, S., Fang, W., Wei, D., Liu, Y., Yin, P., Niu, J., et al. (2018). Dietary Supplementation of Haematococcus pluvialis Improved the Immune Capacity and Low Salinity Tolerance Ability of post-larval white Shrimp, Litopenaeus Vannamei. *Fish Shellfish Immunol.* 80, 452–457. doi:10.1016/j.fsi.2018.06.039

Xie, S., Wei, D., Fang, W., Yin, P., Liu, Y., Niu, J., et al. (2020). Survival and Protein Synthesis of post-larval White Shrimp, Litopenaeus Vannamei Were Affected by Dietary Protein Level. *Anim. Feed Sci. Tech.* 263, 114462. doi:10.1016/j.anifeedsci.2020.114462

Xie, S., Wei, D., Yin, P., Zheng, L., Guo, T., Liu, Y., et al. (2019). Dietary Replacement of Fish-Meal Impaired Protein Synthesis and Immune Response of Juvenile Pacific white Shrimp, Litopenaeus Vannamei at Low Salinity. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 228, 26–33. doi:10.1016/j.cbpb.2018.11.002

Yang, P., He, C., Qin, Y., Wang, W., Mai, K., Qin, Q., et al. (2021). Evaluation of Composite Mixture of Protein Sources in Replacing Fishmeal for Pacific white Shrimp (Litopenaeus Vannamei): Based on the Changing Pattern of Growth Performance, Nutrient Metabolism and Health Status. *Aquacult. Rep.* 21, 100914. doi:10.1016/j.aqrep.2021.100914

Yu, H., Lin, L., Zhang, Z., Zhang, H., and Hu, H. (2020). Targeting NF-Kb Pathway for the Therapy of Diseases: Mechanism and Clinical Study. *Signal. Transduct. Target. Ther.* 5, 209–223. doi:10.1038/s41392-020-00312-6

Yu, Y.-Y., Chen, W.-D., Liu, Y.-J., Niu, J., Chen, M., and Tian, L.-X. (2016). Effect of Different Dietary Levels of Gracilaria Lemaneiformis Dry Power on Growth Performance, Hematological Parameters and Intestinal Structure of Juvenile Pacific white Shrimp (Litopenaeus Vannamei). *Aquaculture* 450, 356–362. doi:10.1016/j.aquaculture.2015.07.037

Zhang, Y., Yu, J., Su, Y., Du, Y., and Liu, Z. (2019). Long-term Changes of Water Quality in Aquaculture-Dominated Lakes as Revealed by Sediment Geochemical Records in Lake Taibai (Eastern China). *Chemosphere* 235, 297–307. doi:10.1016/j.chemosphere.2019.06.179

Zhao, W., Fang, H.-H., Gao, B.-Y., Dai, C.-M., Liu, Z.-Z., Zhang, C.-W., et al. (2020). Dietary Tribonema Sp. Supplementation Increased Growth Performance, Antioxidant Capacity, Immunity and Improved Hepatic Health in golden Pompano (Trachinotus Ovatus). *Aquaculture* 529, 735667. doi:10.1016/j.aquaculture.2020.735667

Zhao, W., Fang, H. H., Liu, Z. Z., Huang, M. Q., Su, M., Zhang, C. W., et al. (2021). A Newly Isolated Strain of Haematococcus pluvialis JNU35 Improves the Growth, Antioxidation, Immunity and Liver Function of golden Pompano (Trachinotus Ovatus). *Aquacult. Nutr.* 27, 342–354. doi:10.1111/anu.13188

Zhao, W., Wang, L., Liu, M., Jiang, K., Wang, M., Yang, G., et al. (2017). Transcriptome, Antioxidant Enzyme Activity and Histopathology Analysis of Hepatopancreas from the white Shrimp Litopenaeus Vannamei Fed with Aflatoxin B1(AFB1). *Dev. Comp. Immunol.* 74, 69–81. doi:10.1016/j.dci.2017.03.031

Zokaeifar, H., Balcázar, J. L., Saad, C. R., Kamarudin, M. S., Sijam, K., Arshad, A., et al. (2012). Effects of Bacillus Subtilis on the Growth Performance, Digestive Enzymes, Immune Gene Expression and Disease Resistance of white Shrimp, Litopenaeus Vannamei. *Fish Shellfish Immunol.* 33, 683–689. doi:10.1016/j.fsi.2012.05.027

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On the Utilization of Dietary Glycerol in Carnivorous Fish - Part I: Insights Into Hepatic Carbohydrate Metabolism of Juvenile Rainbow Trout (*Oncorhynchus mykiss*) and European Seabass (*Dicentrarchus labrax*)

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Glycerol is a 3-carbon sugar alcohol successfully employed as an alternative feed ingredient for land-farmed animals and more recently for farmed fish. While most studies address zootechnical performance, few have delved into the metabolic utilization of dietary glycerol. A growth trial was performed using diets with increasing levels of glycerol (0, 2.5 and 5%) on two relevant species for aquaculture: rainbow trout (8-week trial; 3 tank per diet/25 fish per tank, on a 15 ± 1°C flow-through freshwater system); and European seabass (6-week trial; 5 tank per diet/6-8 fish per tank on a 21°C indoor saltwater RAS system). After this period, fish were subjected to a metabolic trial consisting of a 6-day residence in deuterated water (^2H_2O). Measurements of blood glucose and hepatic glycogen ^2H-enrichments through Nuclear Magnetic Resonance, complemented by mRNA levels of key-enzymes for intermediary metabolism were used to evaluate the catabolic pathways of dietary glycerol. Dietary glycerol had no impact on plasma glucose, but hepatic glycogen levels increased significantly with increasing dietary glycerol levels in both species. While trout was able to regulate circulating glycerol plasma, seabass presented elevated levels on the glycerol-supplemented diets. Despite revealing some significant differences between sampling time (6 and 24 h), none of the enzymes' mRNA levels responded to the dietary treatment. In trout, the main source of blood glucose was not labeled with ^2H (~60%, likely from diet) while other contributors did not differ with glycerol supplementation. In seabass, the unlabeled contribution was approximately half of that observed in trout (~30%), accompanied by a significant increase of gluconeogenic

contributions at the triose-phosphate level to the blood glucose with increasing dietary glycerol. In trout, labeling from ^2H_2O into hepatic glycogen revealed significant differences, with the contribution from the indirect pathway at the triose-phosphate level increasing with increased dietary glycerol. No such differences were found in seabass' glycogen pool. These findings suggest that fish species are able to retain, catabolize glycerol and incorporate it into carbohydrates. The gluconeogenic utilization of exogenous glycerol differed between species and affected the synthesis of hepatic glycogen in trout and the appearance of blood glucose in seabass.

Keywords: ^{2}H NMR, aquaculture, blood glucose, circular economy, deuterated water, glycerol, hepatic glycogen

1 INTRODUCTION

Aquaculture remains highly dependent on fishmeal protein and its procurement still relies on exploiting wild fisheries, a concern heightened by an increasing production of carnivorous fish species. For intensive fish farming, the provision of alternative ingredients that reduce dependence on fishmeal protein yields economic and environmental benefits while improving the sustainability and overall public perception of the industry (Hua et al., 2019). Under the scope of circular economy and zero waste (European Commission, 2020) new purposes have been equated to industrial by-products being used as raw materials for aquafeeds. Glycerol (1,2,3-propanetriol or glycerine; ChEBI: 17754) is the by-product of biodiesel production, which is projected to increase from 36 bln L in 2017 to 39 bln L by 2027 (OECD/FAO, 2019). This water-soluble, almost colorless, odorless, viscous, hygroscopic 3-carbon sugar alcohol is abundant, affordable and available for different purposes. These include pharmaceuticals, cosmetics (Ayoub and Abdullah, 2012), chemical feedstock and animal feeds (Yang et al., 2012). It has been successfully used as an alternative feed ingredient for land-farmed animals including swine (Lammers et al., 2008; Mendoza et al., 2010; Carvalho P.L.D.O. et al., 2012; Oliveira et al., 2014), poultry (Cerrate et al., 2006; Min et al., 2010) and cattle (Chung et al., 2007; Donkin, 2008; Carvalho E.R. et al., 2012).

Its potential as an alternative ingredient for farmed fish feed has been thoroughly assessed in Nile tilapia (*Oreochromis niloticus*) (Neu et al., 2013; Gonçalves et al., 2015; Moesch et al., 2016; Costa et al., 2017; Santos et al., 2019) and occasionally in other fish species like channel catfish (*Ictalurus punctatus*) (Li et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Menton et al., 1986; Magnoni et al., 2021), gilthead seabream (*Sparus aurata*) (Silva et al., 2012) or from the genus *Rhadmia* (Balen et al., 2014; Theisen et al., 2020). While addressing zootechnical performance, few of these studies delved into the actual metabolic utilization of dietary glycerol, an endogenous metabolite at the crossroad of several metabolic pathways. Once absorbed and converted to glycerol-3-phosphate (G3P) by glycerol kinase, glycerol intersects with the glycolytic, gluconeogenic or lipogenic pathways with putative metabolic implications.

When compared with control diets, glycerol-supplemented diets in fish affect glycemia in a species-specific manner with often erratic, non-dose-dependent patterns (if compared at 24 h after last meal). In rainbow trout, while no alterations were reported in inclusions up to 5% glycerol (Panserat et al., 2020), 6% inclusions provoked a significant increase in glycemia (Menton et al., 1986). In channel catfish, glycemia increased at 5% inclusion but remained at control levels up to 20% inclusion (Li et al., 2010). On the contrary, in curimbatá (*Prochilodus lineatus*), glycemia actually decreased at 4% inclusion and then sustained control levels up to 20% inclusion (Balen et al., 2017). Finally, inclusions up to 10% glycerol resulted in unaltered glycemia in Nile tilapia (Neu et al., 2013). To our knowledge, it is only recently that there has been some insight into the molecular and nutritional regulation of hepatic glycerol-related enzymes in fish (Panserat et al., 2020). However, the homeostatic mechanisms triggered in response to circulating dietary glycerol are yet to be addressed, namely the down-stream alterations into a metabolic rearrangement of the energetic substrates to regulate glycemia and store hepatic glycogen.

The use of deuterated water (^2H_2O) is a safe, practical and reliable method to, not only demonstrate the metabolic utilization of glycerol as energy substrate, but also discriminate the metabolic pathways involved. By adding ^2H_2O directly to the tank water, deuterium (^2H) is rapidly incorporated into the fish body water (Viegas et al., 2011). Then, following well-defined metabolic steps, ^2H is incorporated into pathway-dependent stereospecific positions of hepatic glucose 6-phosphate (G-6-P), generating unique labeling patterns, as described in Viegas et al. (2015). This method, when paired with the evaluation of mRNA abundance of the key enzymes involved in hepatic intermediary metabolism, has provided a better understanding of how fish can more efficiently use novel carbohydrate-rich diets (Viegas et al., 2015; Viegas et al., 2016). With this study, we aimed to describe how dietary glycerol affects glycemia, the pathways involved in blood glucose appearance and subsequent rearrangement in the storage of excess glucose as hepatic glycogen. For this purpose,

Abbreviations: ^2H, deuterium; ^2H_2O, deuterated water; AQP, aquaporin; BW, body water; F-6-P, fructose 6-phosphate; *fbp*, fructose-1,6-bisphosphatase (EC 3.1.3.11); G3P, glycerol-3-phosphate; G-6-P, glucose 6-phosphate; *g6pc*, glucose-6-phosphatase (EC 3.1.3.9); *gck*, glucokinase (EC 2.7.1.2); HSI, hepatosomatic index; MAG, monoacetone glucose; *pck*, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); *pk*, pyruvate kinase (EC. 2.7.1.40); RAS, recirculated aquatic system; TW, tank water.

we used as model two representative species of European aquaculture, freshwater rainbow trout (*Oncorhynchus mykiss*) and marine European seabass (*Dicentrarchus labrax*).

2 MATERIAL AND METHODS

2.1 Ethics Statement

This study was conducted under the supervision of accredited experts in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.2 Diet Formulation

Three isoproteic (49% crude protein), isolipid (17% crude lipid) and isoenergetic (~23 kJ kg^{-1}) extruded diets were formulated while fulfilling the known nutritional requirements of both species (Sparos Lda., Olhão, Portugal). An early study with rainbow trout (Menton et al., 1986) reported that 6% supplementation of free glycerol was enough to significantly increase plasma glucose. Hence, a control diet with no glycerol (D0); and two experimental diets, supplemented with 2.5% (D2.5) or 5.0% (D5) glycerol. Further details can be found in **Table 1**. Even if the formulation was the same for both species, for the sake of clarity and context, D0, D2.5 and D5 will hereafter be referred to T0, T2.5, T5 or S0, S2.5, S5, when addressing the rainbow trout or European seabass experiment, respectively.

2.3 Growth Trials – Fish Handling and Sampling

Rainbow trout (hereafter trout; *Oncorhynchus mykiss*) experiments were carried out at the Experimental Research Station of the University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal). The growth trial was started with individuals of 20.2 ± 0.1 g in weight and lasted 8 weeks. Further detail can be found in Magnoni et al. (2021). European seabass (hereafter seabass; *Dicentrarchus labrax*) experiments were carried out at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Porto, Portugal). The growth trial was started with individuals of 51.4 ± 2.6 g in weight and lasted 6 weeks. Further detail can be found in Palma et al. (2019). A summary for both growth trials is presented in **Table 2**.

TABLE 1 | Ingredients and nutrient content of the diets.

Ingredients (g kg^{-1})	Diets		
	D0	**D2.5**	**D5**
Fishmeal Super Prime	125.0	125.0	125.0
Fish protein concentrate	50.0	50.0	50.0
Squid meal	75.0	75.0	75.0
Soy protein concentrate	100.0	100.0	100.0
Pea protein concentrate	50.0	50.0	50.0
Wheat gluten	75.0	75.0	75.0
Corn gluten	75.0	75.0	75.0
Soybean meal 48	100.0	100.0	100.0
Rapeseed meal	50.0	50.0	50.0
Gelatinized starch	50.0	50.0	50.0
Cellulose	50.0	25.0	0.0
Fish oil	130.0	130.0	130.0
Vit & Min Premix PV01	10.0	10.0	10.0
Lutavit C35	1.0	1.0	1.0
Lutavit E50	1.0	1.0	1.0
Soy lecithin	20.0	20.0	20.0
Antioxidant	2.0	2.0	2.0
Sodium propionate	1.0	1.0	1.0
Monocalcium phosphate	10.0	10.0	10.0
Binder	25.0	25.0	25.0
Glycerol	0.0	25.0	50.0
Analyzed nutrient content			
Dry matter (DM, %)	92.12 ± 0.05	91.13 ± 0.04	90.95 ± 0.01
Crude protein, % DM	48.76 ± 0.16	49.20 ± 0.04	50.63 ± 0.03
Crude fat, % DM	17.48 ± 0.04	17.18 ± 0.11	15.87 ± 0.02
Ash, % DM	7.39 ± 0.03	7.22 ± 0.00	7.35 ± 0.03
Crude fiber, % DM	5.13 ± 0.07	3.59 ± 0.04	2.34 ± 0.08
Total carbohydrates, % DM	18.48 ± 0.22	17.53 ± 0.10	17.10 ± 0.02
Gross energy, MJ kg^{-1} DM	23.37 ± 0.02	23.60 ± 0.01	23.62 ± 0.00
Digestible energy, kJ g^{-1}	20.58 ± 0.03	21.38 ± 0.04	21.63 ± 0.04
DP/DE, mg kJ^{-1}	22.26 ± 0.04	21.78 ± 0.02	22.05 ± 0.04

D0, D2.5 and D5 diets including 0, 25 and 50 g kg^{-1} of refined glycerol, respectively. Amino acids in feed as %: Arg 3.03, His 0.98, Ile 1.82, Leu 3.56, Lys 2.73, Thr 1.71, Trp 0.34, Val 2.06, Met 1.03, Cys 0.41, Phe 2.16, Tyr 1.59, Tau 0.17. Asx 3.71, Glx 7.56, Ala 2.24, Gly 2.48, Pro 2.70, and Ser 2.06. Minerals: P 0.96, Ca 0.94, Na 0.39, Mg 0.08, K 0.42 (% feed), Mn 13.01, Zn 42.04, Fe 89.30, Cu 17.02, I 1.26, and Se 0.73 (mg kg^{-1}). Vitamins: Vit A 27617, Vit C 1350, Vit E 600, Vit D 1904 (IU kg^{-1}). DP/DE, digestible protein to digestible energy ratio.

TABLE 2 | Experimental setup and water parameters for the growth trials performed with rainbow trout (*O. mykiss*) and European seabass (*D. labrax*) fed with different levels of glycerol (0, 2.5, and 5%).

	Experimental setup					Water parameters		Initial weight (g)
	Duration	Design	Tank volume	System	Fish per tank	Temperature	Salinity	
Rainbow trout (*Oncorhynchus mykiss*)	8 weeks	3 tanks x 3 diets	300 L	Open (flow-through)	25	15 ± 1°C	0	20.2 ± 0.1
European seabass (*Dicentrarchus labrax*)	6 weeks	5 tanks x 3 diets	80 L	RAS	6-8	22 ± 1°C	30	51.4 ± 2.6

Water temperature and fish weight given as mean ± SD.

2.4 Molecular Analysis of Intermediary Metabolism

At the end of the growth trial, two to three fish per tank were sampled at 6 h and 24 h (n=8 trout and n=7 seabass, per diet, per sampling time) after last meal. Last meal was administered at 08:00, 10 min apart for each tank to compensate for time dedicated to sampling. This is particularly relevant for synchronizing the sampling 6 h after last meal. Fish were anesthetized using well-aerated water from the rearing system containing MS-222 (3-aminobenzoic acid ethyl ester, 0.1 g L^{-1}), buffered with $NaHCO_3$ (0.2 g L^{-1}). Anesthetized fish were weighed, and blood was immediately obtained from the caudal region using heparinized syringes. Fish were euthanized by cervical section, the liver was dissected, weighed, freeze-clamped in liquid nitrogen and stored at −80°C until further analysis. Plasma was separated by centrifugation (3000*g*, 10 min) and stored at −20°C for quantification with commercial kits of glucose (Glucose RTU; BioMerieux), triglycerides (TRG050; Sobioda) and glycerol (MAK117; Sigma-Aldrich), respectively, according to the recommendations of each manufacturer. Glycogen measures were performed in liver as previously detailed in Song et al. (2018).

The relative gene mRNA level was determined by real-time quantitative polymerase chain reaction (qPCR). Liver samples were homogenized using Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France) in tubes containing Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then extracted according to the manufacturer's instructions. The total RNA concentration was determined using the spectrophotometer NanoDrop 2000 whereas the total RNA quality was determined by migration on a 1% agarose gel electrophoresis. Total RNA (1 µg) was used for cDNA synthesis. The Super-Script III RNAse H-Reverse transcriptase kit (Invitrogen, Carlsbad, California, USA) was used with random primers (Promega) to synthesize cDNA. The primer sequences used in real-time qPCR assays for glucose metabolism gene analysis are presented in **Supplementary Tables S1**, **S2** for trout and seabass, respectively. The mRNA level for enzymes of glycolysis (*gck*, glucokinase EC 2.7.1.2; *pk*, pyruvate kinase EC. 2.7.1.40) and gluconeogenesis (*g6pc*, glucose-6-phosphatase EC 3.1.3.9; *fbp*, fructose-1,6-bisphosphatase EC 3.1.3.11; *pck*, phosphoenolpyruvate carboxykinase EC 4.1.1.32) were analyzed. Elongation factor-1 alpha (*ef1α*) was used as the reference gene. As shown in previous studies, ohnologues genes were mostly regulated at the transcription level in the same way (Marandel et al., 2015) so a set of primers that amplify both *g6pcb1* and *g6pcb2* genes (called "glob", respectively) were used. For real-time qPCR assays, the Roche Lightcycler 480 system was used (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using a reaction mix of 6 µL per sample, each containing 2 µL of diluted cDNA template (1:76 for the postprandial and nutritional experiment; 1:25 for ontogenesis experiment), 0.24 µL of each primer (10 µmol L^{-1}) and 3 µL Light Cycler 480 SYBR® Green I Master mix (Roche Diagnostics). The PCR protocol was initiated at 95°C for 10min for initial denaturation of the cDNA and hot-start enzyme activation and continued with 45 cycles of a three-step amplification program (15 s at 95°C followed by 10 s 60°C, for primer hybridization, and 15 s at 72°C to extend DNA). Melting curves were systematically monitored (temperature gradient at 0.11°C/s from 65 to 97°C; five acquisitions/°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and duplicate of PCR amplification) and negative controls (reverse transcriptase-free samples, RNA-free samples).

2.5 Metabolic Trial – Labeling of Blood Glucose and Hepatic Glycogen With ^2H_2O

The fish remaining from the growth trials (n=24 per diet for both species) were transferred to a closed recirculated system consisting of 3 x 150 L tanks for the metabolic trials. Tank water (TW) contained ~4% ^2H-enriched water, achieved by the addition of 99%-enriched ^2H_2O (CortecNet, France) to water from the system where the respective growth trial took place as previously described (Viegas et al., 2011). This water was maintained within previous parameters with an independent closed filtering system (EHEIM eXperience 350) and UV unit (EHEIM reeflexUV 800) as previously described (Palma et al., 2019; Magnoni et al., 2021). Tank water ^2H-enrichment was assessed at the beginning and end of the metabolic trials, lasting 6 days for each species. During the 6-day residence in ^2H-enriched TW, fish were fed once a day (*ad libitum*) and euthanized 24 h after last meal on day 5. Fish were anesthetized, euthanized and sampled for blood and liver as previously described in section 2.4. An aliquot of plasma was processed as described above for quantification with commercial kits of glucose (I.S.E. S.r.l.; ref. A-R0100000601), triglycerides (I.S.E. S.r.l.; A-R0100000901) and glycerol (Sigma-Aldrich; ref. MAK117), as well as assessment of body water (BW) ^2H-enrichment. In order to ensure samples that yielded ^2H Nuclear Magnetic Resonance (NMR) spectra with high signal-to-noise ratios, blood and liver from two fish were pooled (for a final n=12). The blood was deproteinized by the addition of 0.3 M $ZnSO_4$ and 0.15 M

$Ba(OH)_2$, followed by centrifugation (3500*g*, 15 min). The supernatant was desalted by passage through sequential columns containing Dowex® 50WX8 (hydrogen form; Sigma-Aldrich) and Amberlite® IRA-67 (free base, Fluka; Sigma-Aldrich), and lyophilized as previously described (Viegas et al., 2013). Liver was ground with N_2-chilled mortar and pestle and hepatic glycogen was extracted by precipitation with 70% ethanol following an alkaline tissue hydrolysis, as previously described (Viegas et al., 2012). Glycogen was hydrolyzed to glucose by amyloglucosidase from *Aspergillus niger* (glucose-free preparation; Sigma-Aldrich) as described previously (Viegas et al., 2012). In order to optimize the signal resolution of the NMR spectra, glucose (isolated from blood) and glycosyl units (obtained from hepatic glycogen hydrolysis with amyloglucosidase) were converted into their derivative monoacetone glucose (MAG), as previously described (Viegas et al., 2012).

2.6 ^{2}H NMR Acquisition and Metabolic Flux Calculations

Proton-decoupled ^{2}H NMR spectra were obtained with a Bruker Avance III HD 500 spectrometer using a ^{2}H-selective 5 mm probe incorporating a ^{19}F-lock channel. To quantify fish body water (BW) and tank water (TW) ^{2}H-enrichments, duplicates of 10 µL samples were added to 450 µL of calibrated acetone as previously described (Jones et al., 2001) with the addition of 50 µL of hexafluorobenzene (Sigma-Aldrich H8706, Spain) to detect ^{19}F-lock (analyzed in duplicate). MAG samples were resuspended in 0.5 mL 90% acetonitrile/10% ^{2}H-depleted water with the addition of 50 µL of hexafluorobenzene. ^{2}H NMR spectra were obtained following the acquisition parameters described in Rito et al. (2018). Estimation of blood glucose and hepatic glycogen sources from ^2H_2O derive from unique labeling patterns created by their common precursor G-6-P, following the metabolic model for positional ^{2}H-labeling shown in **Figure 1** and described in detail in Palma et al. (2020). The positional ^{2}H-enrichments were quantified by using the MAG methyl signals (MAG-CH_3) as intramolecular standard (Nunes and Jones, 2009) (representative spectrum is presented in **Supplementary Figure S1**). Briefly, all gluconeogenic (=indirect pathway, in the case of glycogen synthesis) contributions are calculated from the ^{2}H-enrichment in position 5 (^{2}H5; **Figure 1** in orange) relative to tank water as follows:

$$\text{All gluconeogenic (= indirect pathway) (\%)} = 100 \times {}^2\text{H5}/\text{TW} \quad (1)$$

G-6-P synthesized from Krebs cycle precursors, is enriched in both positions 5 and 6 while G-6-P derived from substrates entering glycogenesis at the level of triose phosphates, such as glycerol released *via* lipolysis, is only enriched in position 5. Thus, gluconeogenic contributions from the Krebs cycle are calculated from the ^{2}H-enrichment in position 6_S (^2H6_S; **Figure 1** in green) relative to tank water as follows:

$$\text{Contribution from Krebs cycle substrates (\%)} = 100 \times {}^2\text{H6}_S/\text{TW} \quad (2)$$

Gluconeogenic contributions from triose phosphates are estimated as the difference between position 5 and 6_S enrichments relative to tank water:

$$\text{Contribution from trioses (\%)} = 100 \times ({}^2\text{H5} - {}^2\text{H6}_S)/\text{TW} \quad (3)$$

The remaining hepatic glycogen may stem from various sources whose provenance is unreliable to further resolve under the present metabolic model. These include pre-existing glycogen (direct or indirect origin is unknown), cycled glycogen *via* G-6-P, or glycogen derived from the direct pathway (**Figure 1** in blue) and were calculated relatively to eq. 1 as:

$$\text{Pre-existing + cycled + direct pathway (\%)} = 100 - \text{All indirect pathway contributions} \quad (4)$$

Blood glucose originating from glycogenolysis (or cycled from glycogen) would not be labeled in ^{2}H5, being then calculated as:

$$\text{Glycogenolysis (\%)} = 100 - \text{All indirect pathway contributions} \quad (5)$$

^{2}H-enrichment of endogenous G-6-P in position 2 (^{2}H2) is obligatory in gluconeogenesis while dependent on the interconversion between G-6-P and fructose-6-phosphate (F-6-P) by G-6-P isomerase in glycogenolysis. Even if partially incomplete (Martins et al., 2013), labeling in ^{2}H2 (**Figure 1** in grey) is still the most reliable approximation to the labeling of endogenous G-6-P, calculated as

$$\text{Contribution from endogenous G-6-P (\%)} = 100 \times {}^2\text{H2}/\text{TW} \quad (6)$$

As blood glucose is likely to undergo full turnover in both species within the 6-day residence in ^2H_2O (Garin et al., 1987; Haman and Weber, 1996), the unlabeled contributions, were considered to be from dietary sources and calculated as:

$$\text{Contribution from dietary sources (\%)} = 100 - \text{Contribution from endogenous G-6-P} \quad (7)$$

2.7 Statistics

Data are presented as mean ± SD. All spectra were processed in the ACD/NMR Processor Academic Edition from ACD\Labs 12.0 software (Advanced Chemistry Development, Inc.) applying: zero-filling to 65k, line broadening of 1.0 Hz, phasing and baseline correction. For the parameters evaluated following the growth trial, such as final body weight, plasma and hepatic parameters and mRNA levels of carbohydrate-related enzymes, the effects of sampling time and diet were analyzed by two-way ANOVA, with time (6 h and 24 h) and glycerol level (D0, D2.5 and D5) as variables. In the case of significant interactions, one-way ANOVA was performed for each sampling time separately. For the parameters evaluated following the metabolic trial, such as plasma,

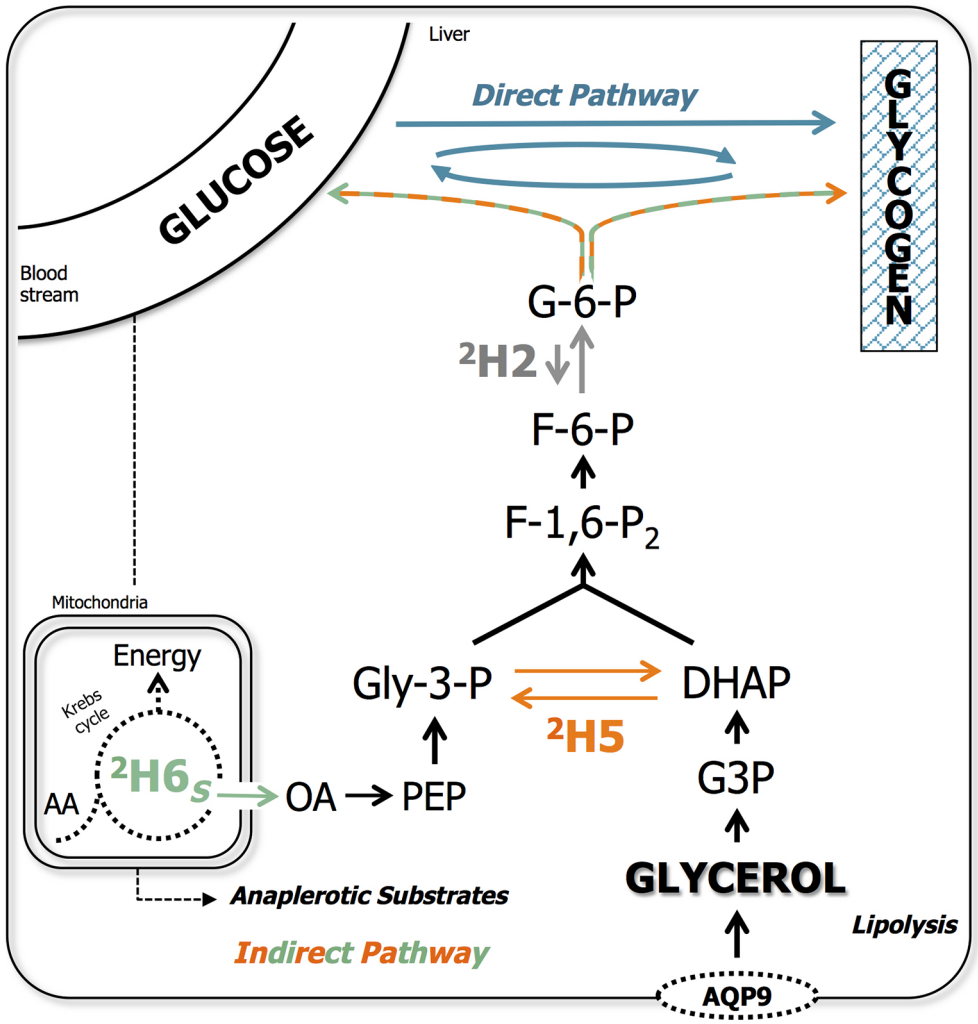

FIGURE 1 | Metabolic model representing metabolic pathways for blood glucose and hepatic glycogen synthesis. Gluconeogenesis/indirect pathway includes: i) precursors that are metabolized *via* the anaplerotic pathways of Krebs cycle (i.e., pyruvate and glucogenic amino acids); ii) glycerol from lipolysis and imported from the blood stream *via* aquaporin 9 (AQP9); and iii) pyruvate derived from glucose, as indicated by the dashed arrow. Metabolic model for ^{2}H-enrichment sites of glucose/glycosyl molecule from $^{2}H_2O$: position 2 (^{2}H2; in grey) *via* glucose-6-phosphate isomerase; position 5 (^{2}H5; in orange) *via* triose phosphate isomerase; and position 6_S ($^{2}H6_S$; in green) *via* fumarase in the Krebs cycle. Several metabolic intermediates were omitted for clarity. G-6-P, glucose-6-phosphate; Gly-3-P, glyceraldehyde-3-phosphate; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; OA, oxaloacetate; PEP, phosphoenolpyruvate; AA, amino acids.

hepatic parameters and metabolic flux calculations were analyzed by one-way ANOVA. In the case of detecting significant differences through the one-way ANOVA, a Tukey multiple range test was performed to discriminate differences between diets. Statistical analysis was performed in GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) and differences were considered statistically significant at $p<0.05$.

3 RESULTS

Tank water (TW) ^{2}H-enrichment was 3.9% and 3.5% for trout and seabass, respectively. Body water (BW) enrichment, assessed using plasma water content as proxy, was consistent with TW with no differences between dietary treatments for both species. Since fish BW and TW enrichments equilibrate within less than 6 h for seabass (Viegas et al., 2011), BW ^{2}H-enrichment level was considered to reflect that of a constant ^{2}H-precursor enrichment level over the 6-day residence time of both species in $^{2}H_2O$.

3.1 Rainbow Trout

After the growth trial, no differences in the final body weight for both diet (T0, T2.5, T5) and sampling time (6 h *vs*. 24 h after last meal) were observed. It was not the case for glucose and glycerol levels in the plasma. These parameters were not significantly affected by diet but responded to the postprandial timing, being significantly higher at 6 h than 24 h. However, since significant interactions were obtained for plasma glycerol, a one-way

ANOVA was performed separately for each variable revealing no further differences between diets in fish 24 h after the last meal. On the contrary, hepatic glycogen was not altered by sampling time but significantly increased alongside dietary levels of glycerol. The hepatosomatic index (HSI) reflected differences from both variables, being significantly higher at 6 h compared with 24 h as expected from a postprandial absorption pattern. Also, and in agreement with the observed increasing hepatic glycogen levels, HSI increased with increasing dietary levels of glycerol. After the metabolic trial, fish were euthanized 24 h after the last meal and the same parameters were measured with the exception of glycogen levels (the liver tissue assigned for the glycogen assay was poorly maintained and this parameter was not measured). HSI did not reveal statistical differences but final body weight, plasma glucose and glycerol followed the same statistical trend as in the growth trial at 24 h postprandial. The results from both trials in trout are summarized in **Table 3**.

In order to assess the impact of glycerol-supplemented diets on hepatic metabolism, mRNA levels of key enzymes involved in the regulation of carbohydrate metabolism were assessed after the growth trial at two different sampling times after last meal (6 and 24 h) by real-time qPCR (**Figure 2**).

None of analyzed transcripts responded significantly to the different levels of dietary glycerol. At 6 h after last meal, and as expected in a postprandial setting, some gluconeogenic enzymes had decreased mRNA levels, namely glucose-6-phosphatase paralogs *g6pca* and *g6pcb1*, fructose-1,6-bisphosphatase paralog *fbp1b2* and phosphoenolpyruvate carboxykinase paralog *pck1*. Curiously, the glucose 6-phosphatase paralog *g6pcb2* was up-regulated at 6 h after last meal. On the other hand, but also consistent with a postprandial setting, the mRNA levels for the glycolytic glucokinase paralogs were significantly down-regulated at 6 h after last meal.

A deeper insight into the impact of glycerol-supplemented diets on hepatic metabolism was achieved through the labeling of blood glucose and hepatic glycogen with ^2H_2O. This approach revealed no differences between diets for the sources of blood glucose pool (**Figure 3**), the majority of which (approximately 60%) were not labeled with ^2H. From the labeled fraction, which corresponded to the remaining 40%, we were able to determine their source from the positional ^2H-enrichment. Most of it, at an average for all dietary treatments of 33% out of total blood glucose, came from gluconeogenic substrates. From this fraction, 57% gluconeogenic substrates came from Krebs cycle and 43% gluconeogenic substrates from triose-phosphate. Only a small fraction, at an average of 6% for all dietary treatments, was derived from the breakdown of hepatic glycogen to total blood glucose. On the other hand, labeling from ^2H_2O revealed significant differences between diets for hepatic glycogen synthesis (**Figure 4**). The majority of the glycogen pool, at an average of 70% for all dietary treatments, derived from pre-existing, cycled or G-6-P directly derived from blood glucose. The contribution from the indirect pathway at the triose-phosphate level statistically increased alongside levels of dietary glycerol from 8.8% at T0 to 17.2% at T5. Apparently this happened at the shared expense of the other fractions since neither the direct contribution (including pre-existing and cycled G-6-P) nor indirect contribution from Krebs cycle revealed statistical differences.

3.2 European Seabass

After the growth trial, no differences in the final body weight and plasma glucose were observed between diets (S0, S2.5, S5) and sampling time (6 h, 24 h after last meal). In fact, none of the parameters assessed were sensitive to postprandial alterations. On the other hand, plasma glycerol increased alongside dietary levels of glycerol, a trend also observed at the metabolic trial. In the growth trial, hepatic glycogen decreased with increasing dietary levels of glycerol but with no observable repercussions in HSI, the results for these same parameters after the metabolic trial revealed another pattern. Glycogen levels were not significantly altered

TABLE 3 | Final weight, blood and liver parameters for rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks sampled at the end of the growth trial at 6 h and 24 h after last meal and the metabolic trial (6-day residence in ^2H-enriched water) 24 h after last meal.

	Growth trial								
Rainbow trout	**6 h**			**24 h**			**2-way ANOVA p-value**		
	T0	**T2.5**	**T5**	**T0**	**T2.5**	**T5**	**time**	**glycerol**	**interaction**
Final body weight (g)	103.41 ± 28.80	84.31 ± 20.05	84.07 ± 25.55	89.66 ± 25.44	95.92 ± 21.19	95.87 ± 28.17	0.659	0.702	0.264
Plasma Glucose (mM)	4.33 ± 0.56	4.61 ± 0.72	4.11 ± 1.00	4.05 ± 0.50	3.77 ± 0.44	3.72 ± 0.44	**0.011**	0.419	0.436
Plasma Glycerol (μM)	47.55 ± 4.02 a	60.05 ± 7.48 b	66.03 ± 16.68 b	42.51 ± 11.53	42.68 ± 6.59	33.69 ± 2.90	**1.01E-7**	0.123	**1.93E-7**
Hepatic Glycogen (mg/g)	32.0 ± 18.7	58.1 ± 32.3	58.1 ± 34.9	22.00 ± 13.9	48.1 ± 22.0	36.7 ± 23.0	0.066	**0.015**	0.767
HSI	1.01 ± 0.10	1.26 ± 0.29	1.33 ± 0.28	0.95 ± 0.09	1.08 ± 0.19	1.06 ± 0.12	**0.005**	**0.006**	0.309
	Metabolic trial - ^2H_2O			**T0**	**T2.5**	**T5**	**1-way ANOVA p-value**		
Final body weight (g)				107.00 ± 30.70	105.90 ± 25.75	98.50 ± 24.97	0.504		
Plasma Glucose (mM)				3.66 ± 0.55	3.65 ± 0.55	3.96 ± 0.45	0.262		
Plasma Glycerol (μM)				98.07 ± 36.71	79.16 ± 19.69	94.22 ± 18.34	0.192		
Hepatic Glycogen (mg/g)				–	–	–	–		
HSI*				0.98 ± 0.16	0.97 ± 0.14	1.00 ± 0.18	0.714		

**Hepatosomatic Index as HSI = (liver weight/body weight) *100.*

Values are mean ± SD. Significant differences in the growth trial (n = 8) were evaluated by two-way ANOVA ($p < 0.05$, values in bold). In case of interaction, a post-hoc Tukey's test was performed separately for each sampling time (indicated by different letters). Significant differences in the metabolic trial (n = 12) between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$, values in bold; followed by Tukey's test).

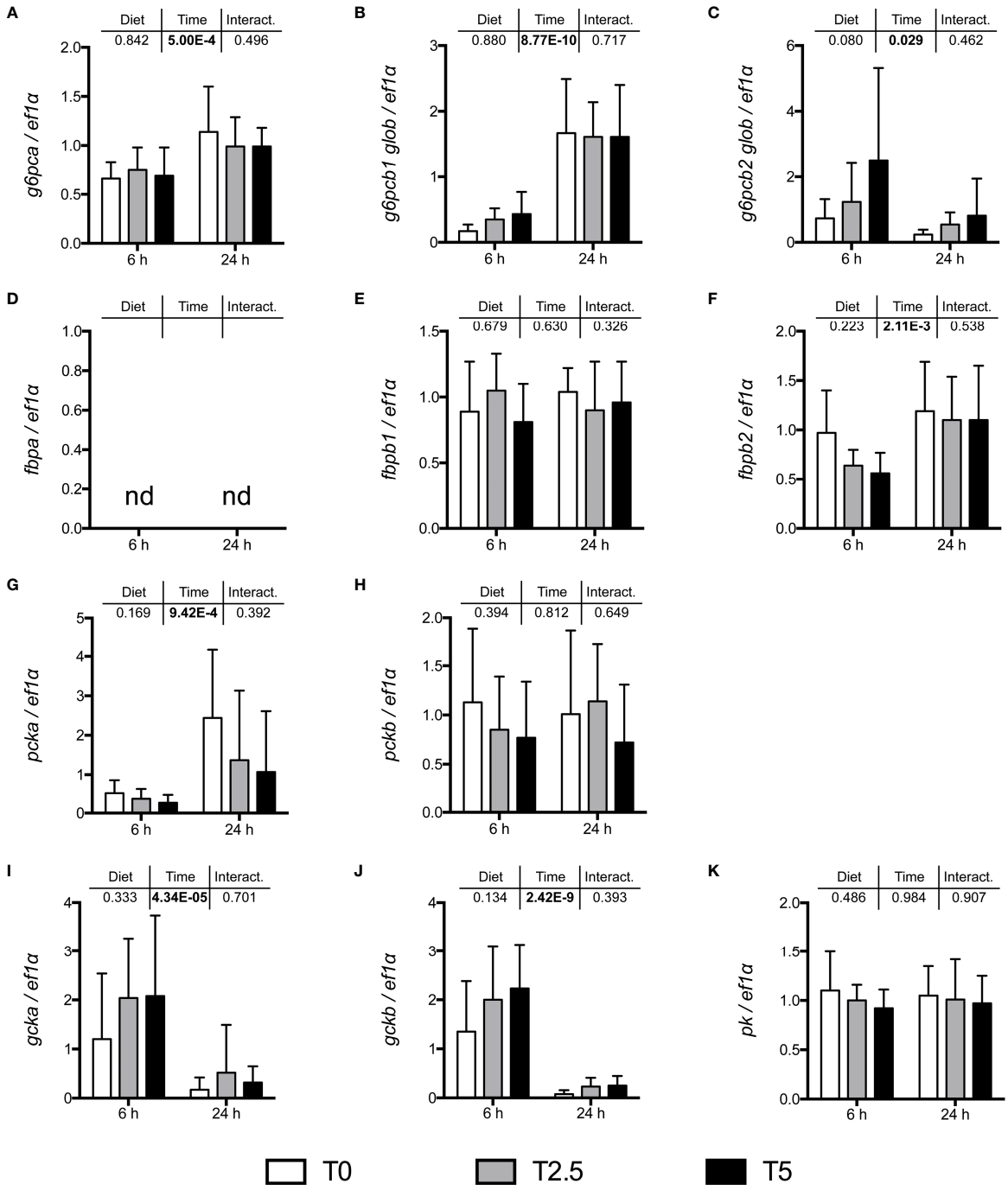

FIGURE 2 | mRNA levels of selected gluconeogenic enzymes **(A)** *g6pca*, **(B)** *g6pcb1*, **(C)** *g6pcb2* (glucose-6-phosphatase paralogs), **(D)** *fbpa*, **(E)** *fbpb1*, **(F)** *fbpb2* (fructose-1,6-bisphosphatase paralogs), **(G)** *pcka*, **(H)** *pckb* (phosphoenolpyruvate carboxykinase paralogs); and glycolytic enzymes **(I)** *gcka*, **(J)** *gckb* (glucokinase paralogs) and **(K)** *pk* (pyruvate kinase), in the liver of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks. Values are mean ± SD (n = 8); nd, not detected. Significant differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

while HSI increased with dietary levels of glycerol. The results from both trials in seabass are summarized in **Table 4**.

Similar to what was observed in trout, mRNA levels of key enzymes involved in the regulation of carbohydrate metabolism did not respond significantly to the different levels of dietary glycerol (**Figure 5**). At 6 h after last meal, and as expected in a postprandial setting, the glycolytic enzymes *gck* and *pk* had increased mRNA levels. In the case of *gck*, despite the barely

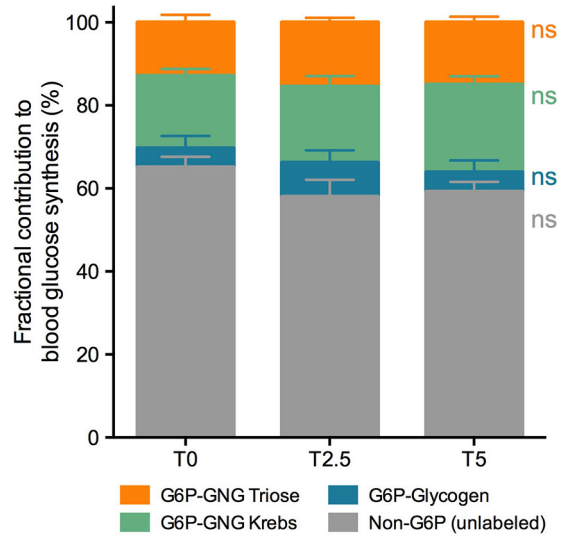

FIGURE 3 | Fractional contribution to blood glucose synthesis of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks. Contributions are represented as percentage of total (%). Values are mean ± SEM (n = 12; one-way ANOVA; followed by Tukey for $p < 0.05$; ns, not significant). The color scheme corresponds to metabolic pathways depicted in **Figure 1**.

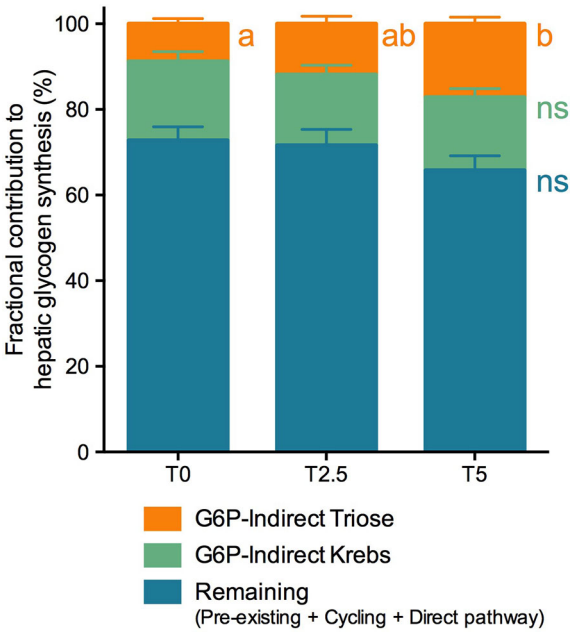

FIGURE 4 | Fractional contribution to hepatic glycogen synthesis of rainbow trout (*Oncorhynchus mykiss*) fed with different levels of glycerol (0, 2.5, and 5%) for 8 weeks. Contributions are represented as percentage of total (%). Values are mean ± SEM (n = 12). Significant differences are indicated by different letters (one-way ANOVA; followed by Tukey for $p < 0.05$; ns, not significant). The color scheme corresponds to metabolic pathways depicted in **Figure 1**.

significant interactions found, *post-hoc* analysis done separately within each sampling time found no differences. For the mRNA levels of gluconeogenic enzymes, only *fbp1* was significantly different and only when considering sampling time.

Labeling of blood glucose and hepatic glycogen with ^2H_2O resulted in opposite results to those found in trout. Sources of blood glucose differed substantially between diets (**Figure 6**), while sources of hepatic glycogen did not (**Figure 7**). Taking into consideration the gluconeogenic substrates the contribution to blood glucose from Krebs cycle decreased, while from triose-phosphate there was an increase as dietary glycerol levels increased. If taken together (Krebs cycle plus triose-phosphate), the contribution of gluconeogenic substrates to blood glucose was significantly lower at S2.5 than with S0 but not statistically different from S5. On the other hand, the contribution from the breakdown of hepatic glycogen significantly increased from 46.7% at S0 to 59.3% at S5. This was accompanied by a significant decrease from 30.0% at S0 to 19.9% at S5 in the unlabeled fraction.

As observed in trout, the majority of the glycogen pool derived from pre-existing, cycled or G-6-P directly derived from blood glucose. Even if reduced to half, from 20% in S0 to 10% in both glycerol-supplemented diets, no statistical differences were found in the indirect contribution, probably due to high variability amongst individuals.

4 DISCUSSION

Both species revealed evidence that carnivorous fish are able to catabolize glycerol as well as retain and incorporate it into carbohydrates. However, whether this incorporation would just add to gluconeogenesis rather than effectively competing with gluconeogenic amino acids for glucose synthesis is yet to be addressed. The latter would potentially spare amino acids for growth, while reducing the conversion of amino acid nitrogen moieties to ammonia with important impact on water quality. In carnivorous fish, gluconeogenic catabolism of amino acids accounts for most endogenous glucose production (Viegas et al., 2012; Viegas et al., 2013). Amongst alternative non-nitrogenous energetic substrates, glycerol is currently recognized as a safe food additive by the main food safety agencies (EFSA et al., 2017; FDA, 2021), thus virtually nontoxic to vertebrate health when administered at physiological levels such as when applied for animal feed. The quantity of nitrogen retained by rainbow trout decreased when fed diets with increasing glycerol levels, showing a lower protein retention. This was reflected as well in higher excretion rates of ammonia (Magnoni et al., 2021). In contrast, the same dietary levels of glycerol inclusion fed to European seabass did not generate differences in excreted ammonia between dietary treatments (Louvado et al., 2020).

4.1 Circulating Glycerol and Hepatic Uptake and/or Synthesis

Normally, the main source of circulating glycerol originates from triacylglycerol de-esterification in adipose tissue which under normal circumstances should be considerably low as was

TABLE 4 | Final weight, blood and liver parameters for European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 6 weeks sampled at the end of the growth trial at 6 h and 24 h after last meal and the metabolic trial (6-day residence in ^{2}H-enriched water) 24 h after last meal.

	Growth trial								
European Seabass	**6 h**			**24 h**			**2-way ANOVA p-value**		
	S0	**S2.5**	**S5**	**S0**	**S2.5**	**S5**	**time**	**glycerol**	**interaction**
Final body weight (g)	128.90 ± 47.08	95.05 ± 40.03	118.61 ± 34.37	87.3 ± 16.3	75.4 ± 9.7	87.6 ± 16.3	0.659	0.221	0.722
Plasma Glucose (mM)	6.03 ± 0.22	6.09 ± 0.44	6.56 ± 0.56	6.61 ± 1.22	5.83 ± 0.78	6.66 ± 1.11	0.125	0.577	0.387
Plasma Glycerol (μM)	60.23 ± 6.60	1479.54 ± 645.99	6789.80 ± 1549.27	93.90 ± 45.83	152.09 ± 101.46	5829.53 ± 2417.60	0.056	**3.16E-15**	0.330
Hepatic Glycogen (mg/g)	114.1 ± 17.9	96.6 ± 26.1	88.8 ± 16.5	118.7 ± 13.7	105.7 ± 10.3	109.3 ± 21.8	0.066	**0.041**	0.547
HSI	1.57 ± 0.39	1.59 ± 0.18	1.68 ± 0.20	1.42 ± 0.24	1.55 ± 0.32	1.53 ± 0.28	0.166	0.664	0.961
	Metabolic trial - $^{2}H_2O$			**S0**	**S2.5**	**S5**	**1-way ANOVA p-value**		
Final body weight (g)				97.13 ± 33.29	92.36 ± 31.07	94.59 ± 29.90	0.832		
Plasma Glucose (mM)				10.18 ± 3.79	11.84 ± 4.67	10.16 ± 3.79	0.522		
Plasma Glycerol (μM)				86.04 ± 40.56 a	228.40 ± 267.00 a	762.50 ± 606.60 b	**3.00E-4**		
Hepatic Glycogen (mg/g)				74.16 ± 31.84	94.28 ± 37.05	104.60 ± 44.41	0.155		
HSI*				1.26 ± 0.42 a	1.72 ± 0.72 b	1.81 ± 0.70 b	**6.20E-4**		

**Hepatosomatic Index as HSI = (liver weight/body weight) *100.*

Values are mean ± SD. Significant differences in the growth trial (n = 7) were evaluated by two-way ANOVA ($p < 0.05$, values in bold). Significant differences in the metabolic trial (n = 12) between dietary treatments are indicated by different letters (one-way ANOVA, $p < 0.05$, values in bold; followed by Tukey's test).

observed in the control fish (~50 μM). However, if provided in the diet, it is necessary to understand the underlying metabolic fluxes that control glycerol utilization. In the present study, the plasma glycerol levels, and its patterns changed substantially between species. In rainbow trout, plasma glycerol levels increased overall 6 h after meal, and did so in response to glycerol supplementation, but returned to control levels regardless of the diet at 24 h. In seabass, the dietary supplementation significantly affected plasma glycerol levels at 6 h with lasting effects at 24 h after last meal, and with values for S5 diet reaching more than 5 mM. After ingestion, glycerol transport across membranes occurs through transmembrane proteins belonging to a subfamily of the aquaporin (AQP) designated as aquaglyceroporins. These include AQP3, 7, 9 and 10, which are permeable to small solutes such as glycerol and urea as well as water. As such, aquaglyceroporins are involved in salinity acclimation and osmoregulation in fish (Deane and Woo, 2006; Giffard-Mena et al., 2007; Tipsmark et al., 2010). The distribution pattern of aquaglyceroporins along the esophagus, stomach and intestine/midgut (Cutler et al., 2007; Tipsmark et al., 2010; Finn and Cerdà, 2011; Madsen et al., 2014) aligns with water recovery capability from the digestive tract, but at the same time may justify the rapid appearance of dietary glycerol circulating in blood after 6 h. High renal expression of AQP3 in saltwater fish, including seabass (Giffard-Mena et al., 2007), also allows reabsorption of large volumes of water in order to compensate for osmotic water loss. The mechanism of glycerol conduction in aquaglyceroporins is driven by an inherent competition between water and glycerol for hydrogen bonds with specific residues. Accordingly, water is essential for glycerol transport at physiological glycerol concentrations (Jensen et al., 2001), allowing for quick, high absorption rates in seabass compared with freshwater trout. Curiously, in most studies using glycerol-supplemented diets in fish, plasma glycerol levels are not reported. From basal 80 μM, a glycerol intraperitoneal injection of 2 g kg^{-1} in seabass led to an increase in plasma glycerol levels to more than 1 mM even 48 h after the injection in both fed and fasted fish (Rito et al., 2019). However, the most extreme increases in plasma glycerol are described in the adaptation of rainbow smelt (*Osmerus mordax*) to cold environments, reaching from 200 to 500 mM (Driedzic et al., 2006; Hall et al., 2015). Once in the bloodstream, the liver has the capacity to import glycerol through AQP9, the major hepatic aquaglyceroporin isoform (Deane and Woo, 2006; Hamdi et al., 2009; Gambardella et al., 2012).

The liver is the primary site for the *de novo* synthesis of glycerol so several metabolic pathways can mobilize and utilize its three carbons. The gene expressions for *aqp9b* during embryogenesis in trout does not appear along the setup of the primitive liver and hepatic portal vein, but rather at a later stage just before the first feeding (Panserat et al., 2020). Similar to that reported in trout, the present glycerol-supplemented diets had no effect on the mRNA levels of AQP9 in juvenile seabass liver at both 6 and 24 h (unpublished). Short-term fasting (48 h) followed by refeeding also had no effect in hepatic AQP9 mRNA levels in juvenile trout (Panserat et al., 2020). On the other hand, the presence and distribution of AQP9 in the membrane of seabass hepatocytes increased in long-term (35 d) fasted fish followed by a decrease in refed fish (Gambardella et al., 2012). As gluconeogenesis is the principal source of endogenous glucose production and a

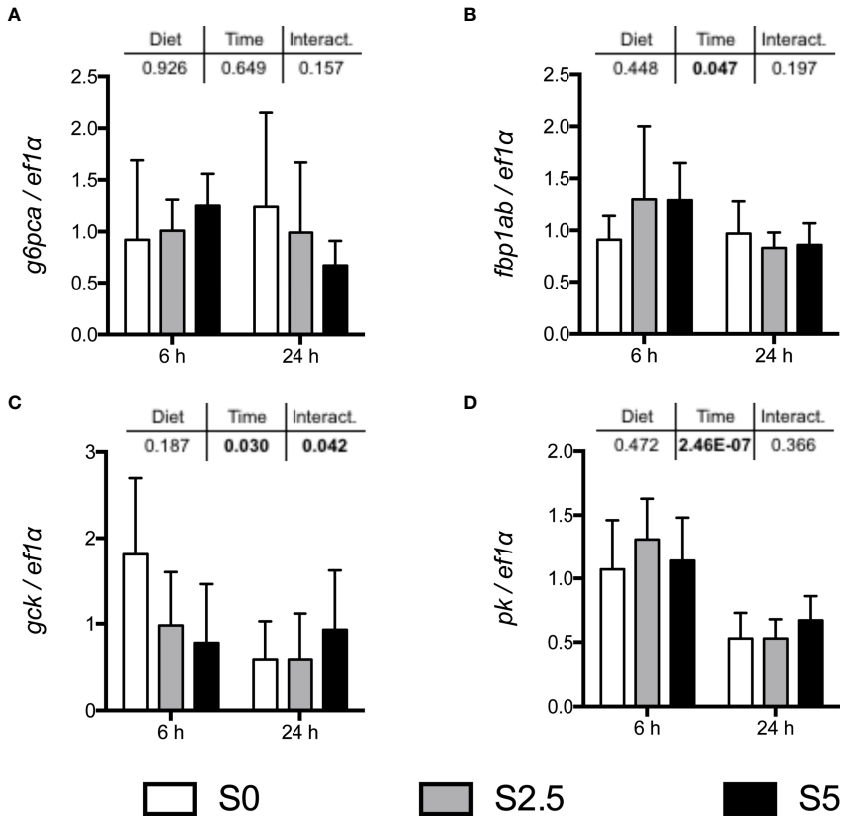

FIGURE 5 | mRNA levels of selected gluconeogenic enzymes **(A)** *g6pca* (glucose-6-phosphatase a), **(B)** *fbp1ab* (fructose-1,6-bisphosphatase with paralogs *fbp1a* and *fbp1b*); and glycolytic enzymes **(C)** *gck* (glucokinase) and **(D)** *pk* (pyruvate kinase), in the liver of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 6 weeks. Values are mean ± SD (n = 7). Significant differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

significant contributor to blood glucose levels during fasting in seabass (Viegas et al., 2013), increased imports of glycerol to the liver would allow for hepatic glycogen replenishment during refeeding. This process is dominated by indirect pathway fluxes (Viegas et al., 2012), a pathway to which glycerol is a precursor to the triose-phosphate. In fact, a glycerol load was responsible for 40% of blood glucose and 28% of hepatic glycogen appearance in fasted seabass. These contributions fall substantially in fed seabass highlighting how lipolysis-derived glycerol carbons are essential gluconeogenic contributors during food deprivation (Rito et al., 2019). Besides, the capacity of the liver to remove glycerol from blood circulation by other mechanisms of disposal should not be ignored. In rainbow smelt, AQP9 is also abundant in red blood cells (RBC) (Hall et al., 2015). In the scenario of cold adaptation mentioned above, a differentially higher glycerol uptake rate by RBC at higher temperatures (4-fold higher at 8°C than at 1.2°C) has been observed, along with a brutal increase in plasma glycerol (Clow and Driedzic, 2012). The loss of glycerol to water by passive diffusion should also not be disregarded and is worthy of further studies (Ditlecadet et al., 2011). The presence of AQP in gill, skin, and kidney (Finn and Cerdà, 2011) underline the possibility of processes for glycerol loss to water and that such mechanisms could affect freshwater and marine fish differently. Glycerol loss through the skin and gills may impact the bacterial community associated with fish mucus. Interestingly, Louvado et al. (2020) have shown an increased diversity and evenness of the gut microbiota of seabass that have been fed diets with increasing glycerol levels (0, 2.5 and 5%).

4.2 Hepatic Glycerol Utilization - Blood Glucose

Upon entrance in the liver, glycerol kinase catalyzes the initial phosphorylation of glycerol into G3P, which intersects with gluconeogenic/glycolityc pathways after being oxidized into dihydroxyacetone phosphate (DHAP) by G3P dehydrogenase. Both glycerol kinase and G3P dehydrogenase were poorly regulated by dietary glycerol levels in trout (Panserat et al., 2020) and Nile tilapia (Costa et al., 2015); even if G3P dehydrogenase and G3P phosphatase (reverse reaction of glycerol kinase) were deemed essential for cold adaptation in rainbow smelt (Driedzic et al., 2006). However, in trout the kinetics of postprandial regulation of glycerol-related enzymes (at first feeding stage and juvenile stage) when fed glycerol-supplemented diets (Panserat et al., 2020) was similar to the previously reported glycolytic and gluconeogenic genes when fed high-carbohydrate diets (Panserat et al., 2019). This suggests that

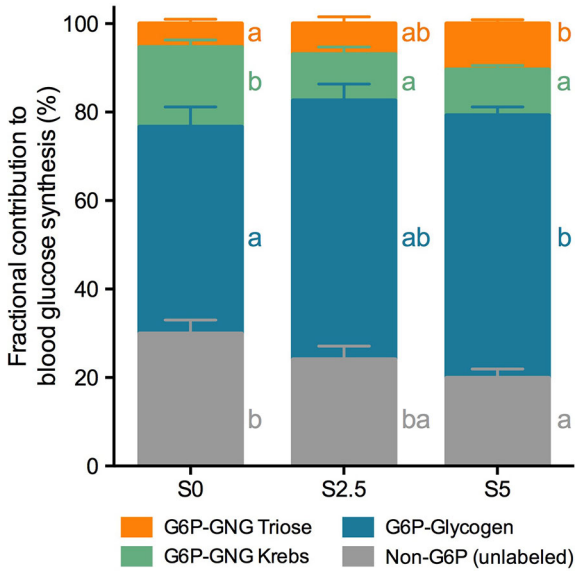

FIGURE 6 | Fractional contribution to blood glucose synthesis of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 6 weeks. Contributions are represented as percentage of total (%). Values are mean ± SEM (n = 12). Significant differences are indicated by different letters (one-way ANOVA; followed by Tukey for $p < 0.05$; ns, not significant). The color scheme corresponds to metabolic pathways depicted in **Figure 1**.

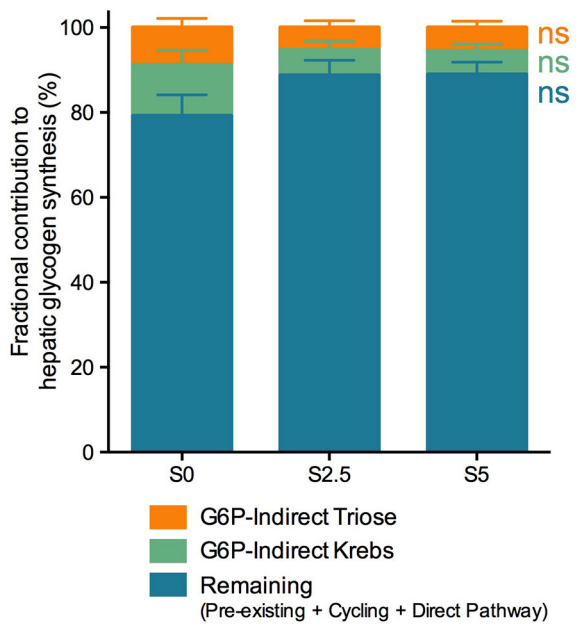

FIGURE 7 | Fractional contribution to hepatic glycogen synthesis of European seabass (*Dicentrarchus labrax*) fed with different levels of glycerol (0, 2.5, and 5%) for 6 weeks. Contributions are represented as percentage of total (%). Values are mean ± SEM (n = 12; one-way ANOVA; followed by Tukey for $p < 0.05$; ns, not significant). The color scheme corresponds to metabolic pathways depicted in **Figure 1**.

glycerol metabolism could be poorly regulated on its own and heavily dependent on the nutritional status. This seems to be corroborated by the mRNA levels of intermediary metabolism enzymes evaluated for both species since most were significantly altered by the time of sampling, but not by dietary treatment. Dietary glycerol supplementation at the expenses of corn in Nile tilapia resulted in lower hyperglycemic peaks at 90 min after last meal and a quicker return to baseline values at higher glycerol concentrations. Despite being a species more tolerant to carbohydrates, Nile tilapia revealed the weak potential of dietary glycerol to induce acute hyperglycemic reactions (Santos et al., 2019). This was not the case for trout where alterations in glycemia were considerably higher at 6 h than at 24 h. Postprandial glycemia in trout significantly increases with carbohydrate-rich diets at 8 h after last meal when compared with control (Kamalam et al., 2012). In contrast, seabass glycemia did not differ between sampling times, even though overall, this species presented higher glycemia when compared with trout. Seabass sampled 6 h after last meal with different starch levels (10, 20 and 30%) and different sources (native, waxy and pregelatinized maize starch) (Enes et al., 2006; Moreira et al., 2008) presented no differences in glycemia relative to control. However, metabolic flux models also based in the use of $^{2}H_2O$ revealed that digestible starch significantly contributes to endogenous glucose fluxes, even in the absence of differences in glycemia. These contributions shift from gluconeogenesis towards contributions of unlabeled (dietary) carbohydrates (Viegas et al., 2015). Nonetheless, if compared with trout, the glucose uptake in this species should still be considered sluggish, as corroborated by the fraction of glucose derived from the diet (unlabeled) observed in the present study. In seabass, the unlabeled fraction of glucose appearance was ~30%, which was approximately half of the fraction found in trout (~60%). Besides, while in trout this contribution was not conditioned by dietary glycerol, in seabass it decreased with increasing glycerol inclusion. This was compensated by the heightened contribution from gluconeogenesis at the trioses phosphate level where glycerol is able to participate, along with glycogenolysis-derived glucose. In agreement with this compensation, and even though considerably higher contributions were found in fasted fish, a IP-injected glycerol load was responsible for 10% of blood glucose appearance in fed seabass (Rito et al., 2019).

4.3 Hepatic Glycerol Utilization - Hepatic Glycogen

Contrary to previous reports on trout (Menton et al., 1986), hepatic glycogen levels significantly increased with glycerol supplementation. This was reinforced by a significant increase in HSI in the growth trial. This had already been the case with crude glycerin supplementation in Nile tilapia (Mewes et al., 2016) or channel catfish at levels above 15% (Li et al., 2010). Using radioactive isotopes, ^{14}C-glycerol incorporation into liver glycogen was described several decades ago in rainbow trout (Lech, 1970) and lampreys (*Lampetra fluviatilis*) (Savina and Wojtczak, 1977). In a more recent study (Costa et al., 2017) a

significantly higher amount of radiolabel from a 5% ^{14}C-glycerol diet was recovered from a fraction defined as "non-protein, non-lipid fraction", from which we speculate most was glycogen. Glycerol utilization for glycogen synthesis has also been observed in gilthead seabream muscle accompanied by an increased energetic status (Silva et al., 2012). Curiously, it has also been observed that the plasma glycerol increases during cold adaptation in rainbow smelt was synthesized primarily from glycogen (Ditlecadet and Driedzic, 2013). On the contrary, during late winter/early spring, plasma glycerol levels decrease accompanied by an increase in glycogen (Driedzic and Short, 2007). A different scenario was revealed in seabass with hepatic glycogen decreasing with increasing levels of glycerol in the growth trial, which was not corroborated in the metabolic trial. In fact, HSI measured in the metabolic trial actually presented the opposite trend raising the question of the metabolic utilization of glycerol. The actual contribution of glycerol as carbon source for gluconeogenesis, which in the context of glycogenesis would be *via* the indirect pathway (at the triose-phosphate level), significantly increased in trout while no alterations were observed in seabass. In seabass an IP-injected glycerol load was responsible for only 2% of hepatic glycogen appearance in fed seabass (Rito et al., 2019). It is worth mentioning that regardless of the levels of hepatic glycogen at the time fish are placed in $^{2}H_2O$, this metabolic model evaluates the fractional contribution. In the present study the overall contributions of the indirect pathway for glycogen synthesis were on average 30% in trout and 14% in seabass. The reliability of this estimate lies in the fact that labeling in ^{2}H5 is mandatory (Jones et al., 2006; Soares et al., 2012) and, in the present study, this estimate was further refined by using $^{2}H6_S$ to distinguish Krebs cycle-derived carbons incorporating $^{2}H_2O$ at the level of fumarase (fumarate hydratase) in the Krebs cycle. This is extremely useful to evaluate the contributions of glycerol, but it is unable to discriminate the remaining contributions from direct pathway, pre-existing and cycled glycogen. This cycling is difficult to discriminate from $^{2}H_2O$ but with the assistance of ^{13}C-labeled glucose this was considered extensive (Rito et al., 2018) with the additional technical advantage that NMR spectroscopy allows for convenient analysis of both isotopes in a same sample. In previous versions of the labeling model, regularly fed seabass presented a very low glycogen turnover (Viegas et al., 2012; Viegas et al., 2015).

5 CONCLUSIONS

In conclusion, we present evidence for gluconeogenic utilization of exogenous glycerol by rainbow trout in the synthesis of hepatic glycogen. In seabass, and despite no clear changes observed in glycemia, metabolic flux calculations confirmed that dietary glycerol was significantly involved in the appearance of blood glucose. The findings of the present study suggest that fish species are able to catabolize glycerol as well as retain and incorporate it into carbohydrates. With the use of stable isotopes, we demonstrate that this incorporation fails to substantially spare gluconeogenic amino acids for hepatic G-6-P synthesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Portuguese Veterinary Authority (1005/92, DGAV-Portugal, according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

AUTHOR CONTRIBUTIONS

IV, LJM, and SP: conceptualization and supervision. LJM, RO, ES, JR, LT, MP, and IV: growth and metabolic trials. SP, EP-J, and LM: molecular analysis of enzymes. JR, LT, ES, MP, and IV: NMR spectral acquisition and processing. IV, LJM, and SP: data treatment and writing of the original draft. IV and LJM: funding acquisition, project administration, and resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.836610/full#supplementary-material

REFERENCES

Ayoub, M., and Abdullah, A. Z. (2012). Critical Review on the Current Scenario and Significance of Crude Glycerol Resulting From Biodiesel Industry Towards More Sustainable Renewable Energy Industry. *Renewable Sustain. Energy Rev.* 16, 2671–2686. doi: 10.1016/j.rser.2012.01.054

Balen, R. E., Junior, G. B., Colpini, L. M. S., Bombardelli, R. A., Silva, L. C. R., and Meurer, F. (2017). Energia Digestível E Inclusão Da Glicerina Bruta Em Dietas Para Juvenis De Curimbatá Concentrations of Ammonia. *B. Inst. Pesca São Paulo* 43347 (3), 347–357. doi: 10.20950/1678-2305.2017v43n3p347

Balen, R. E., Tetu, P. N., Bombardelli, R. A., Pozza, P. C., and Meurer, F. (2014). Digestible Energy of Crude Glycerol for Pacu and Silver Catfish. *Ciec. Rural* 44, 1448–1451. doi: 10.1590/0103-8478cr20131426

Carvalho, P. L. D. O., Moreira, I., Martins, E. N., Piano, L. M., Toledo, J. B., and Costa Filho, C. D. L. (2012). Crude Glycerine in Diets for Piglets. *Rev. Bras. Zootecnia* 41, 1654–1661. doi: 10.1590/S1516-35982012000700014

Carvalho, E. R., Schmelz-Roberts, N. S., White, H. M., Wilcox, C. S., Eicher, S. D., and Donkin, S. S. (2012). Feeding Behaviors of Transition Dairy Cows Fed Glycerol as a Replacement for Corn. *J. Dairy Sci.* 95, 7214–7224. doi: 10.3168/jds.2010-3584

Cerrate, S., Yan, F., Wang, Z., Coto, C., Sacakli, P., and Waldroup, P. W. (2006). Evaluation of Glycerine From Biodiesel Production as a Feed Ingredient for Broilers. *Int. J. Poultry Sci.* 5, 1001–1007. doi: 10.3923/ijps.2006.1001.1007

Chung, Y. H., Rico, D. E., Martinez, C. M., Cassidy, T. W., Noirot, V., Ames, A., et al. (2007). Effects of Feeding Dry Glycerin to Early *Postpartum* Holstein Dairy Cows on Lactational Performance and Metabolic Profiles. *J. Dairy Sci.* 90, 5682–5691. doi: 10.3168/jds.2007-0426

Clow, K. A., and Driedzic, W. R. (2012). Glycerol Uptake is by Passive Diffusion in the Heart But by Facilitated Transport in RBCs at High Glycerol Levels in Cold Acclimated Rainbow Smelt (*Osmerus Mordax*). *Am. J. Physiol. - Regulatory Integr. Comp. Physiol.* 302, R1012–R1021. doi: 10.1152/ajpregu.00645.2011

Costa, D. V. D., Dias, J., Colen, R., Rosa, P. V., and Engrola, S. (2017). Partition and Metabolic Fate of Dietary Glycerol in Muscles and Liver of Juvenile Tilapia. *Arch. Anim. Nutr.* 71, 165–174. doi: 10.1080/1745039X.2017.1281579

Costa, D. V. D., Paulino, R. R., Okamura, D., Oliveira, M. M. D., and Rosa, P. V. E. (2015). Growth and Energy Metabolism of Nile Tilapia Juveniles Fed Glycerol. *Pesquisa Agropecuária Bras.* 50, 347–354. doi: 10.1590/S0100-204X2015000500001

Cutler, C. P., Martinez, A.-S., and Cramb, G. (2007). The Role of Aquaporin 3 in Teleost Fish. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 148, 82–91. doi: 10.1016/j.cbpa.2006.09.022

Deane, E. E., and Woo, N. Y. S. (2006). Tissue Distribution, Effects of Salinity Acclimation, and Ontogeny of Aquaporin 3 in the Marine Teleost, Silver Sea Bream (*Sparus Sarba*). *Marine Biotechnol.* 8, 663–671. doi: 10.1007/s10126-006-6001-0

Ditlecadet, D., and Driedzic, W. R. (2013). Glycerol-3-Phosphatase and Not Lipid Recycling is the Primary Pathway in the Accumulation of High Concentrations of Glycerol in Rainbow Smelt (*Osmerus Mordax*). *Am. J. Physiol. - Regulatory Integr. Comp. Physiol.* 304, R304–R312. doi: 10.1152/ajpregu.00468.2012

Ditlecadet, D., Short, C. E., and Driedzic, W. R. (2011). Glycerol Loss to Water Exceeds Glycerol Catabolism *via* Glycerol Kinase in Freeze-Resistant Rainbow Smelt (*Osmerus Mordax*). *Am. J. Physiol. - Regulatory Integr. Comp. Physiol.* 300, R674–R684. doi: 10.1152/ajpregu.00700.2010

Donkin, S. S. (2008). Glycerol From Biodiesel Production: The New Corn for Dairy Cattle. *Rev. Bras. Zootecnia* 37, 280–286. doi: 10.1590/S1516-35982008001300032

Driedzic, W. R., Clow, K. A., Short, C. E., and Ewart, K. V. (2006). Glycerol Production in Rainbow Smelt (*Osmerus Mordax*) May Be Triggered by Low Temperature Alone and Is Associated With the Activation of Glycerol-3-Phosphate Dehydrogenase and Glycerol-3-Phosphatase. *J. Exp. Biol.* 209, 1016. doi: 10.1242/jeb.02086

Driedzic, W. R., and Short, C. E. (2007). Relationship Between Food Availability, Glycerol and Glycogen Levels in Low-Temperature Challenged Rainbow Smelt *Osmerus Mordax*. *J. Exp. Biol.* 210, 2866. doi: 10.1242/jeb.003749

EFSA and Panel on Food Additives Nutrient Sources Added to Food, Mortensen, A., Aguilar, F., Crebelli, R., Domenico, A. D., et al. (2017). Re-Evaluation of Glycerol (E 422) as a Food Additive. *EFSA J.* 15 (3), 4720. doi: 10.2903/j.efsa.2017.4720

Enes, P., Panserat, S., Kaushik, S., and Oliva-Teles, A. (2006). Effect of Normal and Waxy Maize Starch on Growth, Food Utilization and Hepatic Glucose Metabolism in European Sea Bass (*Dicentrarchus Labrax*) Juveniles. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 143, 89–96. doi: 10.1016/j.cbpa.2005.10.027

European Commission. (2020). Circular Economy Action Plan: for a Cleaner and More Competitive Europe. (Brussels: Directorate-General for Communication, Publications Office). Available at: https://data.europa.eu/doi/10.2779/717149

FDA. (2021). *Electronic Code of Federal Regulations - Title 21, Volume 6, Part 582, Subpart B–General Purpose Food Additives Sec. §582.1320 Glycerin*. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=182.1320 (Accessed 10/02/2021 2021).

Finn, R. N., and Cerdà, J. (2011). Aquaporin Evolution in Fishes. *Front. Physiol.* 2. doi: 10.3389/fphys.2011.00044

Gambardella, C., Gallus, L., Amaroli, A., Terova, G., Masini, M. A., and Ferrando, S. (2012). Fasting and Re-Feeding Impact on Leptin and Aquaglyceroporin 9 in the Liver of European Sea Bass (*Dicentrarchus Labrax*). *Aquaculture* 354-355, 1–6. doi: 10.1016/j.aquaculture.2012.04.043

Garin, D., Rombaut, A., and Fréminet, A. (1987). Determination of Glucose Turnover in Sea Bass *Dicentrarchus Labrax*. Comparative Aspects of Glucose Utilization. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* 87, 981–988. doi: 10.1016/0305-0491(87)90422-6

Giffard-Mena, I., Boulo, V., Aujoulat, F., Fowden, H., Castille, R., Charmantier, G., et al. (2007). Aquaporin Molecular Characterization in the Sea-Bass (*Dicentrarchus Labrax*): The Effect of Salinity on AQP1 and AQP3 Expression. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 148, 430–444. doi: 10.1016/j.cbpa.2007.06.002

Gonçalves, L. U., Cerozi, B. D. S., Silva, T. S. C., Zanon, R. B., and Cyrino, J. E. P. (2015). Crude Glycerin as Dietary Energy Source for Nile Tilapia. *Aquaculture* 437, 230–234. doi: 10.1016/j.aquaculture.2014.12.004

Hall, J. R., Clow, K. A., Rise, M. L., and Driedzic, W. R. (2015). Cloning and Characterization of Aquaglyceroporin Genes From Rainbow Smelt (*Osmerus Mordax*) and Transcript Expression in Response to Cold Temperature. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* 187, 39–54. doi: 10.1016/j.cbpb.2015.05.004

Haman, F., and Weber, J. M. (1996). Continuous Tracer Infusion to Measure *In Vivo* Metabolite Turnover Rates in Trout. *J. Exp. Biol.* 199, 1157–1162. doi: 10.1242/jeb.199.5.1157

Hamdi, M., Sanchez, M. A., Beene, L. C., Liu, Q., Landfear, S. M., Rosen, B. P., et al. (2009). Arsenic Transport by Zebrafish Aquaglyceroporins. *BMC Mol. Biol.* 10, 104. doi: 10.1186/1471-2199-10-104

Hua, K., Cobcroft, J. M., Cole, A., Condon, K., Jerry, D. R., Mangott, A., et al. (2019). The Future of Aquatic Protein: Implications for Protein Sources in Aquaculture Diets. *One Earth* 1, 316–329. doi: 10.1016/j.oneear.2019.10.018

Jensen, M.Ø., Tajkhorshid, E., and Schulten, K. (2001). The Mechanism of Glycerol Conduction in Aquaglyceroporins. *Structure* 9, 1083–1093. doi: 10.1016/S0969-2126(01)00668-2

Jones, J. G., Fagulha, A., Barosa, C., Bastos, M., Barros, L., Baptista, C., et al. (2006). Noninvasive Analysis of Hepatic Glycogen Kinetics Before and After Breakfast With Deuterated Water and Acetaminophen. *Diabetes* 55, 2294–2300. doi: 10.2337/db06-0304

Jones, J. G., Merritt, M., and Malloy, C. (2001). Quantifying Tracer Levels of ^2H_2O Enrichment From Microliter Amounts of Plasma and Urine by ^2H NMR. *Magnetic Resonance Med.* 45, 156–158. doi: 10.1002/1522-2594(200101)45:1<156::AID-MRM1020>3.0.CO;2-Z

Kamalam, B. S., Médale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., and Panserat, S. (2012). Regulation of Metabolism by Dietary Carbohydrates in Two Lines of Rainbow Trout Divergently Selected for Muscle Fat Content. *J. Exp. Biol.* 215, 2567–2578. doi: 10.1242/jeb.070581

Lammers, P. J., Kerr, B. J., Weber, T. E., Dozier, W. A.III, Kidd, M. T., Bregendahl, K., et al. (2008). Digestible and Metabolizable Energy of Crude Glycerol for Growing Pigs. *J. Anim. Sci.* 86, 602–608. doi: 10.2527/jas.2007-0453

Lech, J. J. (1970). Glycerol Kinase and Glycerol Utilization in Trout (*Salmo Gairdneri*) Liver. *Comp. Biochem. Physiol.* 34, 117–124. doi: 10.1016/0010-406X(70)90058-7

Li, M. H., Minchew, C. D., Oberle, D. F., and Robinson, E. H. (2010). Evaluation of Glycerol From Biodiesel Production as a Feed Ingredient for Channel Catfish, *Ictalurus Punctatus*. *J. World Aquaculture Soc.* 41, 130–136. doi: 10.1111/j.1749-7345.2009.00320.x

Louvado, A., Coelho, F. J. R. C., Palma, M., Tavares, L. C., Ozorio, R. O. A., Magnoni, L., et al. (2020). Effect of Glycerol Feed-Supplementation on Seabass Metabolism and Gut Microbiota. *Appl. Microbiol. Biotechnol.* 104, 8439–8453. doi: 10.1007/s00253-020-10809-3

Madsen, S. S., Bujak, J., and Tipsmark, C. K. (2014). Aquaporin Expression in the Japanese Medaka (*Oryzias Latipes*) in Freshwater and Seawater: Challenging

the Paradigm of Intestinal Water Transport? *J. Exp. Biol.* 217, 3108. doi: 10.1242/jeb.105098

Magnoni, L., Rema, P., Silva-Brito, F., Rito, J., Palma, M., Ozorio, R., et al. (2021). Dietary Glycerol Inclusion Decreases Growth Performance and Nitrogen Retention Efficiency in Rainbow Trout (*Oncorhynchus Mykiss*). *Aquaculture* 535, 736383. doi: 10.1016/j.aquaculture.2021.736383

Marandel, L., Seiliez, I., Véron, V., Skiba-Cassy, S., and Panserat, S. (2015). New Insights Into the Nutritional Regulation of Gluconeogenesis in Carnivorous Rainbow Trout (*Oncorhynchus Mykiss*): A Gene Duplication Trail. *Physiol. Genomics* 47, 253–263. doi: 10.1152/physiolgenomics.00026.2015

Martins, F. O., Rito, J., Jarak, I., Viegas, I., Pardal, M. A., Macedo, M. P., et al. (2013). Disposition of [U-$^{2}H_{7}$]Glucose Into Hepatic Glycogen in Rat and in Seabass. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 166, 316–322. doi: 10.1016/j.cbpa.2013.07.002

Mendoza, O. F., Ellis, M., Mckeith, F. K., and Gaines, A. M. (2010). Metabolizable Energy Content of Refined Glycerin and its Effects on Growth Performance and Carcass and Pork Quality Characteristics of Finishing Pigs. *J. Anim. Sci.* 88, 3887–3895. doi: 10.2527/jas.2010-2873

Menton, D. J., Slinger, S. J., and Hilton, J. W. (1986). Utilization of Free Glycerol as a Source of Dietary Energy in Rainbow Trout (*Salmo Gairdneri*). *Aquaculture* 56, 215–227. doi: 10.1016/0044-8486(86)90337-6

Mewes, J. K., Meurer, F., Tessaro, L., Buzzi, A. H., Syperreck, M. A., and Bombardelli, R. A. (2016). Diets Containing Crude Glycerin Damage the Sperm Characteristics and Modify the Testis Histology of Nile Tilapia Broodstock. *Aquaculture* 465, 164–171. doi: 10.1016/j.aquaculture.2016.08.035

Min, Y. N., Yan, F., Liu, F. Z., Coto, C., and Waldroup, P. W. (2010). Glycerin - A New Energy Source for Poultry. *Int. J. Poultry Sci.* 9, 1–4. doi: 10.3923/ijps.2010.1.4

Moesch, A., Meurer, F., Zadinelo, I. V., Carneiro, W. F., Da Silva, L. C. R., and Dos Santos, L. D. (2016). Growth, Body Composition and Hepatopancreas Morphology of Nile Tilapia Fingerlings Fed Crude Glycerol as a Replacement for Maize in Diets. *Anim. Feed Sci. Technol.* 219, 122–131. doi: 10.1016/j.anifeedsci.2016.05.009

Moreira, I. S., Peres, H., Couto, A., Enes, P., and Oliva-Teles, A. (2008). Temperature and Dietary Carbohydrate Level Effects on Performance and Metabolic Utilisation of Diets in European Sea Bass (*Dicentrarchus Labrax*) Juveniles. *Aquaculture* 274, 153–160. doi: 10.1016/j.aquaculture.2007.11.016

Neu, D. H., Furuya, W. M., Boscolo, W. R., Potrich, F. R., Lui, T. A., and Feiden, A. (2013). Glycerol Inclusion in the Diet of Nile Tilapia (*Oreochromis Niloticus*) Juveniles. *Aquaculture Nutr.* 19, 211–217. doi: 10.1111/j.1365-2095.2012.00968.x

Nunes, P. M., and Jones, J. G. (2009). Quantifying Endogenous Glucose Production and Contributing Source Fluxes From a Single ^{2}H NMR Spectrum. *Magnetic Resonance Med.* 62, 802–807. doi: 10.1002/mrm.22062

OECD/FAO (2019). "Chapter 9: Biofuels," in *OECD-FAO Agricultural Outlook (Edition 2019)* (Rome: OECD Publishing, Paris/Food and Agriculture Organization of the United Nations), 191 – 206.

Oliveira, L., Madrid, J., Ramis, G., Martínez, S., Orengo, J., Villodre, C., et al. (2014). Adding Crude Glycerin to Nursery Pig Diet: Effect on Nutrient Digestibility, Metabolic Status, Intestinal Morphology and Intestinal Cytokine Expression. *Livestock Sci.* 167, 227–235. doi: 10.1016/j.livsci.2014.05.013

Palma, M., Tavares, L. C., Rito, J., Henriques, L. F., Silva, J. G., Ozório, R., et al. (2019). Metabolic Effects of Dietary Glycerol Supplementation in Muscle and Liver of European Seabass and Rainbow Trout by ^{1}H NMR Metabolomics. *Metabolites* 9, 202. doi: 10.3390/metabo9100202

Palma, M., Trenkner, L.H., Rito, J., Tavares, L.C., Silva, E., Glencross, B.D., et al. (2020). Limitations to Starch Utilization in Barramundi (Lates calcarifer) as Revealed by NMR-Based Metabolomics. *Frontiers in Physiology* 11, 205. doi: 10.3389/fphys.2020.00205

Panserat, S., Marandel, L., Seiliez, I., and Skiba-Cassy, S. (2019). New Insights on Intermediary Metabolism for a Better Understanding of Nutrition in Teleosts. *Annu. Rev. Anim. Biosci.* 7, 195–220. doi: 10.1146/annurev-animal-020518-115250

Panserat, S., Plagnes-Juan, E., Gazzola, E., Palma, M., Magnoni, L. J., Marandel, L., et al. (2020). Hepatic Glycerol Metabolism-Related Genes in Carnivorous Rainbow Trout (*Oncorhynchus Mykiss*): Insights Into Molecular Characteristics, Ontogenesis, and Nutritional Regulation. *Front. Physiol.* 11. doi: 10.3389/fphys.2020.00882

Rito, J., Viegas, I., Pardal, M. A., Metón, I., Baanante, I. V., and Jones, J. G. (2018). Disposition of a Glucose Load Into Hepatic Glycogen by Direct and Indirect Pathways in Juvenile Seabass and Seabream. *Sci. Rep.* 8, 464. doi: 10.1038/s41598-017-19087-y

Rito, J., Viegas, I., Pardal, M.Â., Metón, I., Baanante, I. V., and Jones, J. G. (2019). Utilization of Glycerol for Endogenous Glucose and Glycogen Synthesis in Seabass (*Dicentrarchus Labrax*): A Potential Mechanism for Sparing Amino Acid Catabolism in Carnivorous Fish. *Aquaculture* 498, 488–495. doi: 10.1016/j.aquaculture.2018.08.066

Santos, L. D. D., Zadinelo, I. V., Moesch, A., Bombardelli, R. A., and Meurer, F. (2019). Crude Glycerol in Diets for Nile Tilapia in the Fattening Stage. *Pesquisa Agropecuária Bras.* 54, e00460. doi: 10.1590/S1678-3921.pab2019.v54.00460

Savina, M. V., and Wojtczak, A. B. (1977). Enzymes of Gluconeogenesis and the Synthesis of Glycogen From Glycerol in Various Organs of the Lamprey (*Lampetra Fluviatilis*). *Comp. Biochem. Physiol. Part B: Comp. Biochem.* 57, 185–190. doi: 10.1016/0305-0491(77)90141-9

Silva, T. S., Matos, E., Cordeiro, O. D., Colen, R., Wulff, T., Sampaio, E., et al. (2012). Dietary Tools to Modulate Glycogen Storage in Gilthead Seabream Muscle: Glycerol Supplementation. *J. Agric. Food Chem.* 60, 10613–10624. doi: 10.1021/jf3023244

Soares, A., Carvalho, R., Veiga, F., Alves, M., Viegas, I., González, J., et al. (2012). Restoration of Direct Pathway Glycogen Synthesis Flux in the STZ-Diabetes Rat Model by Insulin Administration. *Am. J. Physiol. - Endocrinol. Metab.* 303, E875–E885. doi: 10.1152/ajpendo.00161.2012

Song, X., Marandel, L., Skiba-Cassy, S., Corraze, G., Dupont-Nivet, M., Quillet, E., et al. (2018). Regulation by Dietary Carbohydrates of Intermediary Metabolism in Liver and Muscle of Two Isogenic Lines of Rainbow Trout. *Front. Physiol.* 9. doi: 10.3389/fphys.2018.01579

Theisen, M. T., Bombardelli, R. A., Meurer, F., Ferreira, R. L., and Da Silva, L. C. R. (2020). Crude Glycerol Inclusion in Diets for Post-Larvae *Rhamdia Voulezi* and *Rhamdia Branneri*. *Aquaculture Res.* 51, 1313–1316. doi: 10.1111/are.14465

Tipsmark, C. K., Sørensen, K. J., and Madsen, S. S. (2010). Aquaporin Expression Dynamics in Osmoregulatory Tissues of Atlantic Salmon During Smoltification and Seawater Acclimation. *J. Exp. Biol.* 213, 368. doi: 10.1242/jeb.034785

Viegas, I., Jarak, I., Rito, J., Carvalho, R. A., Metón, I., Pardal, M. A., et al. (2016). Effects of Dietary Carbohydrate on Hepatic *De Novo* Lipogenesis in European Seabass (*Dicentrarchus Labrax* L.). *J. Lipid Res.* 57, 1264–1272. doi: 10.1194/jlr.M067850

Viegas, I., Mendes, V. M., Leston, S., Jarak, I., Carvalho, R. A., Pardal, M.Â., et al. (2011). Analysis of Glucose Metabolism in Farmed European Sea Bass (*Dicentrarchus Labrax* L.) Using Deuterated Water. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 160, 341–347. doi: 10.1016/j.cbpa.2011.06.029

Viegas, I., Rito, J., González, J. D., Jarak, I., Carvalho, R. A., Metón, I., et al. (2013). Effects of Food-Deprivation and Refeeding on the Regulation and Sources of Blood Glucose Appearance in European Seabass (*Dicentrarchus Labrax* L.). *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 166, 399–405. doi: 10.1016/j.cbpa.2013.07.013

Viegas, I., Rito, J., Jarak, I., Leston, S., Caballero-Solares, A., Metón, I., et al. (2015). Contribution of Dietary Starch to Hepatic and Systemic Carbohydrate Fluxes in European Seabass (*Dicentrarchus Labrax* L.). *Br. J. Nutr.* 113, 1345–1354. doi: 10.1017/S0007114515000574

Viegas, I., Rito, J., Jarak, I., Leston, S., Carvalho, R. A., Metón, I., et al. (2012). Hepatic Glycogen Synthesis in Farmed European Seabass (*Dicentrarchus Labrax* L.) is Dominated by Indirect Pathway Fluxes. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 163, 22–29. doi: 10.1016/j.cbpa.2012.04.023

Yang, F., Hanna, M. A., and Sun, R. (2012). Value-Added Uses for Crude Glycerol - a Byproduct of Biodiesel Production. *Biotechnol. Biofuels* 5, 13. doi: 10.1186/1754-6834-5-13

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Hepatic Metabolomics Analysis of Hybrid Grouper (*Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) Fed With Quercetin and Sodium Quercetin-5′-Sulfonates

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Combining feeding trials and metabolomics analyses of tissues and biofluids could shed light on nutrient effects and changes in feed intake. In the present study, hybrid grouper (F1 hybrid *Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) was used as the marine fish model to quantify the impacts of quercetin and sodium quercetin-5′-sulfonates on serum biochemistry and the status of hepatic lipid accumulation and the changes of metabolites in the liver using ultra-performance liquid chromatography-electrospray triple quadrupole mass spectrometry (UPLC-QTRAP/MS/MS). The study shows that total protein, albumin, alkaline phosphatase and glucose were not significantly different among the three groups ($P > 0.05$). Total cholesterol, triglyceride, and alanine aminotransferase of fish fed quercetin, and sodium quercetin-5′-sulfonates were significantly lower than fish fed without additives ($P < 0.05$). Low-density lipoprotein cholesterol and aspartate aminotransferase of fish fed quercetin were significantly lower than that of fish fed without additives and sodium quercetin-5′-sulfonates ($P < 0.05$). The high-density lipoprotein cholesterol of fish fed quercetin, and sodium quercetin-5′-sulfonates were significantly higher than that of fish fed without additives ($P < 0.05$). Lipid accumulation in the quercetin and sodium quercetin-5′-sulfonates groups decreased significantly. Quercetin and sodium quercetin-5′-sulfonates were effective by increasing hypolipidemic and hepatoprotective compounds that are known for reducing blood lipid levels and liver fat accumulation. As a result of this study, we provide international data for metabolic adaptations during the additives feeding using the fish liver as the study model. By understanding the metabolic effects of these feed additives, this study provides a first step toward understanding the molecular mechanisms of these additives and how they function.

Keywords: Quercetin, Sodium quercetin-5′-sulfonates, oxylipins, metabolomics, marine fish

INTRODUCTION

Aquaculture nutrition has evolved into a more efficient and sustainable industry with continuous feed formulation innovation over the last two decades. It usually depends on the fish oil (FO) and fish meal (FM) for fish nutrition at higher trophic levels, such as salmonids and marine species, since these nutrition resources provide adequate nutrition for the fish. Natural prey consumed by wild fish has the most similar nutritional composition to FM and FO diets. FM indeed contains highly digestible, high-quality proteins with an appropriate essential amino acid profile, and FO contains a high fatty acid profile suitable for many fish species, especially marine fish (Oliva-Teles et al., 2015; Roques et al., 2020). A shortage of marine resources for aquaculture will result from increased aquaculture production and other markets for pharmaceutics, cosmetics, and human nutrition (FAO, 2016; Roques et al., 2020). As a result, the aquaculture industry's FM and FO market values will not be sustainable in the future. As a result of these constraints, FM and FO in fish feed need to be replaced by more sustainable raw materials. In the past decade, incorporating plant ingredients to replace marine ingredients has resulted in many enhancements in the formulation of sustainable diets. Since plant resources are now being used as feed for fish, persistent organic pollutants found in feed and fish have decreased (Berntssen et al., 2015). FO substitution is largely dependent on the supply of essential fatty acids. In fact, a number of marine species (Bell and Koppe, 2011; Roques et al., 2020) are unable to produce long-chain PUFAs from the nutritional poly-unsaturated fatty acids (PUFAs) linolenic and linoleic acid because of their enzymatic limitations. Furthermore, a decrease in the docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content in fish flesh results from substituting vegetable oils, while the other qualities of fish flesh remain the same (Oliva-Teles et al., 2015).

FM and FO are only about 10–15% of the total FM, and FO needed for fish feed (Medale et al., 2013; Ytrestøyl et al., 2015) to meet carnivorous and marine species' nutritional needs. In this regard, replacing fish feed with fish resources is complicated for carnivorous fishes, and earlier researches have resulted in decreased growth rates and metabolic changes which are not compatible with the production of aquaculture (Geay et al., 2011; Collins et al., 2013). Hence, there is a need to include new feedstuffs in fish diets to compensate for these shortcomings, but researchers must first accurately characterise them to fully comprehend their impacts on fish metabolism.

Known as the most abundant flavonoid in foods (Petersen et al., 2016), quercetin (QE) has been linked to healthy and functional diets (Spagnuolo et al., 2012; Bigliardi and Galati, 2013). According to previous studies, QE mostly occurs in its glycosylated form in fruits and vegetables (Wang et al., 2020), exhibiting anti-cancer antiviral and anti-bacterial properties, neuroprotective effects, and anti-inflammatory properties beneficial to human health (Wang et al., 2020). Selective sulfonation of QE has been shown to exert liver-protective effects against heavy metal and carbon tetrachloride-induced liver damage in rodents and has potential as a chemopreventive and chemotherapeutic agent for liver disease (Cui et al., 2014). Notably, compared with the parent compound QE, sodium quercetin-5′-sulfonates (QS) showed stronger biological activity than Czerwonka et al. (2020) reported. Furthermore, QS was shown to have antioxidant (Robak and Kopacz, 1989), anti-bacterial (Woznicka et al., 2013) and antitumor activities (Krol et al., 2002) and exhibited hepatoprotective activity in rodent models stimulated by heavy metals (Szelag et al., 2003; Magdalan et al., 2007; Chlebda et al., 2010; Trocha et al., 2012). However, no studies have examined the impact of QE and QS on hybrid grouper (F1 hybrid *Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) metabolism, which needs to be studied further.

Typically, new feed formulae are evaluated by determining their analytic composition and digestibility and then evaluating their influences on growth performance, food intake, and further zootechnical indices. In spite of this, these techniques may not be complete enough to understand the effects of fish feed on the metabolism of fish. Chemical analyses of fish diets give a rough idea of the composition; nonetheless, they do not offer data about the profiles of small compounds, like non-essential nutrients such as creatine and taurine and other nutrition-related factors, and several under nutrition-related factors like phytic acid, polyphenols, or mycotoxins found in plant feeds (Glencross et al., 2007; Roques et al., 2020). An extensive and expensive analysis is needed for each of these factors and compounds. In metabolomics, the global set of metabolites in a biological system is investigated in order to gain insight into the system's metabolic state. Combining feeding trials and metabolomics analyses of tissues and biofluids could shed light on nutrient effects and changes in feed intake.

Hepatic is a multifunctional organ with a high metabolic rate and biotransformation capacity, making it highly susceptible to drugs, feed, pollutants and toxins (Cuykx et al., 2018; Song et al., 2019; Zhou et al., 2019). Interestingly, hepatic is involved in the metabolism of QE and its derivatives and represents one of the essential metabolic organs (Wang et al., 2016b). Hybrid grouper is an essential marine aquaculture fish-breed developed through hybridisation technology and widely cultured in the southeast coast of China and other countries (Amenyogbe et al., 2020; Xie et al., 2021). The *in vivo* absorption, distribution, metabolism, and bioavailability of QE have been extensively studied in animal models and humans (Wang et al., 2016b). After digestion and absorption by the digestive system in vertebrates, QE forms glucuronic acid or sulfate derivatives in the liver (Wang et al., 2016b). The content and structure type of QE derivatives play a key role in their absorption and distribution (Spencer et al., 2008). Based on high performance liquid chromatography (HPLC) detection method, it was found that QE was mainly deposited in the body in the form of aglycone after being absorbed by tilapia, and the changes of QE concentration were found in plasma, liver and whole-body homogenate (Park et al., 2009). For a better insight into metabolic adjustment, serum biochemical parameters, and liver fat accumulation through

feeding additives (QE and QS) to hybrid groupers, the liver hepatic metabolic profile was examined after 56 days of feeding.

MATERIALS AND METHODS

Chemical Synthesis of QS

All chemicals and reagents are analytically pure and used without further purification. QE (97%, *w/w*) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). ^{1}H NMR and ^{13}C NMR spectra were measured at room temperature on a Bruker Avance III 400 MHz NMR spectrometer. Tetramethylsilane (TMS) served as the internal standard.

QS were prepared using the method outlined by Czerwonka et al. (2020). This method includes the following two steps: synthesis of quercetin-5′-sulfonic acid was carried out; the product of sulfonic acid was neutralised with NaOH solution to get the targeted material QS. In brief, the QE powder was added to the appropriate 98% sulfuric acid and put in an 80°C water bath for 2 h to heated with stirring. After the reaction, the mixture was cooled down to room temperature (25°C), and purified water was added while stirring continuously. The quercetin-5′-sulfonic acid precipitate was collected by filtration and then double crystalised from the saturated aqueous solution. The final product of QS was obtained by neutralising the synthesised acid in NaOH aqueous solution and double crystallising the resulting precipitate from the saturated water solution.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.47 (1H, s, 5-OH), 10.98 (1H, s, 4'-OH), 10.78 (1H, s, 7-OH), 9.46 (1H, s, 3-OH), 9.26 (1H, s, 3'-OH), 7.87(1H, d, H-2'), 7.62(1H, d, H-6'), 6.40 (1H, d, H-8), 6.19(1H, d, H-6).

^{13}C NMR (101 MHz, DMSO-d_6) δ: 175.84 (C-4), 163.99 (C-7), 160.76 (C-5), 156.12 (C-9), 145.96 (C-2), 145.46 (C-3'), 144.20 (C-4'), 136.10 (C-3), 131.19 (C-5'), 120.81 (C-1'), 117.76 (C-2'), 115.37 (C-6'), 103.04 (C-10), 98.22 (C-6), 93.26 (C-8).

The molecular structure of the compound is shown in **Figure 1**.

Experimental Design and Diets

Referring to the study of grouper (Ye et al., 2020; Xie et al., 2021), the ingredients and proximate composition of the primary feed are presented in **Tables 1**, **2**. We opted for QE dosage based on a previous study (Shin et al., 2010; Zhai and Liu, 2013; Xu et al., 2019). Group CT was fed a non-additive (QE or QS) diet; group QE was fed a diet containing 0.8 mmol/kg of QE; group QS was fed a diet containing 0.8 mmol/kg of QS. Before the feed was prepared, all the raw materials had to be crushed, sieved and weighed precisely according to the formula. Mixed all the powder ingredients, and then added oil and water mixed thoroughly with

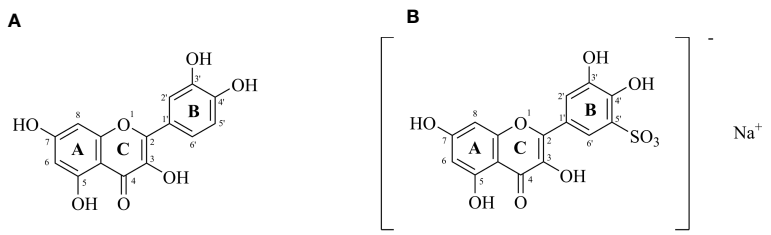

FIGURE 1 | **(A)** Structure of quercetin (3,3′,4′,5,7-pentahydroxyflavone); **(B)** Sodium Quercetin -5'-Sulfonates (sodium 3,3',4',5,7-pentahydroxyflavon-5'-sulfonate) (Czerwonka et al., 2020).

TABLE 1 | Ingredients and proximate composition analysis (on dry weight basis) of the experimental diets.

Ingredients	Percentage	Proximate composition	Percentage
Fish meal	50.00	Moisture	9.70
Vital wheat gluten	12.00	Crude protein	48.74
Wheat flour	17.47	Crude fat	10.60
Corn gluten meal	8.00	Crude ash	9.90
Fish oil	4.00		
soybean oil	1.50		
Soybean lecithin	1.50		
Microcrystalline cellulose	1.00		
Calcium monophosphate	2.00		
Choline chloride	0.50		
Ethoxyquin	0.03		
Vitamin and mineral premix	2.00		
Total	100.00		

Two kg premix contained the following: VB_1, 20.00 g; VB_2, 20.50g; VB_6, 45.00 g; VB_{12}, 0.10 g; VK_3, 4.55 g; VE, 90.00 g; VA, 10 g; VD_3, 40.00 g; nicotinic acid, 82 g; D–calcium pantothenate, 60.67 g; biotin, 2.0 g; folic acid, 6.17 g; inositol, 140.04 g; cellulose, 478.97g; ferric citrate, 14.35g; $ZnSO_4{\cdot}7H_2O$, 29.12g; $MgSO_4{\cdot}H_2O$, 12.19g; $MnSO_4{\cdot}7H_2O$, 0.11g; $CuSO_4{\cdot}5H_2O$, 18.85 g; $CoCl_2{\cdot}6H_2O$, 4.35 g; $Ca(H_2PO_4)_2{\cdot}H_2O$, 50 g; KIO_3, 0.03 g; KCl, 17.74 g; Na_2SeO_3, 2.0 g; zeolite powder, 851.26 g.

TABLE 2 | Preparation of quercetin mixture for the different additive levels used in experimental diets.

Groups	Additive levels (mmol/kg diet)	QE or QS (g/kg diet)	Microcellulose (g/kg diet)
CT	0.0	0.000	10.000
QE	0.8	0.271	9.729
QS	0.8	0.381	9.619

a groove-type mixer (CDH-100, Sichuan Chuanda Drying Technology Engineering Co., Ltd., China). In this study, dough with a diameter of 4 mm was wet extruded with a pelletiser (F-26, South China University of Technology, Guangzhou, China) and dried under air conditioning at 16°C, stored in a sealed bag at -20°C until feeding.

Experimental Conditions and Fish

Experimental fish were taken from the self-breeding and self-raising seedlings of Zhanjiang Ocean High-tech Park, Guangdong Ocean University. The fish were selected and transported with a special fish transportation vehicle to the breeding base of Guangdong Evergreen Feed Industry Co., Ltd., Zhanjiang, China. They were given the basal diet (crude protein ≥ 40%, crude fibre ≤ 5.0%, crude ash ≤ 16%, crude lipid ≥ 6%, moisture ≤ 12%, total phosphorus 0.90-1.60 and lysine ≥ 2.10) for two weeks to become accustomed to the experimental diets and farming conditions. After 24 hours of fasting, 225 fish (weighed 10.10 ± 0.02 g) were distributed evenly among 9 fibreglass tanks (500L). 25 fish per tank, 3 replicates per treatment. The experiment lasted eight weeks; fish were fed twice a day at 9:00 and 16:00 at 5 - 8% of their body weight. During this experimentation, the rearing water (flow-through natural seawater processed with sand filtration and sedimentation) was constantly aerated and maintained under the following conditions: temperature 25 - 29°C, pH 7.5 - 8.0, salinity 28.0 - 32.0g/L, dissolved oxygen 6 - 8 mg/L, and ammonia stayed 0.03 ± 0.01 mg/L. The fish were reared in a continuous 24-h ventilated flowing water culture system.

Sample Collection

Before sampling, the fish fasted for 24 hours at the end of the feeding trial and were weighed with the average fish size (54.36 ± 3.95 g); then, they were anesthetised with 8 mg/L methane-sulfonate (MS-222, Sigma-Aldrich). Blood samples were collected from the caudal vein of fish (n = 6 per tank) randomly selected from each tank and pooled together. The collected blood (approximately 0.7 mL per fish) was transferred into 1.5 mL anticoagulant-free centrifuge tubes and then stored at 4°C for 12 h. After centrifugation at 3500 rpm and 4°C for 15 min, the separated serum was obtained and immediately stored at -80°C.

Eighteen individuals of hybrid grouper were randomly selected for each treatment to collect livers for metabolomic analysis. Six (n = 6 per tank) liver samples from each replicate tank were pooled together in two tubes each. For histological analysis (Oil red O staining in the liver), the livers of two fishes from each tank were sampled. We immediately froze liver tissues in liquid nitrogen and stored them at -80°C. During sample collection, low temperatures and rapidity were maintained.

Biochemical Parameters in Serum

Cryopreserved serum samples were thawed on ice. The serum total protein (TP, A045-4), albumin (ALB, A028-2-1), total cholesterol (T-CHO, A111-1-1), triglyceride (TG, A110-1-1), alkaline phosphatase (AKP, A059-2), glucose (GLU, A154-1-1), low-density lipoprotein cholesterol (LDL, A113-1-1), high-density lipoprotein cholesterol (HDL, A112-1-1), aspartate aminotransferase (AST, C010-2-1) and alanine aminotransferase (ALT, C009-2-1) levels were measured using the methods described in the detection kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) through the use of a VICTOR Nivo Multimode Microplate Reader (PerkinElmer).

Oil Red O Staining

The trimmed liver tissue was embedded with an embedding agent (OCT) and then placed in a frozen sectioning machine for cryofixation. The sectioning was started after waiting for solidification. Frozen sections were stained in fresh oil red O staining solution for 8-10 min at room temperature, rinsed in tap water, and counterstained with hematoxylin until the desired degree of staining (Servicebio, China). Finally, glycerol jelly mounting medium was added to the sections, and the slides were sealed with cover glass. Lipid droplets were examined with the Nikon Eclipse microscope (Nikon, Japan). Quantification of the relative area of oil red O solution staining was performed using Image-Pro Plus 6.0 software. Six images of each sample were randomly selected, and the average relative area of Oil Red O staining was calculated to determine the relative content of lipid droplets.

Metabolomics Analysis

Extraction and Preparation of Samples

Hepatic samples were thawed on ice. Take 50 ± 2 mg of one sample, add cold steel balls to the mixture, and homogenate at 30 Hz for 3 min. Add 1 mL 70% methanol with internal standard extract to the homogenised centrifuge tube, whirl the mixture for 5 min, and then centrifuge it with 12,000 rpm at 4°C for 10 min. After centrifugation, draw 400 uL of supernatant into the corresponding EP tube and store in a -20°C refrigerator overnight, centrifuge at 12000 r/min at 4°C for 3 min, and take 200 uL of supernatant in the liner of the corresponding injection bottle for on-board analysis.

HPLC Conditions

Liver sample extracts were analyzed using an LC-ESI-MS/MS system (UPLC, ExionLC AD, https://sciex.com.cn/; MS, QTRAP® System, https://sciex.com/).The analytical conditions

were as follows (Zhang et al., 2021), UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm*100 mm); column temperature, 40°C; flow rate, 0.4 mL/min; injection volume, 2μL; solvent system, water (0.1% formic acid): acetonitrile (0.1% formic acid); gradient program, 95:5 V/V at 0 min, 10:90 V/V at 11.0 min, 10:90 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 14.0 min.

ESI-QTRAP-MS/MS

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). The ESI source operation parameters were as follows (Li et al., 2022): source temperature 500°C; ion spray voltage (IS) 5500 V (positive), -4500 V (negative); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Data Processing and Statistical Analysis

MS data acquisition and processing were performed previously described (Chen et al., 2013). Metabolites were annotated using the Metware in-house MS2 spectral tag (MS2T) library (Wuhan Metware Biotechnology Co., Ltd.; http://www.metware.cn, Wuhan, China). Differential variables of the metabolites were analysed using orthogonal partial least squares-discriminant analysis (OPLS-DA). VIP ≥ 1 with fold change ≥ 2 or fold change ≤ 0.5 was considered statistically significant. The results of the biochemical indexes were presented as the mean ± standard error (SEM). The experimental data were subjected to one-way ANOVA. Multiple treatment groups were compared by Duncan's honestly significant difference test at $P \leq 0.05$. Statistical analysis of all data was performed using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Serum Biochemical Indices

Table 3 presents the effects of different diets on serum biochemical parameters in grouper. The result shows that TP, ALB, AKP and GLU were not significantly different among the three groups ($P > 0.05$). T-CHO, TG and ALT of fish fed QE and QS were significantly lower than those of fish fed CT ($P < 0.05$). LDL and AST of fish fed QE were significantly lower than that of fish fed CT and QS ($P < 0.05$). The HDL of fish fed QE and QS was significantly higher than those fish fed CT ($P < 0.05$).

Lipid Accumulation in the Liver of Fish Fed Different Diets

For the comparison of lipid accumulation in the liver, oil red O staining was performed, shown in **Figure 2**. Lipid droplets and nuclei were stained red and blue for oil red O staining, respectively. The CT group showed more red spots and lipid droplets than the QE and QS groups in the present study. Compared to the CT group (25.67%), lipid accumulation in the QE and QS groups decreased significantly to 19.55% and 19.32%, respectively ($P < 0.05$).

Differential Metabolites in the Liver in Response to QE Treatment

To investigate changes in liver metabolites in QE-treated hybrid grouper, livers from the CT and QE groups were collected for an extensive targeted metabolomic analysis. OPLS-DA model was used to determine metabolite differences between the CT and QE groups. OPLS-DA combines orthogonal signal correction (OSC) and PLS-DA methods to screen for differential variables by removing uncorrelated differences, allowing further indication of differences between groups. OPLS-DA scores clearly classify samples into distinct clusters, indicating significant differences in metabolites between the CT and QE groups. The number of differential metabolites is clearly shown in the OPLS-DA S plot. A total of 91 differential metabolites (**Supplementary Table S1**) were detected in the CT and QE groups (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5). After qualitative and quantitative analysis of the detected differential metabolites, the results of the metabolites with the top-ranked changes are presented in **Figure 3C**.

TABLE 3 | Serum biochemical indices of hybrid grouper fed with experimental diets.

Parameters	Experimental diets		
	CT	QE	QS
TP (g L^{-1})	30.03 ± 0.78	30.28 ± 1.18	30.55 ± 0.73
ALB (g L^{-1})	5.67 ± 0.27	5.67 ± 0.36	5.72 ± 0.28
T-CHO (mmol L^{-1})	3.49 ± 0.16^{a}	2.66 ± 0.08^{b}	2.71 ± 0.10^{b}
TG (mmol L^{-1})	0.37 ± 0.03^{a}	0.24 ± 0.03^{b}	0.26 ± 0.02^{b}
AKP (U L^{-1})	40.65 ± 4.84	40.77 ± 1.90	53.05 ± 5.51
GLU (mmol L^{-1})	4.85 ± 0.50	4.43 ± 0.28	4.84 ± 0.15
LDL (mmol L^{-1})	0.65 ± 0.02^{a}	0.35 ± 0.03^{b}	0.57 ± 0.01^{a}
HDL (mmol L^{-1})	2.35 ± 0.15^{b}	3.54 ± 0.11^{a}	3.49 ± 0.19^{a}
AST (U L^{-1})	73.06 ± 1.03^{a}	50.57 ± 3.10^{b}	65.86 ± 4.82^{a}
ALT (U L^{-1})	165.26 ± 8.32^{a}	133.84 ± 2.61^{b}	138.10 ± 3.36^{b}

Values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).

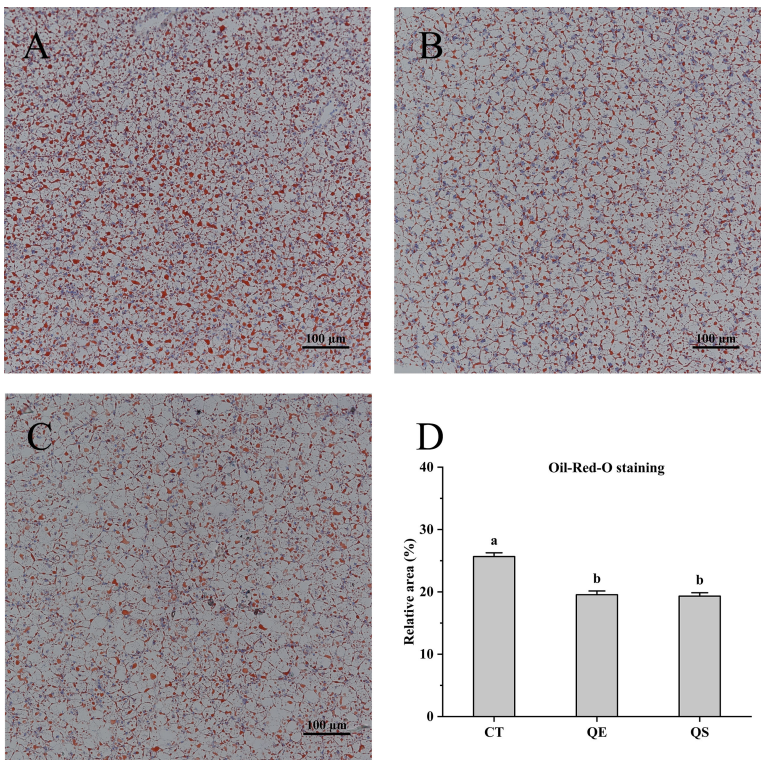

FIGURE 2 | Hepatocyte oil red O (ORO) staining section of hybrid grouper treated with different diets (Magnification × 20). **(A)** CT; **(B)** QE; **(C)** QS; **(D)** The relative area of red lipid droplets in the hepatic section area (%). Values were presented as means ± SEM, and bars with different letters are significantly different ($P < 0.05$).

Differential metabolites from CT vs. QE could be divided into 11 categories, mainly from fatty acyl groups (FA; 42.86%) and amino acids and their metabolites (21.98%). Oxylipins accounted for 71.05% of FA.

Table 4 classifies 28 different oxylipins, with the most derived from arachidonic acid (ARA, 35.71%), docosahexaenoic acid (DHA, 21.43%) derived oxylipins ranking second, and linoleic acid (LA, 14.29%), alpha-linolenic acid (ALA, 10.71%) and eicosapentaenoic acid (EPA, 10.71%) derived oxylipins as follows. Further KEGG analysis showed that arachidonic acid metabolism, inflammatory mediator regulation of TRP channels and linoleic acid metabolism were significantly enriched after QE supplementation (**Figure 3D**).

Effect of QS on Metabolites in the Liver of Hybrid Grouper

To further investigate the effect of QS on the liver metabolites of hybrid grouper, liver tissues from the QS group were also analysed. A total of 83 differential metabolites (**Supplementary Table S2**) were identified (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5), and the results of OPLS-DA and OPLS-DA S plots are shown in **Figures 4A**, **4B**. Among the differential metabolite, 11β-Prostaglandin E2 (11β-PGE2) was the top metabolite that increased the most (**Figure 4C**). The total differential metabolites CT vs. QS could be divided into 10 main categories, of which 42.17% belonged to FA and 36.14% to amino acids and their metabolites. Among the 35 FA species, 29 belonged to oxylipins.

Table 5 classifies 29 different oxylipins, of which the most derived from ARA (31.03%), DHA (27.59%) derived oxylipins ranked second, and LA (13.79%), ALA (10.34%) and EPA (10.34%) derived oxylipins were as follows. Further KEGG analysis showed that arachidonic acid metabolism, inflammatory mediator regulation of TRP channels and linoleic acid metabolism were significantly enriched after QE supplementation (**Figure 4D**).

DISCUSSION

Changes in serum biochemical indicators reflect the physiological and metabolic status of fish, which is helpful for us to determine the response of fish to dietary supplements (Tan et al., 2018; Xie et al., 2021). In general, if lipid parameters including TG, T-CHO, and LDL increase and HDL in fish decreases, is an indication that fish may have some lipid metabolic disorders and liver damage (Zhai and Liu, 2013). In our results, QE and QS decreased serum TG, T-CHO, LDL levels and increased HDL levels in hybrid grouper. Consistent with our findings, Shin et al. (2010) reported that feeding diets supplemented with 0.25% or 0.50% QE significantly reduced T-CHO levels in flounder, with 60 days of feeding better than 30

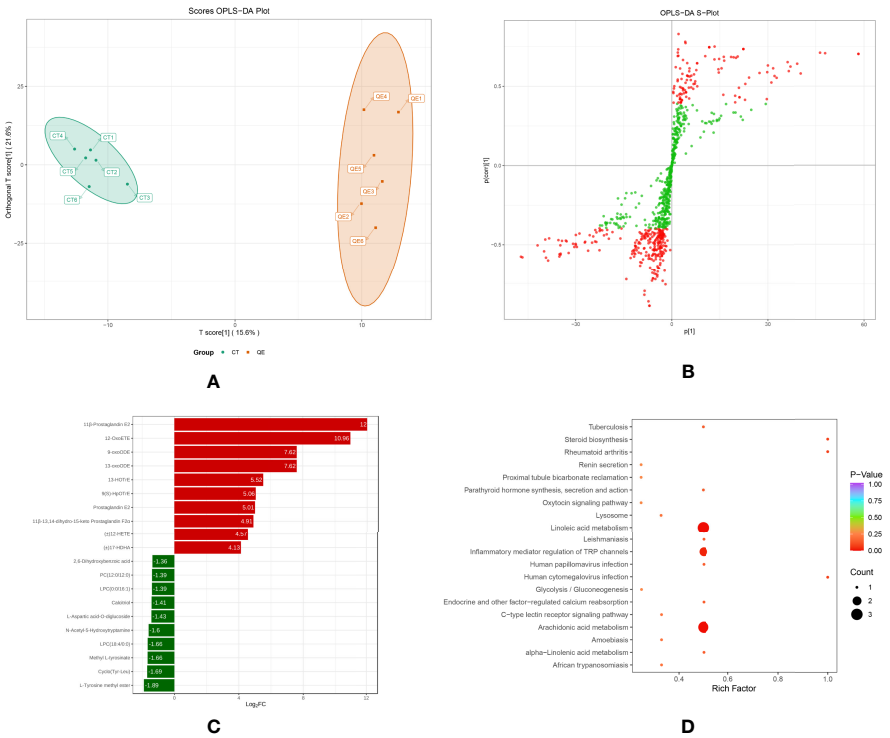

FIGURE 3 | Effect of QE on oxylipins in the liver of hybrid grouper. **(A)** The OPLS-DA scores of the CT group (green) and QE group (red). **(B)** The OPLS-DA S-plot. The X-axis indicates the correlation coefficient between the principal components and oxylipins, and the Y-axis indicates the correlation coefficient between the principal components and oxylipins. The red dot represents VIP ≥ 1, and the green dot represents VIP ≤ 1. **(C)** Bar chart showing the differential oxylipins in the CT group (green) and QE group (red). **(D)** Differential oxylipins enrich KEGG pathways in the CT group and QE group. (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5, n = 6).

days. Repeated use significantly increases the bioavailability of QE (Rangel-Ordonez et al., 2010). Zhai and Liu (2013) indicated that QE could decrease serum TG levels and increase HDL level in tilapia. In addition, QE has a modulating effect on low-density lipoprotein receptor expression (Moon et al., 2012). It reduces blood lipids by increasing the rate of LDL clearance from the blood. Similar results with QE for lowering serum or liver lipids have been reported in rats (Padma et al., 2012), chickens (Qureshi et al., 2011), and rabbits (Kamada et al., 2005), and humans (Egert et al., 2010). In this study, the sulfonated product QS obtained consistent results with QE, indicating that QE and QS showed potential activity in lowering cholesterol and triglycerides and helping to avoid pathological changes in the fatty liver.

QE has preventive and therapeutic effects on hepatic damage, such as fatty liver, cirrhosis and liver fibrosis (Miltonprabu et al., 2017). Cui et al. (2014) revealed that QE significantly reduced the activity of serum AST and ALT in rats. AST and ALT plays an important role in the liver's metabolic function. Increased ALT enzyme activity is an indicator of the degree of liver cell membrane damage, and increased AST level is another indicator of liver mitochondrial damage (Xu et al., 2010; Yang et al., 2011). In this study, QE and QS significantly reduced the levels of AST and ALT in the serum of hybrid grouper and had the effect on protecting the liver. On this basis, it is suggested that both QE and QS can stabilise the liver cell membrane and have a protective effect on mitochondria. Cui et al. (2014) believe that QE and quercetin-5',8-disulfonate (QDS) effectively attenuate the increase of AST and ALT in the serum of mice induced by carbon tetrachloride, and the selective sulfonation of QE increases the hepatoprotection effect. Water-soluble QDS was more effective than QE in reducing AST and ALT release.

In contrast, QE was more potent than QS in our results, probably due to the high dose of QS. Remarkably, Significant increases in markers of hepatotoxicity (AST and ALT) were found in mice injected with high doses of QE (1500 and 2000 mg/kg), mediated in part through oxidative stress (Singh et al., 2022). Related findings were found in acute doses of apigenin (Singh et al., 2012) and genistein (flavonoids) (Singh et al., 2014). From a molecular point of view, the toxic effects of QE are likely related to the oxidation of QE to potentially toxic products during ROS scavenging, namely quercetin-quinine, which is highly reactive with thiols, and GSH might be the principal reactant (Galati et al., 2001; Awad et al., 2002; Boots et al., 2007).

In fish, the liver is the primary site for lipid synthesis and storage (Martin et al., 2017; Zhou et al., 2019). Lipid droplets (LDs) are intracellular organelles dedicated to storing energy in the form of neutral lipids and are involved in lipid metabolism (Welte and Gould, 2017). According to previous studies, QE treatment attenuate inflammation and fibrosis in mice

TABLE 4 | Significant differences in oxylipins between the CT and QE groups.

Compounds	Class II	VIP	Fold Change	Log2FC
(±)15-HETE	ARA	1.55	2.04	1.03
(±)12-HETE	ARA	1.74	23.70	4.57
(±)16-HETE	ARA	1.89	5.00	2.32
(±)17-HETE	ARA	1.89	5.00	2.32
(±)18-HETE	ARA	1.89	5.00	2.32
12-OxoETE	ARA	1.80	1991.47	10.96
11β-Prostaglandin E2	ARA	1.79	4100.47	12.00
11β-13,14-dihydro-15-keto Prostaglandin F2α	ARA	1.55	30.10	4.91
14,15-Leukotriene C4 (ExC4)	ARA	1.11	4.32	2.11
15-deoxy-δ-12,14-PGJ2	ARA	1.51	8.09	3.02
(±)17-HDHA	DHA	1.05	17.52	4.13
(±)4-HDHA	DHA	1.22	3.01	1.59
14 (S)-HDHA	DHA	1.72	14.62	3.87
10-HDoHE	DHA	1.75	3.82	1.93
8-HdoHE	DHA	1.40	12.02	3.59
11-HdoHE	DHA	1.73	14.92	3.90
13-oxoODE	LA	1.78	197.23	7.62
9-oxoODE	LA	1.78	197.23	7.62
13®-HODE	LA	1.74	14.08	3.82
9 (S),12 (S),13 (S)-TriHOME	LA	1.70	10.34	3.37
13-HOTrE	ALA	1.60	45.84	5.52
9 (S)-HpOTrE	ALA	1.43	33.29	5.06
9 (S)-HOTrE	ALA	1.90	6.28	2.65
(±)12-HEPE	EPA	1.86	16.65	4.06
(±)15-HEPE	EPA	1.86	16.65	4.06
(±)18-HEPE	EPA	1.86	16.65	4.06
11-HEDE	Uncategorized	1.63	2.83	1.50
15-HEDE	Uncategorized	1.63	2.83	1.50

nonalcoholic fatty liver disease (NAFLD), alleviate hepatic fat accumulation (Marcolin et al., 2012; Liu et al., 2018). Thus, the current study also observed the lipid accumulation status in hepatic by oil red sections. ORO results show that QE and QS treatments significantly reduced the lipid droplet area in the liver of hybrid grouper, indicating that QE and QS helped to reduce liver fat accumulation. Derivatives of QE have also revealed consistent results in *in vitro* and *in vivo* research (Yu et al., 2016; Qin et al., 2018). Amelioration of fat deposition by QE and its derivatives was probably related to the regulation of adipogenic gene expression and antioxidant function (Jung et al., 2013; Wang et al., 2016a; Liu et al., 2018).

This study provides basic metabolic profiling data for studying the marine fish liver response to QE supplementation by ultra-performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (UPLC-QTRAP/MS/MS). These data increased our understanding of QE. Indeed, a study has explored the metabolism of QE (Wang et al., 2016b). On this basis, it has been suggested that high phenolic intake increases intestinal hippurate production (Mullen et al., 2008; de Mello et al., 2017; Ulaszewska et al., 2020). However, only dogs produce hippuric acid primarily in the kidneys; in other species, such as mice and rats, hippuric acid production depends on hepatocytes (Toromanovic et al., 2008; Lees et al., 2013). In particular, most QE-derived metabolites are identified as 3-hydroxyphenylacetic acid, benzoic acid and hippuric acid (Mullen et al., 2008). In this experiment, hepatic hippuric acid was significantly increased after dietary QE and QS

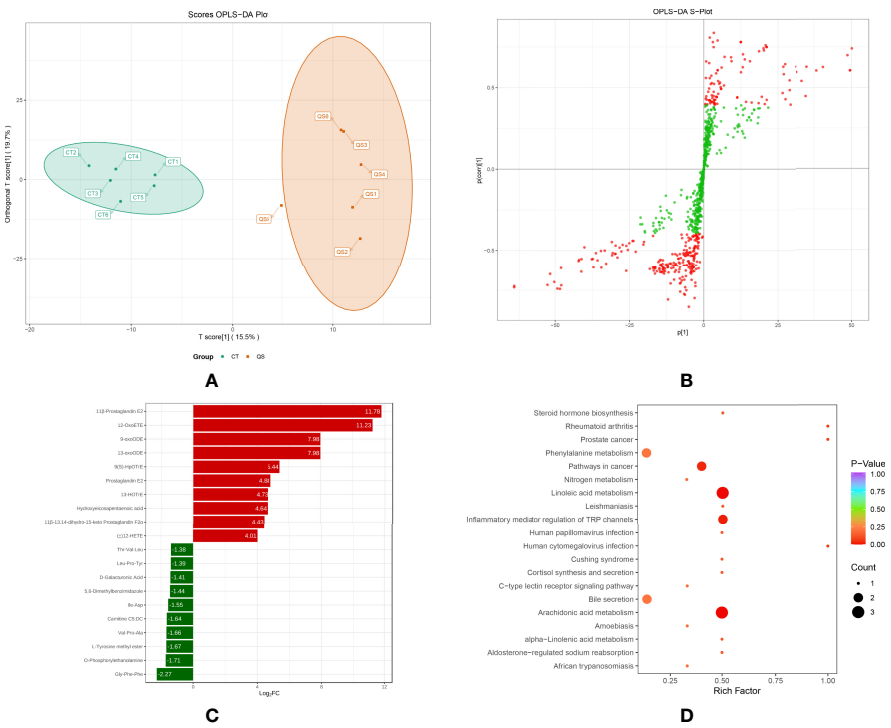

FIGURE 4 | Effect of QS on oxylipins in the liver of hybrid grouper. **(A)** OPLS-DA scores **(B)** OPLS-DA S-plot. **(C)** Bar chart showing the differential oxylipins in the CT group (green) and QS group (red). **(D)** Differential oxylipins enriched KEGG pathways in the CT group and QS group. (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5, n = 6).

TABLE 5 | Significant differences in oxylipins between the CT and QS groups.

Compounds	Class II	VIP	Fold Change	Log2FC
(±)15-HETE	ARA	1.75	2.50	1.32
(±)12-HETE	ARA	1.93	16.13	4.01
(±)16-HETE	ARA	1.98	4.91	2.29
(±)17-HETE	ARA	1.98	4.91	2.29
(±)18-HETE	ARA	1.98	4.91	2.29
12-OxoETE	ARA	1.53	2398.98	11.23
11β-Prostaglandin E2	ARA	1.88	3517.59	11.78
11β-13,14-dihydro-15-keto Prostaglandin F2α	ARA	1.60	21.52	4.43
15-deoxy-δ-12,14-PGJ2	ARA	1.37	12.31	3.62
(±)17-HDHA	DHA	1.48	12.97	3.70
(±)4-HDHA	DHA	1.31	2.66	1.41
14 (S)-HDHA	DHA	1.85	11.65	3.54
10-HdoHE	DHA	1.74	3.08	1.62
8-HdoHE	DHA	1.64	11.22	3.49
11-HdoHE	DHA	1.88	12.92	3.69
16-HdoHE	DHA	1.02	2.14	1.10
20-HdoHE	DHA	1.03	3.78	1.92
13-oxoODE	LA	1.54	252.99	7.98
9-oxoODE	LA	1.54	252.99	7.98
13®-HODE	LA	1.71	9.99	3.32
9 (S),12 (S),13 (S)-TriHOME	LA	1.51	9.47	3.24
13-HOTrE	ALA	1.60	26.49	4.73
9 (S)-HpOTrE	ALA	1.59	43.52	5.44
9 (S)-HOTrE	ALA	1.68	3.43	1.78
(±)12-HEPE	EPA	1.90	11.80	3.56
(±)15-HEPE	EPA	1.90	11.80	3.56
(±)18-HEPE	EPA	1.90	11.80	3.56
11-HEDE	Uncategorized	1.25	2.50	1.32
15-HEDE	Uncategorized	1.25	2.50	1.32

(**Tables S1, S2**), indicating that the liver is directly or indirectly involved in the metabolism of QE and QS in fish.

Analysing the metabolomic data, supplementation with QE and QS had the greatest effect on oxylipins metabolites *in vivo*. Oxylipins are a class of bioactive lipids that play important roles in regulating the intensity and duration of inflammatory responses as well as tissue repair, blood coagulation, vascular permeability and energy regulation (Dennis and Norris, 2015). Therefore, we focused on further analysis of oxylipins in FA. Oxylipins are oxidised PUFAs; depending on the source of their parent PUFAs, oxylipins can be divided into two categories: oxylipins derived from omega-6 PUFAs, such as ARA and LA; and those derived from omega-3 PUFAs, such as α-Linolenic acid (18:3n-3) (ALA), eicosapentaenoic acid (20:5n-3) (EPA) and docosahexaenoic acid (22:6n-3) (DHA) (Gabbs et al., 2015; Picklo and Newman, 2015). Studies showed that oxylipins are rapidly produced and eliminated in the body, and the detectable oxylipins represent their biologically active form (Shaik et al., 2013; Barquissau et al., 2017). A large number of oxylipins were detected in this experiment, indicating that the basic physiological reactions requiring the participation of oxylipins may have been going on in the fish liver all the time.

ARA-derived oxylipins are more diverse and are thought to play important roles in most tissues and organs (Leng et al., 2018; Gao et al., 2021). In the present study, 28 and 29 oxylipins were found in the livers of QE and QS, respectively, most of which were ARA-derived (**Tables 4**, **5**). KEGG annotation also showed that ARA metabolism was mainly enriched (**Figures 3**, **4**), suggesting that QE and QS may be involved in the body's basal metabolism by regulating ARA levels. Studies on oxylipins in fish are relatively scarce; however, a recent study found 57 targeted lipids of five major n-6 and n-3 PUFAs in the plasma of large yellow croaker and rainbow trout, approximately 42.1% from ARA (Gao et al., 2021). In addition, Leng et al. (2018) identified the 70 oxylipins quantified in the rat liver, about two-thirds of the oxylipins were derived from n-6 PUFAs, and ARA-derived oxylipins accounted for about two-thirds of them. Of the 87 oxylipins present in the rat brain, half (51%) were derived from ARA, accounting for 81-90% of the total oxylipins mass (Ferdouse et al., 2019b). It is speculated that the predominant oxylipins in animals may be roughly similar even with different dietary conditions; that is, ARA-type oxylipins generally have more types and qualities, suggesting that they may play an important role in life activities.

Oxylipins show a wide range of functions, most of which are still being discovered. Interestingly, oxylipins obtained from different PUFA substrates and pathways can have similar or opposite effects so it is important to fully identify the entire oxylipins profile to properly understand their overall biological effects (Deline et al., 2015). In this study, in addition to ARA, EPA and DHA were also significantly increased after QE and QS treatment. Studies have shown that n-3 EPA and DHA have antithrombotic and antiarrhythmic properties (Endo and Arita, 2016; Lee et al., 2016a). In addition, the n-3 fatty acid EPA has a

strong anti-inflammatory effect and can affect the T cell response to infection (Wu and Meydani, 1998; Harris et al., 2000). Based on the significant health benefits of EPA and DHA, many studies have begun using EPA and DHA dietary supplements to increase the content of n-3 PUFA oxylipins. Dietary supplements EPA and DHA increased anti-inflammatory n-3 LC-PUFA oxylipins and decreased pro-inflammatory n-6 oxylipins in plasma, liver, kidney, heart, brain, and other tissues (Leng et al., 2018; Ferdouse et al., 2019a; Rey et al., 2019; Kutzner et al., 2020). As a low-toxic natural flavonoid, QE will find more meaningful value and far-reaching effects if further studies on ARA, EPA, and DHA respond to treatments.

Furthermore, prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) have inhibitory effects on adipogenesis and can inhibit the early differentiation of adipocytes (Stewart and Fisher, 2015; Barquissau et al., 2017). In the study, PGE2 was significantly increased after QE and QS treatment (**Table S1, S2**). Increased synthesis and release of PGE2 and PGF2α activate prostaglandin membrane receptors that bind to PGE2 and PGF2α, resulting in sustained calcium oscillations that inhibit the expression of PPARγ target genes, including the uncoupling protein 1 (UCP1) gene, and ultimately inhibit the white adipose tissue browning (Stewart and Fisher, 2015). In this study, 11β-PGE2 was significantly increased after treatment, presumably related to the significant increase in PGE2. Due to its properties, including activation of endogenous stem cells, immunoregulation, and angiogenesis, PGE2 plays a vital role in regenerating various organ systems following injury. As a result, PGE2 can help to reduce inflammation, fibrosis, necrosis, and other adverse effects of liver ischemia or reperfusion injury (Cheng et al., 2021). There is convincing evidence *in vitro* and *in vivo* that PGE2 signalling pathways protect various organs from inflammation, oxidative stress, and fibrosis. Hence the present study suggests that QE and QS help to increase the PGE2 production, which could be beneficial to the hybrid grouper, but further studies are needed to elucidate the exact role.

CONCLUSION

In conclusion, UPLC-QTRAP/MS/MS study of hybrid grouper liver samples brought to light universal information concerning the metabolic metabolism of the liver in response to nutritional additives QE and QS. In addition to providing new ideas for studying feeding additives, these data confirmed the existing research results. This study showed that QE and QS effectively increase hypolipidemic and hepatoprotective compounds that are known for reducing blood lipid levels and liver fat accumulation in groupers. Additionally, we found that QE and QS altered oxylipin levels in the liver of grouper fish, suggesting that the QE and QS are partly mediated through the regulation of oxylipins. There are similarities in the biological effects of quercetin and its sulfonated derivatives. It is believed that sulfation in the structural transformation of flavonoids may provide a novel mechanism to regulate their bioavailability and bioactivity, and further research is needed to evaluate the mechanism underlying the hepatoprotective effects of QE and QS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was conducted following the regulations for the administration of laboratory animals in Guangdong province, China, reviewed and approved by the Guangdong Ocean University Research Council for the care and use of laboratory animals (approval number: GDOU-LAE-2021-021).

AUTHOR CONTRIBUTIONS

JL was involved in project administration, data collection, formal analysis, processing, and writing of the original draft. EA was involved in writing – original draft and writing – reviewing and editing. J-SH supervision and involvement in resources. GC: conceptualised, involved in methodology, acquired funding, supervision and involved in resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.891080/full#supplementary-material

REFERENCES

Amenyogbe, E., Chen, G., and Wang, Z. (2020). Identification, Characterisation, and Expressions Profile Analysis of Growth Hormone Receptors (GHR1 and GHR2) in Hybrid Grouper (Epinephelus Fuscoguttatus Female X Epinephelus Polyphekadion Male). *Genomics* 112 (1), 1–9. doi: 10.1016/j.ygeno.2019.05.012

Awad, H. M., Boersma, M. G., Boeren, S., van Bladeren, P. J., Vervoort, J., and Rietjens, I. (2002). The Regioselectivity of Glutathione Adduct Formation With Flavonoid Quinone/Quinone Methides is pH-Dependent. *Chem. Res. Toxicol.* 15 (3), 343–351. doi: 10.1021/tx010132l

Barquissau, V., Ghandour, R. A., Ailhaud, G., Klingenspor, M., Langin, D., Amri, E.-Z., et al. (2017). Control of Adipogenesis by Oxylipins, GPCRs and PPARs. *Biochimie.* 136, 3–11. doi: 10.1016/j.biochi.2016.12.012

Bell, J., and Koppe, W. (2011). "Lipids in Aquafeeds," in *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*. Eds. G. M. Turchini, W.-K. Ng and D. R. Tocher (Boca Raton Fla: CRC Press), 21–59.

Berntssen, M. H. G., Ornsrud, R., Hamre, K., and Lie, K. K. (2015). Polyaromatic Hydrocarbons in Aquafeeds, Source, Effects and Potential Implications for Vitamin Status of Farmed Fish Species: A Review. *Aquacult Nutr.* 21 (3), 257–273. doi: 10.1111/anu.12309

Bigliardi, B., and Galati, F. (2013). Innovation Trends in the Food Industry: The Case of Functional Foods. *Trends Food Sci. Technol.* 31, 118–129. doi: 10.1016/j.tifs.2013.03.006

Boots, A. W., Li, H., Schins, R. P. F., Duffin, R., Heemskerk, J. W. M., Bast, A., et al. (2007). The Quercetin Paradox. *Toxicol. Appl. Pharmacol.* 222 (1), 89–96. doi: 10.1016/j.taap.2007.04.004

Cheng, H., Huang, H., Guo, Z., Chang, Y., and Li, Z. (2021). Role of Prostaglandin E2 in Tissue Repair and Regeneration. *Theranostics* 11 (18), 8836–8854. doi: 10.7150/thno.63396

Chen, W., Gong, L., Guo, Z., Wang, W., Zhang, H., Liu, X., et al. (2013). A Novel Integrated Method for Large-Scale Detection, Identification, and Quantification of Widely Targeted Metabolites: Application in the Study of Rice Metabolomics. *Mol. Plant* 6 (6), 1769–1780. doi: 10.1093/mp/sst080

Chlebda, E., Magdalan, J., Merwid-Lad, A., Trocha, M., Kopacz, M., Kuzniar, A., et al. (2010). Influence of Water-Soluble Flavonoids, Quercetin-5 '-Sulfonic Acid Sodium Salt and Morin-5 '-Sulfonic Acid Sodium Salt, on Antioxidant Parameters in the Subacute Cadmium Intoxication Mouse Model. *Exp. Toxicol. Pathol.* 62 (2), 105–108. doi: 10.1016/j.etp.2009.02.118

Collins, S. A., Øverland, M., Skrede, A., and Drew, M. D. (2013). Effect of Plant Protein Sources on Growth Rate in Salmonids: Meta-Analysis of Dietary Inclusion of Soybean, Pea and Canola/Rapeseed Meals and Protein Concentrates. *Aquaculture* 400–401, 85–100. doi: 10.1016/j.aquaculture.2013.03.006

Cui, Y., Han, Y., Yang, X., Sun, Y., and Zhao, Y. (2014). Protective Effects of Quercetin and Quercetin-5 ',8-Disulfonate Against Carbon Tetrachloride-Caused Oxidative Liver Injury in Mice. *Molecules* 19 (1), 291–305. doi: 10.3390/molecules19010291

Cuykx, M., Rodrigues, R. M., Laukens, K., Vanhaecke, T., and Covaci, A. (2018). *In Vitro* Assessment of Hepatotoxicity by Metabolomics: A Review. *Arch. Toxicol.* 92 (10), 3007–3029. doi: 10.1007/s00204-018-2286-9

Czerwonka, A., Maciolek, U., Kalafut, J., Mendyk, E., Kuzniar, A., and Rzeski, W. (2020). Anti-Cancer Effects of Sodium and Potassium Quercetin-5 '-Sulfonates Through Inhibition of Proliferation, Induction of Apoptosis, and Cell Cycle Arrest in the HT-29 Human Adenocarcinoma Cell Line. *Bioorg. Chem.* 94, 103426. doi: 10.1016/j.bioorg.2019.103426

Deline, M., Keller, J., Rothe, M., Schunck, W.-H., Menzel, R., and Watts, J. L. (2015). Epoxides Derived From Dietary Dihomo-Gamma-Linolenic Acid Induce Germ Cell Death in C. Elegans. *Sci. Rep.* 5, 15417. doi: 10.1038/srep15417

de Mello, V. D., Lankinen, M. A., Lindstrom, J., Puupponen-Pimia, R., Laaksonen, D. E., Pihlajamaki, J., et al. (2017). Fasting Serum Hippuric Acid Is Elevated After Bilberry (Vaccinium Myrtillus) Consumption and Associates With Improvement of Fasting Glucose Levels and Insulin Secretion in Persons at High Risk of Developing Type 2 Diabetes. *Mol. Nutr. Food Res.* 61 (9), 1700019. doi: 10.1002/mnfr.201700019

Dennis, E. A., and Norris, P. C. (2015). Eicosanoid Storm in Infection and Inflammation. *Nat. Rev. Immunol.* 15 (8), 511–523. doi: 10.1038/nri3859

Egert, S., Boesch-Saadatmandi, C., Wolffram, S., Rimbach, G., and Mueller, M. J. (2010). Serum Lipid and Blood Pressure Responses to Quercetin Vary in Overweight Patients by Apolipoprotein E Genotype. *J. Nutr.* 140 (2), 278–284. doi: 10.3945/jn.109.117655

Endo, J., and Arita, M. (2016). Cardioprotective Mechanism of Omega-3 Poly-Unsaturated Fatty Acids. *J. Cardiol.* 67 (1-2), 22–27. doi: 10.1016/j.jjcc.2015.08.002

FAO. (2016). *La Situation Mondiale Des Pêches Et De L'aquaculture 2016* (Rome (I: Contribuer à la sécurité alimentaire et à la nutrition de tous FAO). FAO. 2016.

Ferdouse, A., Leng, S., Winter, T., and Aukema, H. M. (2019a). Dietary N-6 and N-3 PUFA Alter the Free Oxylipin Profile Differently in Male and Female Rat Hearts. *Br. J. Nutr.* 122 (3), 252–261. doi: 10.1017/s0007114519001211

Ferdouse, A., Leng, S., Winter, T., and Aukema, H. M. (2019b). The Brain Oxylipin Profile Is Resistant to Modulation by Dietary N-6 and N-3 Polyunsaturated Fatty Acids in Male and Female Rats. *Lipids* 54 (1), 67–80. doi: 10.1002/lipd.12122

Gabbs, M., Leng, S., Devassy, J. G., Monirujjaman, M., and Aukema, H. M. (2015). Advances in Our Understanding of Oxylipins Derived From Dietary PUFAs. *Adv. Nutr.* 6 (5), 513–540. doi: 10.3945/an.114.007732

Galati, G., Moridani, M. Y., Chan, T. S., and O'Brien, P. J. (2001). Peroxidative Metabolism of Apigenin and Naringenin Versus Luteolin and Quercetin: Glutathione Oxidation and Conjugation. *Free Radical Biol. Med.* 30 (4), 370–382. doi: 10.1016/s0891-5849(00)00481-0

Gao, S., Cui, K., Li, Y., Pang, Y., Fang, W., Mai, K., et al. (2021). Comparation of Oxylipin Profiles as Well as Their Substrates and Synthetic Enzymes Transcriptional Expression Between Marine Fish Larimichthys Crocea and Freshwater Fish Oncorhynchus Mykiss. *Aquaculture* 539, 736641. doi: 10.1016/j.aquaculture.2021.736641

Geay, F., Ferraresso, S., Zambonino-Infante, J. L., Bargelloni, L., Quentel, C., Vandeputte, M., et al. (2011). Effects of the Total Replacement of Fish-Based Diet With Plant-Based Diet on the Hepatic Transcriptome of Two European Sea Bass (Dicentrarchus Labrax) Half-Sibfamilies Showing Different Growth Rates With the Plant-Based Diet. *BMC Genomics* 12, 522. doi: 10.1186/1471-2164-12-522

Glencross, B. D., Booth, M., and Allan, G. L. (2007). A Feed is Only as Good as its Ingredients - A Review of Ingredient Evaluation Strategies for Aquaculture Feeds. *Aquacult. Nutr.* 13 (1), 17–34. doi: 10.1111/j.1365-2095.2007.00450.x

Harris, H. W., Gosnell, J. E., and Kumwenda, Z. L. (2000). The Lipemia of Sepsis: Triglyceride-Rich Lipoproteins as Agents of Innate Immunity. *J. endotoxin Res.* 6 (6), 421–430. doi: 10.1179/096805100101532351

Jung, C. H., Cho, I., Ahn, J., Jeon, T.-I., and Ha, T.-Y. (2013). Quercetin Reduces High-Fat Diet-Induced Fat Accumulation in the Liver by Regulating Lipid Metabolism Genes. *Phytother Res.* 27 (1), 139–143. doi: 10.1002/ptr.4687

Kamada, C., Da Silva, E. L., Ohnishi-Kameyama, M., Moon, J. H., and Terao, J. (2005). Attenuation of Lipid Peroxidation and Hyperlipidemia by Quercetin Glucoside in the Aorta of High Cholesterol-Fed Rabbit. *Free Radical Res.* 39 (2), 185–194. doi: 10.1080/10715760400019638

Krol, W., Dworniczak, S., Pietsz, G., Czuba, Z. P., Kunicka, M., Kopacz, M., et al. (2002). Synthesis and Tumoricidal Activity Evaluation of New Morin and Quercetin Sulfonic Derivatives. *Acta Poloniae pharm.* 59 (1), 77–79. doi: 12026118

Kutzner, L., Esselun, C., Franke, N., Schoenfeld, K., Eckert, G. P., and Schebb, N. H. (2020). Effect of Dietary EPA and DHA on Murine Blood and Liver Fatty Acid Profile and Liver Oxylipin Pattern Depending on High and Low Dietary N6-PUFA. *Food Funct.* 11 (10), 9177–9191. doi: 10.1039/d0fo01462a

Lee, J. M., Lee, H., Kang, S., and Park, W. J. (2016a). Fatty Acid Desaturases, Polyunsaturated Fatty Acid Regulation, and Biotechnological Advances. *Nutrients* 8 (1), 23. doi: 10.3390/nu8010023

Lees, H. J., Swann, J. R., Wilson, I. D., Nicholson, J. K., and Holmes, E. (2013). Hippurate: The Natural History of a Mammalian-Microbial Cometabolite. *J. Proteome Res.* 12 (4), 1527–1546. doi: 10.1021/pr300900b

Leng, S., Winter, T., and Aukema, H. M. (2018). Dietary ALA, EPA and DHA Have Distinct Effects on Oxylipin Profiles in Female and Male Rat Kidney, Liver and Serum. *J. Nutr. Biochem.* 57, 228–237. doi: 10.1016/j.jnutbio.2018.04.002

Li, H., Lv, Q., Liu, A., Wang, J., Sun, X., Deng, J., et al. (2022). Comparative Metabolomics Study of Tartary (Fagopyrum Tataricum (L.) Gaertn) and Common (Fagopyrum Esculentum Moench) Buckwheat Seeds. *Food Chem.* 371, 131125. doi: 10.1016/j.foodchem.2021.131125

Liu, P., Lin, H., Xu, Y., Zhou, F., Wang, J., Liu, J., et al. (2018). Frataxin-Mediated PINK1-Parkin-Dependent Mitophagy in Hepatic Steatosis: The Protective Effects of Quercetin. *Mol. Nutr. Food Res.* 62 (16), e1800164. doi: 10.1002/mnfr.201800164

Magdalan, J., Szeląg, A., Chlebda, E., Merwid-Ląd, A., and PR, D. (2007). Quercetin-5'-Sulfonic Acid Sodium Salt and Morin-5'-Sulfonic Acid Sodium Salt as Antidotes in the Subacute Cadmium Intoxication in Mice. *Pharmacol. Rep.* 59, suppl. 1, 210–216. doi: 236844549

Marcolin, E., San-Miguel, B., Vallejo, D., Tieppo, J., Marroni, N., Gonzalez-Gallego, J., et al. (2012). Quercetin Treatment Ameliorates Inflammation and Fibrosis in Mice With Nonalcoholic Steatohepatitis. *J. Nutr.* 142 (10), 1821–1828. doi: 10.3945/jn.112.165274

Martin, B. T., Heintz, R., Danner, E. M., and Nisbet, R. M. (2017). Integrating Lipid Storage Into General Representations of Fish Energetics. *J. Anim. Ecol.* 86 (4), 812–825. doi: 10.1111/1365-2656.12667

Medale, F., Le Bouche, R., Dupont-Nivet, M., Quillet, E., Aubin, J., and Panserat, S. (2013). Des Aliments a Base De V_Eg_Etaux Pour Les Poissons D'_Elevage. *INRA Productions Animales* 26, 303–316. doi: 10.20870/productions-animales.2013.26.4.3159

Miltonprabu, S., Tomczyk, M., Skalicka-Wozniak, K., Rastrelli, L., Daglia, M., Nabavi, S. F., et al. (2017). Hepatoprotective Effect of Quercetin: From Chemistry to Medicine. *Food Chem. Toxicol.* 108, 365–374. doi: 10.1016/j.fct.2016.08.034

Moon, J., Lee, S.-M., Do, H. J., Cho, Y., Chung, J. H., and Shin, M.-J. (2012). Quercetin Up-Regulates LDL Receptor Expression in HepG2 Cells. *Phytother Res.* 26 (11), 1688–1694. doi: 10.1002/ptr.4646

Mullen, W., Rouanet, J.-M., Auger, C., Teissedre, P.-L., Caldwell, S. T., Hartley, R. C., et al. (2008). Bioavailability of 2-C-14 Quercetin-4 '-Glucoside in Rats. *J. Agric. Food Chem.* 56 (24), 12127–12137. doi: 10.1021/jf802754s

Oliva-Teles, A., Enes, P., and Peres, H. (2015). Replacing Fishmeal and Fish Oil in Industrial Aquafeeds for Carnivorous Fish. *Feed Feeding Practices Aquacult*, 203–233. doi: 10.1016/B978-0-08-100506-4.00008-8

Padma, V. V., Lalitha, G., Shirony, N. P., and Baskaran, R. (2012). Effect of Quercetin Against Lindane Induced Alterations in the Serum and Hepatic Tissue Lipids in Wistar Rats. *Asian Pacific J. Trop. Biomed.* 2 (11), 910–915. doi: 10.1016/s2221-1691(12)60252-4

Park, K. H., Rodriguez-Montes de Oca, G. A., Bonello, P., Lee, K.-J., and Dabrowski, K. (2009). Determination of Quercetin Concentrations in Fish Tissues After Feeding Quercetin-Containing Diets. *Aquacult. Int.* 17 (6), 537–544. doi: 10.1007/s10499-008-9222-6

Petersen, B., Egert, S., Bosywestphal, A., Müller, M. J., Wolffram, S., Hubbermann, E. M., et al. (2016). Bioavailability of Quercetin in Humans and the Influence of Food Matrix Comparing Quercetin Capsules and Different Apple Sources. *Food Res. Int.* 88, 159–165. doi: 10.1016/j.foodres.2016.02.013

Picklo, M. J., and Newman, J. W. (2015). Antioxidant Supplementation and Obesity Have Independent Effects on Hepatic Oxylipin Profiles in Insulin-Resistant, Obesity-Prone Rats. *Free Radical Biol. Med.* 89, 182–191. doi: 10.1016/j.freeradbiomed.2015.07.152

Qin, G., Ma, J., Huang, Q., Yin, H., Han, J., Li, M., et al. (2018). Isoquercetin Improves Hepatic Lipid Accumulation by Activating AMPK Pathway and Suppressing TGF-β Signaling on an HFD-Induced Nonalcoholic Fatty Liver Disease Rat Model. *Int. J. Mol. Sci.* 19 (12), 4126. doi: 10.3390/ijms19124126

Qureshi, A. A., Reis, J. C., Qureshi, N., Papasian, C. J., and Schaefer, D. M. (2011). δ-Tocotrienol and Quercetin Reduce Serum Levels of Nitric Oxide and Lipid Parameters in Female Chickens. *Lipids Health Dis.* 10 (1), 39. doi: 10.1186/1476-511X-10-39

Rangel-Ordonez, L., Noeldner, M., Schubert-Zsilavecz, M., and Wurglics, M. (2010). Plasma Levels and Distribution of Flavonoids in Rat Brain After Single and Repeated Doses of Standardised Ginkgo Biloba Extract EGb 761 (R). *Planta Med.* 76 (15), 1683–1690. doi: 10.1055/s-0030-1249962

Rey, C., Delpech, J. C., Madore, C., Nadjar, A., Greenhalgh, A. D., Amadieu, C., et al. (2019). Dietary N-3 Long Chain PUFA Supplementation Promotes a Pro-Resolving Oxylipin Profile in the Brain. *Brain Behav. Immun.* 76, 17–27. doi: 10.1016/j.bbi.2018.07.025

Robak, J., and Kopacz, M. (1989). The Influence of Sulfonated Bioflavonoids on Enzymatic Oxidation of Arachidonic Acid and on Non-Enzymatic Lipid Oxidation. *Polish J. Pharmacol. Pharm.* 41 (5), 469–473. doi: 2518221

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., and Fauconneau, B. (2020). Metabolomics and Fish Nutrition: A Review in the Context of Sustainable Feed Development. *Rev. Aquacult.* 12 (1), 261–282. doi: 10.1111/raq.12316

Shaik, J. S. B., Ahmad, M., Li, W., Rose, M. E., Foley, L. M., Hitchens, T. K., et al. (2013). Soluble Epoxide Hydrolase Inhibitor Trans-4- 4-(3-Adamantan-1-Yl-Ureido)-Cyclohexyloxy -Benzoic Acid is Neuroprotective in Rat Model of Ischemic Stroke. *Am. J. Physiol-Heart Circulatory Physiol.* 305 (11), H1605–H1613. doi: 10.1152/ajpheart.00471.2013

Shin, H. S., Yoo, J. H., Min, T. S., Lee, K. Y., and Choi, C. Y. (2010). The Effects of Quercetin on Physiological Characteristics and Oxidative Stress Resistance in Olive Flounder, Paralichthys Olivaceus. *Asian-Australasian J. Anim. Sci.* 23 (5), 588–597. doi: 10.5713/ajas.2010.90624

Singh, P., Mishra, S. K., Noel, S., Sharma, S., and Rath, S. K. (2012). Acute Exposure of Apigenin Induces Hepatotoxicity in Swiss Mice. *PloS One* 7 (2), e31964. doi: 10.1371/journal.pone.0031964

Singh, P., Sharma, S., and Rath, S. K. (2014). Genistein Induces Deleterious Effects During Its Acute Exposure in Swiss Mice. *BioMed. Res. Int.* 2014, 1–14. doi: 10.1155/2014/619617

Singh, P., Sharma, S., and Rath, S. K. (2022). A Versatile Flavonoid Quercetin: Study of its Toxicity and Differential Gene Expression in the Liver of Mice. *Phytomed Plus.* 2 (1), 100148. doi: 10.1016/j.phyplu.2021.100148

Song, H., Xu, D., Tian, L., Chen, R., Wang, L., Tan, P., et al. (2019). Overwinter Mortality in Yellow Drum (Nibea Albiflora): Insights From Growth and Immune Responses to Cold and Starvation Stress. *Fish Shellfish Immunol.* 92, 341–347. doi: 10.1016/j.fsi.2019.06.030

Spagnuolo, C., Russo, M., Bilotto, S., Tedesco, I., Laratta, B., and Russo, G. L. (2012). Dietary Polyphenols in Cancer Prevention: The Example of the Flavonoid Quercetin in Leukemia. *Ann. N. Y. Acad.* 1259, 95–103. doi: 10.1111/j.1749-6632.2012.06599.x

Spencer, J. P. E., El Mohsen, M. M. A., Minihane, A.-M., and Mathers, J. C. (2008). Biomarkers of the Intake of Dietary Polyphenols: Strengths, Limitations and Application in Nutrition Research. *Br. J. Nutr.* 99 (1), 12–22. doi: 10.1017/s0007114507798938

Stewart, A., and Fisher, R. A. (2015). "Introduction: G Protein-Coupled Receptors and RGS Proteins," in *Rgs Protein Physiology and Pathophysiology*. Ed. R. A. Fisher, 1–11. doi: 10.1016/bs.pmbts.2015.03.002

Szelag, A., Magdalan, J., Kopacz, M., Kuzniar, A., Kowalski, P., and Piesniewska, M. (2003). Assessment of Efficacy of Quercetin-5 '-Sulfonic Acid Sodium Salt in the Treatment of Acute Chromium Poisoning: Experimental Studies. *Polish J. Pharmacol.* 55 (6), 1097–1103.

Tan, X., Sun, Z., Liu, Q., Ye, H., Zou, C., Ye, C., et al. (2018). Effects of Dietary Ginkgo Biloba Leaf Extract on Growth Performance, Plasma Biochemical Parameters, Fish Composition, Immune Responses, Liver Histology, and Immune and Apoptosis-Related Genes Expression of Hybrid Grouper (Epinephelus Lanceolatus Male X Epinephelus Fuscoguttatus Female) Fed High Lipid Diets. *Fish Shellfish Immunol.* 72, 399–409. doi: 10.1016/j.fsi.2017.10.022

Toromanovic, J., Kovac-Besovic, E., Sapcanin, A., Tahirovic, I., Rimpapa, Z., Kroyer, G., et al. (2008). Urinary Hippuric Acid After Ingestion of Edible Fruits. *Bosnian J. Basic Med. Sci.* 8 (1), 38–43. doi: 10.17305/bjbms.2008.2994

Trocha, M., Merwid-Lad, A., Szuba, A., Sozanski, T., Magdalan, J., Szelag, A., et al. (2012). Effect of Quercetin-5 '-Sulfonic Acid Sodium Salt on SOD Activity and ADMA/DDAH Pathway in Extracorporeal Liver Perfusion in Rats. *Adv. Clin. Exp. Med.* 21 (4), 423–431. doi: 23240447

Ulaszewska, M., Garcia-Aloy, M., Vazquez-Manjarrez, N., Soria-Florido, M. T., Llorach, R., Mattivi, F., et al. (2020). Food Intake Biomarkers for Berries and Grapes. *Genes Nutr.* 15 (1), 17. doi: 10.1186/s12263-020-00675-z

Wang, W., Sun, C., Mao, L., Ma, P., Liu, F., Yang, J., et al. (2016b). The Biological Activities, Chemical Stability, Metabolism and Delivery Systems of Quercetin: A Review. *Trends Food Sci. Technol.* 56, 21–38. doi: 10.1016/j.tifs.2016.07.004

Wang, L. L., Zhang, Z. C., Hassan, W., Li, Y., Liu, J., and Shang, J. (2016a). Amelioration of Free Fatty Acid-Induced Fatty Liver by Quercetin-3-O-β-D-Glucuronide Through Modulation of Peroxisome Proliferator-Activated Receptor-Alpha/Sterol Regulatory Element-Binding Protein-1c Signaling. *Hepatol. Res.* 46 (2), 225–238. doi: 10.1111/hepr.12557

Wang, J., Zhang, C., Zhang, J., Xie, J., Yang, L., Xing, Y., et al. (2020). The Effects of Quercetin on Immunity, Antioxidant Indices, and Disease Resistance in

Zebrafish (Danio Rerio). *Fish Physiol. Biochem.* 46, 759–770. doi: 10.1007/s10695-019-00750-2

Welte, M. A., and Gould, A. P. (2017). Lipid Droplet Functions Beyond Energy Storage. *Biochim. Et Biophys. Acta-Molecular Cell Biol. Lipids* 1862 (10), 1260–1272. doi: 10.1016/j.bbalip.2017.07.006

Woznicka, E., Kuzniar, A., Nowak, D., Nykiel, E., Kopacz, M., Gruszecka, J., et al. (2013). Comparative Study on the Anti-Bacterial Activity of Some Flavonoids and Their Sulfonic Derivatives. *Acta Poloniae Pharm.* 70 (3), 567–571. doi: 23757948

Wu, D., and Meydani, S. N. (1998). N-3 Poly-Unsaturated Fatty Acids and Immune Function. *Proc. Nutr. Soc.* 57 (4), 503–509. doi: 10.1079/pns19980074

Xie, R.-T., Amenyogbe, E., Chen, G., and Huang, J.-s. (2021). Effects of Feed Fat Level on Growth Performance, Body Composition and Serum Biochemical Indices of Hybrid Grouper (Epinephelus Fuscoguttatus X Epinephelus Polyphekadion). *Aquaculture* 530, 735813. doi: 10.1016/j.aquaculture.2020.735813

Xu, Z., Li, X., Yang, H., Liang, G., Gao, B., and Leng, X. (2019). Dietary Quercetin Improved the Growth, Antioxidation, and Flesh Quality of Grass Carp (Ctenopharyngodon Idella). *J. World Aquacult. Soc.* 50 (6), 1182–1195. doi: 10.1111/jwas.12663

Xu, J.-Y., Su, Y.-Y., Cheng, J.-S., Li, S.-X., Liu, R., Li, W.-X., et al. (2010). Protective Effects of Fullerenol on Carbon Tetrachloride-Induced Acute Hepatotoxicity and Nephrotoxicity in Rats. *Carbon* 48 (5), 1388–1396. doi: 10.1016/j.carbon.2009.12.029

Yang, X., Dong, C., and Ren, G. (2011). Effect of Soyasaponins-Rich Extract From Soybean on Acute Alcohol-Induced Hepatotoxicity in Mice. *J. Agric. Food Chem.* 59 (4), 1138–1144. doi: 10.1021/jf103749r

Ye, G., Dong, X., Yang, Q., Chi, S., Liu, H., Zhang, H., et al. (2020). Low-Gossypol Cottonseed Protein Concentrate Used as a Replacement of Fish Meal for Juvenile Hybrid Grouper (Epinephelus Fuscoguttatus Female X Epinephelus Lanceolatus Male) Effects on Growth Performance, Immune Responses and Intestinal Microbiota. *Aquaculture* 524, 735309. doi: 10.1016/j.aquaculture.2020.735309

Ytrestøyl, T., Aas, T. S., and Asgard, T. (2015). Utilisation of Feed Resources in Production of Atlantic Salmon (Salmo Salar) in Norway. *Aquaculture* 448, 365–374. doi: 10.1016/j.aquaculture.2015.06.023

Yu, Z., Ming, G., Cai, W., Yu, L., and Cheng, H. (2016). Dietary Component Isorhamnetin Is a Pparγ Antagonist and Ameliorates Metabolic Disorders Induced by Diet or Leptin Deficiency. *Sci. Rep.* 6, 19288. doi: 10.1038/srep19288

Zhai, S.-W., and Liu, S.-L. (2013). Effects of Dietary Quercetin on Growth Performance, Serum Lipids Level and Body Composition of Tilapia. *Ital. J. Anim. Sci.* 12 (4), e85. doi: 10.4081/ijas.2013.e85

Zhang, Z., Zhou, C., Fan, K., Zhang, L., Liu, Y., and Liu, P. (2021). Metabolomics Analysis of the Effects of Temperature on the Growth and Development of Juvenile European Seabass (Dicentrarchus Labrax). *Sci. Total Environment.* 769, 145155. doi: 10.1016/j.scitotenv.2021.145155

Zhou, W., Rahimnejad, S., Lu, K., Wang, L., and Liu, W. (2019). Effects of Berberine on Growth, Liver Histology, and Expression of Lipid-Related Genes in Blunt Snout Bream (*Megalobrama Amblycephala*) Fed High-Fat Diets. *Fish Physiol. Biochem.* 45 (1), 83–91. doi: 10.1007/s10695-018-0536-7

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Dietary Use of the Microalga *Chlorella fusca* Improves Growth, Metabolism, and Digestive Functionality in Thick-Lipped Grey Mullet (*Chelon labrosus*, Risso 1827) Juveniles

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In recent years, a clear emphasis has been placed on replacing fishmeal and fish oil in aquafeeds with other alternative ingredients, including algae, particularly in low trophic omnivorous fish species. This work aimed at evaluating the effects of moderate dietary supplementation with the green microalga *Chlorella fusca* on growth, metabolism, and digestive functionality in juvenile thick-lipped grey mullet (*Chelon labrosus*). Fish were fed a control diet (CT) or a diet containing 15% *C. fusca* (C-15) biomass during 90 days. *C. labrosus* fed with the C-15 diet showed higher growth performance (in terms of final weight and length, weight gain, and specific growth rate) than the control group. Somatic indices and muscle proximate composition were similar at the end of the feeding trial. Regarding fatty acids profile, *C. fusca*-fed fish showed a selective retention of docosahexaenoic acid (DHA) in the liver, and arachidonic acid (ARA), eicosapentaenoic acid (EPA), and DHA in the muscle. Dietary inclusion of this microalga significantly increased intestinal total alkaline protease, leucine aminopeptidase, and alkaline phosphatase activities in specimens fed with C-15 diet. Furthermore, intestine histological analysis revealed the absence of damage signs on gut morphology in fish fed the microalgae supplemented diet. Thick-lipped grey mullets fed the C-15 diet increased plasma glucose and decreased plasma lactate. Overall, the effects observed on liver (lipid metabolism, glycolysis and glycogenolysis) enzyme activities, together with adequate fatty acid profile, metabolic response, and gut morphology, and a significant

increase in the intestinal mucosa's digestive and absorptive capacity, could explain the positive effects on growth performance obtained in fish fed the microalgae-supplemented diet. In conclusion, the results obtained showed that *C. fusca* is suitable as dietary ingredient for feeding thick-lipped grey mullet juveniles.

Keywords: absorptive capacity, aquafeed, Chlorophyta, enzymatic activity, fatty acids, fish quality, low-trophic species, Mugilidae

INTRODUCTION

Fishmeal represents a vital protein source for cultured fish species in aquafeeds (Jannathulla et al., 2019; Oliva-Teles et al., 2020). However, constraints related to high prices, limited availability, and environmental concerns have promoted extensive research efforts focused on assessing lower-cost alternative protein sources (Tibaldi et al., 2015; Minjarez-Osorio et al., 2016).

Microalgae are a promising alternative to fishmeal and crop-based ingredients since they have several features that make them attractive for the aquafeed industry. From an economical point of view, a model facility for microalgae production of 111 ha would produce 2,750 tonnes yr^{-1} of protein and 2,330 tonnes yr^{-1} of algal oil, at a capital cost of $29.3 M, such a facility would generate $5.5 M in average annual net income over its 30-year lifetime (Beal et al., 2018). From a nutritional perspective, microalgae have high levels of protein (30 to 60%, dry matter basis), relatively well-balanced amino acid profile, essential fatty acid (specifically eicosapentaenoic and docosahexaenoic acids, EPA and DHA, respectively) (Shah et al., 2018; Tibbetts, 2018) and bioactive compounds, including minerals, vitamins, and pigments (Camacho et al., 2019).

In this sense, the suitability of microalgae in practical diets for different fish species has been previously studied (Vizcaíno et al., 2014; Tibaldi et al., 2015; Yeganeh et al., 2015; Sørensen et al., 2016; Perera et al., 2020). Although depending on species tested and levels of inclusion (Shah et al., 2018), low, moderate, and even total substitution of fishmeal by microalgae in aquafeeds evidenced beneficial effects on growth, nutrient utilization, digestibility, metabolism, or survival rate (Rahimnejad et al., 2017; Vizcaíno et al., 2018; García-Márquez et al., 2020; Perera et al., 2020). Microalgae also have health-promoting effects in fish, modulate the fatty acid profile and the quality of the fish flesh and also improve the stress resistance, which is also of great interest today for both producers and consumers (Sarker et al., 2016; Gong et al., 2019; Silveira Júnior et al., 2019).

Overall, herbivorous and omnivorous fish tolerate higher inclusion levels of algae than carnivorous species. However, results reported in the literature indicate that the optimum dietary algae inclusion level should vary depending on the algae and the farmed fish species (Shah et al., 2018). Therefore, specific research should be carried out on each particular algae strain and fish species. Owing to the high nutritional value, species of the genus *Chlorella* have been used as dietary protein source, and abundant literature assessing their effects on a wide range of farmed fish species is available (Atlantic salmon, *Salmo salar*: Tibbetts et al., 2017; common carp, *Cyprinus carpio*: Abdulrahman et al., 2018; European sea bass, *Dicentrarchus labrax*: Hasanein et al., 2018; grey mullet, *Mugil cephalus*: Akbary and Aminikhoei, 2019). The microalga *Chlorella fusca* shows a high content of various high-value compounds, including carotenoids, specifically lutein (Becker, 2013). Moreover, this species can accumulate a high content of essential fatty acids such as linoleic, and α-linolenic acids (Pratoomyot et al., 2005). However, it is well-known that chlorophyte microalgae of the *Chlorella* genus possess recalcitrant cell walls (Domozych et al., 2012; Liu and Hu, 2013), and in fact, itself may act as protective barrier, diminishing the digestibility and assimilation of intracellular nutrients (Lavecchia et al., 2016). Moreover, the nutritional effects of *Chlorella* species depend on its inclusion levels in aquaculture feeds. In this sense, Ahmad et al. (2020) reported controversial effects on growth performance and feed utilization in different species of fish, which may be attributed to the levels of inclusion of the microalga, as levels above 20% compromises growth performance (Hasan and Chakrabarti, 2009; Lupatsch and Blake, 2013).

The thick-lipped grey mullet (*Chelon labrosus*, Risso 1827) has been described as an easily cultivable species and could constitute a low-trophic level new candidate for aquaculture diversification (Zouiten et al., 2008; García-Márquez et al., 2021). Recent studies have been focused on practical aspects of its culture, like its sensitivity to stress or its digestive physiology (de las Heras et al., 2015; Pujante et al., 2015; Pujante et al., 2017; Pujante et al., 2018). Nevertheless, there is a lack of information regarding feed utilization or growth performance in *C. labrosus* fed compound diets.

Thus, the aim of this study is to assess the potential of the microalga *C. fusca* as dietary ingredient for feeding *C. labrosus* juveniles. We hypothesise that *C. fusca* might improve growth performance, nutrient utilization, and several parameters related to physiological metabolism, and digestive functionality in juvenile *C. labrosus* when tested at moderate dietary inclusion level (15%) through a 90-day feeding trial.

MATERIALS AND METHODS

Microalgae

The microalga *Chlorella fusca* was produced in pilot-scale photobioreactors (PBR) at the SABANA facilities of the University of Almeria (Spain). The inoculum was produced in a bubble column photobioreactor (100-L water capacity, 0.20 m

diameter, 2.0 m height) with automatic temperature and pH control, and air bubbling of 0.2 vol vol^{-1} min^{-1} (volume of air per volume of reactor per time). After that, 3.0 m^3 tubular photobioreactors (0.10 m tube diameter) were used to produce the final amount of biomass required. The pH, temperature, and dissolved oxygen were continuously monitored on these reactors using specific probes (Crison Instruments, Spain). The pH was controlled automatically by the on-demand injection of CO_2. The temperature was kept within the range required for optimal growth of *C. fusca* (20-25°C) by controlling the greenhouse's temperature on which the reactors were located. The culture medium used was the one described by Sorokin and Krauss (1958), which was prepared by dissolving fertilizers in tap water and then sterilized by filtration (0.02 μm) and ozone (1 mg L^{-1}). Microalgal biomass was harvested by centrifugation (SSD6 GEA Westfalia, Germany). Cell disruption was performed using a high-pressure homogenizer (GEA Ariete NS3015H) at 600 bars in a single pass, these operational conditions being previously optimized. For drying, a spray-dryer was utilized (GEA Mobile MinorTM Spray dryer), performed at an inlet temperature of 160 °C, while the outlet temperature was kept below 80 °C. The final powder was stored in the dark at −20°C until further preparation of the experimental diet.

Experimental Diets and Feeding Trial

Two iso-nitrogenous and isolipidic (40% and 7%, respectively, on a dry weight basis) experimental diets were formulated and elaborated by the Service of Experimental Diets (CEIMAR-University of Almeria, Spain, grant EQC2019-006380-P) using standard aquafeed processing procedures to obtain 3 mm floating pellets. The diet designed as C-15 included 15% (w/w) dry *C. fusca* biomass, and an algae-free diet was used as control (CT). The ingredient composition and fatty acid profile of the experimental diets and *C. fusca* are shown in **Tables 1**, **2**, respectively. Feed ingredients were finely ground and mixed in a vertical helix ribbon mixer (Sammic BM-10, 10-L capacity, Sammic, Azpeitia, Spain) before fish oil and diluted choline chloride were added. All the ingredients were mixed together for 15 min, and then water (350 mL kg^{-1}) was added to the mixture to obtain a homogeneous dough. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JSConwell Ltd, New Zealand). The extruder barrel consisted of four sections, and the temperature profile in each section (from inlet to outlet) was 95°C, 98°C, 100°C, and 110°C, respectively. Finally, pellets were dried at 27°C in a drying chamber (Airfrio, Almeria, Spain) for 24 h, and kept in sealed plastic bags at −20°C until use.

Thick-lipped grey mullet (*Chelon labrosus*) specimens (n = 180) were provided by the Centro Integrado de Formación Profesional C.I.F.P. Marítimo Zaporito (San Fernando, Cadiz, Spain), and transferred to the Centro de Experimentación de Ecología y Microbiología de Sistemas Acuáticos Controlados Grice-Hutchinson (CEMSAC) of the University of Malaga (Malaga, Spain; Spanish Operational Code REGA ES290670002043). The fish were acclimated to experimental conditions and fed with a commercial diet (32% protein, 6% fat, TI-3 Tilapia, Skretting, Spain) for 30 days before starting the feeding trial. Six homogeneous groups of 30 fish (84.7 ± 0.3 g) were randomly distributed in 1000 L tanks coupled to a recirculation aquaculture system (RAS), equipped with physical and biological filters, and maintained under natural photoperiod (November 2019 – February 2020), in the range of 17.9–23.8°C, and salinity 1.0–1.2 ‰. Supplemental aeration was provided to maintain dissolved oxygen at 6.8 ± 0.4 mg L^{-1}. Ammonia (<0.1 mg L^{-1}), nitrite (<0.2 mg L^{-1}), and nitrate (<50 mg L^{-1}) were determined weekly at 9:00 AM. The two experimental dietary groups (CT and C-15) were then established in triplicates. Fish were hand-fed twice per day (9:00 AM and 5:00 PM) at a rate of 1.5% of their body weight for 90 days. The uneaten pellets were collected after 1 h and then dried and weighed.

TABLE 1 | Ingredient composition of the experimental diets used in the feeding trial.

	CT	C-15
Ingredients **(g kg^{-1} dry weight, DW)**		
Fishmeal LT94[1]	75	64
Chlorella fusca **biomass[2]**	0	150
Pea protein concentrate[3]	75	64
Soybean protein concentrate[4]	175	149
Soybean meal	188	159
Sunflower meal	20	20
Wheat gluten[5]	60	51
Wheat meal[6]	210	170
Potato starch	25	25
Fish oil	40	35
Vitamin and mineral premix[7]	10	10
Binder (guar gum)	15	15
Proximate composition **(g kg^{-1}, DW)**		
Crude protein	404.5	376.2
Total lipid	70.4	68.0
Ash	122.9	143.1
Nitrogen-free extracts[8]	402.2	412.7

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet.
[1](protein: 69.4%; lipid: 12.3%), Norsildemel (Bergen, Norway); [2](protein: 15.2%; lipid: 1.1%); [3](protein: 85.5%; lipid: 1.3%); [4](protein: 51.5%; lipid: 8.0%); [5](protein: 76.0%; lipid: 1.9%); [6](protein: 12.0%; lipid: 2.0%); [7]Vitamin & Mineral Premix: Vitamins (IU or mg kg−1 premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2500 mg; vitamin B1 (thiamine hydrochloride), 3000 mg; vitamin B2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg; vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg−1 premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain); [8]Calculated as 100 – (% crude protein + % ether extract + % ash).

Fish Sampling

Fish were counted and group-weighed every 3 weeks, and feed intake was recorded for each experimental replicate to calculate growth performance parameters. At the end of the trial (day 90), overnight fasted fish (3 fish per replicate, 9 per experimental diet) were randomly selected, deeply anesthetized with 2-phenoxyethanol (1 mL L^{-1}, Sigma-Aldrich 77699), and then sampled for blood and tissue collection. Blood was drawn from caudal vessels with heparinized syringes, centrifuged at 3000 × g for 5 min at 4°C, and plasma samples were snap-frozen in liquid

TABLE 2 | Fatty acid composition (% of total fatty acids) of microalga and experimental diets used in the feeding trial.

Fatty acids	*C. fusca*	Experimental diets	
		CT	C-15
14:0	–	2.62	2.37
16:0	44.36	19.45	20.45
16:1n7	–	3.20	3.93
16:2n4	–	0.66	0.60
16:3n4	4.51	–	0.68
18:0	5.16	4.86	4.53
18:1n9	17.25	17.76	17.26
18:1n7	1.49	–	–
18:2n6	13.13	20.99	19.70
18:3n3	14.09	2.34	4.76
18:4n3	–	0.70	0.97
20:1n9	–	1.74	1.79
20:4n6	–	0.96	0.91
20:5n3	–	5.58	4.94
22:5n3	–	1.12	0.99
22:6n3	–	13.66	12.36
SFA	49.53	26.93	27.35
MUFA	18.75	22.70	22.98
PUFA	–	21.33	19.20
Other FA	–	4.35	3.75
n - 3	14.09	23.41	24.03
n - 6	13.13	21.95	20.60
n - 9	17.25	1.74	1.79
n - 3/n - 6	1.07	1.07	1.17
EPA/DHA	–	0.41	0.40

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet.

nitrogen. Immediately, whole viscera were obtained, the intestines were separated from the other organs, and all visible perivisceral fat was removed. Liver, total viscera, and perivisceral fat were weighed for hepatosomatic, viscerosomatic, and perivisceral indices, respectively. Plasma, liver, and white muscle samples were stored at −80 °C until biochemical analysis.

Moreover, for the enzymatic analysis intestines from four fish per each experimental tank were randomly grouped, which allowed obtaining four different enzymatic extracts per experimental tank (12 enzymatic extracts per dietary treatment). Intestinal samples were homogenized in distilled water at 4°C (w/v 1:2). Supernatants were obtained after centrifugation (13,000 × g, 12 min, 4°C) and stored at −20°C until further analysis. In parallel, 1 cm length portions of the proximal intestine of three specimens from each tank (9 fish per dietary treatment) were collected for further examination under light (LM), transmission (TEM), and scanning (SEM) electron microscopy.

Growth Performance and Biometric Parameters

The following growth parameters were evaluated: (1) weight gain (WG) = ((final fish weight – initial fish weight) x 100); (2) specific growth ratio (SGR) = (100 x [(ln final fish weight) – (ln initial fish weight)]/experimental days); (3) feed conversion ratio (FCR) = dry feed intake (g)/weight gain (g); (4) protein efficiency ratio (PER) = weight gain/intake of particular protein; (5) condition factor (K, %) = ((fish weight/fish length^3) x 100); (6) hepatosomatic index (HSI, %) = ((liver weight/body weight) x 100); (7) viscerosomatic index (VSI, %) = ((total viscera weight/body weight) x 100); (8) perivisceral index (PVI, %) = ((total fat viscera weight/total viscera weight) x 100).

Proximate Composition, Fatty Acid Profile, and Indices of Lipid Metabolism and Quality

Proximate analyses of feeds and fish muscle samples were carried out according to AOAC (2000) for dry matter and ash. Crude protein content (N × 6.25) was determined using elemental analysis (C:H:N) (Fisons EA 1108 analyzer, Fisons Instruments, USA), and total lipid content was quantified according to the methods described by Folch et al. (1957). Fatty acid composition of feeds, liver, and muscle samples was determined by gas chromatograph following the procedure described by Rodríguez-Ruiz et al. (1998).

From the fatty acid profile of fish muscle, different indices were calculated (Arakawa and Sagai, 1986; Senso et al., 2007): (1) peroxidability index (PI)= (% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8); (2) index of thrombogenicity (IT) = (14:0 + 16:0 + 18:0)/[(0.5 × 18:1) + (0.5 × ΣMUFAs) + (0.5 × n-6 PUFAs) + (3 × n-3 PUFAs) + (n-3/n6)]; (3) index of atherogenicity (IA) = (12:0 + 4 × 14:0 + 16:0)/[(n-6 + n-3) PUFAs + 18:1 + other MUFAs]; (4) fish lipid quality (FLQ, %) = [(20:5n-3 + 22:6n-3)/total lipid] × 100. MUFAs and PUFAs stand for monounsaturated fatty acids and polyunsaturated fatty acids, respectively.

Determination of Digestive Enzyme Activities

Total alkaline protease activity was measured using buffered 5 g L^{-1} casein (50 mM Tris-HCl, pH 9.0) as substrate following the method described by Alarcón et al. (1998). One unit of activity (UA) was defined as the amount of enzyme releasing 1 μg tyrosine per minute measured spectrophotometrically at 280 nm (extinction coefficient for tyrosine of 0.008 μg^{-1} cm^{-1} mL^{-1}). Trypsin and chymotrypsin activities were determined using 0.5 mM BAPNA (N-α-benzoyl-DL-arginine-4-nitroanilide) as substrate according to Erlanger et al. (1961) and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-P-nitroanilide) according to DelMar et al. (1979), respectively, in 50 mM Tris–HCl buffer, pH 8.5, containing 10 mM $CaCl_2$. Leucine aminopeptidase activity was assayed using buffered 2 mM L-leucine -p-nitroanilide (LpNa) (100 mM Tris-HCl, pH 8.8) as substrate according to Pfleiderer (1970), and alkaline phosphatase activity was determined using buffered p-nitrophenyl phosphate (pH 9.5) as substrate, according to the methodology described by Bergmeyer (1974). For trypsin, chymotrypsin, and leucine aminopeptidase activities, one UA was defined as the amount of enzyme that released 1 μmol of p-nitroanilide (pNA) per minute (extinction coefficient 8,800 M cm^{-1} at 405 nm). For alkaline phosphatase, one UA was defined as the amount of enzyme that released 1 μg of nitrophenyl per min (extinction coefficient 17,800 M cm^{-1} at 405 nm). All assays were performed in triplicate in each one of the 12 enzymatic

extracts obtained per dietary treatment, and specific enzyme activities were expressed as U g tissue^{-1} (Galafat et al., 2020).

In addition, a substrate-SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) electrophoresis gel was carried out to visualize the active proteases present in intestinal extracts of fish. Intestinal extracts were mixed with SDS sample buffer (1:1), and SDS-PAGE was performed according to Laemmli (1970) using 11% polyacrylamide (100 V per gel, 60 min, 4°C). Zymograms revealing protease active bands were made according to Alarcón et al. (1998). After electrophoresis, gels were washed with distilled water and incubated for 30 min at 4°C in buffer 0.75% (w/v) casein solution (50 mM Tris–HCl buffer, pH 9). Then, gels were incubated in the same solution for 90 min at 37°C. After the incubation, gels were washed and fixed in 12% trichloroacetic acid solution (TCA) for 105 min to stop the reaction prior to staining with Coomassie Brilliant Blue R-250 in a solution of methanol-acetic acid-water overnight. Finally, gels were destained using a methanol–acetic acid–water solution. Clear gel zones revealed the presence of active proteases with caseinolytic activity.

Histological and Ultrastructural Study of the Intestinal Mucosa

Anterior intestinal samples from three fish per tank (9 per dietary treatment) were collected for examination by light and electron microscopy. For light microscopy examination, intestine samples were fixed for 24 h in phosphate-buffered formalin (4% v/v, pH 7.2), dehydrated, and embedded in paraffin according to standard histological techniques described in Vizcaíno et al. (2014). Briefly, samples were cut in 5 μm transversal sections, and the slides were stained with hematoxylin-eosin (H&E). The stained preparations were examined under a light microscope (Olympus ix51, Olympus, Barcelona, Spain) equipped with a digital camera (CC12, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Images were analyzed with specific software (Image J, National Institutes of Health, USA). The length and diameter of mucosal folds, total enterocyte height, as well as the thickness of the lamina propria of the submucosa, muscular and serous layer were analyzed (50 independent measurements per treatment) in intestinal samples.

Samples for transmission (TEM) and scanning (SEM) electron microscopy were processed as described in Vizcaíno et al. (2014). For TEM, intestine samples were fixed with glutaraldehyde 25 g L^{-1} and formaldehyde 40 g L^{-1} in phosphate buffer saline (PBS) pH 7.5 for 4 h at 4°C. Then, they were washed three times with PBS, and a 2 h post-fixation with 20 g L^{-1} osmium tetroxide was carried out. After that, samples were dehydrated by consecutive immersions in gradient ethanol solutions (from 50% to 100%; v/v). The dehydrated tissue was embedded in a mixture of 1:1 ethanol 100% (v/v) and EPON resin for 2 h with stirring. Then, it was included in pure EPON resin for 24 h and polymerized at 60°C. The ultra-fine cuts were placed on 700 copper mesh and stained with uranyl acetate and lead citrate. The observation was performed with a transmission electron microscope Zeiss 10C at 100 kV (Carl Zeiss, Barcelona, Spain). For SEM, samples were fixed for 24 h in phosphate-buffered formalin (4% v/v, pH 7.2). Then, they were washed and progressively dehydrated in graded ethanol. Then samples were dried by critical point (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain) with absolute ethanol as the intermediate fluid and CO_2 as the transition fluid. Dried samples were mounted on supports, fixed with graphite (PELCO® Colloidal Graphite, Ted Pella Inc., Ca, USA), and gold sputter-coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). All digital images were analyzed with UTHSCA ImageTool software and morphometric analysis to determine the microvilli length (ML), the microvilli diameter (MD), the number of microvilli over 1 μm distance, and the enterocyte apical area (EA) was carried out. Finally, the total absorption surface per enterocyte (TAS) was estimated following the procedure described byVizcaíno et al. (2014).

Tissue Metabolites

For assessing tissue metabolite levels, samples from liver and muscle (three fish per tank, 9 per dietary treatment) were individually minced on an ice-cold Petri dish and subsequently homogenized by mechanical disruption (Ultra-Turrax®, T25basic with an S25N-8G dispersing tool, IKA®-Werke) with 7.5 vol. (w/v) of ice-cold 0.6 N perchloric acid and neutralized after adding the same volume of 1 M $KHCO_3$. Subsequently, the homogenates were centrifuged (3500 × g, 30 min, 4°C), and the supernatants were recovered in different aliquots. The aliquots were then stored at −80°C until used in metabolite assays. Metabolite concentrations in plasma (glucose, lactate, and triglycerides) and liver (glucose and triglycerides) were determined using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides ref. 1001311) with reactions adapted to 96-well microplates. Liver glycogen levels were assessed using the method from Keppler and Decker (1974). After subtracting free glucose levels, the glucose obtained after glycogen was determined using the commercial kit described above for glucose. Plasma total protein concentration was determined using bovine serum albumin (BSA) as the standard with BCA Protein AssayKit (PIERCE, Thermo Fisher Scientific, USA, #23225). Total α-amino acid levels were assessed colorimetrically using the ninhydrin method from Moore (1968) adapted to 96-well microplates. All standards and samples were measured in duplicate. All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, VT, USA) using KCjunior™ software.

Activity of Metabolic Enzymes in the Liver

Frozen liver tissues (three fish per tank, 9 per dietary treatment) for enzyme activity assays were homogenized by mechanical disruption (Ultra-Turrax®) with 10 vol. (w/v) of ice-cold homogenization buffer (in mM: 50 imidazole, 1 2-mercaptoethanol, 50 NaF, 4 EDTA, 0.5 phenylmethylsulfonyl fluoride (PMSF), and 250 sucrose; pH 7.5). Homogenates were centrifuged for 30 min at 3220 × g and 4°C, and supernatants were stored at −80°C for further analysis. The assays of several enzymes involved in glycogenolysis (GPase [active]: glycogen phosphorylase, EC 2.4.1.1), glycolysis (HK: hexokinase,

EC 2.7.1.1; PK: pyruvate kinase, EC 2.7.1.40), gluconeogenesis (LDH: lactate dehydrogenase, EC 1.1.1.27; FBP: fructose 1,6-bisphosphatase, EC 3.1.3.11) and lipid metabolism (HOAD: 3-hydroxyacyl-CoA dehydrogenase, EC 1.1.1.35) were performed as previously described for gilthead seabream (*Sparus aurata*) tissues (Perera et al., 2020). All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, VT, USA) using KCjunior™ software. Activities were expressed as specific activities per mg of protein in the homogenate (U mg $prot^{-1}$). Proteins were assayed in duplicate, as described above for plasma samples.

Statistical Analysis

Results are reported as means ± SEM (n = 9). Normal distribution was checked for all data with the Shapiro–Wilk test, while the homogeneity of the variances was obtained using the Levene test. When necessary, an arcsin transformation was performed. Differences between the two experimental diets (CT and C-15) were tested using Student's t-test. In all statistical tests performed, $p < 0.05$ was considered significantly different. All analyses were performed with SPSS Statistics 25 software (SPSS Inc, IBM Company, NY, USA).

RESULTS

Growth Performance, Nutrient Utilization, and Proximate Composition

No mortality occurred during the experimental period. The inclusion of *C. fusca* statistically stimulated growth performance of *C. labrosus* juveniles (**Table 3**). After 90 days, C-15 group animals enhanced significantly body weight and length as well as weight gain and specific growth ratio respect to fish fed CT diet. Fish fed with *Chlorella* increased protein efficiency and decreased feed conversion ratio compared to the control group; however, no statistical differences were found between experimental groups. Furthermore, no significant differences were found in Fulton's Condition Factor (K) and somatic indices (HSI, VSI, and PVI).

TABLE 3 | Growth performance and somatic indices of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

Parameters	CT	C-15	*p*
Initial weight (g)	84.5 ± 0.6	85.0 ± 0.5	n.s.
Final weight (g)	144.8 ± 5.1	158.8 ± 3.6*	0.018
Initial length (cm)	18.0 ± 0.1	17.9 ± 0.2	n.s.
Final length (cm)	21.6 ± 0.4	22.3 ± 0.2*	0.041
WG (%)	171.8 ± 7.2	185.5 ± 3.3*	0.039
SGR (%)	0.6 ± 0.0	0.7 ± 0.0*	0.045
FCR	3.5 ± 1.2	2.6 ± 0.1	n.s.
PER	0.8 ± 0.2	1.0 ± 0.0	n.s.
K	1.4 ± 0.0	1.4 ± 0.0	n.s.
HSI (%)	0.8 ± 0.2	0.8 ± 0.1	n.s.
VSI (%)	9.1 ± 1.7	7.6 ± 1.8	n.s.
PVI (%)	43.4 ± 14.9	39.7 ± 9.5	n.s.

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE of triplicate groups. Asterisks denote significant differences (p < 0.05). n.s., not significant.

The proximate composition of *C. labrosus* specimens is presented in **Table 4**. Body composition of fish did not show any significant variation between fish fed CT and C-15 diets.

Tissue Fatty Acids

Fatty acid profiles results are shown in **Table 5**. The fatty acid profiles of liver and muscle differed between experimental groups. Palmitic acid (16:0) and oleic acid (18:1n-9) were both tissues' most abundant fatty acids. Liver from the CT group had significantly higher proportions of oleic acid, linolenic acid (18:3n-3), and arachidonic acid (ARA, 20:4n-6). However, liver from the C-15 group showed significantly higher proportions of vaccenic acid (18:1n-7), eicosenoic acid (20:1n-9), and docosahexaenoic acid (DHA, 22:6n-3). In muscle, C-15 specimens presented statistically higher proportions of palmitic acid, stearic acid (18:0), ARA, eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (22:5n-3), and DHA. On the other hand, muscle from the control group had higher oleic acid, vaccenic acid, linoleic acid (18:2n6), linolenic acid, and eicosenoic acid. Hepatic percentage of total saturated, monounsaturated and polyunsaturated fatty acids (SFA, MUFA, and PUFA, respectively) was not modified by the inclusion of *C. fusca*. In contrast, while a significantly higher proportion of SFA, PUFA, and n-3 content was found in muscle of the C-15 diet, higher content of MUFA, n-6, and n-9 were detected in muscle of control specimens. Altogether, the n-3/n-6 ratio enhanced significantly in both tissues from C-15-fed fish, while EPA/DHA ratio decreased significantly. As a result of these differences due to the inclusion of *C. fusca*, C-15 specimens showed a significantly higher peroxidability index (PI), flesh lipid quality (FLQ), and atherogenicity index (IA), as well as a lower index of thrombogenicity (IT).

Digestive Functionality

Enzyme activities measured in the intestinal extracts of *C. labrosus* specimens fed with the experimental diets are shown in **Table 6**. Total alkaline protease activity was significantly higher in the C-15 group than fish fed the control diet, whilst trypsin and chymotrypsin activity levels were similar between both groups. Regarding brush border enzymes, dietary inclusion of *C. fusca* significantly increased both leucine aminopeptidase and alkaline phosphatase activity levels compared to CT group. In addition, the zymogram of the intestinal proteases revealed the same profile of active fractions in both CT and C-15 fed fish (**Figure 1**).

The histological characteristics of intestinal sections from fish receiving the two dietary treatments are shown in **Figure 2**. Overall, no signs of intestinal damage were found as all specimens presented intestinal mucosa without evidence of

TABLE 4 | Muscle proximate composition (% dry weight) of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

	CT	C-15	*p*
Protein	79.31 ± 0.27	80.06 ± 0.71	n.s.
Lipid	8.75 ± 0.83	8.81 ± 0.20	n.s.
Ash	7.41 ± 0.35	7.38 ± 0.53	n.s.

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE (n = 9 fish per dietary treatment). n.s., not significant.

TABLE 5 | Liver and muscle fatty acid profile (% of total fatty acids) of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

Fatty acids	Liver			Muscle		
	CT	C-15	*p*	CT	C-15	*p*
14:0	3.59 ± 0.46	3.08 ± 0.04	n.s.	1.94 ± 0.03	1.99 ± 0.08	n.s.
16:0	27.43 ± 0.93	28.55 ± 0.07	n.s.	23.54 ± 0.05	24.63 ± 0.31*	0.001
16:1n7	8.51 ± 1.04	10.18 ± 1.13	n.s.	6.69 ± 0.02	6.97 ± 0.19	n.s.
18:0	3.27 ± 0.59	3.50 ± 0.10	n.s.	3.36 ± 0.05	3.74 ± 0.20*	0.010
18:1n9	33.67 ± 0.92*	29.17 ± 0.08	0.006	34.31 ± 0.50*	30.30 ± 0.34	<0.001
18:1n7	3.88 ± 0.09	4.98 ± 0.08*	<0.001	1.34 ± 0.00*	1.10 ± 0.04	0.004
18:2n6	8.45 ± 0.91	6.91 ± 0.09	n.s.	13.46 ± 0.19*	11.80 ± 0.01	0.002
18:3n3	1.60 ± 0.16*	1.22 ± 0.03	0.004	2.60 ± 0.12*	2.38 ± 0.01	0.045
20:1n9	0.48 ± 0.18	1.05 ± 0.03*	0.002	1.31 ± 0.03*	1.10 ± 0.08	0.004
20:4n6, ARA	0.63 ± 0.12*	0.40 ± 0.02	0.009	1.20 ± 0.01	1.38 ± 0.05*	0.001
20:5n3, EPA	1.43 ± 0.53	1.80 ± 0.03	n.s.	2.63 ± 0.06	2.95 ± 0.06*	<0.001
22:5n3	–	–	–	1.21 ± 0.06	1.42 ± 0.08*	0.007
22:6n3, DHA	2.46 ± 1.32	4.95 ± 0.26*	0.010	4.87 ± 0.29	8.64 ± 0.54*	<0.001
SFA	34.29 ± 0.79	35.13 ± 0.22	n.s.	28.84 ± 0.03	30.35 ± 0.19*	0.002
MUFA	46.53 ± 2.55	45.38 ± 1.32	n.s.	43.64 ± 0.52*	39.46 ± 0.65	<0.001
PUFA	5.12 ± 2.80	8.23 ± 0.31	n.s.	9.92 ± 0.16	14.40 ± 0.73*	<0.001
Other FA	3.51 ± 0.18*	2.32 ± 0.93	0.036	1.04 ± 0.65	1.14 ± 0.08	n.s.
n - 3	6.32 ± 2.86	9.05 ± 0.30	n.s.	11.32 ± 0.05	15.40 ± 0.68*	0.004
n - 6	9.08 ± 1.03	7.32 ± 0.12	n.s.	14.66 ± 0.21*	13.18 ± 0.04	0.001
n - 9	34.15 ± 1.59*	30.22 ± 0.11	0.026	35.61 ± 0.53*	31.40 ± 0.42	<0.001
n - 3 PUFA	4.49 ± 2.68	7.83 ± 0.33	n.s.	8.72 ± 0.17	13.02 ± 0.68*	<0.001
n - 3/n - 6	0.68 ± 0.24	1.24 ± 0.06*	0.005	0.77 ± 0.01	1.17 ± 0.05*	<0.001
EPA/DHA	0.61 ± 0.11*	0.36 ± 0.01	0.033	0.54 ± 0.04*	0.34 ± 0.01	<0.001
PI[1]	–	–	–	87.11 ± 1.10	118.91 ± 5.37*	<0.001
IT[2]	–	–	–	0.39 ± 0.00*	0.37 ± 0.00	0.031
IA[3]	–	–	–	0.47 ± 0.00	0.49 ± 0.01*	0.004
FLQ[4]	–	–	–	7.50 ± 0.23	11.60 ± 0.60*	<0.001

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE (n = 9 fish per experimental diet). Asterisks denote significant differences (p < 0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n.s., not significant. [1]PI, peroxidability index; [2]IT, index of thrombogenicity; [3]IA, index of atherogenicity; [4]FLQ, fish lipid quality.

TABLE 6 | Enzyme activities (U g tissue^{-1}) measured in the whole intestinal extracts of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

	CT	C-15	*p*
Pancreatic enzymes			
Total alkaline protease	490.31 ± 105.13	763.20 ± 173.66*	0.002
Trypsin	0.16 ± 0.05	0.21 ± 0.04	n.s.
Chymotrypsin	2.88 ± 0.90	3.01 ± 0.76	n.s.
Brush border enzymes			
Leucine aminopeptidase	0.36 ± 0.07	0.46 ± 0.07*	0.008
Alkaline phospatase	3.72 ± 0.45	5.21 ± 0.74*	<0.001

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE of triplicate determinations (n = 12 extracts per dietary treatment). Asterisks denote significant differences (p < 0.05). n.s., not significant.

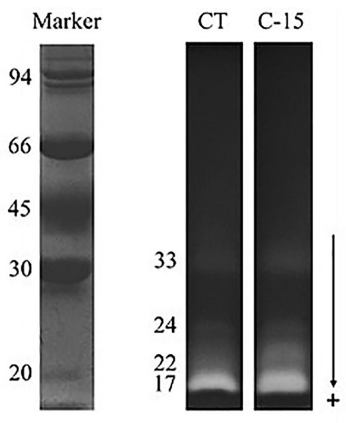

FIGURE 1 | Zymogram showing total proteolytic activity from pooled intestinal extracts of fish. Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Protein standards employed were phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20). The molecular mass (in kDa) of proteins was measured using a linear plot of log Mr. of protein standards (M) vs. relative mobility (Rf). Five microliters of molecular weight marker (M) were loaded.

abnormality. The morphometric analysis (**Table 7**) revealed a significant increase of fold length in fish fed the C-15 diet, while fold diameter and enterocyte height were similar in both dietary groups. Additionally, thickness of the serosa and muscular layers, as well as of the lamina propria was significantly reduced in specimens fed with C-15 diet.

TEM and SEM observations confirmed a well-defined and organized intestinal brush border membrane in both dietary groups (**Figure 3**). Morphometric analysis of TEM and SEM

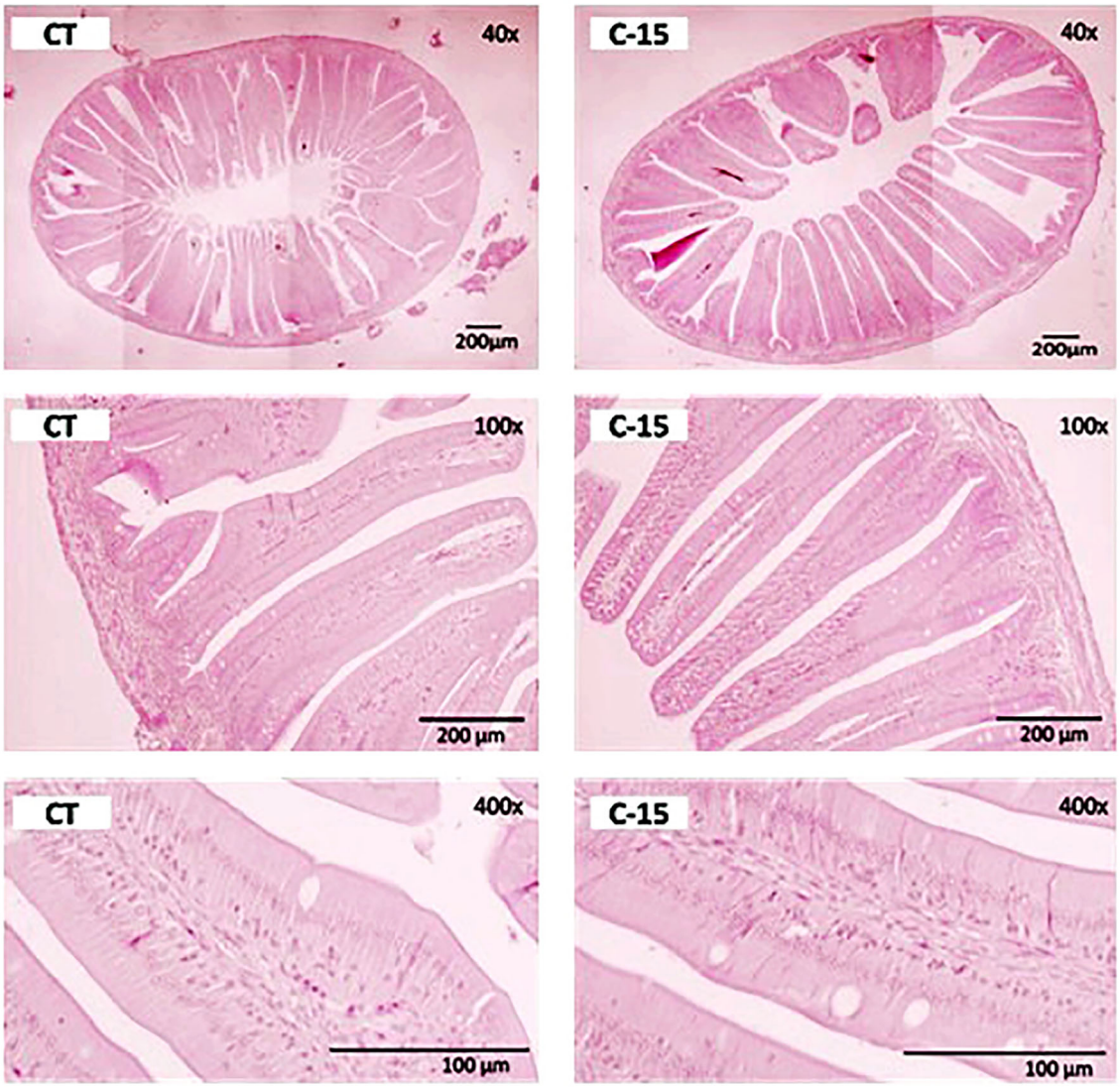

FIGURE 2 | Transversal sections from the proximal intestine of C. labrosus juveniles fed control (CT) or experimental (C-15) diets during 90 days. H&E stain, scale bar 200 µm, and 100 µm. Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet.

TABLE 7 | Quantification of the histological parameters assessed in the intestine of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

	CT			C-15			*p*
Fold length (µm)	677.39	±	62.28	728.30	±	52.68*	<0.001
Fold diameter (µm)	120.33	±	21.05	112.01	±	13.53	n.s.
Enterocyte height (µm)	40.93	±	4.25	41.55	±	2.63	n.s.
Serosa layer (µm)	31.22	±	5.90*	20.97	±	2.65	<0.001
Muscular layer (µm)	33.75	±	3.33*	30.63	±	2.53	<0.001
Submucosa layer (µm)	29.75	±	3.27*	24.39	±	1.63	0.024
Lamina propria (µm)	39.41	±	5.13*	33.32	±	3.90	<0.001

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE of triplicate determinations (n = 9 fish per dietary treatment). Asterisks denote significant differences (p < 0.05). n.s., not significant.

images revealed that enterocytes from proximal intestine of fish fed with C-15 diet increased significantly microvilli length, apical area, as well as total absorption surface compared to the CT group (**Table 8**).

Plasma, Liver, and Muscle Metabolites and Enzyme Metabolic Activities

Plasma, liver, and muscle metabolites are shown in **Table 9**. The experimental diets did not cause significant plasma triglycerides and protein changes. A statistical increase in plasma glucose and a reduction in lactate values were observed in C-15 group compared to the CT group. In the liver, triglycerides levels decreased statistically in C-15 group respect to CT group, while amino acid levels enhanced significantly. No significant differences were detected among experimental groups in hepatic free glucose, glycogen, or lactate values. In muscle, no significant differences were detected among experimental groups for any metabolite assessed.

The effect of the dietary inclusion of *C. fusca* was also evaluated in several metabolic enzymes related to glycogenolysis, glycolysis, gluconeogenesis, and lipid metabolism in the liver (**Figure 4**). HOAD (lipid metabolism), HK (glycolysis), and GPact (glycogenolysis) enzymes displayed a significant increase in C-15 group after 90 days of feeding. In contrast, hepatic activity of the FBP (gluconeogenesis) enzyme decreased in this group. No effects were found between experimental groups on the hepatic activity of LDH (gluconeogenesis) and PK (glycolysis) enzymes.

DISCUSSION

Microalgae have been evaluated as functional dietary ingredients or for replacement of fishmeal in different aquaculture species, generally with positive effects on growth and fatty acid profile of fish fillet (Vizcaíno et al., 2014; Pakravan et al., 2017; Sarker et al., 2018; García-Márquez et al., 2020), thereby not only improving

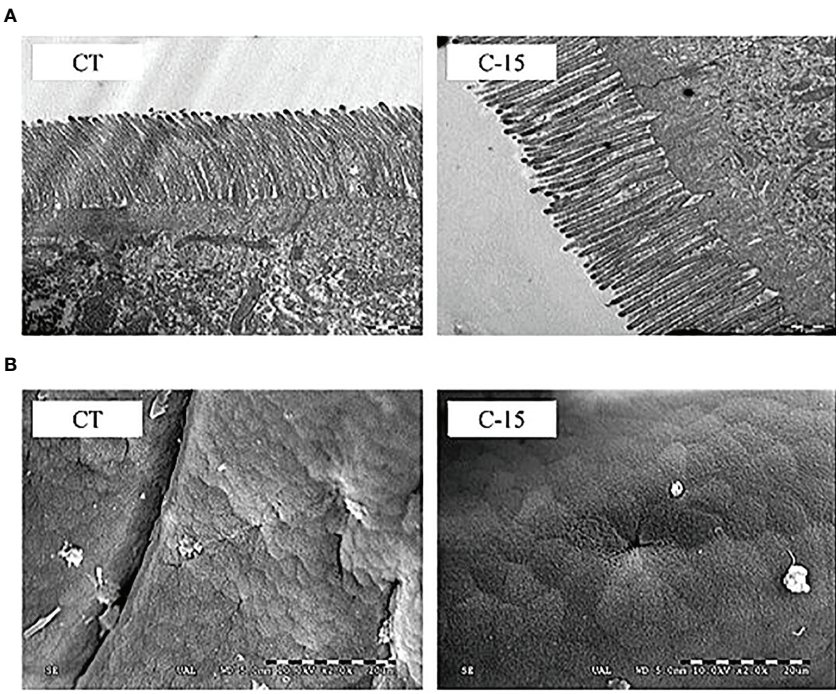

FIGURE 3 | Comparative TEM **(A)** and SEM **(B)** micrographs from the anterior intestine of *C. labrosus* juveniles at the end of the feeding trial. Dietary codes: CT, control diet; C-15: 15% *C. fusca* supplemented-diet. (TEM bar: 1 mm, SEM bar: 20 mm).

TABLE 8 | Microvillar morphology of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

	CT	C-15	*p*
Microvilli length (μm)	1.95 ± 0.12	2.55 ± 0.36*	<0.001
Microvilli diameter (μm)	0.11 ± 0.01	0.11 ± 0.01	n.s.
Density (microvilli per μm^2)	43.22 ± 6.85	46.11 ± 5.73	n.s.
Enterocyte apical área (μm^2)	18.13 ± 3.25	31.63 ± 2.58*	<0.001
Enterocyte absorption Surface (μm^2)	521.49 ± 93.47	1312.33 ± 97.50*	<0.001

Dietary codes: CT, control diet; C-15, 15% C. fusca supplemented-diet. Values are expressed as mean ± SE (n = 9 fish per dietary treatment). Asterisks denote significant differences (p < 0.05); n.s., not significant.

TABLE 9 | Tissue metabolites content of juvenile *C. labrosus* fed control (CT) and C-15 diets during 90 days.

	CT	C-15	*p*
Plasma			
Glucose (mM)	6.91 ± 0.34	7.42 ± 0.31*	0.038
Triglycerides (mM)	8.26 ± 0.80	7.06 ± 1.11	n.s.
Lactate (mM)	2.07 ± 0.16*	1.54 ± 0.18	0.001
Protein (mM)	37.44 ± 4.63	35.97 ± 3.68	n.s.
Liver			
Glucose (mmol g^{-1}w.w.)	101.82 ± 0.99	101.25 ± 1.41	n.s.
Glycogen (mmol g^{-1}w.w.)	33.70 ± 4.26	38.22 ± 4.49	n.s.
Triglycerides (mmol g^{-1}w.w.)	2.51 ± 0.59*	1.28 ± 0.13	0.018
Lactate (mmol g^{-1}w.w.)	2.35 ± 0.68	2.09 ± 0.44	n.s.
Amino acid (mg g^{-1})	5.30 ± 0.93	7.18 ± 0.35*	0.002
Muscle			
Glucose (mmol g^{-1}w.w.)	96.04 ± 2.22	96.75 ± 1.71	n.s.
Glycogen (mmol g^{-1}w.w.)	1.24 ± 0.73	2.33 ± 1.09	n.s.
Triglycerides (mmol g^{-1}w.w.)	0.18 ± 0.05	0.18 ± 0.04	n.s.
Lactate (mmol g^{-1}w.w.)	65.41 ± 2.58	69.08 ± 9.97	n.s.
Amino acid (mg g^{-1}w.w)	7.47 ± 0.85	7.44 ± 1.19	n.s.

Dietary codes: CT, control diet; C-15: 15% C. fusca supplemented-diet. Values are expressed as mean ± SE (n = 9 fish per dietary treatment). Asterisks denote significant differences (p < 0.05); n.s., not significant.

fish health but also increasing productivity and the value of the commercial product obtained. The result of this work revealed that the inclusion of 15% of *C. fusca* stimulated specific growth rate and body weight, which is in broad agreement with that previously published for other fish species fed with different microalgae (Vizcaíno et al., 2018; Gong et al., 2019; Sarker et al., 2020). Furthermore, the feed efficiency (PER and FCR) was not negatively affected by the inclusion of *C. fusca*. Different studies have reported both favourable and unfavourable effects of *Chlorella* species at varying supplementation levels in aquaculture feeds on growth performance and feed utilization in different fish species. For instance, Rahimnejad et al. (2017) reported that dietary inclusion of 10–15% of *Chlorella vulgaris* enhanced significantly growth performance and specific growth rate in Olive flounder (*Paralichthys olivaceus*). Similarly, Teuling et al. (2017) reported the overall performance in both Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarus gariepinus*) were similar or superior compared to fish fed the reference diet when *C. vulgaris* meal was included at 30% in the aquafeeds. In contrast, when *Chlorella* sp. meal was supplemented at 1.6% and 2% in diets for gibel carp (*Carassius auratus gibelio*) feed conversion ratio was significantly lower than the control group (Xu et al., 2014). To resume, the varied effects on fish performance reported at higher and lower dietary *Chlorella* inclusion levels could be attributed to the different inclusion levels, the specific microalgae strain and fish species, and/or the duration of the experiment. Even so, the positive effects observed in the present study strongly suggest its use as suitable ingredient in herbivorous/omnivorous marine species, at least at 15% of biomass. However, it has been reported that *C. labrosus* changes its digestive enzyme profile according to the size/age of specimens (Pujante et al., 2017). For this reason, the beneficial effects observed of dietary inclusion of *C. fusca* (i.e. 15%) could be confirmed using specimens with different stages of development.

Similar to that reported previously in feeding trials using aquafeed with inclusion of other microalgae, no differences in proximate muscle composition attributable to the dietary inclusion of *C. fusca* were observed (Vizcaíno et al., 2014; Vizcaíno et al., 2018; Sales et al., 2021). However, disparate effects of microalgae inclusion on muscle proximate composition can be described. Thus, some authors described that microalgal biomass inclusion may increase protein and decrease lipid content in different farmed fish species owing to the presence of several bioactive compounds in microalgae biomass that may activate fish metabolism, and particularly the use of lipid as energy source (Roohani et al., 2019; Galafat et al., 2020).

Regarding fatty acids profile, the inclusion of *C. fusca* induced selective retention of DHA in the liver, and ARA, EPA, and DHA in the muscle. This fact has been previously pointed out in other studies, which evidenced a relationship between microalgae inclusion and higher efficiency of mobilizing lipids from liver (He et al., 2018; Vizcaíno et al., 2018; García-Márquez et al., 2020). Besides this, the relative increase of structural fatty acids in muscle (ARA, EPA, and DHA) is also reflected in the significant fish lipid quality index (FLQ), n3/n6 ratio, and index of atherogenicity (IA) enhancement in fish fed on C-15 diet, which could be beneficial from a human nutrition standpoint (Abdelhamid et al., 2018; Román et al., 2019).

According to Furné et al. (2008), adequate feed efficiency is highly related to the physiological capacity of fish to digest and transform the ingested nutrients, and therefore to the presence of an appropriate set of digestive enzymes. Indeed, any change in their activity levels could reveal a significant impact on fish growth and proper nutrient utilization. Previous works evidenced that dietary inclusion of different microalgal biomasses may exert substantial changes in the activity of several enzymes involved in the digestive and absorptive processes at intestinal level (Vizcaíno et al., 2014; Vizcaíno et al., 2018; Gong et al., 2019; Galafat et al., 2020; Galafat et al., 2022). In agreement with these studies, the results obtained in this piece of research evidenced noticeable differences in enzyme activity levels between fish fed C-15 diet and those fed on the control diet. Dietary inclusion of 15% *C. fusca* did not cause any adverse effects on enzymatic activities from the pancreatic secretion, as it can be observed in the zymogram, which evidenced that microalgae-fed fish showed the same pattern of intestinal proteases that control (CT) specimens. However, protease activity was significantly higher in fish fed C-15 diet than the CT group. Similar results were shown by Akbary and Raeisi (2020), who found an increase of total alkaline protease activity in grey mullet (*M. cephalus*) specimens fed on diets supplemented with up to 15% *C. vulgaris*. This increase in proteolytic activity seems to be related to an improvement in digestive functionality that could lead to better absorption and utilization of nutrients (Engrola et al., 2007), in line with the higher growth performance observed.

On the other hand, there are previous works focused on evaluating the changes in the digestive enzyme profile in fish that modify their feeding habits during the ontogeny such as mullets, which showed an omnivorous feeding habits during their early

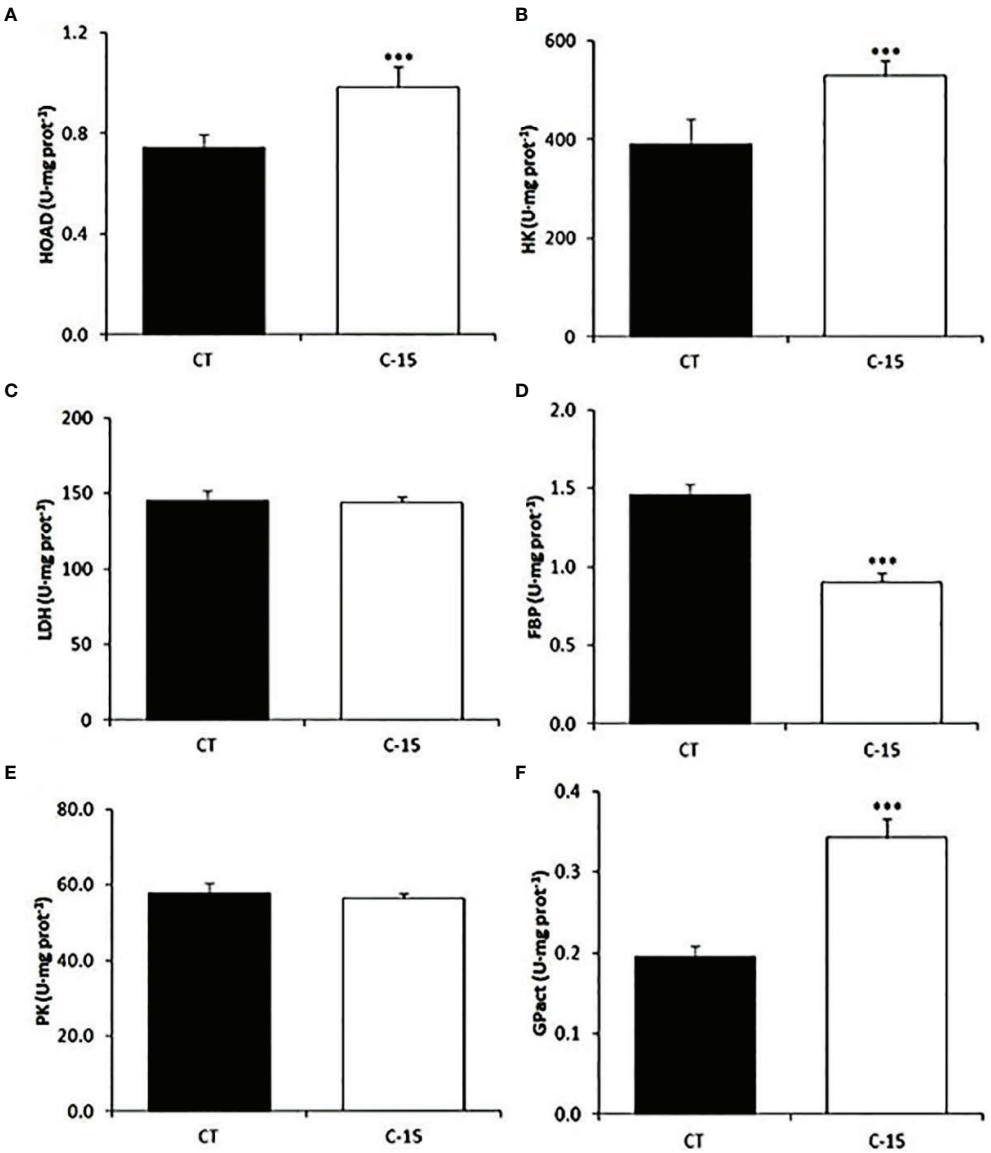

FIGURE 4 | Specific activity (U mg protein−1 as mean ± SEM) of metabolic enzymes in the liver of juvenile **(C)** labrosus fed control (CT) and C-15 diets during 90 days. Asterisks denote significant differences at $p < 0.001$ (***). **(A)** HOAD, 3-hydroxyacyl-CoA dehydrogenase; **(B)** HK, hexokinase; **(C)** LDH, lactate dehydrogenase; **(D)** FBP, fructose 1,6-bisphosphatase; **(E)** PK, pyruvate kinase; **(F)** GPase, glycogen phosphorylase (active).

stages with a trend to become herbivorous with age (Wassef et al., 2001). In this regard, Pujante et al. (2017), found a noticeable increase in the protease activity levels in *C. labrosus* as consequence of the changes in the fish-feeding habits. These modifications might reflect a possible compensation mechanism (i.e. by increasing enzyme secretion) that ensure efficient digestive processes, as it has been described in other herbivorous fish species such as carp or tilapia (Uscanga-Martínez et al., 2011; Hernández-Sámano et al., 2017). Regarding brush border enzymes, a significant increase in leucine aminopeptidase and alkaline phosphatase activity levels was observed in fish fed the diets supplemented with 15% *C. fusca*. Both enzymes play a key role in the final stages of protein digestion, hydrolysing the oligopeptides released by pancreatic enzymes into free amino acids, dipeptides or tripeptides (Gisbert et al., 2018) and allowing absorption or transport of amino acids through the enterocytes (Cahu and Zambonino Infante, 2001). These enzymes, especially alkaline phosphatase, can be used as indicator of intestinal integrity and nutrient absorption, so increased activity levels seem to be related to an improvement in digestive process efficiency and absorptive capability of the intestinal mucosa (Vizcaíno et al., 2014).

In addition, it was observed that these positive changes in enzymatic intestinal activities concur with the histological and

ultrastructural measurements carried out on the intestinal mucosa. In herbivorous fish, intestines are generally larger than in carnivorous ones, which is thought to allow for additional processing of relatively difficult-to-digest items (Wilson and Castro, 2010). The structural condition of the intestine is considered a reliable nutritional and physiological biomarker since it reflects any physiological disorder caused by unbalanced diets or inadequate feeding conditions (Gisbert et al., 2008). Similar to other previous studies (Vizcaíno et al., 2014; Galafat et al., 2022), histological observations indicated that microalgae inclusion did not cause adverse effects on gut morphology. Besides the increase in mucosal fold length observed in fish fed on *C. fusca*-supplemented diet, the histological analysis revealed the absence of inflammatory processes in the lamina propria or accumulation of lipid vacuoles inside the enterocytes that could evidence intestinal enteritis and/or steatosis (Uran et al., 2008). Indeed, the serosa, muscular, and submucosa layers, as well as the lamina propria, were significantly thinner in fish fed with *C. fusca* than those fed with the microalgae-free diet. Those results pointed out that dietary microalgae inclusion might be useful to prevent intestinal pro-inflammatory processes, but further research is required to elucidate if longer feeding period or if higher microalgae dietary inclusion level can produce morphological alteration on the intestinal morphology in this species.

Electron microscopy analysis did not evidence structural alterations or signs of damage on intestinal brush border attributable to dietary inclusion of *C. fusca* biomass. It was observed that all the specimens showed a particular morphology and disposition of their intestinal folds, which was different from that observed in other fish species such as the carnivorous seabream (*S. aurata*), seabass (*D. labrax*), or Senegalese sole (*Solea senegalensis*) (Vizcaíno et al., 2014; Vizcaíno et al., 2018). *C. labrosus* folds were characterized by the presence of numerous laminar ridges with a flattened apex and a random distribution similar to that observed in other Mugilidae species such as gold grey mullet (*Liza aurata*) (Ferrando et al., 2006) (see **Supplementary Material 1**). Regarding the morphometric analysis of TEM and SEM images, microvilli length, enterocyte apical area, and enterocyte absorption surface significantly increased in fish fed *C. fusca*-supplemented diet. These results are consistent with those observed in previous studies performed in *S. aurata* (Vizcaíno et al., 2014; Galafat et al., 2020; Galafat et al., 2022), so it seems that the use of this microalga in aquafeeds enhanced absorption capacity, reinforcing the idea of the function of the intestinal mucosa as a physical barrier.

The response of the metabolism concerning the experimental diets was also characterized. The C-15 diet significantly mobilized carbohydrate metabolism by increasing plasma glucose and concomitantly decreasing plasma lactate. Thus, we suggest that *C. fusca* inclusion in the aquafeed may promote depletion of plasma lactate, which is previously originated in white muscle due to anaerobic metabolism, for later be partially incorporated into the liver according to what Perera et al. (2020) observed. However, the conversion of lactate to pyruvate by LDH in the liver (i.e. Cori cycle), and its further conversion to glycogen, are not supported neither by LDH activity nor glucose-glycogen trends in the present study, which remained unchanged regardless of the experimental diet. In this regard, high plasma glucose levels might indicate a proficient digestive breakdown of carbohydrates from the diet in this species and a potential source for glycogen reservoirs (Omlin et al., 2014). It has also been found that *C. fusca* inclusion increased hepatic HK activity, whereas PK activity was unaltered. HK is the first step in glycolysis, phosphorylating glucose to be used by cells, while PK catalyzes the last step of glycolysis, producing pyruvate and ATP. Even though hepatic glycogen content was not significantly higher, its increase supports that the C-15 diet enhanced hepatic glucose uptake capacity to be stored as glycogen instead of oxidized for energy, as previously observed in gilthead seabream (Perera et al., 2020).

The above-mentioned metabolic stage agrees with the observed reduction of hepatic gluconeogenic enzyme (FBP) activity in C-15 group, further supporting subsequent non-significant increases in muscle and hepatic glycogen within this same group. Although the metabolic significance of higher glycogen phosphorylase activity remains unknown in fish, it could be related to the turnover of liver glycogen for glucose in other metabolic pathways, such as synthesizing specific fatty acids as occurs in humans (Adeva-Andany et al., 2016). In this regard, the significant decrease in liver triglycerides values (the fundamental unit of lipid metabolism) in the C-15 group supports this hypothesis.

Increased glucose uptake by the liver, or production of glucose from glycogen, is known to have a stimulatory effect on the lipogenic enzymes, glucose-6-phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH) (Perera et al., 2020), which provide NADPH for the biosynthesis of fatty acids, and subsequently lead to higher lipid storage or export from the liver (Alvarez et al., 2000; Laliotis et al., 2010). However, we cannot rule out the possibility that some of the changes in specific fatty acids depositions are due to the existence of other nutrients in this microalga. The higher muscular HOAD activity (the third step of beta-oxidation) observed in specimens of C-15 group is remarkable. In higher vertebrates, lipid availability increases mitochondrial fatty acid oxidative capacity in muscle (Turner et al., 2007). Thus, we suggest that HOAD activity in the present study may be a compensatory mechanism to control excessive fat accumulation in fish muscles supplemented with microalgae or might be involved in lipid muscle deposition remodeling. Additionally, we also observed a significant increase in hepatic α-amino acids. The liver is an important organ for protein synthesis, degradation, and detoxification as well as amino acid metabolism. Overall hepatic amino acids are involved in various cellular metabolisms, the synthesis of lipids and nucleotides as well as detoxification reactions (Lee and Kim, 2019).

In conclusion, the results obtained in this work are in accordance with previous studies using microalgae-based aquafeeds and confirmed that *C. fusca* biomass is suitable for using as dietary ingredient in *C. labrosus* juveniles. The effects

observed on digestive and metabolic enzyme activities, together with adequate metabolic response and gut morphology, as well as a significant increase in intestinal mucosa's digestive and absorptive capacity, could explain the positive effects on growth performance obtained in fish fed the microalgae. However, given the scarce information related to the optimization of specific aquafeeds for this species and the changes in intestinal digestive capacity associated with developmental stages (Pujante et al., 2017), further studies to determine optimal inclusion levels of *C. fusca* in a long-term feeding trial and/or different developmental stages in this fish species are needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee from the Autonomous Andalusian Government (Junta de Andalucía reference number 11/07/2020/082).

AUTHOR CONTRIBUTIONS

Conceptualization: JG-M, SA, and RA-D; Methodology: JG-M, JM-S, FA, and GA; Validation: JG-M, JM-S, FA, JM, SA, and RA-D; Formal analysis: JG-M, AG, and AV; Resources: JM-S, FA, JM, and FF; Data curation: JG-M, AG, AV, AB, and JM-S; Visualization: JG-M, AG, AV, AB, and JM-S; Supervision: FA, JM, SA, and RA-D; Project administration: SA and RA-D; Funding acquisition: FA, JM, GA, and FF; Writing-original draft: JG-M; Writing-review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.902203/full#supplementary-material

REFERENCES

Abdelhamid, A. S., Brown, T. J., Brainard, J. S., Biswas, P., Thorpe, G. C., Moore, H. J., et al. (2018). Omega-3 Fatty Acids for the Primary and Secondary Prevention of Cardiovascular Disease. *Cochrane Database Syst. Rev.* 11, CD003177. doi: 10.1002/14651858.cd003177.pub4

Abdulrahman, N., Hoshyar Abid, S., Aziz Khidir, A., Bakhtyar Omer, B., Bahman Hama Rasheed, D., and Hıwa Baha Alddin, L. (2018). Effect of Adding Microalgae Chlorella Sp. On Some Biological Parameters and Proximate Analysis of Common Carp Cyprinus Carpio L. *Iran. J. Vet. Med.* 12 (3), 199–206. doi: 10.22059/IJVM.2018.244747.1004856

Adeva-Andany, M. M., Pérez-Felpete, N., Fernández-Fernández, C., Donapetry-García, C., and Pazos-García, C. (2016). Liver Glucose Metabolism in Humans. *Biosci. Rep.* 36 (6), 416. doi: 10.1042/BSR20160385/56473

Ahmad, M. T., Shariff, M., Md. Yusoff, F., Goh, Y. M., and Banerjee, S. (2020). Applications of Microalga Chlorella Vulgaris in Aquaculture. *Rev. Aquac.* 12 (1), 328–346. doi: 10.1111/RAQ.12320

Akbary, P., and Aminikhoei, Z. (2019). Impact of Dietary Supplementation of Chlorella Vulgaris (Beijerinck 1890) on the Growth, Antioxidant Defense and Immune Status of the Grey Mullet, Mugil Cephalus (Linnaeus 1758). *Iran. J. Aquat. Anim. Heal.* 5 (1), 57–70. doi: 10.29252/IJAAH.5.1.57

Akbary, P., and Raeisi, E. (2020). Optimum Dietary Level of Chlorella Vulgaris Powder as a Feed Additive for Some Blood Parameters and Digestive Enzymatic Activities of Grey Mullet, Mugil Cephalus. *Iran. J. Fish. Sci.* 19 (3), 1130–1139. doi: 10.22092/IJFS.2019.119600.0

Alarcón, F. J., Díaz, M., Moyano, F. J., and Abellán, E. (1998). Characterization and Functional Properties of Digestive Proteases in Two Sparids; Gilthead Seabream (Sparus Aurata) and Common Dentex (Dentex Dentex). *Fish Physiol. Biochem.* 19 (3), 257–267. doi: 10.1023/A:1007717708491

Alvarez, M. J., Díez, A., López-Bote, C., Gallego, M., and Bautista, J. M. (2000). Short-Term Modulation of Lipogenesis by Macronutrients in Rainbow Trout (Oncorhynchus Mykiss) Hepatocytes. *Br. J. Nutr.* 84 (5), 619–628. doi: 10.1017/S0007114500001951

AOAC. (2000). *Official Methods of Analysis. 17th Edition* (Washington: Association of Official Analytical Chemists).

Arakawa, K., and Sagai, M. (1986). Species Differences in Lipid Peroxide Levels in Lung Tissue and Investigation of Their Determining Factors. *Lipids* 21, 769–775. doi: 10.1007/BF02535410

Beal, C. M., Gerber, L. N., Thongrod, S., Phromkunthong, W., Kiron, V., Granados, J., et al. (2018). Marine Microalgae Commercial Production Improves Sustainability of Global Fisheries and Aquaculture. *Sci. Rep.* 8 (1), 15064. doi: 10.1038/s41598-018-33504-w

Becker, E. W. (2013). *Microalgae for Aquaculture: Nutritional Aspects. Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, *2nd ed.* UK: John Wiley & Sons, Ltd. 671–691. doi: 10.1002/9781118567166.CH36

Bergmeyer, H. U. (1974). *Methods of Enzymatic Analysis. Volume 2* (New York: Academic Press), 682 pp.

Cahu, C., and Zambonino Infante, J. (2001). Substitution of Live Food by Formulated Diets in Marine Fish Larvae. *Aquaculture* 200 (1–2), 161–180. doi: 10.1016/S0044-8486(01)00699-8

Camacho, F., Macedo, A., and Malcata, F. (2019). Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review. *Mar. Drugs* 17 (6), 312. doi: 10.3390/MD17060312

de las Heras, V., Martos-Sitcha, J. A., Yúfera, M., Mancera, J. M., and Martínez-Rodríguez, G. (2015). Influence of Stocking Density on Growth, Metabolism and Stress of Thick- Lipped Grey Mullet (Chelon Labrosus) Juveniles. *Aquaculture* 448, 29–37. doi: 10.1016/J.AQUACULTURE.2015.05.033

DelMar, E. G., Largman, C., Brodrick, J. W., and Geokas, M. C. (1979). A Sensitive New Substrate for Chymotrypsin. *Anal. Biochem.* 99 (2), 316–320. doi: 10.1016/S0003-2697(79)80013-5

Domozych, D. S., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P., and Willats, W. G. T. (2012). The Cell Walls of Green Algae: A Journey Through Evolution and Diversity. *Front. Plant Sci.* 3. doi: 10.3389/FPLS.2012.00082/BIBTEX

Engrola, S., Conceição, L. E. C., Dias, L., Pereira, R., Ribeiro, L., and Dinis, M. T. (2007). Improving Weaning Strategies for Senegalese Sole: Effects of Body Weight and Digestive Capacity. *Aquac. Res.* 38 (7), 696–707. doi: 10.1111/J.1365-2109.2007.01701.X

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961). The Preparation and Properties of Two New Chromogenic Substrates of Trypsin. *Arch. Biochem. Biophys.* 95 (2), 271–278. doi: 10.1016/0003-9861(61)90145-X

Ferrando, S., Maisano, M., Parrino, V., Ferrando, T., Girosi, L., and Tagliafierro, G. (2006). Gut Morphology and Metallothionein Immunoreactivity in Liza Aurata From Different Heavy Metal Polluted Environments. *Ital. J. Zool.* 73 (1), 7–14. doi: 10.1080/11250000500502228

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A Simple Method for the Isolation and Purification of Total Lipides From Animal Tissues. *J. Biol. Chem.* 226 (1), 497–509. doi: 10.3989/scimar.2005.69n187

Furné, M., García-Gallego, M., Hidalgo, M. C., Morales, A. E., Domezain, A., Domezain, J., et al. (2008). Effect of Starvation and Refeeding on Digestive Enzyme Activities in Sturgeon (Acipenser Naccarii) and Trout (Oncorhynchus Mykiss). *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 149 (4), 420–425. doi: 10.1016/J.CBPA.2008.02.002

Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Arizcun, M., Chaves-Pozo, E., et al. (2022). Assessment of Dietary Inclusion of Crude or Hydrolysed Arthrospira Platensis Biomass in Starter Diets for Gilthead Seabream (Sparus Aurata). *Aquaculture* 548, 737680. doi: 10.1016/J.AQUACULTURE.2021.737680

Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Jérez-Cepa, I., Mancera, J. M., et al. (2020). Evaluation of Arthrospira Sp. Enzyme Hydrolysate as Dietary Additive in Gilthead Seabream (Sparus Aurata) Juveniles. *J. Appl. Phycol.* 32 (5), 3089–3100. doi: 10.1007/S10811-020-02141-0/TABLES/6

García-Márquez, J., Galafat, A., Alarcón, F. J., Figueroa, F. L., Martínez-Manzanares, E., Arijo, S., et al. (2021). Cultivated and Wild Juvenile Thick-Lipped Grey Mullet, Chelon Labrosus: A Comparison From a Nutritional Point of View. *Animals* 11 (7), 2112. doi: 10.3390/ANI11072112

García-Márquez, J., Rico, R. M., del Pilar Sánchez-Saavedra, M., Gómez-Pinchetti, J. L., Acién, F. G., Figueroa, F. L., et al. (2020). A Short Pulse of Dietary Algae Boosts Immune Response and Modulates Fatty Acid Composition in Juvenile Oreochromis Niloticus. *Aquac. Res.* 51, 4397–4409. doi: 10.1111/are.14781

Gisbert, E., Fournier, V., Solovyev, M., Skalli, A., and Andree, K. B. (2018). Diets Containing Shrimp Protein Hydrolysates Provided Protection to European Sea Bass (Dicentrarchus Labrax) Affected by a Vibrio Pelagius Natural Infection Outbreak. *Aquaculture* 495, 136–143. doi: 10.1016/J.AQUACULTURE.2018.04.051

Gisbert, E., Ortiz-Delgado, J. B., and Sarasquete, C. (2008). Nutritional Cellular Biomarkers in Early Life Stages of Fish. *Histol. Histopathol.* 23 (12), 1525–1539. doi: 10.14670/HH-23.1525

Gong, Y., Bandara, T., Huntley, M., Johnson, Z. I., Dias, J., Dahle, D., et al. (2019). Microalgae Scenedesmus Sp. As a Potential Ingredient in Low Fishmeal Diets for Atlantic Salmon (Salmo Salar L.). *Aquaculture* 501, 455–464. doi: 10.1016/j.aquaculture.2018.11.049

Hasan, M. R., and Chakrabarti, R. (2009). *Use of Algae and Aquatic Macrophytes as Feed in Small-Scale Aquaculture. A Review. Fisheries and Aquaculture Technical Paper 531* (Rome: FAO), 123.

Hasanein, S. S., Saleh, N. E., El-Sayed, H. S., and Helal, A. M. (2018). The Effect of Dietary Supplementation of Spirulina Platensis and Chlorella Vulgaris Algae on the Growth and Disease Resistance of the Sea Bass (Dicentrarchus Labrax). *Egypt. J. Aquat. Biol. Fish.* 22 (4), 249–262. doi: 10.21608/ejabf.2018.17160

He, Y., Lin, G., Rao, X., Chen, L., Jian, H., Wang, M., et al. (2018). Microalga Isochrysis Galbana in Feed for Trachinotus Ovatus: Effect on Growth Performance and Fatty Acid Composition of Fish Fillet and Liver. *Aquac. Int.* 26 (5), 1261–1280. doi: 10.1007/S10499-018-0282-Y

Hernández-Sámano, A. C., Guzmán-García, X., García-Barrientos, R., and Guerrero-Legarreta, I. (2017). Actividad Enzimática De Proteasas De Cyprinus Carpio (Cypriniformes: Cyprinidae) Extraídas De Una Laguna Contaminada En México. *Rev. Biol. Trop.* 65 (2), 589–597. doi: 10.15517/RBT.V65I2.24486

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., and Dayal, J. S. (2019). Fishmeal Availability in the Scenarios of Climate Change: Inevitability of Fishmeal Replacement in Aquafeeds and Approaches for the Utilization of Plant Protein Sources. *Aquac. Res.* 50 (12), 3493–3506. doi: 10.1111/ARE.14324

Keppler, D., and Decker, K. (1974). "Glycogen Determination With Amyloglucosidase," in *Methods of Enzymatic Analysis*. Ed. H. U. Bergmeyer (New York: Academic Press), 1127–1131.

Laemmli, U. K. (1970). Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* 227 (5259), 680–685. doi: 10.1038/227680A0

Laliotis, G. P., Bizelis, I., and Rogdakis, E. (2010). Comparative Approach of the *De Novo* Fatty Acid Synthesis (Lipogenesis) Between Ruminant and Non Ruminant Mammalian Species: From Biochemical Level to the Main Regulatory Lipogenic Genes. *Curr. Genomics* 11 (3), 168–183. doi: 10.2174/138920210791110960

Lavecchia, R., Maffei, G., and Zuorro, A. (2016). Evaluation of Dissolved Organic Carbon (DOC) as a Measure of Cell Wall Degradation During Enzymatic Treatment of Microalgae. *Chem. Eng. Trans.* 49, 373–378. doi: 10.3303/CET1649063

Lee, D.-Y., and Kim, E.-H. (2019). Therapeutic Effects of Amino Acids in Liver Diseases: Current Studies and Future Perspectives. *J. Cancer Prev.* 24 (2), 72. doi: 10.15430/JCP.2019.24.2.72

Liu, J., and Hu, Q. (2013). *Chlorella: Industrial Production of Cell Mass and Chemicals. Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, *2nd ed.* UK: John Wiley & Sons, Ltd. 327–338. doi: 10.1002/9781118567166.CH16

Lupatsch, I., and Blake, C. (2013). Algae Alternative: Chlorella Studied as Protein Source in Tilapia Feeds. *Global Aquacul. Advocate* 16, 78–79.

Minjarez-Osorio, C., Castillo-Alvarado, S., Gatlin, D. M., González-Félix, M. L., Perez-Velazquez, M., and Rossi, W. (2016). Plant Protein Sources in the Diets of the Sciaenids Red Drum (Sciaenops Ocellatus) and Shortfin Corvina (Cynoscion Parvipinnis): A Comparative Study. *Aquaculture* 453, 122–129. doi: 10.1016/J.AQUACULTURE.2015.11.042

Moore, S. (1968). Amino Acid Analysis: Aqueous Dimethyl Sulfoxide as Solvent for the Ninhydrin Reaction. *J. Biol. Chem.* 243 (23), 6281–6283. doi: 10.1016/S0021-9258(18)94488-1

Oliva-Teles, A., Couto, A., Enes, P., and Peres, H. (2020). Dietary Protein Requirements of Fish – A Meta-Analysis. *Rev. Aquac.* 12 (3), 1445–1477. doi: 10.1111/RAQ.12391

Omlin, T., Langevin, K., and Weber, J. M. (2014). Exogenous Lactate Supply Affects Lactate Kinetics of Rainbow Trout, Not Swimming Performance. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 307 (8), R1018–R1024. doi: 10.1152/AJPREGU.00200.2014

Pakravan, S., Akbarzadeh, A., Sajjadi, M. M., Hajimoradloo, A., and Noori, F. (2017). Partial and Total Replacement of Fish Meal by Marine Microalga Spirulina Platensis in the Diet of Pacific White Shrimp Litopenaeus Vannamei: Growth, Digestive Enzyme Activities, Fatty Acid Composition and Responses to Ammonia and Hypoxia Stress. *Aquac. Res.* 48 (11), 5576–5586. doi: 10.1111/are.13379

Perera, E., Sánchez-Ruiz, D., Sáez, M. I., Galafat, A., Barany, A., Fernández-Castro, M., et al. (2020). Low Dietary Inclusion of Nutraceuticals From Microalgae Improves Feed Efficiency and Modifies Intermediary Metabolisms in Gilthead Sea Bream (Sparus Aurata). *Sci. Rep.* 10 (1), 1–14. doi: 10.1038/s41598-020-75693-3

Pfleiderer, G. (1970). "Particle-Bound Aminopeptidase From Pig Kidney," in *Methods in Enzymology, 19th edn.* Eds. G. E. Penman and L. Lowland (London: Academic Press), 514–521. doi: 10.1016/0076-6879(70)19038-0

Pratoomyot, J., Srivilas, P., and Noiraksar, T. (2005). Fatty Acids Composition of 10 Microalgal Species. *Songklanakarin J. Sci. Technol.* 27, 1179–1187.

Pujante, I. M., Díaz-López, M., Mancera, J. M., and Moyano, F. J. (2017). Characterization of Digestive Enzymes Protease and Alpha-Amylase Activities in the Thick-Lipped Grey Mullet (Chelon Labrosus, Risso 1827). *Aquac. Res.* 48 (2), 367–376. doi: 10.1111/ARE.13038

Pujante, I. M., Martos-Sitcha, J. A., Moyano, F. J., Ruiz-Jarabo, I., Martínez-Rodríguez, G., and Mancera, J. M. (2015). Starving/re-Feeding Processes Induce Metabolic Modifications in Thick-Lipped Grey Mullet (Chelon Labrosus, Risso 1827). *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* 180, 57–67. doi: 10.1016/J.CBPB.2014.10.005

Pujante, I. M., Moyano, F. J., Martos-Sitcha, J. A., Mancera, J. M., and Martínez-Rodríguez, G. (2018). Effect of Different Salinities on Gene Expression and Activity of Digestive Enzymes in the Thick-Lipped Grey Mullet (Chelon Labrosus). *Fish Physiol. Biochem.* 44, 349–373. doi: 10.1007/s10695-017-0440-6

Rahimnejad, S., Park, H.-G., Lee, S.-M., and Choi, J. (2017). Effects of Dietary Inclusion of Chlorella Vulgaris on Growth, Blood Biochemical Parameters and Antioxidant Enzymes Activity in Olive Flounder, Paralichthys Olivaceus. *Fish Shellfish Immunol.* 53, 103–112. doi: 10.1016/j.fsi.2016.04.067

Rodríguez-Ruiz, J., Belarbi, E. H., Sanchez, J. L. G., and Alonso, D. L. (1998). Rapid Simultaneous Lipid Extraction and Transesterification for Fatty Acid Analyses. *Biotechnol. Tech.* 12 (9), 689–691. doi: 10.1023/A:1008812904017

Román, G. C., Jackson, R. E., Gadhia, R., Román, A. N., and Reis, J. (2019). Mediterranean Diet: The Role of Long-Chain ω-3 Fatty Acids in Fish; Polyphenols in Fruits, Vegetables, Cereals, Coffee, Tea, Cacao and Wine; Probiotics and Vitamins in Prevention of Stroke, Age-Related Cognitive Decline, and Alzheimer Disease. *Rev. Neurol.* 175, 724–741. doi: 10.1016/j.neurol.2019.08.005

Roohani, A. M., Abedian Kenari, A., Fallahi Kapoorchali, M., Borani, M. S., Zoriezahra, S. J., Smiley, A. H., et al. (2019). Effect of Spirulina Spirulina Platensis as a Complementary Ingredient to Reduce Dietary Fish Meal on the Growth Performance, Whole-Body Composition, Fatty Acid and Amino Acid Profiles, and Pigmentation of Caspian Brown Trout (Salmo Trutta Caspius) Juveniles. *Aquac. Nutr.* 25 (3), 633–645. doi: 10.1111/ANU.12885

Sørensen, M., Berge, G. M., Reitan, K. I., and Ruyter, B. (2016). Microalga Phaeodactylum Tricornutum in Feed for Atlantic Salmon (Salmo Salar) — Effect on Nutrient Digestibility, Growth and Utilization of Feed. *Aquaculture* 460, 116–123. doi: 10.1016/J.AQUACULTURE.2016.04.010

Sales, R., Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Cerón-García, M. C., et al. (2021). Effects of Dietary Use of Two Lipid Extracts From the Microalga Nannochloropsis Gaditana (Lubián 1982) Alone and in Combination on Growth and Muscle Composition in Juvenile Gilthead Seabream, Sparus Aurata. *Algal Res.* 53, 102162. doi: 10.1016/J.ALGAL.2020.102162

Sarker, P. K., Kapuscinski, A. R., Bae, A. Y., Donaldson, E., Sitek, A. J., Fitzgerald, D. S., et al. (2018). Towards Sustainable Aquafeeds: Evaluating Substitution of Fishmeal With Lipid-Extracted Microalgal Co-Product (Nannochloropsis Oculata) in Diets of Juvenile Nile Tilapia (Oreochromis Niloticus). *PloS One* 13 (7), e0201315. doi: 10.1371/journal.pone.0201315

Sarker, P. K., Kapuscinski, A. R., Lanois, A. J., Livesey, E. D., Bernhard, K. P., and Coley, M. L. (2016). Towards Sustainable Aquafeeds: Complete Substitution of Fish Oil With Marine Microalga Schizochytrium Sp. Improves Growth and Fatty Acid Deposition in Juvenile Nile Tilapia (Oreochromis Niloticus). *PloS One* 11 (6), e0156684. doi: 10.1371/JOURNAL.PONE.0156684

Sarker, P. K., Kapuscinski, A. R., McKuin, B., Fitzgerald, D. S., Nash, H. M., and Greenwood, C. (2020). Microalgae-Blend Tilapia Feed Eliminates Fishmeal and Fish Oil, Improves Growth, and is Cost Viable. *Sci. Rep.* 10 (1), 1–14. doi: 10.1038/s41598-020-75289-x

Senso, L., Suarez, M. D., Ruiz-Cara, T., and García-Gallego, M. (2007). On the Possible Effects of Harvesting Season and Chilled Storage on the Fatty Acid Profile of the Fillet of Farmed Gilthead Sea Bream (Sparus Aurata). *Food Chem.* 101 (1), 298–307. doi: 10.1016/j.foodchem.2006.01.036

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaeimehr, A., et al. (2018). Microalgae in Aquafeeds for a Sustainable Aquaculture Industry. *J. Appl. Phycol.* 30 (1), 197–213. doi: 10.1007/s10811-017-1234-z

Silveira Júnior, A. M., Faustino, S. M. M., and Cunha, A. C. (2019). Bioprospection of Biocompounds and Dietary Supplements of Microalgae With Immunostimulating Activity: A Comprehensive Review. *PeerJ* 2019 (10), e7685. doi: 10.7717/PEERJ.7685/TABLE-4

Sorokin, C., and Krauss, R. W. (1958). The Effects of Light Intensity on the Growth Rates of Green Algae. *Plant Physiol.* 33 (2), 109–113. doi: 10.1104/PP.33.2.109

Teuling, E., Schrama, J. W., Gruppen, H., and Wierenga, P. A. (2017). Effect of Cell Wall Characteristics on Algae Nutrient Digestibility in Nile Tilapia (Oreochromis Niloticus) and African Catfish (Clarus Gariepinus). *Aquaculture* 479, 490–500. doi: 10.1016/J.AQUACULTURE.2017.06.025

Tibaldi, E., Chini Zittelli, G., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., et al. (2015). Growth Performance and Quality Traits of European Sea Bass (D. Labrax) Fed Diets Including Increasing Levels of Freeze-Dried Isochrysis Sp. (T-ISO) Biomass as a Source of Protein and N-3 Long Chain PUFA in Partial Substitution of Fish Derivatives. *Aquaculture* 440, 60–68. doi: 10.1016/j.aquaculture.2015.02.002

Tibbetts, S. M. (2018). The Potential for 'Next-Generation', Microalgae-Based Feed Ingredients for Salmonid Aquaculture in Context of the Blue Revolution. *Microalgal Biotechnol.* 151–175. doi: 10.5772/INTECHOPEN.73551

Tibbetts, S. M., Mann, J., and Dumas, A. (2017). Apparent Digestibility of Nutrients, Energy, Essential Amino Acids and Fatty Acids of Juvenile Atlantic Salmon (Salmo Salar L.) Diets Containing Whole-Cell or Cell-Ruptured Chlorella Vulgaris Meals at Five Dietary Inclusion Levels. *Aquaculture* 481, 25–39. doi: 10.1016/j.aquaculture.2017.08.018

Turner, N., Bruce, C. R., Beale, S. M., Hoehn, K. L., So, T., Rolph, M. S., et al. (2007). Excess Lipid Availability Increases Mitochondrial Fatty Acid Oxidative Capacity in Muscle Evidence Against a Role for Reduced Fatty Acid Oxidation in Lipid-Induced Insulin Resistance in Rodents. *Diabetes* 56 (8), 2085–2092. doi: 10.2337/DB07-0093

Uran, P. A., Schrama, J. W., Rombout, J. H. W. M., Obach, A., Jensen, L., Koppe, W., et al. (2008). Soybean Meal-Induced Enteritis in Atlantic Salmon (Salmo Salar L.) at Different Temperatures. *Aquac. Nutr.* 14 (4), 324–330. doi: 10.1111/J.1365-2095.2007.00534.X

Uscanga-Martínez, A., Perales-García, N., Álvarez-González, C. A., Moyano, F. J., Tovar-Ramírez, D., Gisbert, G. E., et al. (2011). Changes in Digestive Enzyme Activity During Initial Ontogeny of Bay Snook Petenia Splendida. *Fish Physiol. Biochem.* 37 (3), 667–680. doi: 10.1007/S10695-011-9467-2

Vizcaíno, A. J., López, G., Sáez, M. I., Jiménez, J. A., Barros, A., Hidalgo, L., et al. (2014). Effects of the Microalga Scenedesmus Almeriensis as Fishmeal Alternative in Diets for Gilthead Sea Bream, Sparus Aurata, Juveniles. *Aquaculture* 431, 34–43. doi: 10.1016/j.aquaculture.2014.05.010

Vizcaíno, A. J., Rodiles, A., López, G., Sáez, M. I., Herrera, M., Hachero, I., et al. (2018). Growth Performance, Body Composition and Digestive Functionality of Senegalese Sole (Solea Senegalensis Kaup 1858) Juveniles Fed Diets Including Microalgae Freeze-Dried Biomass. *Fish Physiol. Biochem.* 44, 661–677. doi: 10.1007/s10695-018-0462-8

Wassef, E. A., El Masry, M. H., and Mikhail, F. R. (2001). Growth Enhancement and Muscle Structure of Striped Mullet, Mugil Cephalus, L., Fingerlings by Feeding Algal Mealbased Diets. *Aquac. Res.* 32, 315–322. doi: 10.1046/j.1355-557x.2001.00043.x

Wilson, J. M., and Castro, L. F. C. (2010). Morphological Diversity of the Gastrointestinal Tract in Fishes. *Fish Physiol.* 30 (C), 1–55. doi: 10.1016/S1546-5098(10)03001-3

Xu, W., Gao, Z., Qi, Z., Qiu, M., Peng, J., and Shao, R. (2014). Effect of Dietary Chlorella on the Growth Performance and Physiological Parameters of Gibel Carp, Carassius Auratus Gibelio. *Turkish J. Fish. Aquat. Sci.* 14, 53–57. doi: 10.4194/1303-2712-v14_1_07

Yeganeh, S., Teimouri, M., and Amirkolaie, A. K. (2015). Dietary Effects of Spirulina Platensis on Hematological and Serum Biochemical Parameters of Rainbow Trout (Oncorhynchus Mykiss). *Res. Vet. Sci.* 101, 84–88. doi: 10.1016/J.RVSC.2015.06.002

Zouiten, D., Khemis, I.B., Besbes, R., and Cahu, C. (2008). Ontogeny of the Digestive Tract of Thick Lipped Grey Mullet (Chelon Labrosus) Larvae Reared in "Mesocosms". *Aquaculture* 279 (1–4), 166–172. doi: 10.1016/J.AQUACULTURE.2008.03.039

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Atlantic Salmon (*Salmo salar*) Performance Fed Low Trophic Ingredients in a Fish Meal and Fish Oil Free Diet

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To evolve fish farming in an eco-efficient way, feed production must become less dependent on forage fish-based ingredients and make more use of low trophic level organisms, including microalgae, higher plants, as filter feeding organisms and other ingredients with low competition to established food value chains. Diets nearly free of fish meal and fish oil are not a novelty but are often composed of complex mixtures, containing supplements to meet the farmed animal's nutritional requirements. Sustaining a growing aquaculture production, maintaining at the same time fish health, welfare, and profitability, and meeting strict environmental and food safety demands, is challenging and requires new technologies, great investments, and more knowledge. A benchmarking feeding trial was performed to demonstrate the main effects of four low trophic raw materials on Atlantic salmon (*Salmo salar*) growth, metabolism, skin health and fillet quality. To this end, a diet was produced to contain commercially relevant levels of fresh high quality organic FM and FO and was used as a control in the trial (FMFO). Heterotrophically produced *Schizochytrium limacinum* biomass was used to replace organic FO (HM diet). Spray dried cell wall disrupted biomass of the phototrophically cultured diatom *Phaeodactylum tricornutum* replaced partly FM and FO (PM diet). Black soldier fly (*Hermetia illucens*) larvae meal and tunicate (*Ciona intestinalis*) meal, were used to produce the diets BSFL and TM, respectively, replacing large parts of FM as compared to the FMFO. A fifth test diet was produced combining all test raw materials and removing all FM and FO (0FM0FO diet). All test ingredients were well accepted sustaining high growth rates (TGC values near 4) and feed efficiency (FCR values below 0.9) in salmon showing good gut health and normal metabolic responses. However, none of the treatments reached the growth performance of FMFO. Additional differences between test and control treatments were identified in dietary nutrient apparent digestibility, fish biometrics, blood metabolites and fillet and skin composition. Extensive raw material and dietary chemical characterisation was performed to provide insight on potential shortcomings in the novel low trophic level ingredients which can possibly be overcome combining complementary raw materials.

Keywords: Atlantic salmon, black soldier fly larvae, microalgae, tunicates, *Schizochytrium sp.*, low trophic level feed ingredients

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INTRODUCTION

A plethora of different raw materials are currently being explored as candidates to replace finite marine resources (Heal and Schlenker 2009; Beal et al., 2018) used to produce fish meal (FM) and fish oil (FO) for aquaculture diets. Contemporary Atlantic salmon farming trends focus on using low trophic level organisms as feed ingredients, preferably produced locally, with potential to achieve high production volumes, with low environmental effects, low carbon footprint and based on circularity principles (e.g., PhI et al., 2020). Moreover, the increasing consumer awareness on food safety and sustainability, has introduced a steady demand for organic products in the market (Polymeros et al., 2014; Lernoud and Willer, 2018), adding to the complexity of successful and sustainable fish farming growth strategies. Several of the so far novel feed raw materials considered have different nutritional and technical performance from FM, FO, and high protein higher plant meals. This is an additional practical challenge that the sector is facing in replacing established value chains with new local ones, at adequate amounts and competitive costs. New low trophic raw materials often contain high levels of complex carbohydrates, such as those present in rigid bacterial, yeast and algal cell wall structures (e.g., Harrison et al., 1991; Safi et al., 2013; Wu et al., 2015). Ingredients produced from arthropods contain significant exoskeleton chitin amounts shown to limit performance in fish species like rainbow trout (Lindsay et al., 1984) whereas being well digested by others, like cod (Toppe et al., 2006) possessing lower gastric pH (Danulat, 1987), and high chitinolytic enzyme activity both in the stomach, and in the intestinal contents (Danulat and Kausch, 1984). *Schizochytrium limacinum* (*S. limacinum*) is a DHA rich fungal protist, often referred to as microalgae or microalgae - like organism, known to store fully saturated triglycerides, such as tripalmitin, whereas only one or two but not 3 saturated fatty acids are present in FO triglycerides (Bogevik et al., 2018). Fully saturated fatty acids are less digestible in fish farmed in low water temperatures, such as Atlantic salmon (Kousoulaki et al., 2016). New ingredients may also have very different rheological properties affecting the technical quality of extruded feeds (Samuelsen et al., 2018; 2022). Thus, considering new ingredients for aquatic diets, their nutritional value, best use practices and safety must be thoroughly studied before establishing their commercial production and use.

Microalgae, being in the bottom of the trophic pyramid, and primary producers of essential nutrients (Ahlgren et al., 1992; Helliwell et al., 2016; Duncan and Petrou, 2022) are popular candidates as new feed ingredients and supplements (Beal et al., 2018; Tocher et al., 2019). Phototrophic microalgae and microalgae - like heterotrophic biomass can be produced practically anywhere in closed photobioreactors (Chen et al., 2011) or dark fermentation containers (Turon et al., 2016), respectively. They require minimal amounts of fresh water and can be produced in conjunction with other industrial food production activities, using sidestream biomasses as cultivation inputs, as for instance CO_2, waste heat and organic by products (Ren et al., 2018). Not surprisingly, entrepreneurship on the development of microalgal based industries is in focus with heterotrophic production showing the lowest environmental impacts, mainly due to high production densities and favourable oil content and quality (Barba et al., 2014; Lu et al., 2021). The chemical composition of the phototrophic microalgal strains varies considerably in terms of total protein, carbohydrate, and lipids, but their amino acid profile is similar to FM (Tibbetts, 2018). In the present study we used cell wall disrupted diatom biomass of the species *Phaeodactylum tricornutum* (*P. tricornutum*) as in whole biomass the presence of intact rigid cell walls may compromise the bioavailability of micro algal nutrients in carnivorous fish (Sørensen et al., 2016). Heterotrophically produced *Schizochytrium* sp. biomass (**Tables 1–3**) contains higher levels of lipids and yields good performance results in Atlantic salmon (*Salmo salar*) at low and moderately high dietary levels (Kousoulaki et al., 2015), providing benefits also in low FM diets (Kousoulaki et al., 2016). Moreover, in a life - long feeding trial in Atlantic salmon, replacement of FO by heterotrophically produced *S. limacinum* biomass yielded encouraging results including better growth, higher flesh eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels and improved pigmentation (Kousoulaki et al., 2020b).

Tunicates belong to a group of filter-feeding sea invertebrates (ascidians, also known as sea squirts) growing in shallow ocean waters worldwide. They trap prey particles by pumping water through the oral siphon and thus functions as a biological water filtration system that can remove microalgae from eutrophic plankton - rich waters. Though tunicates have historically not been viewed as a valuable fish feed source, in the recent years, the interest in this organism has grown. In the present work we tested a tunicate meal produced by the tunicate species *Ciona intestinalis*, produced by Marine Feed AB. The tunics (45% of total animal dry weight) of *Ciona intestinalis*, composed mainly of valuable animal cellulose (Angles and Dufresne, 2000; Mathew and Dufresne, 2002), protein, and ash, act as a skeletal structure, whereas the inner animal (55% of total animal weight) consists mainly of protein and ω - 3 fatty acids and may be used as feed ingredients (Kousoulaki et al., 2020a).

Insects are part of salmon parr's natural pray (Henry et al., 2015). In a study in the Louvenga River, Kola Peninsula, Russia, aerial insects represented 24% of the food items found in wild Atlantic salmon parr together with aquatic insect larvae and pupae that represented 68.2% of their diet (Orlov et al., 2006). In the same study, the feeding behaviour of farmed Atlantic salmon parr that were released in the same river differed from that of the wild fish with aquatic insect larvae and pupae representing lower (49%) and terrestrial insects, higher (32%) proportion of their diet. Protein rich insect meals can be produced converting low value feedstuffs, including co - products of the agricultural industry, into high value protein and oils in a circular economy manner. Replacing FM and FO in aquafeeds by insect - based ingredients can reduce the economic fish - in - fish - out value and land use of the aquaculture and provide in the future a sustainable solution for further growth of the sector (Quang Tran et al., 2022). Insect meals can vary largely in nutrient composition, e.g., ranging between 9.3 and 76% in protein and 7.9–40% in fat content (as reviewed by Nogales-

Mérida et al., 2019). Recent studies on the use of insect meals in diets for aquaculture fish species including Atlantic salmon, have shown encouraging results replacing up to 100% of dietary FM with some types of insect meals and revealed shortcomings of other (e.g., Lock et al., 2016). Atlantic salmon performed equally when fed a FM - based control diet and diets where FM was substituted by black soldier fly meal at lower dietary inclusion levels (8–16% in the diet) with a trend to deteriorating performance at increasing black soldier fly larvae dietary inclusion level (32%) (Weththasinghe et al., 2019). Belghit et al. (2018) saw lower protein digestibility followed by non - statistically significant growth reduction and elevated hepatosomatic index in Atlantic salmon fed 60% of black soldier fly larvae meal in the diet.

In the present study we benchmarked four different low trophic level organism - based ingredients, namely, tunicate meal, insect meal, phototrophic microalgae biomass and heterotrophically produced *S. limacinum* biomass against a high - quality organic FM and FO diet. Our aim was to identify opportunities and limitations in using these raw materials in salmon diets alone or combined in a FM and FO free diet, in terms of nutrient availability, and effects on general fish performance, metabolism, skin health and nutritional quality of flesh.

MATERIALS AND METHODS

Test Ingredient Sourcing

Freshly produced organic FM, made from herring by-product (51%), Norway Pout (25%), sprat (18%) and blue whiting (6%), and organic FO produced from Norwegian spring spawning herring by product, Norwegian spring spawning herring, herring by-product and herring, were provided by Pelagia factories in Egersund (Norway) and Måløy (Norway), respectively. Organic FM and FO must be stabilised with different tocopherol extracts (E 306) and its origin must be either from offal from organic aquaculture or from cuts from fish already caught for human consumption in sustainable fisheries, the latter was the case in this study. The *P. tricornutum* (ND58; Prestegard et al., 2009) biomass used in this trial was provided by the NORCE, Bergen, Norway, and produced at the National Algae pilot Mongstad (NAM; Mongstad, Norway) in a fed-batch process using four 800 L photobioreactors (GemTube MK2-750 from LGem, Rotterdam, Netherlands). The *P. tricornutum* culture was harvested and concentrated by centrifugation (Evodos 50, Evodos b. v) and the concentrated paste (dry matter (DM) content 22–35%) was delivered vacuum packed and frozen to Nofima in Bergen, Norway, and kept at −23°C until further use. The biomass was thawed by heating to 60°C, disrupted to an approximate 92% cell wall disruption degree (estimated microscopically), and spray dried to a fine powder. Cell wall disruption was performed using a Dyno-Mill Multi Lab (Willy A. Bachofen, Muttenz, Switzerland) at 80% chamber filling rate in a 1.4 L milling chamber, using glass beads of 0.75 mm diameter. The mill was operated at 12 m s^{-1} tip speed (2,865 rpm). The biomass was diluted with tap water when necessary to approx. 22% dry matter and processed at an approximate flow rate of 7–9 kgh^{-1} and processing temperature of ca. 25–27°C. Spray dried *S. limacinum* biomass was provided by Alltech Inc. (Dunboyne, Ireland) and was the same batch as the one used in Kousoulaki et al. (2020b). Tunicate meal (*Ciona intestinalis*) was provided by Marine Feed AB (Stenungsund, Sweden) and black soldier fly larvae meal (*Hermetia illucens*) was supplied from INNOVAFEED (Gouzeaucourt, France). Krill hydrolysate was added as attractant in the test diets with lower FM or/and FO as compared to the high FM and FO diet used as control, with the aim to reduce the risk of obtaining physiological differences between the treatments due to low feed intake rates (Kousoulaki et al., 2013). The test raw materials were extensively characterised for their content in protein, lipids, water, ash, trace minerals (Cu, Fe, Mn, Zn and Se), total and soluble phosphorus (P), vitamins, total and free amino acid and fatty acid profile, lipid class composition, soluble protein level and soluble protein peptide size distribution, nucleotides, lipid oxidation and presence of undesirable compounds.

Experimental Diets

A control diet was produced containing relatively high organic FM (25% in the diet) and FO (10% in the diet) levels (FMFO) (**Table 1**). Two more test diets were produced containing high inclusion levels of either black soldier fly larvae or tunicate heat and air-dried meals (BSFL and TM, respectively), partly substituting FM as compared to the FMFO diet. Two more test diets were produced containing heterotrophically produced *S. limacinum* spray dried biomass substituting FO (HM), or cell wall disrupted and spray dried *P. tricornutum* biomass partly substituting FM and FO (PM), as compared to the FMFO diet. A fifth test diet was produced combining all test ingredients at equal levels present in the respective single ingredient replacement test diets, substituting all dietary FM and FO (0FM0FO). The diets were balanced for protein, non-dispensable amino acids, EPA ± DHA and n-3/n-6 ratio, phospholipids, and soluble P, using wheat and horse beans, crystalline amino acids, plant oils, lecithin, and monosodium phosphate, respectively. The reasoning behind our test feed formulation choices was to use high levels of the test ingredients to be able to substitute FM and FO in the FO0FM0 diet with low risk of inducing negative health and production performance effects, and at the same time reveal potential positive or negative effects from the special micro and macro-elements that were not specially balanced for, and their in between interactions.

The experimental diets were produced at the Feed Technology Centre of Nofima in Bergen, Norway, in the same production series, using a Wenger TX-52 co-rotating twin-screw extruder with 150 kgh^{-1} capacity. The settings of the extruder were close to commercial i.e., the production can be scaled up to a feed factory, slightly adjusted during production from diet to diet. The screw configuration of the extruder was D, die opening 2.5 mm, knife speed varied between 1,000 and 1,450 rpm to yield pellets of similar length, SME was 6.2–6.9 kW, feed rate approx. 110–120 kg h^{-1}, amount of steam added in the DDC conditioner was 11.7–12.3 kg h^{-1} and 0 h^{-1} in the extruder, water added in the DDC conditioner was 0.120–0.140 kg min^{-1} and in the extruder 0.080–0.160 kg min^{-1}. The set temperatures in the extruder heads (H) 2-7 were H2: 83°C

TABLE 1 | Formulation of the experimental diets used in the current trial. Raw material levels are given in g 100^{-1} of total raw material mix.

	FMFO	TM	BSFL	PM	HM	0FM0FO
Fish meal	25.00	13.60	8.01	20.30	24.50	
Insect meal			20			20
Tunicate meal		20				20
Schizochytrium limacinum					7.7	7.7
Phaeodactylum tricornutum				7.5		7.5
Wheat gluten	16.92	18.49	16.51	15.95	15.16	14.77
Horse beans	16.00	14.00	16.00	16.00	16.00	2.00
Wheat meal	16.10	5.00	11.40	13.00	13.30	5.00
Krill hydrolysate (60% dry matter)		2.00	2.00	2.00	2.00	2.00
Fish oil	10.10	9.20	11.80	9.10		
Rapeseed oil	5.75	5.95	4.75	5.43	9.60	9.50
Linseed oil	0.75	1.50	0.60	1.00	2.70	2.50
Rapeseed lecithin	1.00	1.70	0.85	1.20	0.80	1.30
Vitamin premix[a]	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix	0.50	0.50	0.50	0.50	0.50	0.50
NaH2PO4	2.20	2.40	2.00	2.30	2.20	1.75
Biomoss	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Cholesterol	0.50	0.50	0.50	0.50	0.50	0.50
Lysine	0.67	0.70	0.68	0.70	0.62	0.73
Methionine	0.24	0.29	0.35	0.25	0.23	0.34
Threonine	0.39	0.29	0.38	0.36	0.33	0.20
Histidine	0.77	0.77	0.56	0.80	0.75	0.60
Carop. Pink (35%)	0.05	0.05	0.05	0.05	0.05	0.05
Stay-C	0.05	0.05	0.05	0.05	0.05	0.05
Yttrium oxide	0.01	0.01	0.01	0.01	0.01	0.01
Sum	100	100	100	100	100	100
Analysed composition in DM						
Protein%	44.6	43.9	43.8	43.8	44.6	43.7
Lipid%	23.6	23.9	24.3	24.6	24.2	25.1
Ash%	6.5	11.1	5.6	7.4	6.9	11.9
Total P%	1.29	1.09	1.09	1.31	1.31	0.97
Soluble P%	0.84	0.83	0.87	0.87	0.81	0.83
Starch%	20	12.6	16.7	18.2	17	7.4
Combustion value kJ g^{-1}	23.84	22.79	24.31	23.64	23.69	22.78
Yttrium mg kg^{-1}	82.99	88.71	78.94	86.93	83.15	85.44
Pellet technical quality[b]						
Water stability index% (WSI)	79.48 ^{bc} ±0.03	74.99^{b} ± 3	83.06^{c} ±0.24	79.72 ^{bc} ±1.02	81.01 ^{bc} ±0.1	17.58^{a} ±3.79

[a]*Provides vitamins to a final feed concentration of 2000 IU, vitamin A, 2500 IU, vitamin D3, 200 mg/kg vitamin E, 20 mg/kg vitamin K3, 20 mg/kg vitamin B1, 30 mg/kg vitamin B2, 30 mg/kg vitamin B6 pyridoxin-HCl, 25 mg/kg vitamin B6 pyridoxin, 0.05 mg/kg vitamin B12, 10 mg/kg folic acid, 60 mg/kg Ca-D-pantothenic acid, 200 mg/kg niacin, and 1 mg/kg vitamin H (biotin).*

[b]*Water stability index values with different small superscript letter are significantly different (p = 0.000) following Tukey post hoc test. Each value is a mean of 3 replicate analyses.*

(achieved 75–90°C), H3: 110–120°C (achieved 100–112°C), H4: 120–130°C (achieved 88–109°C), H5: 115–140°C (not measured), H7: 70–80°C (achieved 71–80°C). The diets were analysed for water stability index (WSI) and their content in crude protein, crude lipid, gross energy, crude starch, phosphorus (P), yttrium, total amino acid, and fatty acid profile, vitamins and soluble peptide molecular size distribution (**Tables 1–5**).

Atlantic Salmon Smolt Feeding Trial

The experimental diets were fed to triplicate Atlantic salmon smolt groups of 50 fish per tank (500 L) in indoor tanks at Nofima facilities in Sunndalsøra, Norway. The experimental fish used were Atlantic salmon smolt of organic production provided by SalMar ASA (Frøya, Norway), with initial mean fish body weight 141.7 ± 5.6 g. The experimental tanks were equipped with continuous light and flow through sea water systems using UV-treated filtrated water from 40 m depth with 32ppt salinity. At trial start 100 random fish were weighed and measured to determine the ranges of fish sizes that would be accepted (70% of the initial population) or rejected (lowest and highest 15%) during distribution in the experimental tanks. Each experimental diet was randomly assigned to 3 tanks and fish were fed 15–20% above saturation using automatic belt feeders, distributed in two meals per day for a period of 12 weeks. Uneaten feed was collected and weighed daily for the estimation of total daily feed intake of the experimental fish

TABLE 2 | Fatty acid profile and lipid oxidation status of the experimental diets used in the current trial.

		FMFO	TM	BSFM	PM	HM	0FM0FO
14:0	g/100 g B&D extract	3.70	3.30	4.40	3.60	1.30	1.60
16:0	g/100 g B&D extract	8.50	8.10	9.00	8.60	13.80	14.50
18:0	g/100 g B&D extract	1.30	1.30	1.20	1.30	1.80	1.70
20:0	g/100 g B&D extract	0.30	0.30	0.20	0.30	0.30	0.30
22:0	g/100 g B&D extract	0.10	0.10	0.10	0.10	0.20	0.20
Saturated fatty acids	**g/100 g B&D extract**	**13.90**	**13.10**	**14.90**	**13.90**	**17.40**	**18.30**
16:1 n-7	g/100 g B&D extract	2.00	1.90	2.20	3.00	0.50	1.60
18:1 (n-9)±(n-7)±(n-5)	g/100 g B&D extract	24.60	26.10	22.70	24.80	32.90	29.60
20:1 (n-9)±(n-7)	g/100 g B&D extract	7.30	6.50	7.50	6.80	1.60	0.70
22:1 (n-11)±(n-9)±(n-7)	g/100 g B&D extract	10.90	9.40	11.40	10.00	1.40	0.20
24:1 n-9	g/100 g B&D extract	0.40	0.40	0.40	0.40	0.10	<0.1
Monounsaturated fatty acids	**g/100 g B&D extract**	**45.20**	**44.30**	**44.20**	**45.00**	**36.50**	**32.10**
16:2 n-4	g/100 g B&D extract	0.20	0.10	0.20	0.30	<0.1	0.20
16:3 n-4	g/100 g B&D extract	0.10	0.10	0.10	0.30	<0.1	0.20
18:2 n-6	g/100 g B&D extract	9.80	10.50	9.80	10.00	13.80	13.50
18:3 n-6	g/100 g B&D extract	0.10	0.10	0.10	0.10	<0.1	<0.1
20:2 n-6	g/100 g B&D extract	0.10	0.10	0.10	0.10	0.10	0.10
20:3 n-6	g/100 g B&D extract	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
20:4 n-6	g/100 g B&D extract	0.10	0.10	0.10	0.20	0.10	0.10
22:4 n-6	g/100 g B&D extract	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
PUFA (n-6) fatty acids	**g/100 g B&D extract**	**10.10**	**10.80**	**10.10**	**10.40**	**14.00**	**13.70**
18:3 n-3	g/100 g B&D extract	4.80	6.60	4.30	5.50	11.00	9.10
18:4 n-3	g/100 g B&D extract	1.30	1.10	1.40	1.20	0.20	0.10
20:3 n-3	g/100 g B&D extract	0.10	<0.1	<0.1	<0.1	<0.1	<0.1
20:4 n-3	g/100 g B&D extract	0.30	0.20	0.30	0.20	0.10	0.10
20:5 n-3 (EPA)	g/100 g B&D extract	3.00	2.50	3.00	3.40	0.70	0.90
21:5 n-3	g/100 g B&D extract	0.10	0.10	0.10	0.10	<0.1	<0.1
22:5 n-3	g/100 g B&D extract	0.50	0.50	0.50	0.50	0.30	0.20
22:6 n-3 (DHA)	g/100 g B&D extract	3.90	3.10	3.50	3.60	5.90	4.90
PUFA (n-3) fatty acids	**g/100 g B&D extract**	**14.00**	**14.10**	**13.10**	**14.50**	**18.20**	**15.30**
Omega-6/omega-3 ratio		0.73	0.77	0.78	0.71	0.76	0.90
EPA ± DHA	g/100 g B&D extract	6.90	5.60	6.50	7.00	6.60	5.80
Identified fatty acids	g/100 g B&D extract	83.50	82.50	82.60	84.40	86.10	79.80
Unidentified fatty acids	g/100 g B&D extract	2.60	2.40	5.50	2.70	2.30	5.60
Free fatty acids	% in B&D extract	6.40		6.10	10.10	5.70	8.80
Anisidine value		12.00		1.70	49.00	22.00	62.00
Peroxide value	meq peroxide/kg fat	15.00		12.00	22.00	11.00	21.00

TABLE 3 | Protein bound amino acid profile of the experimental diets used in the current trial. Tryptophane and cysteine analyses were not performed.

		FMFO	TM	BSFM	PM	HM	0FM0FO
Aspartic acid	%	2.50	2.50	2.40	2.50	2.60	2.70
Glutamic acid	%	9.20	9.00	8.40	8.70	8.60	7.80
Hydroxyproline	%	0.17	0.14	0.13	0.17	0.19	<0.10
Serine	%	1.70	1.70	1.50	1.60	1.70	1.70
Glycine	%	1.90	1.80	1.70	1.80	1.80	1.80
Histidine	%	1.30	1.30	0.84	1.30	1.30	1.20
Arginine	%	2.00	1.90	1.80	2.00	2.00	1.80
Threonine	%	1.60	1.50	1.40	1.60	1.60	1.50
Alanine	%	1.70	1.50	1.70	1.70	1.70	1.70
Proline	%	2.80	2.80	2.80	2.80	2.60	2.80
Tyrosine	%	1.10	1.20	1.40	0.98	0.99	1.50
Valine	%	1.70	1.70	1.70	1.60	1.60	1.80
Methionine	%	0.98	0.97	0.94	0.93	0.94	0.97
Isoleucine	%	1.50	1.50	1.40	1.50	1.50	1.50
Leucine	%	2.80	2.70	2.70	2.70	2.70	2.60
Phenylalanine	%	1.60	1.60	1.60	1.60	1.60	1.60
Lysine	%	2.50	2.30	2.20	2.40	2.50	2.20
Total amino acids	%	35.2	35.9	35.9	34.6	36.1	37.1
Total EAA	%	15.98	15.47	14.58	15.63	15.74	15.17

populations. Water oxygen levels were maintained above 80% saturation and water circulation speed was set at 0.8 L body length^{-1}. Mean temperature during the trial was 9.3°C.

At trial end fish in each tank were anaesthetised, individually weighed, and stripped for faeces. Faeces samples were set at −25°C and kept frozen before being freeze dried and analysed for inert marker content (yttrium), proximate composition (crude protein and fat) and gross energy content. Skin (1 × 1 cm right below dorsal fin), Norwegian quality cut (NQC) filet, and blood serum samples were collected from 5 fish per tank. Livers were individually weighed. Blood was withdrawn from the caudal artery using a heparinised vacutainer and centrifuged at 3,000 rpm for 10 min to separate the serum, which was collected and stored at −20°C until analysis. Levels of cortisol were analysed with ELISA using a commercially available kit (Demeditic Diagnostics GmbH, Kiel, Germany), while the rest of the serum analyses were performed by the Pentra Clinical Chemistry Analyzer (Pentra C400, HORIBA ABX SAS, Montpellier, France). Skin samples were analysed for essential trace minerals (Cu, Fe, Mn, Zn and Se), total amino acids and

TABLE 4 | Vitamin content of experimental diets used in the current trial. Empty cells represent missing values.

		FMFO	TM	BSFM	PM	HM	0FM0FO
Vitamin A	mg/kg	4.19	1.87	3.87	3.49	2.27	0.62
Vitamin D3	mg/kg	0.191	0.137	0.2	0.177	0.0788	0.0601
Vitamin K1	ug/kg	149	172	121	132	189	
Vitamin C (ascorbyl-2-phosphate)	mg/kg	233	206	252	262	230	237
Vitamin C (ascorbic acid)	mg/kg	<5	<5	<5	<5	<5	
alpha Tocopherol	mg/kg	256	224	279	253	263	242
beta Tocopherol	mg/kg	<5	<5	<5	<5	<5	5.14
gamma Tocopherol	mg/kg	41.8	7.11	48.8	35.8	60.9	18.7
delta Tocopherol	mg/kg	8.11	<5	8.37	15.3	7.66	12.4
Vitamin E (sum tocopherols)	mg/kg	306	231	337	304	332	278

TABLE 5 | Peptide size distribution of the water-soluble proteins in the current trial's experimental diets. Values are given in% of water soluble peptides unless otherwise specified.

	FMFO	TM	BSFM	PM	HM	0FM0FO
Soluble protein% in sample	9.5	9.2	8.85	10.2	10.7	9.5
>20,000	1.2	1.2	1.0	0.8	0.8	0.8
15,000–20,000	2.7	2.7	2.5	2.1	2.1	1.9
10,000–15,000	6.8	7.0	6.5	5.2	5.3	4.9
8,000–10,000	3.8	3.5	4.4	2.9	3.0	3.2
6,000–8,000	4.7	4.1	5.6	3.6	3.7	4.0
4,000–8,000	6.2	5.3	6.3	5.0	5.2	4.4
2000–4,000	8.0	6.9	7.6	6.9	7.5	5.4
1,000–2000	4.7	5.2	5.5	5.2	5.7	5.0
500–1,000	3.1	4.7	4.8	4.3	4.6	5.4
200–500	4.5	6.8	7.1	5.9	3.2	8.5
<200	54.3	52.6	48.5	58.0	55.9	56.5

evaluated histologically. NQC samples were analysed for protein, lipids, fatty acid profile and total amino acids.

The feeding experiment followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20 December 1974, amended 19th of June 2009. The trial facilities were granted permission by the Norwegian Food Safety Authority to run the experiments. The decision was made on the basis of Regulations 18th of June 2015 on the use of animals in experiments, §§ 6, 7, 9, 10 and 11.

Chemical and Pellet Structure Analyses

The experimental diets, and where relevant fish tissues and faeces, were analysed for protein (Kjeldahl method N x 6.25; ISO 5983–1997), moisture (ISO 6496–1999), ash (ISO 5984–2002) and lipid (Bligh and Dyer, 1959) followed by determination of the oxidation state of the oil by the analysis of peroxide number (AOCS Cd 8b-90) and anisidine number (AOCS cd 18–90). Dietary gross energy was determined in a Parr adiabatic bomb calorimeter. For total amino acid profile determination, samples were hydrolysed in 6 M HCl for 22 h at 110°C and analysed by HPLC using a fluorescence technique for detection (Cohen and Michaud, 1993). Free amino acids, taurine (Tau) and anserine were analysed as described in Bidlingmeyer et al. (1987). The water-soluble fraction of the marine protein meals and the diets was extracted with boiling water, the extract was then filtered using Whatman black ribbon filter paper, and the crude protein content in the water-phase was determined by the Kjeldahl method. Total starch was measured using a modified glucoamylase method described by Chiang and Johnson (1977) and Samuelsen and Oterhals (2016). Total phosphorous (P) was determined by a spectrophotometric method (ISO 6491–1998). Undesirable compounds such as aldrin, dioxins, PCBs, DDT, DDE, TDE, PAH 4 and heavy metals (Hg, Pb, Cd and As) and vitamins in raw materials as well as trace minerals and in raw materials and tissues were analysed by an external laboratory (Eurofins, Hamburg, Germany). The analyses performed externally (Cu, Se, Mn, Zn, Fe, vitamins, and undesirable compounds) were performed in single samples, whereas the remaining analyses were performed in duplicate samples. If differences between parallels exceeded standardised values, new duplicate analyses were carried out according to accredited procedures. More in detail (as provided by Eurofins): For the test trace mineral analyses the sample preparation was realised according to §64 LFGB L.00.00–19/1, CON-PV 00001 (2019–03) with microwave digestion. Copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn) were analysed using ICP-OES according to EN ISO 11885 (modified). Selenium (Se) was analysed using ICP-MS according to an analogue method to §64 LFGB L 00.00–19/3. Retinol (vitamin A) was analysed according to EN 12823–1 2014, alpha tocopherol (vitamin E) was analysed according to EN 12822:2014 and DJCPH L-ascorbyl-2-phosphate (stay-C form of vitamin C added in the diets) was analysed by LC-DAD.

Pellet water stability index (WSI) was used to determine pellet technical quality and was determined by a slightly modified method described by Baeverfjord et al. (2006). Triplicate samples of each diet (20 g each) were added in custom made steel-mesh container placed inside 1,000 ml glass beakers filled with 500 ml distilled water. The beakers were incubated in a thermostat-controlled water bath at 23°C and shaken (160°min^{-1}) for 120 min, and the remaining amount of dry matter (DM) was determined.

Sampling and Calculations

At the end of the trial all fish from each tank, except for 10, of which 5 fish were used for further biological studies and 5 more kept in store, were stripped and their faeces separated from urine and collected in 1 pre-weighed box per tank. Following sampling of each tank the collected faeces were frozen immediately at

−20°C prior to further freeze drying and analysis. Apparent digestibility coefficient (ADC) of nutrients and energy in the test diets was calculated from the following formula: ADC = 100 – 100 × [Yd/Yf] × [Nf/Nd] where d is diet, f is faeces, Y yttrium content and N nutrient content.

Fish growth rate, survival, feed intake rates and feed efficiency (TGC: thermal growth coefficient, FCR: feed conversion ratio) and fish biometrics (D%: dress out percentage, HSI: hepatosomatic index, CF: condition factor) were measured. Feed intake was expressed as the total feed consumed per tank, mean feed intake per fish, or mean daily feed consumption per fish expressed as% of its body weight. Feed conversion ratio is feed consumed/biomass increase. Thermal growth coefficient is TGC = ($w_{final}^{1/3}$- $w_{start}^{1/3}$) x 1,000/degree-days where w is mean fish body weight (Cho, 1992). Condition factor is CF = fish weight (g) x fish fork length^{-3} x 100. Dress out percentage is D% = gutted fish weight/whole fish weight x 100. Hepatosomatic index (HSI) is the% of liver weight/whole fish weight.

Tissue samples were stored in 10% formalin pots (CellStore™ 20 ml Pots, CellPath). Embedding, sectioning, and staining of the tissue samples were done at the Norwegian Veterinary Institute in Harstad, Norway. In brief, the tissue sections were hydrated in water and stained with 1% Alcian blue (Alfa Aesar), 3% acetic acid for 15°min, transferred to 1% periodic acid (VWR) for 10°min, followed by Schiffs (Sigma-Aldrich®) reagent for 15°min, 30 s in heamatoxylin (VWR) before dehydration and mounting. The stained tissue sections were scanned with a Hamamatsu slide scanner (Hamamatsu) and uploaded to the Aiforia® platform and analysed according to Sveen et al. (2021).

Statistics

Biological and analytical data were subjected to one way analysis of variance (ANOVA) tests using IBM SPSS statistics 27 to detect dietary effects. When differences among treatments were identified, means were ranked using the Tukey post hoc test. Equality of error variances was tested with Levene's test. Effects were considered at a significance level of $p < 0.05$, and tendencies are discussed at $p < 0.1$.

RESULTS AND DISCUSSION

Raw Material Chemical Characterisation

The analysed fi FM is superior in crude protein content as compared to the rest of the analysed ingredients used in the present trial, followed by black soldier fly larvae meal, *P. tricornutum* biomass, tunicate meal, and *S. limacinum* biomass (**Supplementary Table S1**). On the other hand, *S. limacinum* meal is a lipid rich source, followed by *P. tricornutum*, FM, black soldier fly meal and tunicate meal (**Supplementary Table S6**). The FM used in this trial was a rich source of water-soluble peptides, of known growth promoting nutritional value in Atlantic salmon fed low FM diets (Kousoulaki et al., 2009), richer than *P. tricornutum* and black soldier fly larvae meal with lowest values analysed in *S. limacinum* biomass (**Supplementary Table S5**). Nevertheless, expressed in % of total protein the two microalgal biomasses have equally high levels of water-soluble protein (approx. 40% of total protein) whereas FM and black soldier fly larvae meal contain approx. 23–25% water soluble protein of total protein.

The used test meals had great differences in terms of amino acid composition compared to FM. The FM used in this trial had similar amino acid profile as other conventional FMs, and nearly or more than double the amounts of lysine (Lys) and methionine (Met), respectively, as % in total protein compared to the test ingredients. The black soldier fly larvae meal was also low in cysteine and cystine (Cys), unlike the three other materials which were richer or as rich in both Cys and tryptophane (Trp) as compared to FM. Except tunicate meal, the test ingredients were also lower in arginine (Arg) as compared to FM. The black soldier fly larvae meal contained the highest histidine (His) level, similar to that in FM, and had also similar levels of isoleucine (Ile) and valine (Val) as compared to FM (**Supplementary Table S2**). The non-dispensable amino acid His is important for osmoregulation (Bjerkås and Sveier, 2004) and prevention of cataract (Breck et al., 2003; Bjerkås and Sveier, 2004) in Atlantic salmon and thus considered in formulating transfer (from fresh to salt water) diets. A considerable part of His in black soldier fly larvae meal was in free form, unlike the remaining test ingredients, where His was either exclusively or mostly in protein bound form. The experimental diets were balanced based on the analysed raw material compositions to be equal in His levels. However, there were analysed lower levels of His in the BSFM diet which may be due to for instance loss of free His due to Maillard reactions during the extrusion process involving the black soldier fly larvae meal. Free His was found to be the most effective free amino acid in breaking down plant polysaccharide chains and reactive with the generated sugars, as it exerted buffering effect eliminating the inhibiting effects of certain compounds (organic acids) on Maillard reaction (Liu et al., 2019). Accordingly, the ADC of His was lowest in BSFM as compared to FMFO, TM, PM, and HM (**Table 6**). Else, the diets were well balanced in protein bound amino acids, even though we only balanced Lys, Met, Thr, and His in the formulations adding crystalline amino acids. Fishmeal was the raw material with the highest % of non-dispensable amino acids of total protein, followed by tunicate meal, black soldier fly larvae meal and *P. tricornutum* biomass with between them equal levels, and last *S. limacinum* biomass.

Phaeodactylum tricornutum biomass was the raw material with the highest % of free amino acids in the water-soluble protein fraction (**Supplementary Table S2**), and other nitrogenous compounds of low molecular weight (<200 Da), followed by *S. limacinum* biomass, FM, black soldier fly larvae meal, and tunicate meal. Tunicate meal and *S. limacinum* biomass had the highest % of small peptides (200–2000 Da). Black solider fly larvae meal, tunicate meal and FM had the highest levels of medium sized peptides (2000–15,000 Da). Finally, the two analysed FMs and the tunicate meal had the highest levels of larger soluble peptides (>20,000 Da) (**Supplementary Table S3**).

In terms of free amino acids, FM was richest in free creatinine and Tau and had in total similar total free amino acid levels as those analysed in black soldier fly larvae meal and *S. limacinum* biomass. Tunicate meal had the lowest level of free amino acids

TABLE 6 | Experimental diet apparent digestibility coefficient (ADC) of nutrients in Atlantic salmon. Values are in%.

Nutrient	FMFO	TM	BSFM	PM	HM	0FM0FO	*p* value[a]
Protein	91.7 ^{d} ± 0.1	85.3^{ab} ± 1.4	86.6 ^{bc} ±1.9	90.4 ^{cd} ± 0.6	90.5 ^{cd} ± 0.1	81.7^{a} ±3.2	0.000
Fat	95.4^{c} ±0.5	93.3 ^{bc} ±1.2	93.9 ^{bc} ±0.8	93.4 ^{bc} ±0.7	88.4^{a} ±0.9	90.7^{ab} ± 2.8	0.001
Asp	84.8^{b} ± 0.4	79.5^{ab} ± 1.9	78.8^{ab} ± 4.3	84.6^{b} ± 1.1	84^{b} ± 0.7	75.1^{a} ±4	0.002
Glu	97.5^{b} ± 0.2	95.6^{b} ± 0.4	95.9^{b} ± 0.9	96.8^{b} ± 0.4	96.6^{b} ± 0.3	92.8^{a} ±1.3	0.000
Ser	93.5^{c} ±0.6	87.5^{ab} ± 2	89.3 ^{bc} ±2.7	91.3 ^{bc} ±1.2	92.2 ^{bc} ±0.6	84^{a} ±3	0.001
Gly	90.8 ^{d} ± 0.7	84.2 ^{bc} ±1.9	82.6^{b} ± 3.5	88.9 ^{cd} ± 1	88.9 ^{cd} ± 0.8	77.2^{a} ±3.8	0.000
His	96.8^{c} ±0.8	91.7^{b} ± 1	86.3^{a} ±3.2	95.4 ^{bc} ±0.7	95.3 ^{bc} ±0.8	85.1^{a} ±2.7	0.000
Arg	96.2^{b} ± 0.6	90.7^{a} ±1.7	94.4^{b} ± 1.5	95.8^{b} ± 0.2	94.9^{b} ± 0.5	89.6^{a} ±1.8	0.000
Thr	94.3^{c} ±0.3	86.2^{b} ± 1.9	90.3 ^{bc} ±2.01	92.2^{c} ±1	92.5^{c} ±0.7	81.4^{a} ±3.3	0.000
Ala	94.1^{c} ±0.2	88.6^{ab} ± 1.8	90.7 ^{bc} ±1.7	92.8^{c} ±0.7	93.4^{c} ±0.3	86.9^{a} ±2	0.000
Pro	96.3^{b} ± 0.5	93.9^{ab} ± 1.1	94.1^{ab} ± 1.2	95.8^{b} ± 0.5	95.3^{b} ± 0.3	92.1^{a} ±1.3	0.001
Tyr	93.1^{c} ±1	88.4^{ab} ± 0.9	90.7 ^{bc} ±1.9	90.4 ^{bc} ±0.1	91.7 ^{bc} ±0.6	86.1^{a} ±2.3	0.001
Val	93.6^{c} ±0.1	88.9^{b} ± 0.8	89.2^{b} ± 1.7	92 ^{bc} ±0.7	92.5 ^{bc} ±0.6	84.2^{a} ±2.9	0.000
Met	94^{b} ± 0.3	90.2^{b} ± 0.9	93.8^{b} ± 0.5	93.2^{b} ± 0.6	93.3^{b} ± 0.7	89.1^{a} ±1.8	0.000
Ile	94.2 ^{d} ± 0.2	89.5^{b} ± 0.9	90.3 ^{bc} ±1.7	93.1 ^{cd} ± 0.6	93.4 ^{cd} ± 0.6	86.3^{a} ±2.4	0.000
Leu	95.2^{c} ±0	91.5^{b} ± 0.9	92.73 ^{bc} ±1.1	94.3^{c} ±0.3	94.4^{c} ±0.4	88.4^{a} ±1.9	0.000
Phe	93.1^{c} ±0.1	89.2^{ab} ± 0.9	91.2 ^{bc} ±1.4	92.7^{c} ±0.4	92.8^{c} ±0.4	86.6^{a} ±2.3	0.000
Lys	95.3^{c} ±0.3	92.1^{b} ± 0.8	92.5 ^{bc} ±1.8	94 ^{bc} ±0.6	94.1 ^{bc} ±0.4	88.2^{a} ±1.9	0.000
14:0	97.5^{c} ±0.7	95.2^{b} ± 1	96.3^{b} ± 0.8	96.1^{b} ± 0.8	69.6^{a} ±3.1	78.8^{b} ± 7.3	0.000
16:0	96^{c} ±0.8	93.9^{c} ±1	94.8^{c}±0.7	94.6^{c} ±0.8	66.5^{a} ±3.2	75.8^{b} ± 8.2	0.000
18:0	95.7^{b} ± 0.8	92.9^{b} ± 1	93.6^{b} ± 0.6	94.4^{b} ± 1	88^{a} ±1.1	91.7^{b} ± 3	0.001
20:0	97.5^{b} ± 1	94.2^{b} ± 1.1	93.9^{b} ± 0.8	97.2^{b} ± 2.4	88.4^{a} ±0.9	93.8^{b} ± 1.9	0.000
22:0	94 ± 2.5	89.2 ± 3.3	90 ± 2.8	93.4 ± 0.7	88.4 ± 0.9	71.3 ± 35.6	Ns
Saturated fatty acids	**96.4 ^{c} ±0.7**	**94.1 ^{c} ±1**	**95.1 ^{c} ±0.7**	**95 ^{c} ±0.8**	**69.6 ^{a} ±2.9**	**78 ^{b} ± 7.5**	**0.000**
16:1 n-7	98.2^{b} ± 0.3	97.8^{b} ± 0.5	97.4^{b} ± 0.7	96.8^{b} ± 0.4	94.6^{a} ±1.1	97.7^{b} ± 0.7	0.000
18:1 (n-9)±(n-7)±(n-5)	98.1^{b} ± 0.3	97.7^{ab} ± 0.5	96.9^{a} ±0.6	97.4^{ab} ± 0.4	96.8^{a} ±0.2	99.1^{c} ±0.2	0.000
20:1 (n-9)±(n-7)	97.6 ^{cd} ± 0.3	96.4 ^{bc} ±0.7	96.2^{b} ± 0.7	96.5 ^{bc} ±0.5	94.9^{a} ±0.4	97.9 ^{d} ± 0.3	0.000
22:1 (n-11)±(n-9)±(n-7)	97.2^{c} ±0.3	95.8 ^{bc} ±0.7	95.7 ^{bc} ±0.6	95.8 ^{bc} ±0.8	92.3^{a} ±0.5	94^{ab} ± 2.5	0.003
24:1 n-9	95.1^{b} ± 0.4	92.9^{b} ± 1.6	93.3^{b} ± 1.7	93.4^{b} ± 1.7	88.4^{a} ±0.9		0.001
Monounsaturated fatty acids	**97.8 ^{b} ± 0.3**	**97.1 ^{ab} ±0.5**	**96.5 ^{a} ±0.6**	**96.8 ^{ab} ±0.5**	**96.5 ^{a} ±0.2**	**99 ^{c} ±0.2**	**0.000**
18:2 n-6	97.4^{b} ± 0.2	97.4^{b} ± 0.4	96.9^{ab} ± 0.3	96.9^{ab} ± 0.3	96.5^{a} ±0.3	98.9^{c} ±0.4	0.000
PUFA (n-6) fatty acids	**97.5 ^{b} ± 0.2**	**97.5 ^{b} ± 0.4**	**96.9 ^{ab} ±0.3**	**97 ^{ab} ±0.3**	**96.6 ^{a} ±0.3**	**98.9 ^{c} ±0.3**	**0.000**
18:3 n-3	99^{c} ±0	99.2^{c} ±0.3	98.5^{b} ± 0.3	98.4^{ab} ± 0.3	98^{a} ±0.2	99.7 ^{d} ± 0.1	0.000
20:5 n-3 (EPA)	98.9 ^{bc} ±0.1	98.7 ^{bc} ±0.3	98.6 ^{bc} ±0.2	98.3^{b} ± 0.2	96.7^{a} ±0.2	99^{c} ±0.3	0.000
22:5 n-3	96.1^{b} ± 0.6	95.3^{b} ± 1.4	96.7^{b} ± 2.9	94.2^{b} ± 2	88.4^{a} ±0.9	94^{b} ± 2.5	0.002
22:6 n-3 (DHA)	97.9^{b} ± 0.2	97.7^{b} ± 0.4	97.7^{b} ± 0.1	97.4^{b} ± 0.3	95.4^{a} ±0.5	98^{b} ± 0.7	0.000
PUFA (n-3) fatty acids	**98.6 ^{ab} ±0.1**	**98.7 ^{ab} ±0.4**	**98.3 ^{ab} ±0.3**	**98 ^{b} ± 0.3**	**97 ^{a} ±0.3**	**99 ^{c} ±0.3**	**0.000**
PUFA fatty acids	98.2^{b} ± 0.1	98.2^{b} ± 0.3	97.8^{b} ± 0.3	97.6^{b} ± 0.3	96.8^{a} ±0.2	99^{c} ±0.3	0.000
EPA ± DHA	98.4^{b} ± 0.1	98.1^{b} ± 0.4	98.2^{b} ± 0.2	97.8^{b} ± 0.3	95.5^{a} ±0.5	98.1^{b} ± 0.6	0.000
Identified fatty acids	97.7^{c} ±0.3	96.9^{c} ±0.5	96.6^{c} ±0.5	96.8^{c} ±0.5	91.2^{a} ±0.8	94.2^{b} ± 1.9	0.000
Unidentified fatty acids	98.3^{c} ±0.8	96.6^{c} ±0.4	97.5^{c} ±0.4	97.1^{c} ±0.8	82.6^{a} ±1.6	92.4^{b} ± 2.6	0.000
Cu	19.9 ± 3.1	17.5 ± 18.4	21.2 ± 11.9	15.5 ± 0.9	8.8 ± 3.04	14.3 ± 21.1	ns
Fe	−8.0^{a} ±10.8	12.2^{ab} ± 6.5	15.4^{ab} ± 10.1	−2.3^{a} ±5.7	5.0^{ab} ± 5.7	24.0^{b} ± 15.2	0.015
Zn	3.1 ± 10.4	-4.6 ± 17.1	11.2 ± 14.3	−1.8 ± 1.7	−5.26 ± 10.5	1.6 ± 21.8	ns
Se	50.0 ± 11.8				46.0 ± 11.7	28.2 ± 25.3	ns
Mn	−21.1 ± 24.2	10.1 ± 61.9	6 ± 13.8	1.7 ± 6.6	−20.8 ± 16.4	−12.9 ± 25	ns

[a]*Values in the same line with different small superscript letter are significantly different (p < 0.05) following Tukey post hoc test.*

among the analysed raw materials. The predominant free amino acids in black soldier fly larvae meal were alanine (Ala), Arg, proline (Pro), and tyrosine (Tyr) (**Supplementary Table S2**). *Phaeodactylum tricornutum* biomass was the richest source of free amino acids among the analysed raw materials. The most abundant free amino acids in *P. tricornutum* biomass were the dispensable amino acids glutamic acid (Glu; 1.245% in the diet), Pro (0.955% in the diet), Ala (0.85% in the diet), glycine (Gly; 0.485% in the diet), ornithine (Orn; 0.435% in the diet) all but Orn also found overrepresented in krill hydrolysate and correlating with increased feed intake levels (Kousoulaki et al., 2013) and feed searching activity (Hara, 2006; Hara et al., 1993) in salmonids. All free amino acids analysed in *S. limacinum* biomass were found in concentrations between 0.0 and 0.1% (**Supplementary Table S2**). Fishmeal was richest in Tau and creatinine compared to the rest of the test ingredients, which are water soluble nitrogenous compounds present in the water-soluble fraction of FM (stickwater) and their dietary level was found to correlate with feed intake rates and growth in Atlantic salmon (Aksnes et al., 2006; Kousoulaki et al., 2009).

EPA-DHA levels ranged between 9.5 and 27% of total lipids in the assessed marine dietary ingredients. *S. limacinum* contained the highest levels of sum EPA ± DHA (mainly DHA) in the lipid fraction of the biomass as compared to the other marine sources analysed, followed by FM, *P. tricornutum* biomass, FO and tunicate meal. Other interesting facts regarding the assessed

marine raw material lipids are the high level saturated fatty acids in *S. limacinum* biomass (approx. 62% of total lipids), the high levels of monounsaturated fatty acids of FO (48.5% of total lipids) and the high levels of unidentified fatty acids in the tunicate meal lipid extract (76.3% of total Bligh & Dyer (B&D) extract (1959)) which deserves further exploration.

Black soldier fly larvae meal contained approx. 10% lipids, with a rather high levels of unidentified fatty acids too. The main fatty acids present in black soldier fly larvae meal were saturated fatty acids, followed by n-6 PUFA and monounsaturated fatty acids with no EPA or DHA present at any significant amounts (EPA ± DHA = 0.1% of total lipid extract) (**Supplementary Table S6**).

The lipids analysed in *P. tricornutum* biomass were mostly in free fatty acid form with presence of cholesterol esters. Though absent from higher plant oils, marine microalgae (Vernon et al., 1998; Volkman et al., 1989) as well as the freshwater species *Nannochloropsis limnetica* (Martin-Creuzburg and Merkel, 2016) can produce and store cholesterol, besides other sterols. Sterols are essential nutrients for crustacean zooplankton, but different phytosterols were found to support better growth in *Dafnia magna* than cholesterol (Martin-Creuzburg et al., 2014). Aquafeed cholesterol levels are reducing by substitution of FO with plant oils, as for instance soy and rapeseed oil that are devoid of cholesterol (Norambuena et al., 2013). Cholesterol has vital physiological roles in animals and though it can be endogenously synthesized by fish including Atlantic salmon (Leaver et al., 2008), it has been stipulated that dietary supplementation of cholesterol may provide benefits (Norambuena et al., 2013). Polar lipids in *P. tricornutum* were also analysed at higher levels as compared to *S. limacinum* which was in turn richer in triacylglycerols (**Supplementary Table S7**). Only 36% of the extracted lipid fraction in *P. tricornutum* was identified in lipid classes as compared to over 55% identified as fatty acids in the same raw material. Almost 100% of FO and *S. limacinum* biomass B&D extract were accounted for in the different identified lipid classes. The analysed FM B&D extract was richest in polar lipids as compared to the other analysed ingredients, with high levels of phosphatidylcholine. *S. limacinum* B&D extract was rich in tripalmitin (over 30% of the lipid extract), which is assumed to be highly indigestible in Atlantic salmon (Kousoulaki et al., 2015).

Aerobic microorganisms were present at highest levels in the black soldier fly larvae meal (410,000 KDE/g), followed by the processed *P. tricornutum* biomass (1,600 KDE/g), *S. limacinum* (460 KDE/g) and FM (230 KDE/g) (**Supplementary Table S5**). Bacteria are originally present at high levels in fish and seafood (Nickelson and Finne, 1992) and are growing in the cultivation medium together with microalgae or in the insect feed medium but are expected to be drastically reduced by heat treatment or cell wall disruption in the case of microalgae (e.g., Doucha and Lívanský, 2008). The Norwegian Food Authorities guidelines limit the number of aerobic bacteria in feed exported to countries withing the Euro-Asiatic Economic Zone to 500,000 KDE/g, which is higher than that analysed in the test ingredients used in this study. Though no official limits are defined, the presence of high aerobic bacterial count may indicate lower raw material quality and shelf-life due to contamination or insufficient heat treatment. The *P. tricornutum* biomass used in our study was a fresh and mild processed (bead milled and spray dried) biomass with nearly no detectable levels of histamine, putrescin or cadaverine. The *S. limacinum* biomass was heat processed to kill the cells in culture followed by spray drying and was low in microbial load, but it contained the highest analysed levels of putrescine followed by FM and black soldier fly larvae meal. The levels of biogenic amines putrescine, cadaverine and histamine detected in FM and *S. limacinum* biomass were 10 or more times lower than those analysed in FM produced by stale herring but higher than those analysed in FM produced by fresh herring (Opstvedt et al., 2000). Putrescine, cadaverine and histamine do not appear to be a hazard for fish and do not affect Atlantic salmon performance when added in the diet at levels equivalent to those present in FM produced by stale fish, but they provide indication of raw material quality significantly and negatively affecting fish performance (Aksnes and Mundheim, 1997; Opstvedt et al., 2000).

The tested FM was richer in vitamin A compared to the other analysed test meals, and contained significant amounts of vitamin E, which is probably due to the use of tocopherols as antioxidant in the raw material against ethoxyquin that was previously used in conventional FM. In June 2017 the EU commission suspended the organic authorisation of ethoxyquin for all animal species and categories with a transition period to introduce alternative antioxidants until 2020 (EU Regulation 2017/962). The levels of vitamin A in FM (5.47 mg/kg) were approx. 10 times the amount provided in the trial diets by the vitamin mixed used intended to cover the requirement of Atlantic salmon (**Table 1**). Vitamin A was analysed also in *S. limacinum* biomass (1.23 mg/kg) which was the only vitamin of those analysed present in this raw material. Vitamin D3 was identified only in FM (0.053 mg/kg) at levels equivalent to those added by the dietary vitamin mix (0.0625 mg/kg diet), which is just above the minimum level of the estimated requirement of Atlantic salmon in sea water (0.06–0.09 mg/kg diet) (Antony Jesu Prabhu et al., 2019). The *P. tricornutum* biomass was naturally rich in both tocopherols (vitamin E) (156 mg/kg) and vitamin C (1,050 mg/kg ascorbic acid). The dietary vitamin mix provides approx. 200 ppm vitamin E in the final diet, which is equivalent to that present in the algal biomass and 4 times that present in the test FM. The requirement of fish in vitamin E depends on and can be covered by adequate levels of vitamin C and Se in the diet (El-Sayed and Izquierdo, 2021) and was found to be higher than 60 mg/kg in Hamre et al. (1994). Vitamin C was only analysed in *P. tricornutum* biomass, where it appears to be present in a stable form, unlike in fish where it is known to perish rapidly during processing during fishmeal or compound feed production (Putnam, 1976). The minimum requirement of Atlantic salmon fry in vitamin C at start feeding was estimated to be 10–20 mg/kg dry diet (Sandnes et al., 1992), which is covered in commercial and research feeds by dietary addition of a stable form of ascorbic acid (stay-C: phosphorylated L-ascorbic acid) (Grant et al., 1989) and could alternatively also be covered by 1–2% dietary inclusion of the *P. tricornutum* biomass used in our study or other microalgal biomasses with similar properties.

Excessive levels of vitamin C may be present when using more than 2% phototrophic microalgae biomass in the diet such as is the case in the present study. Inconsistent data exist in the literature regarding the safety of high vitamin C levels in fish diets, including suspected negative effects in Atlantic salmon survival and growth by inclusion of approx. 1,580 mg/kg vitamin C combined with 450 mg/kg vitamin E in the diet (Kousoulaki et al., 2021), improved immune responses and disease resistance of Atlantic salmon pre-smolt fed up to 4,000 mg/kg diet vitamin C (Waagbo et al., 1993) or no effect by feeding juvenile eel a diet with 1,137 mg/kg vitamin C (Bae et al., 2012). Regarding K vitamins, K1 was analysed only in tunicate meal and *P. tricornutum* at comparable levels (**Supplementary Table S8**).

In terms of essential trace minerals, all analysed ingredients were found to be good sources of Fe (**Supplementary Table S8**). However, tunicate meal was found to contain excessive Fe levels, whereas black soldier fly larvae meal in turn, contained very high Mn levels, as also appears to be the case in Belghit et al. (2018) where diets containing 60% insect meal were analysed to have 160 mg kg^{-1} higher Mn levels as compared to those containing FM and soy protein concentrate instead. In the same study it appears that the insect meal used also contained higher levels of Fe than the replaced ingredients, which was not the case in our study. The maximum allowed levels of Mn in aqua feeds for Atlantic salmon is 100 mg kg^{-1} and that of Fe is 750 mg kg^{-1} (Regulation (EC) No 1831, 2003), which would render the supplementation of these trace minerals in diets containing significant amounts of insect meal and tunicate meal unnecessary. Organic fishmeal and tunicate meals were found to be good sources of Se. Zn was found in higher levels in tunicate meal and black soldier fly larvae meal whereas it was present in significantly lower levels in the FM.

The analysed test raw materials contained generally low levels of undesirable compounds and within the limits defined by the Directive 2002/32/EC and the amended Regulation (EC) No 1881/2006 (**Supplementary Table S9**). Organic fishmeal had higher levels of undesirable compounds as for instance dioxins and non-dioxin like PCBs and heavy metals (Pb, As, Hg, Cd) as compared to the other test raw materials analysed. This does not come as a surprise, as *P. tricornutum*, *S. limacinum* and insects are grown in controlled environment and can be produced on cleaner nutrient sources than wild fish foraging in proximity of industrial areas (Nøstbakken et al., 2018). The level of heavy metals and persistent organic pollutants (POPs) in organisms growing wild vary largely by genera. The mechanisms of heavy metal accumulation also differ from that of POP accumulation (Landis et al., 1993) but there are a few ecotoxicological generalisations that can be applied to tunicates as a feed-ingredient. Tunicates are grown in the sea and are, generally, considered to eat mostly phytoplankton and are as such low in the trophic food chain. Short-lived organisms accumulate less pollutants than the long-lived organisms. Tunicates are harvested as feed ingredient when they are half-a-year to maximum 1 year old (Grzimek 1972). The production period for tunicates in East Atlantic waters at ~58°N is 1 year. The species used in this study (*Ciona intestinalis*) spawn in mid-May to mid-June (Dybern 1965) and are ready to be harvested in September. Harvest starts when the animals reach ~5 cm in length. After spawning, one-year-old individuals die (Fredrik Norén unpublished data). Forage fish of higher trophic level have longer life span, e.g., up to 6 years for Pacific sandfish caught in southeaster Alaska (Thedinga et al., 2006) and over 4 years that is the reproduction age of Atlantic herring (https://www.fisheries.noaa.gov/species/atlantic-herring) before capture, thus resulting in lower accumulation levels of contaminants in their tissues.

The test FM, the *P. tricornutum* and *S. limacinum* biomass and the black soldier fly larvae meal used were also analysed for their content in nucleotides which can be used as indication of muscle degradation in fish (ATP and metabolites) (**Supplementary Table S4**). The test FM contained larger amounts of hypoxanthine, IMP, and inosine, due to the potential larger relative amounts of intestines with higher enzymatic activity prior to heat treatment in FM production as compared to the remaining ingredients. Black soldier fly larvae meal contained higher levels AMP (Adenosine monophosphate) compared to the other ingredients and similar levels ADP (Adenosine diphosphate) as *P. tricornutum* biomass. Nucleotides can have bioactive function in diets, acting as growth promoters or appetite stimulators (Dias et al., 1997; Yilmaz, 2005; Li and Gatlin, 2006). Nevertheless, Ikeda et al. (1991) found that, in jack mackerel, only some nucleotides exert chemoattractant effect, including IMP present in higher amounts in FM as compared to the other test ingredients in our study, but not ADP or AMP that were present in higher amounts in black soldier fly larvae meal as compared to FM. Accordingly, Rumsey et al. (1992) found negative effects in feed intake and growth in rainbow trout (*Oncorhynchus mykiss*) given a diet supplemented with free adenine (purine), whereas no such negative effect was seen by respective dietary increases in whole yeast extract or other purines such as guanine and xanthine, that exerted positive feeding response, and hypoxanthine that had no effect. Nevertheless, differences in dietary crude protein levels among the different test diets in the above-mentioned study, render, according to the authors, the conclusions on the direct negative effect of adenine ambiguous. Uauy et al. (1990) found growth increase and differentiation of the developing gastrointestinal tract in rats added AMP in the diet, and Kousoulaki et al. (2013) found no effect in growth or feed intake adding 0.18% AMP in the diet of Atlantic salmon. In any case, raw material freshness and the quality and amount of non-protein nitrogen fraction in novel single cell raw materials should be critically considered when incorporated at high levels in the diets of fish, and more specific studies are needed to reveal potential negative effects.

Pellet Technical Quality of Experimental Diets

Highest water stability index (WSI) (approx. 20%) was analysed in the BSFL diet, followed by PM and HM diets, then the FMFO diet, the TM diet and last, the 0FM0FO diet. The FM0FM0 diet had too low WSI to estimate the amounts of uneaten feed in a reliable way. Thus, uneaten feed collection was not performed in this treatment. Pellet quality and technical characteristics depend on among other the chemical properties of the ingredients used

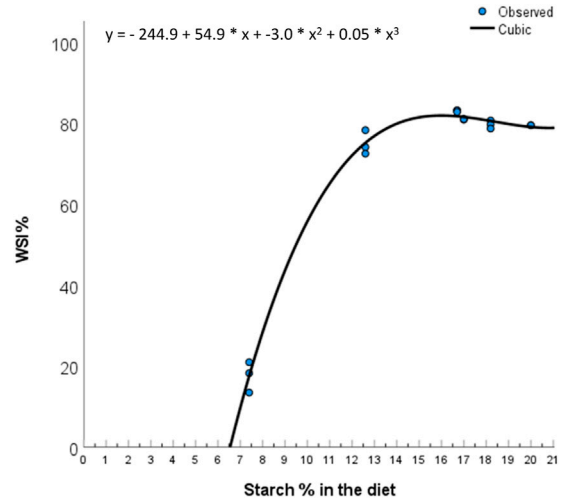

FIGURE 1 | Relation between dietary starch levels and dry matter water stability. Best fit of data was achieved by a cubic model (Total degrees of freedom = 16; R = 0.997; R^2 = 0.994, adjusted R^2 = 0.993, standard error of the estimate = 2.112; ANOVA $p < 0.001$).

(Sørensen 2012). The low water stability of extruded pellets, as is the case in our study, relates well with the amounts of starch present in the diet (**Figure 1**), which varied due to balancing the diets, using ingredients with very different proximate composition, for adequate and equal protein and lipid levels for Atlantic salmon smolt to the expense of mainly carbohydrates. As a rule of thumb in feed production in Aquafeed Technology Center (ATC) (Nofima, Bergen, Norway) (Personal communication with Dr. Odd Helge Romarheim) we consider 10% starch to be enough to produce water stable pellets. Based on **Figure 1**, we see that the increases in WSI are low for dietary starch levels above 12.5%. The TM diet contained lower levels of starch as compared to BSFM, FMFO, PM and HM diets, and had lower WSI. Dietary starch levels may have not been the only reason for the observed effect, as in Samuelsen et al. (2022) high levels of tunicate meal inclusion tended to result in lower pellet water stability irrespective of dietary starch levels. On the other hand, the FMFO diet contained the highest levels of starch but did not have the highest WSI. Fishmeal physicochemical and rheological properties are known to greatly affect pellet quality characteristics (Samuelsen et al., 2013; Samuelsen et al., 2014; Samuelsen and Oterhals, 2016). The relationship between technical and nutritional quality of extruded fish feed pellets is a little studied research area that deserves more attention.

Fish Performance and Blood Metabolites

We saw significantly higher growth rate in the FMFO treatment as compared to the rest. Fish TGC was similar and high (approx. 4) among the single test ingredient experimental groups and higher as compared to the 0FM0FO treatment. Growth mostly correlated with feed intake, thus FCR was similar among treatments, except for the TM treatment where FCR was higher than in the other treatments (but also in general rather low) which may be due to the higher inert ash levels present in the TM diet. TGC correlated significantly and positively with serum K, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and creatitine cinase CK. Thought higher blood levels of these liver and skeletal muscle metabolites are considered in context of physiological stress as for instance during exposure to pathogens (e.g., Rojas et al., 2018) or contaminants (e.g., Yancheva et al., 2014), it appears that they may also correlate with higher cellular and metabolic turnover among equally healthy fish. ASAT and ALAT are enzymes involved in amino acid metabolism, and similarly to our study, Gaye-Siessegger et al. (2007) measured higher liver ASAT values in Nile Tilapia of higher growth rates.

Belghit et al. (2018) used 60% black soldier fly larvae meal replacing 29% of FM and 24.5% soy protein concentrate in three different diets for Atlantic salmon and saw no significant reduction in fish growth performance, though the ADC of protein and nearly all amino acids considered in the insect meal-based diets were significantly lower than that of the respective FM-based diets. This may be because the insect meal-based diets in the study of Belghit et al. (2018) contained significantly higher levels of lipids and gross energy compared to the high FM diets which may have contributed to increased growth rate, and also may have contributed to the increase in relative liver weight that the authors report. Similar inclusion levels of *S. limacinum* (Kousoulaki et al., 2020b) and *Ciona intestinalis* (Kousoulaki et al., 2020a) have yielded similar or better (in the case of *S. limacinum*) performance results in Atlantic salmon as compared to FM/FO based diets, but then the experimental diets and FM and FO raw material qualities were different.

The superior performance of the FMFO treatment is in agreement with the ADC results of this study, showing that FM had higher ADC of protein as compared to the alternative protein sources (black soldier fly larvae and tunicate meals) and FO had higher ADC of total lipids and most fatty acids as compared to the lipids that the microalgal ingredients contributed to the diet with. Dietary nutrient ADC was significantly affected by dietary treatment for most nutrients analysed except Cu, Zn, Se, Mn and gross energy and 22:0. The diets with alternative protein sources (black soldier fly larvae meal and tunicate meal) had lower ADC of protein (86.6 and 85.3%, respectively) than the FMFO (91.7%) diet. Their combination and complete removal of the higher digestible FM reduced ADC of protein further in the 0FM0FO treatment (81.7%). Similar picture was shown for ADC of most amino acids, though their dietary levels were balanced, which may indicate suboptimal processing of these new raw materials still in development (e.g., excess heating). Ramos-Elorduy et al. (1981) reported protein digestibility in different insect meal between 45 and 66.9%. Nogales-Mérida et al. (2019) reviewed the ADC of nutrients of different insect meals in fish and reported higher ADC of crude protein in black soldier fly larvae meal (81.1–97.0%) as compared to than those obtained in *Tenebrio molitor* larvae meal (79.2–92.0%) or *Zophobas morio* larvae meal (50.5%). Belghit et al. (2018) reported lower amino acid ADC and a 3% decrease in ADC of protein in Atlantic salmon fed 60% black soldier fly larvae meal in the diet against a control diet with 35%

FM inclusion. Moreover, in Belghit et al. (2018) ADC of protein in the insect rich diet was higher (approx. 93–94%) than in our study (87%), probably due to fish size difference (50–143 g fish in Belghit et al. (2018) against 140–450 g in the current study) but also differences in raw material quality and dietary formulation. According to Jonas-Levi and Martinez (2017), the observed differences in apparent crude protein digestibility of insect meals in the literature are due to overestimation of dietary protein level due to the presence of chitin. However, this explanation does not cover the observed differences in individual amino acids seen e.g., in both Belghit et al. (2018) and our current study. Though arthropods, including insects have been part of fish's natural pray since prehistoric times (Maisey 1994), lower insect meal protein digestibility is probably also due to the presence of high levels of the crude fibre chitin. Chitin is part of the insects' exoskeleton, previously shown to limit rainbow trout growth performance as it was little digested despite the presence of relatively high levels of chitinase activity in the stomachs and of chitobiase in the intestines of the fish (Lindsay et al., 1984; Matsumiya et al., 2006). ADC of protein, some non-dispensable amino acids (Lys, Ile, Val and His) and lipids in the *P. tricornutum* treatment reduced as compared to the FMFO diet, but not statistically significantly.

In the present study, we observed lower fish growth performance when replacing FM with phototrophic microalga *P. tricornutum* cell wall disrupted biomass, contrary to Kiron et al. (2016) where only FCR but not growth was affected by 10 and 20% dietary inclusion of defatted *Desmodesmus* sp. biomass. In our study lower growth was related to lower feed intake rates in the PM treatment. The FCR in the PM treatment was numerically lowest among that of the other treatments with single novel ingredient dietary inclusion, though not statistically significantly, and equal to that of the FMFO. ADC of lipids was lowest in the HM diet, as reported in short term trials before (Kousoulaki et al., 2015) due to the high levels of tripalmitin in *S. limacinum* oil (Bogevik et al., 2018). The HM diet had also slightly lower ADC of MUFA, n-6PUFA, n-3PUFA and EPA ± DHA as compared to the FMFO and other test diets, except the 0FM0FO which had surprisingly the highest levels among all the groups (**Table 6**). In previous studies where *S. limacinum* was used in test diets to replace FO, the control diets included higher levels of palm oil and lower levels of linen oil to balance all experimental diets for total saturated fatty acids and n-3/n-6 dietary profiles. This resulted, in short term to lower saturated fatty acid ADC (Miller et al., 2007; Kousoulaki et al., 2015; Kousoulaki et al., 2016), but in longer term, to higher lipid and protein ADC in the *S. limacinum* groups (Kousoulaki et al., 2020b). The reasoning behind this design was to identify physiological dietary effects in Atlantic salmon from decreasing EPA/DHA ratio in the dietary lipids, which is the consequence of replacement of FO by *S. limacinum*, without the confounding effects of other lipid group imbalances. In our present trial, we aimed to reveal novel raw material limitations and balanced essential nutrients such as for instance Lys, Met and EPA + DHA, as well as for nutrients and dietary characteristics with known effects on fish health, such as the ratio of proinflammatory n-6 PUFA to anti-inflammatory PUFA. Nevertheless, despite the differences in ADC, fish in the *S. limacinum* treatment performed equally to those in BSFL and TM, and better than those in 0FM0FO treatments, showing that ADC is not always a good indicator for predicting growth performance or FCR and that differences in dietary saturated fatty acid amount and quality can affect protein and energy metabolism in a not always predictable manner.

Lipid ADC was significantly higher in PM as compared to the HM, with higher ADC of all fatty acids analysed (SFA, MUFA, n-3 and n-6 PUFAs), possibly due to the relatively higher FO level in the PM diet combined with the higher ADC of *P. tricornutum* lipids as compared to those in *S. limacinum* biomass. The *P. tricornutum* biomass used in PM was cell wall disrupted according to an optimised process using bead milling (Kokkali et al. manuscript) and spray dried before feed production. Spray drying is a mild drying process safeguarding raw material quality properties (Ameri and Maa, 2006). Still, using *P. tricornutum* biomass in the diet of Atlantic salmon in our study, as when using other autotrophically produced microalgal biomasses, yielded lower fish performance as compared to FM and FO, which may be due to different factors, such as for instance partial cell wall integrity though the biomass was both pre-disrupted and the diet produced by extrusion which is also known to improve microalgae nutrient availability (Wang et al., 2018; Gong et al., 2020), digestion disturbance (Jafri, 1998; Refstie et al., 1999) and gut microbial imbalance by microalgal carbohydrates (Desai et al., 2012). Even the presence of too high levels of vitamins and other antioxidant compounds such as phenolics present in phototrophic microalgae (Khan et al., 2018) may disrupt normal digestive physiology in fish, which can be approached by biorefinery processes (Bongiorno et al., 2022) separating and using the different biomass fractions in appropriate applications and dietary levels.

Apparent digestibility of minerals was, as often seen, difficult to relate to performance. Statistically significant differences were only seen in ADC of Fe, with higher values in the best and lower values (negative) in the lowest performing groups.

Fish Biometrics and Tissue Composition

The fish biometric indices showed little variation and small significant differences among the treatments (**Table 7**). Fish in 0FM0FO treatment were the smallest and also had the lowest CF and D%. A characteristic difference among treatments was the higher relative liver size (HSI) in BSFL treatment as compared to the FMFO which agreed also with the analysed higher lipid levels in the fillets (NQC) of fish in this treatment. This result may be related to the lower levels of the non-dispensable amino acid His in the BSFL diet and the generally lower ADC of non-dispensable amino acids in this diet as compared to FM, which may in turn have induced higher amino acid catabolism for use as energy source resulting in higher lipid deposition in body tissues. Nevertheless, according to this theory we should have observed the same effects in the TM treatment which was not the case. Belghit et al. (2018) and Lock et al. (2016) also observed increased HSI in Atlantic salmon fed diets with high inclusion levels of black soldier fly larvae meal and

TABLE 7 | Atlantic salmon performance, biometrics and blood metabolite profile when fed the experimental diets of the present study. As uneaten feed collection was not reliable in the 0FM0FO groups, total feed intake and FCR values are not calculated for this treatment.

	FMFO	TM	BSFM	PM	HM	0FM0FO	*p* value[a]
Fish number per tank	50	50	50	50	50	50	-
Start BW g	142 ± 1.5	142 ± 2.9	142 ± 0.2	141 ± 0.7	142 ± 0.5	141 ± 1.4	-
End BW g	475^{c} ±13.1	432^{b} ± 8.7	449^{b} ± 3.1	432^{b} ± 4.7	439^{b} ± 10.9	372^{a} ±9.7	0.000
BW increase g	333^{c} ±13.2	291^{b} ± 7.6	306^{b} ± 3.2	291^{b} ± 4.3	297^{b} ± 10.5	231^{a} ±9.6	0.000
TGC	4.16^{c} ±0.12	3.79^{b} ± 0.07	3.93^{b} ± 0.03	3.80^{b} ± 0.04	3.85^{b} ± 0.09	3.21^{a} ±0.10	0.000
FCR	0.74^{a} ±0.03	0.81^{b} ± 0.02	0.77^{a} ±0.03	0.74^{a} ±0.01	0.76^{a} ±0.02		0.017
Cumulative feed intake g $fish^{-1}$	12,336 ± 862	11,842 ± 480	11,785 ± 604	10,793 ± 276	11,224 ± 470		0.065
D%	87.0 ^{abc} +0.6	86.3 ^{abc} +0.6	86.0^{ab} + 0.3	87.2^{c} +0.1	87.1 ^{bc} +0.5	85.8^{a} +0.2	0.007
CF	1.5^{b} + 0.04	1.49^{b} + 0.06	1.47^{ab} + 0.05	1.44^{ab} + 0.02	1.45^{ab} + 0	1.36^{a} +0.05	0.014
HSI	1.36^{a} +0.06	1.37^{a} +0.06	1.54^{b} + 0.02	1.45^{ab} + 0.07	1.34^{a} +0.03	1.38^{ab} + 0.09	0.014
Serum cortisol (nmol/L)	98.8 ± 38	93.5 ± 17	126.5 ± 74	117.0 ± 71	89.3 ± 37	182.4 ± 110	Ns
Serum K (mmol/L)	1.94 ± 1.25	0.91 ± 0.34	1.37 ± 0.79	1.54 ± 0.50	1.40 ± 0.74	0.71 ± 0.13	Ns
Serum glucose (mmol/L)	4.95 ^{abc} ±0.25	4.87^{ab} ± 0.38	5.54 ^{cd} ± 0.16	5.40 ^{bc} ±0.24	4.73^{a} ±0.34	6.13 ^{d} ± 0.51	0.003
Serum total protein (g/L)	48.7 ± 1.95	49.4 ± 2.93	48.6 ± 3.93	45.1 ± 4.29	48.9 ± 2.13	49.8 ± 2.63	ns
Serum cholesterol (mmol/L)	19.9 ± 0.87	19.2 ± 2.85	19.2 ± 1.68	15.1 ± 1.89	18.6 ± 2.09	16.7 ± 1.82	0.074
Serum ASAT U/L	778^{c} ±90	617 ^{bc} ±146	544^{ab} ± 175	559^{ab} ± 121	538^{ab} ± 67	308^{a} ±140	0.020
Serum ALAT U/L	7.4^{b} ± 0.85	5.2^{a} ±1.42	6.2^{a} ±2.40	5.1^{a} ±1.13	4.8^{a} ±0.18	3.4^{a} ±0.86	0.026
Serum CK U/L	36,509 ± 6,521	27,670 ± 6,215	17,281 ± 5,737	24,284 ± 10,715	21,489 ± 6,392	12,456 ± 8,351	ns

[a]*Values in the same line with different small superscript letter are significantly different (p < 0.05) following Tukey post hoc test.*

attributed the effect to suboptimal lipid metabolism due to lack of Tau in the low FM diets. This hypothesis does not appear to be true in the case of our study. The trial diets of the current study contained equal levels Met. The 0FM0FO and BSFL diets contained the same dietary level of black soldier fly larvae meal (20%) and as such the lowest and equally among them (calculated) Tau levels among the experimental diets. However, only in the BSFL treatment fish had significantly higher HSI values as compared to the remaining treatments, but not in the 0FM0FO, which had equal HSI values as e.g., the FMFO treatment containing the highest dietary Tau level.

Drawing conclusions on simple correlations entails significant risk of ending up with false conclusions. For instance, HSI values in this study correlate significantly (two tailed Pearson's correlation; $p < 0.05$) and negatively with dietary soluble P, 18:1 and 20:0 fatty acids, dietary serine, glycine, His and isoleucine, and also with the response parameter fillet protein, and no other parameter. This occurs obviously because the BSFL treatment has the highest HSI and also the lowest levels, through only marginally of the above listed dietary factors, that are equal among the control and remaining test diets. On the other hand, the negative correlation between fillet protein levels and fish HSI is more interesting. Fillet protein levels also correlate (two tailed Pearson's correlation; $p < 0.05$) significantly with dietary His, which was low in the BSFL diet, despite the originally analysed high levels in the raw material, dietary isoleucine and serine, differing only marginally in the diets. Interestingly fillet protein also correlates positively with fillet Zn levels and negatively with ADC of dietary Zn. This fact, combined with the much higher Mn levels in BSFL diet, may indicate that Zn uptake was hindered by competition with Mn, possibly disturbing both lipid and protein metabolism, where both Zn and Mn are involved as cofactors in key enzymatic processes (Greenwood and Earnshaw, 1997). Prabhakaran et al. (2008) propose that in humans, the mechanism behind manganese toxicity induced by high levels of Mn inhalation is dysregulation of physiological processes involving oxidative stress, mitochondrial dysfunction, glutamate-mediated excitotoxicity, and aggregation of proteins. It was previously reported that organic minerals, including Zn improve dietary acid digestibility, and reduce fillet gaping in Atlantic salmon (Kousoulaki et al., 2016). Eder and Kirchgessner (1994a;b) in their work in mammalian model animals show the role of dietary Zn in the elongation of alpha linolenic acid to EPA and DHA and that Zn deficiency is demonstrated in both brain and liver lipid profile of the animals. In our study. This agrees with the observations in our study where Atlantic salmon in the BSFL treatment, had the lowest EPA + DHA levels in the fillet, though the respective diet did not contain the lowest amounts of EPA and DHA. It is thus a plausible assumption that some of the main effects we have seen using black soldier fly larvae meal are due to its very high level of Mn and there by inhibition of Zn uptake and related functions. If this is true, insect meal producers should aim to reduce the level of this trace element in their product.

The rest of the treatment differences in NQC fatty acid profile can be explained by the differences in the fatty acid composition of the dietary lipids (**Table 2**). Nevertheless, at trial end, filets lipids in all treatments contained approx. 16% saturated fatty acids (SFA), whereas SFA spanned from 13.1 to 18.3% of total dietary fatty acids. The monounsaturated fatty acid (MUFA) levels in Atlantic salmon NQC were lower than in the respective diets when those contained approx. 45% of total lipids MUFA and higher than in the respective diets when dietary MUFA levels were below 40%. Filet and dietary n-6 PUFA fatty acids were the same. Last, filet n-3 PUFA were slightly higher than in the respective diets, except for the BSFL treatment where it was slightly reduced (**Table 7**).

The total amino acid levels (66–67%) and profile of filet were similar in all treatments except for the BSFL treatment where fish filets contained significantly lower levels of His and total amino acids (63%) (**Table 8**). Last, though the levels

TABLE 8 | NQC fillet nutritional quality of Atlantic salmon fed diets containing low trophic organism-based ingredients as FM and/or FO replacement, in terms of total lipids (% in freeze dried NQC fillet), fatty acids profile (% in B&D extract), protein, total amino acids and essential trace elements (% in freeze dried NQC fillet). The values are means of 3 values, representing the mean of each replicate group for each treatment ± standard variation.

	FMFO	TM	BSFM	PM	HM	0FM0FO	*p* value[a]
Moisture wet%	71.1 ± 0.56	71.1 ± 0.21	70.9 ± 0.56	71.8 ± 0.49	71.8 ± 0.1	71.9 ± 0.91	0.107
Lipids (Bligh & Dyer)	24.57^{ab} ± 1.21	24.73^{ab} ± 1.4	28.83^{c} ±2.55	24.73 ^{abc} ±3.25	23.8^{ab} ± 0.82	22.4^{a} ±1.76	0.037
Protein	74.23^{ab} ± 0.^{75}	74.3^{ab} ± 1.21	69.93^{a} ±2.10	74.43^{ab} ± 2.67	75.2^{b} ± 0.50	75.33^{b} ± 2.05	0.023
Moisture freeze dried%	0.23 ± 0.4	0.17 ± 0.29	0.1 ± 0.17	0.17 ± 0.29	0 ± 0	0.2 ± 0.35	Ns
Freeze drying factor	0.288^{ab} ± 0.004	0.288^{ab} ± 0.005	0.290^{b} ± 0.005	0.280^{ab} ± 0.005	0.282^{ab} ± 0.001	0.279^{a} ±0.006	0.026
C14:0	3.27^{c} ±0.12	2.93^{b} ± 0.06	3.63 ^{d} ± 0.15	3.1 ^{bc} ±0.00	1.27^{a} ±0.06	1.43^{a} ±0.06	0.000
C16:0	11.20^{a} ±0.20	10.67^{a} ±0.21	11.13^{a} ±0.45	11.2^{a} ±0.26	12.50^{b} ± 0.00	12.2^{b} ± 0.20	0.000
C18:0	2.10^{a} ±0.10	2.23^{a} ±0.06	2.13^{a} ±0.06	2.2^{a} ±0.1	2.57^{b} ± 0.12	2.6^{b} ± 0.10	0.000
C20:0	0.20 ± 0.00	0.20 ± 0.00	0.17 ± 0.06	0.2 ± 0.00	0.2 ± 0.00	0.23 ± 0.06	Ns
C22:0	0.10 ± 0.00	0.10 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.10 ± 0.00	Ns
Saturated fatty acids	**16.87 ± 0.4**	**16.13 ± 0.23**	**17.17 ± 0.70**	**16.80 ± 0.35**	**16.63 ± 0.06**	**16.57 ± 0.25**	**0.099**
C16:1n-7	2.27^{c} ±0.06	2.13^{c} ±0.06	2.47 ^{d} ± 0.12	2.87^{e} ±0.06	1.07^{a} ±0.06	1.77^{b} ± 0.06	0.000
C18:1 (n-9) + (n-7) + (n-5)	24.87^{ab} ± 0.25	26.6^{b} ± 0.66	23.07^{a} ±1.06	24.17^{a} ±0.38	30.87^{c} ±0.15	30.27^{c} ±1.01	0.000
C20:1 (n-9) + (n-7)	7.33 ^{d} ± 0.06	6.67^{c} ±0.12	6.9^{c} ±0.17	6.67^{c} ±0.06	3.00^{b} ± 0.10	2.53^{a} ±0.06	0.000
C22:1 (n-11) + (n-9) + (n-7)	8.17^{c} ±0.12	7.37^{b} ± 0.21	8.03^{c} ±0.4	7.4^{b} ± 0.10	2.13^{a} ±0.06	1.67^{a} ±0.06	0.000
C24:1 n-9	0.50^{b} ± 0.00	0.47^{b} ± 0.06	0.47^{b} ± 0.06	0.50^{b} ± 0.00	0.30^{a} ±0.00	0.23^{a} ±0.06	0.000
Monounsaturated fatty acids	**43.13 ^{b} ± 0.21**	**43.23 ^{b} ± 0.95**	**40.93 ^{b} ± 1.60**	**41.60 ^{b} ± 0.53**	**37.37 ^{a} ±0.29**	**36.47 ^{a} ±1.11**	**0.000**
C16:2 n-4	0.10 ± 0.00	0.10 ± 0.00	0.1 ± 0.00	0.20 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	ns
C16:3 n-4	0.07 ± 0.06	0.00 ± 0.00	0.07 ± 0.06	0.07 ± 0.06	0.00 ± 0.00	0.07 ± 0.06	ns
C18:2 n-6	9.60^{a} ±0.10	10.27^{a} ±0.21	9.2^{a} ±0.4	9.5^{a} ±0.17	12.27^{b} ± 0.21	12.1^{b} ± 0.87	0.000
C18:3 n-6	0.10 ± 0.00	0.17 ± 0.06	0.13 ± 0.06	0.13 ± 0.06	0.10 ± 0.00	0.13 ± 0.06	ns
C20:2 n-6	0.80^{a} ±0.00	0.77^{a} ±0.06	0.73^{a} ±0.06	0.73^{a} ±0.06	1.00^{b} ± 0.00	0.80^{a} ±0.00	0.000
C20:3 n-6	0.20^{a} ±0.00	0.30^{b} ± 0.00	0.30^{b} ± 0.00	0.30^{b} ± 0.00	0.23^{a} ±0.06	0.30^{b} ± 0.00	0.000
C20:4 n-6	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.20 ± 0.00	0.10 ± 0.00	0.20 ± 0.00	ns
PUFA (n-6) fatty acids	**10.80 ^{a} ±0.10**	**11.60 ^{a} ±0.30**	**10.47 ^{a} ±0.50**	**10.87 ^{a} ±0.23**	**13.7 ^{b} ± 0.17**	**13.53 ^{b} ± 0.90**	**0.000**
C18:3 n-3	3.70^{ab} ± 0.00	4.90^{c} ±0.20	3.13^{a} ±0.15	3.87^{b} ± 0.15	7.57^{a} ±0.12	6.23 ^{d} ± 0.55	0.000
C18:4 n-3	0.83^{b} ± 0.06	0.90^{b} ± 0.00	0.83^{b} ± 0.06	0.87^{b} ± 0.06	0.43^{a} ±0.06	0.47^{a} ±0.06	0.000
C20:3 n-3	0.30 ± 0.00	0.30 ± 0.00	0.20 ± 0.00	0.30 ± 0.00	0.70 ± 0.00	0.40 ± 0.00	ns
C20:4 n-3	0.90 ^{d} ± 0.00	0.80^{c} ±0.00	0.90 ^{d} ± 0.00	0.80^{c} ±0.00	0.60^{b} ± 0.00	0.47^{a} ±0.06	0.000
C20:5 n-3 (EPA)	2.13^{c} ±0.06	1.77^{b} ± 0.12	2.00 ^{bc} ±0.10	2.10^{c} ±0.10	0.83^{a} ±0.06	0.80^{a} ±0.10	0.000
C21:5 n-3	0.10 ± 0.00	0.10 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	ns
C22:5 n-3	0.93^{b} ± 0.06	0.80^{b} ± 0.00	0.9 ^{bc} ±0	0.97^{c} ±0.06	0.47^{a} ±0.06	0.47^{a} ±0.06	0.000
C22:6 n-3 (DHA)	6.00 ^{bc} ±0.10	5.50^{ab} ± 0.10	5.07^{a} ±0.23	6 ^{bc} ±0.36	7.93 ^{d} ± 0.25	6.47^{c} ±0.32	0.000
PUFA (n-3) fatty acids	**14.9 ^{b} ± 0.17**	**15.07 ^{b} ± 0.29**	**13.13 ^{a} ±0.50**	**15.00 ^{b} ± 0.52**	**18.53 ^{c} ±0.35**	**15.3 ^{b} ± 1.13**	**0.000**
Total-PUFA fatty acids	25.87^{ab} ± 0.21	26.77 ^{bc} ±0.57	23.77^{a} ±1.050	26.13^{ab} ± 0.45	32.23 ^{d} ± 0.51	29.00^{c} ±2.08	0.000
Omega-6/omega-3 ratio	0.73^{a} ±0.01	0.77 ^{bc} ±0.01	0.80^{c} ±0.01	0.72^{a} ±0.03	0.74^{ab} ± 0.01	0.88 ^{d} ± 0.010	0.000
EPA ± DHA	8.13^{c} ±0.15	7.27^{ab} ± 0.15	7.07^{a} ±0.32	8.10 ^{bc} ±0.46	8.77 ^{d} ± 0.25	7.27^{ab} ± 0.42	0.000
Total identified fatty acids	85.87 ± 0.75	86.13 ± 1.74	81.87 ± 3.35	84.53 ± 1.17	86.23 ± 0.78	82.03 ± 3.37	0.079
Total unidentified fatty acids	3.97^{b} ± 0.12	3.80^{b} ± 0.20	5.63^{c} ±0.38	4.17^{b} ± 0.15	2.97^{a} ±0.06	4.07^{b} ± 0.12	0.000
Aspartic acid	7.27 ± 0.12	7.17 ± 0.06	6.77 ± 0.25	7.03 ± 0.21	7.23 ± 0.06	7.20 ± 0.44	ns
Glutamic acid	10.3 ± 0.36	10.30 ± 0.30	9.7 ± 0.36	10.27 ± 0.46	10.17 ± 0.38	10.40 ± 0.20	ns
Serine	2.87 ± 0.06	2.93 ± 0.06	2.8 ± 0.00	2.87 ± 0.06	2.90 ± 0.00	2.93 ± 0.15	ns
Glycine	3.60 ± 0.20	3.60 ± 0.10	3.47 ± 0.15	3.63 ± 0.15	3.57 ± 0.06	3.63 ± 0.06	ns
Histidine	2.33^{b} ± 0.15	2.27^{b} ± 0.06	1.87^{a} ±0.06	2.23^{b} ± 0.06	2.27^{b} ± 0.06	2.20^{b} ± 0.10	0.000
Arginine	4.1 ± 0.10	4.07 ± 0.12	3.8 ± 0.17	4.00 ± 0.30	4.00 ± 0.00	4.00 ± 0.10	ns
Threonine	3.33 ± 0.06	3.37 ± 0.06	3.13 ± 0.06	3.23 ± 0.21	3.33 ± 0.06	3.30 ± 0.20	ns
Alanine	3.93 ± 0.40	3.80 ± 0.40	3.7 ± 0.30	4.00 ± 0.30	4.27 ± 0.51	3.93 ± 0.49	ns
Proline	2.33 ± 0.15	2.33 ± 0.06	2.23 ± 0.15	2.43 ± 0.15	2.60 ± 0.36	2.37 ± 0.29	ns
Tyrosine	2.50 ± 0.00	2.47 ± 0.15	2.33 ± 0.15	2.47 ± 0.06	2.50 ± 0.17	2.43 ± 0.15	ns
Valine	3.87 ± 0.15	3.87 ± 0.21	3.67 ± 0.21	3.80 ± 0.20	3.90 ± 0.10	3.90 ± 0.20	ns
Methionine	2.3 ± 0.00	2.30 ± 0.10	2.17 ± 0.06	2.30 ± 0.10	2.23 ± 0.29	2.30 ± 0.17	ns
Isoleucine	3.37 ± 0.12	3.33 ± 0.15	3.13 ± 0.15	3.27 ± 0.21	3.30 ± 0.10	3.30 ± 0.10	ns
Leucine	5.57 ± 0.06	5.43 ± 0.15	5.17 ± 0.12	5.43 ± 0.21	5.53 ± 0.06	5.50 ± 0.26	ns
Phenylalanine	2.93 ± 0.06	2.87 ± 0.06	2.77 ± 0.06	2.87 ± 0.15	2.97 ± 0.06	2.93 ± 0.23	ns
Lysine	6.80 ± 0.17	6.63 ± 0.25	6.40 ± 0.17	6.67 ± 0.31	6.77 ± 0.12	6.63 ± 0.32	ns
Total amino acids	67.40 ± 1.15	66.73 ± 1.47	63.1 ± 1.68	66.5 ± 2.66	67.53 ± 0.70	66.97 ± 3.32	ns
Fe	9.47 ± 0.21	9.70 ± 1.21	11.67 ± 2.08	11.33 ± 0.58	10.80 ± 1.31	11.33 ± 0.58	ns
Zn	15.67ab ± 0.58	16.67^{b} ± 0.58	14.67^{a} ±0.58	16.67^{b} ± 0.58	16.00^{ab} ± 0.00	17.00^{b} ± 1.00	0.006
Se	0.70 ± 0.00	0.67 ± 0.06	0.60 ± 0.00	0.63 ± 0.06	0.63 ± 0.06	0.63 ± 0.06	ns
Mn	0.37 ± 0.12	0.30 ± 0.00	0.33 ± 0.06	0.30 ± 0.00	0.37 ± 0.06	0.37 ± 0.06	ns

[a]*Values in the same line with different small superscript letter are significantly different (p < 0.05) following Tukey post hoc test.*

TABLE 9 | Skin moisture, amino acid profile (% in freeze dried skin sample) and trace mineral content (mg kg^{-1} freeze dried skin sample) in Atlantic salmon fed diets containing low trophic organism-based ingredients as FM and/or FO replacement. The values for moisture are in wet samples whereas those for amino acids and minerals are in freeze dried samples. Values are means of 3 values, representing the mean of each replicate group for each treatment ± standard variation.

	FMFO	TM	BSFM	PM	HM	0FM0FO	*p* value[a]
Moisture wet%	61.4 ± 1.9	60.2 ± 4.3	63.5 ± 1.08	62 ± 0.7	62.5 ± 0.63	63.1 ± 0.97	Ns
Freeze drying factor	0.386 ± 0.019	0.398 ± 0.043	0.365 ± 0.011	0.38 ± 0.007	0.375 ± 0.006	0.369 ± 0.01	Ns
Aspartic acid	4.97 ± 0.23	5.10 ± 0.10	5.03 ± 0.23	5.13 ± 0.21	5.00 ± 0.10	5.07 ± 0.12	Ns
Glutamic acid	7.37 ± 0.32	7.43 ± 0.15	7.3 ± 0.44	7.5 ± 0.35	7.27 ± 0.21	7.47 ± 0.21	Ns
Hydroxyproline	3.97 ± 0.57	4.27 ± 0.32	4.10 ± 0.44	4.37 ± 0.49	4.07 ± 0.21	3.97 ± 0.21	Ns
Serine	3.10 ± 0.10	3.17 ± 0.15	3.200 ± 0.1	3.23 ± 0.06	3.17 ± 0.06	3.17 ± 0.06	Ns
Glycine	13.67 ± 0.86	14.00 ± 0.50	13.33 ± 1.01	13.87 ± 0.78	13.30 ± 0.61	13.57 ± 0.38	Ns
Histidine	1.63 ± 0.15	1.63 ± 0.06	1.53 ± 0.15	1.67 ± 0.15	1.53 ± 0.06	1.60 ± 0.00	Ns
Arginine	4.8 ± 0.2	4.9 ± 0.20	4.70 ± 0.35	4.87 ± 0.25	4.73 ± 0.15	4.8 ± 0.17	Ns
Threonine	2.1 ± 0.00	2.13 ± 0.06	2.10 ± 0.10	2.07 ± 0.06	2.07 ± 0.06	2.13 ± 0.06	Ns
Alanine	5.17 ± 0.35	5.43 ± 0.38	5.30 ± 0.61	5.40 ± 0.20	5.20 ± 0.40	5.33 ± 0.15	Ns
Proline	6.33 ± 0.38	6.83 ± 0.23	6.60 ± 0.62	6.57 ± 0.23	6.40 ± 0.36	6.37 ± 0.35	Ns
Tyrosine	1.13 ± 0.06	1.20 ± 0.00	1.23 ± 0.06	1.17 ± 0.06	1.13 ± 0.06	1.17 ± 0.06	Ns
Valine	2.00 ± 0.00	2.00 ± 0.00	2.03 ± 0.06	2.03 ± 0.06	2.00 ± 0.00	2.00 ± 0.10	Ns
Methionine	2.17 ± 0.06	2.13 ± 0.06	2.10 ± 0.10	2.17 ± 0.06	2.10 ± 0.10	2.10 ± 0.10	ns
Isoleucine	1.40 ± 0.00	1.37 ± 0.06	1.43 ± 0.06	1.4 ± 0.00	1.4 ± 0.00	1.43 ± 0.06	ns
Leucine	2.60 ± 0.10	2.57 ± 0.06	2.63 ± 0.06	2.63 ± 0.06	2.63 ± 0.06	2.60 ± 0.10	ns
Phenylalanine	1.83 ± 0.12	1.83 ± 0.06	1.83 ± 0.06	1.87 ± 0.06	1.80 ± 0.00	1.83 ± 0.06	ns
Lysine	2.77 ± 0.21	2.93 ± 0.12	3.00 ± 0.26	2.97 ± 0.23	2.87 ± 0.15	2.90 ± 0.3	ns
Total amino acids	**67 ± 3.04**	**68.93 ± 1.57**	**67.47 ± 4.3**	**68.9 ± 3.16**	**66.67 ± 1.8**	**67.5 ± 2.25**	**ns**
Cu	1.37 ± 0.23	1.57 ± 0.21	1.5 ± 0.17	1.87 ± 0.46	1.60 ± 0.10	1.53 ± 0.15	ns
Fe	16.5 ± 3.54	16.00 ± 2.00	16.67 ± 2.52	21.33 ± 7.77	16.33 ± 1.53	16.67 ± 2.52	ns
Zn	116.67 ± 11.55	116.67 ± 15.28	110 ± 20	133.33 ± 11.55	120.00 ± 10.00	126.67 ± 11.55	ns
Se	1.03 ± 0.06	0.97 ± 0.06	0.90 ± 0.00	0.97 ± 0.06	1.03 ± 0.06	0.90 ± 0.10	0.071
Mn	17.33^{b} ± 0.58	15.33^{ab} ± 1.53	16.67^{b} ± 1.15	13.33^{a} ±0.58	15.00^{ab} ± 1.00	13.00^{a} ±0.00	0.001

[a]*Values in the same line with different small superscript letter are significantly different (p < 0.05) following Tukey post hoc test.*

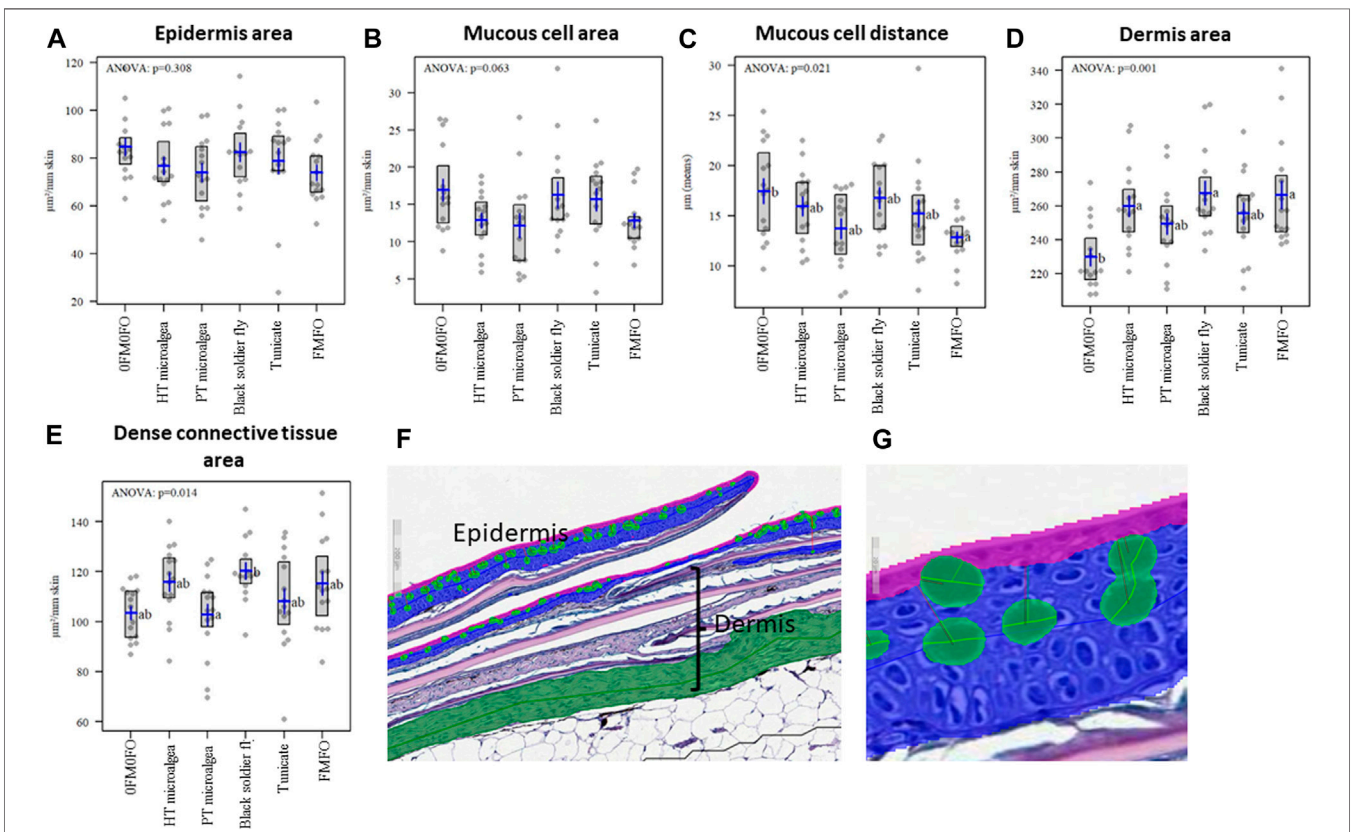

FIGURE 2 | Aiforia® results for **(A)** Epidermis area, **(B)** Mucous cell area, **(C)** Mucous cell distance, **(D)** Dermis area, and **(E)** Dense connective tissue area in histological samples of skin **(F,G)** from Atlantic salmon in the experimental treatments of the current study.

of essential trace minerals varied significantly among the 6 test diets, in the filet they were very similar in all treatments except BSFL were filets contained lower levels of Zn (**Table 8**).

Skin Composition and Histology

The amino acid composition of the skin was similar in fish from all treatments with typical profile of a collagen rich tissue, containing relatively high levels of the dispensable amino acids Gly, Hyp, and Pro and lower relative levels of non-dispensable amino acids (**Table 9**). Atlantic salmon skin was rich in Zn, followed by Mn and Fe, and contained also higher levels Se but lower levels of Cu as compared to the fillets (**Tables 8** and **9**). Minerals are involved in a great number of reactions, i. e, related to their antioxidant function. For instance, superoxide dismutase (SOD), part of the cellular antioxidant defence, are associated with essential trace metals. In the skin, there are found SODs associated with Cu-Zn, Fe and Mn, and their activities are found to be higher in the dark than in the light parts of skin (Nakano et al., 1993) which explain their mineralisation pattern. As for amino acids, and as in filet, the levels of essential trace minerals in the skin were very similar among fish from different dietary treatments, unlike the large differences among the diets, introduced by the great raw material differences in this respect. Nevertheless, there was some tendencies and significant but small differences in Se and Mn levels in skin. The skin of Atlantic salmon in PM and 0FM0FO treatments had lower levels Mn than fish in the FMFO and BSFL treatments. Moreover, there was analysed less Se in the skin of fish in BSFL and 0FM0FO treatments as compared to the rest.

Histological quantification of skin tissues showed a thinner dermis for the 0FM0FO fish, which can be because those fish were the smallest among all trial treatments. The dense connective tissue of the skin was thicker for the BSFL as compared to the PM group, and these two groups where those that differed from the others in terms of their skin content in Se and Mn. The scale areas followed a similar pattern as dermis with lowest values for the 0FM0FO fish (**Figure 2**). There was no effect on the epidermal area or number and area of mucous cells, and the epidermis of the fish studied looked healthy.

CONCLUSION

Our study shows that *S. limacinum* biomass, disrupted *Phaeodactylum tricornutum* biomass, black soldier fly larvae (*Hermetia illucens*) meal, and tunicate (*Ciona intestinalis*) meal are well accepted raw materials and can sustain a healthy Atlantic salmon with high growth rate and low FCR fed low or no FM and FO in the diet. Nevertheless, the performance of fish fed diets containing high quality fresh and organic FM and FO was superior to each one, and even more when all four test raw materials were combined. The performance of novel raw materials as ingredients is aquafeeds may vary according to the marine raw materials they are compared to, ranging from performing equally or better than an average quality FM and FO to performing inferiorly to high quality fresh FM and FO. Besides being sources of proteins and lipids, the studied ingredients can be used as rich natural sources of limiting non-dispensable amino acids, trace minerals and vitamins. However, the degree of stability and availability of these non-dispensable nutrients in extruded diets for fish should be established and safeguarded. Possible physiological effects of high Mn level in black soldier fly larvae meal are worth further investigation. Fish filet lipids, amino acid and trace mineral profile and level as well as the profile and levels of amino acids and trace mineral in the skin were conservative to a great degree unlike the compositional differences among the experimental diets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Mattilsynet, The Norwegian Food Authority.

AUTHOR CONTRIBUTIONS

KK contributed with funding aquisition, trial planing, execution, statistics and article writing. LS contributed with trial planing, analyses, statistics and article writing and editing. FN contributed with funding, funding aquisition, raw material production and supply and article writing and editing. ÅE contributed with funding aquisition, trial management, trial execution, article writing and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.884740/full#supplementary-material

REFERENCES

Ahlgren, G., Gustafsson, I.-B., and Boberg, M. (1992). Fatty Acid Content and Chemical Composition of Freshwater Microalgae1. *J. Phycol.* 28, 37–50. doi:10.1111/j.0022-3646.1992.00037.x

Aksnes, A., Hope, B., and Albrektsen, S. (2006). Size-fractionated Fish Hydrolysate as Feed Ingredient for Rainbow Trout (*Oncorhynchus mykiss*) Fed High Plant Protein Diets. II: Flesh Quality, Absorption, Retention and Fillet Levels of Taurine and Anserine. *Aquaculture* 261, 318–326. doi:10.1016/j.aquaculture.2006.07.026

Aksnes, A., and Mundheim, H. (1997). The Impact of Raw Material Freshness and Processing Temperature for Fish Meal on Growth, Feed Efficiency and Chemical Composition of Atlantic Halibut (*Hippoglossus hippoglossus*). *Aquaculture* 149, 87–106. doi:10.1016/s0044-8486(96)01438-x

Ameri, M., and Maa, Y.-F. (2006). Spray Drying of Biopharmaceuticals: Stability and Process Considerations. *Dry. Technol.* 24, 763–768. doi:10.1080/03602550600685275

Angles, M. N., and Dufresne, A. (2000). Plasticized Starch/tunicin Whiskers Nanocomposites. 1. Structural Analysis. *Macromolecules* 33, 8344–8353. doi:10.1021/ma0008701

Antony Jesu Prabhu, P., Lock, E.-J., Hemre, G.-I., Hamre, K., Espe, M., Olsvik, P. A., et al. (2019). Recommendations for Dietary Level of Micro-minerals and Vitamin D3 to Atlantic Salmon (*Salmo salar*) Parr and Post-smolt when Fed Low Fish Meal Diets. *PeerJ* 7, e6996. doi:10.7717/peerj.6996

Bae, J.-Y., Park, G.-H., Yoo, K.-Y., Lee, J.-Y., Kim, D.-J., and Bai, S. C. (2012). Re-evaluation of the Optimum Dietary Vitamin C Requirement in Juvenile Eel, *Anguilla Japonica* by Using L-Ascorbyl-2-Monophosphate. *Asian Australas. J. Anim. Sci.* 25, 98–103. doi:10.5713/ajas.2011.11201

Baeverfjord, G., Refstie, S., Krogedal, P., and Åsgård, T. (2006). Low Feed Pellet Water Stability and Fluctuating Water Salinity Cause Separation and Accumulation of Dietary Oil in the Stomach of Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture* 261, 1335–1345. doi:10.1016/j.aquaculture.2006.08.033

Barba, F. J., Grimi, N., and Vorobiev, E. (2014). New Approaches for the Use of Non-conventional Cell Disruption Technologies to Extract Potential Food Additives and Nutraceuticals from Microalgae. *Food Eng. Rev.* 7, 45–62. doi:10.1007/s12393-014-9095-6

Beal, C. M., Gerber, L. N., Thongrod, S., Phromkunthong, W., Kiron, V., Granados, J., et al. (2018). Marine Microalgae Commercial Production Improves Sustainability of Global Fisheries and Aquaculture. *Sci. Rep.* 8, 15064. doi:10.1038/s41598-018-33504-w

Belghit, I., Liland, N. S., Waagbø, R., Biancarosa, I., Pelusio, N., Li, Y., et al. (2018). Potential of Insect-Based Diets for Atlantic Salmon (*Salmo salar*). *Aquaculture* 491, 72–81. doi:10.1016/j.aquaculture.2018.03.016

Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L., and Frost, B. (1987). A New, Rapid, High-Sensitivity Analysis of Amino Acids in Food Type Samples. *J. Assoc. Off. Anal. Chem.* 70, 241–247. doi:10.1093/jaoac/70.2.241

Bjerkås, E., and Sveier, H. (2004). The Influence of Nutritional and Environmental Factors on Osmoregulation and Cataracts in Atlantic Salmon (*Salmo salar* L). *Aquaculture* 235, 101–122. doi:10.1016/j.aquaculture.2003.10.005

Bligh, E. G., and Dyer, W. J. (1959). A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi:10.1139/o59-099

Bogevik, A. S., Nygren, H., Balle, T., Haugsgjerd, B. O., and Kousoulaki, K. (2018). Enzymatic Interesterification of Heterotrophic Microalgal Oil with Rapeseed Oil to Decrease the Levels of Tripalmitin. *Eur. J. Lipid Sci. Technol.* 2018, 1800063. doi:10.1002/ejlt.201800063

Bongiorno, T., Foglio, L., Proietti, L., Vasconi, M., Moretti, V. M., Lopez, A., et al. (2022). Hydrolyzed Microalgae from Biorefinery as a Potential Functional Ingredient in Siberian Sturgeon (*A. baerii* Brandt) Aquafeed. *Algal Res.* 62, 102592. doi:10.1016/j.algal.2021.102592

Breck, O., Bjerkas, E., and Campbell, P. (2003). Cataract Preventative Role of Mammalian Blood Meal, Histidine, Iron and Zinc in Diets for Atlantic Salmon (Salmo salar L.) of Different Strains. *Aquaculture Nutrition* 9, 341–350. doi:10.1046/j.1365-2095.2003.00269.x

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., and Chang, J.-S. (2011). Cultivation, Photobioreactor Design and Harvesting of Microalgae for Biodiesel Production: A Critical Review. *Bioresour. Technol.* 102, 71–81. doi:10.1016/j.biortech.2010.06.159

Chiang, B.-Y., and Johnson, J. A. (1977). Measurement of Total and Gelatinized Starch by Glucoamylase and O-Toluidin Reagent. *Cereal Chem.* 54, 429–435.

Cho, C. Y. (1992). Feeding Systems for Rainbow Trout and Other Salmonids with Reference to Current Estimates of Energy and Protein Requirements. *Aquaculture* 100, 107–123. doi:10.1016/0044-8486(92)90353-M

Danulat, E. (1987). Digestibility of Chitin in cod,*Gadus morhua*, *In Vivo*. *Helgol. Meeresunters* 41, 425–436. doi:10.1007/bf02365402

Danulat, E., and Kausch, H. (1984). Chitinase Activity in the Digestive Tract of the Cod, *Gadus morhua* (L.). *J. Fish. Biol.* 24, 125–133. doi:10.1111/j.1095-8649.1984.tb04784.x

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., Drew, M. D., van Kessel, A. G., et al. (2012). Effects of Plant-Based Diets on the Distal Gut Microbiome of Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture* 350-353, 134–142. doi:10.1016/j.aquaculture.2012.04.005

Dias, J., Gomes, E. F., and Kaushik, S. J. (1997). Improvement of Feed Intake through Supplementation with an Attractant Mix in European Seabass Fed Plant-Protein Rich Diets. *Aquat. Living Resour.* 10, 385–389. doi:10.1051/alr:1997043

Doucha, J., and Lívanský, K. (2008). Influence of Processing Parameters on Disintegration of Chlorella Cells in Various Types of Homogenizers. *Appl. Microbiol. Biotechnol.* 81, 431–440. doi:10.1007/s00253-008-1660-6

Duncan, R. J., and Petrou, K. (2022). Biomolecular Composition of Sea Ice Microalgae and its Influence on Marine Biogeochemical Cycling and Carbon Transfer through Polar Marine Food Webs. *Geosciences* 12, 38. doi:10.3390/geosciences12010038

Dybern, B. I. (1965). The Life Cycle of *Ciona Intestinalis* (L.) *F. Typica* in Relation to the Environmental Temperature. *Oikos* 16, 109–131. doi:10.2307/3564870

Eder, K., and Kirchgessner, M. (1994a). The Effect of Zinc Deficiency on Heart and Brain Lipids in Rats Force-Fed with Coconut Oil or Fish Oil Diets. *Z. Ernahrungswiss* 33, 136–145. (in German). doi:10.1007/BF01622226

Eder, K., and Kirchgessner, M. (1994b). Dietary Fat Influences the Effect of Zinc Deficiency on Liver Lipids and Fatty Acids in Rats Force-Fed Equal Quantities of Diet. *J. Nutr.* 124, 1917–1926.

El-Sayed, A.-F. M., and Izquierdo, M. (2021). The Importance of Vitamin E for Farmed Fish – A Review. *Rev. Aquac.* 14, 688–703. doi:10.1111/raq.12619

Gaye-Siessegger, J., Focken, U., Abel, H., and Becker, K. (2007). Influence of Dietary Non-essential Amino Acid Profile on Growth Performance and Amino Acid Metabolism of Nile tilapia, *Oreochromis niloticus* (L.). *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 146, 71–77. doi:10.1016/j.cbpa.2006.09.025

Gong, Y., Sørensen, S. L., Dahle, D., Nadanasabesan, N., Dias, J., Valente, L. M. P., et al. (2020). Approaches to Improve Utilization of *Nannochloropsis Oceanica* in Plant-Based Feeds for Atlantic Salmon. *Aquaculture* 522. doi:10.1016/j.aquaculture.2020.735122

Grant, B. F., Seib, P. A., Liao, M.-L., and Corpron, K. E. (1989). Polyphosphorylated L-Ascorbic Acid: A Stable Form of Vitamin C for Aquaculture Feeds. *J. World Aquac. Soc.* 20, 143–157. doi:10.1111/j.1749-7345.1989.tb00568.x

Greenwood, N. N., and Earnshaw, A. (1997). *Chemistry of the Elements*. 2nd ed. Oxford: Butterworth-Heinemann. ISBN 978-0-7506-3365-9.

Grzimek, B. (1972). *Grzimek's Animal Life Encyclopedia: Volume 3-Mollusks and Echinoderms*. New York: Van Nostrand Reinhold Company.

Hamre, K., Hjeltnes, B., Kryvi, H., Sandberg, S., Lorentzen, M., and Lie, Ø. (1994). Decreased Concentration of Hemoglobin, Accumulation of Lipid Oxidation Products and Unchanged Skeletal Muscle in Atlantic Salmon (*Salmo salar*) Fed Low Dietary Vitamin E. *Fish. Physiol. Biochem.* 12, 421–429. doi:10.1007/bf00004306

Hara, T. J. (2006). Feeding Behaviour in Some Teleosts Is Triggered by Single Amino Acids Primarily through Olfaction. *J. Fish. Biol.* 68, 810–825. doi:10.1111/j.0022-1112.2006.00967.x

Hara, T. J., Sveinsson, T., Evans, R. E., and Klaprat, D. A. (1993). Morphological and Functional Characteristics of the Olfactory and Gustatory Organs of Three Salvelinus Species. *Can. J. Zool.* 71, 414–423. doi:10.1139/z93-058

Harrison, S. T., Dennis, J. S., and Chase, H. A. (1991). Combined Chemical and Mechanical Processes for the Disruption of Bacteria. *Bioseparation* 2, 95–105.

Heal, G., and Schlenker, W. (2009). Economics: Sustainable Fisheries. *Nature* 455, 1044–1045.

Helliwell, K. E., Lawrence, A. D., Holzer, A., Kudahl, U. J., Sasso, S., Kräutler, B., et al. (2016). Cyanobacteria and Eukaryotic Algae Use Different Chemical Variants of Vitamin B12. *Curr. Biol.* 26, 999–1008. doi:10.1016/j.cub.2016.02.041

Henry, M., Gasco, L., Piccolo, G., and Fountoulaki, E. (2015). Review on the Use of Insects in the Diet of Farmed Fish: Past and Future. *Animal Feed Sci. Technol.* 203, 1–22. doi:10.1016/j.anifeedsci.2015.03.001

Ikeda, I., Hosokawa, H., Shimeno, S., and Takeda, M. (1991). Studies on Feeding Stimulants for Jack Mackerel-III. Feeding Stimulant Activity of Nucleotides, Tryptophan, and Their Related Compounds for Jack Mackerel. *Nippon. Suisan Gakkaishi* 57, 1539–1542. doi:10.2331/suisan.57.1539

Jafri, A. K. (1998). Growth, Feed Conversion, Body Composition and Nutrient Retention Efficiencies in Fingerling Catfish, *Heteropneustes Fossilis* (Bloch), Fed Different Sources of Dietary Carbohydrate. *Aquac. Res.* 30, 43–49.

Jonas-Levi, A., and Martinez, J.-J. I. (2017). The High Level of Protein Content Reported in Insects for Food and Feed Is Overestimated. *J. Food Compos. Analysis* 62, 184–188. doi:10.1016/j.jfca.2017.06.004

Khan, M. I., Shin, J. H., and Kim, J. D. (2018). The Promising Future of Microalgae: Current Status, Challenges, and Optimization of a Sustainable and Renewable Industry for Biofuels, Feed, and Other Products. *Microb. Cell. Fact.* 17, 36. doi:10.1186/s12934-018-0879-x

Kiron, V., Sørensen, M., Huntley, M., Vasanth, G. K., Gong, Y., Dahle, D., et al. (2016). Defatted Biomass of the Microalga, *Desmodesmus Sp. Can Replace Fishmeal Feeds Atl. salmon Front. Mar. Sci.* 3, 2016. doi:10.3389/fmars.2016.00067

Kousoulaki, K., Albrektsen, S., Langmyhr, E., Albrektsen, S., Olsen, H. J., Campbell, P., et al. (2009). The Water Soluble Fraction in Fish Meal (Stickwater) Stimulates Growth in Atlantic Salmon (*Salmo salar* L.) Given High Plant Protein Diets. *Aquaculture* 289, 74–83. doi:10.1016/j.aquaculture.2008.12.034

Kousoulaki, K., Baeverfjord, G., Nygaard, H., and Norén, F. (2020a). *Cultivation of Tunicates as Novel Ingredient in Fish Feed*, 35. Nofima Report K-33/2020, April 2020.

Kousoulaki, K., Berge, G. M., Mørkøre, T., Krasnov, A., Baeverfjord, G., Ytrestøyl, T., et al. (2020b). Microalgal *Schizochytrium Limacinum* Biomass Improves Growth and Filet Quality when Used Long-Term as a Replacement for Fish Oil, in Modern Salmon Diets. *Front. Mar. Sci.* 7, 57. 14 February 2020. doi:10.3389/fmars.2020.00057

Kousoulaki, K., Krasnov, A., Ytteborg, E., Sweetman, J., Pedersen, M. E., Høst, V., et al. (2021). A Full Factorial Design to Investigate Interactions of Variable Essential Amino Acids, Trace Minerals and Vitamins on Atlantic Salmon Smoltification and Post Transfer Performance. *Aquac. Rep.* 20, 100704. doi:10.1016/j.aqrep.2021.100704

Kousoulaki, K., Mørkøre, T., Nengas, Y., Nengas, I., Berge, R. K., and Sweetman, J. (2016). Microalgae and Organic Minerals Enhance Lipid Retention Efficiency and Fillet Quality in Atlantic Salmon (*Salmo salar* L.). *Aquaculture* 451, 47–57. doi:10.1016/j.aquaculture.2015.08.027

Kousoulaki, K., Østbye, T-K. K., Krasnov, A., Torgersen, J. S., Mørkøre, T., and Sweetman, J. (2015). Microalgae Feed for Future Omega-3 Rich Farmed Fish: Fish Metabolism, Health and Fillet Nutritional Quality. *J. Nutr. Sci.* 4 (e24), 1–13. doi:10.1017/jns.2015.14

Kousoulaki, K., Rønnestad, I., Olsen, H. J., Rathore, R., Campbell, P., Nordrum, S., et al. (2013). Krill Hydrolysate Free Amino Acids Responsible for Feed Intake Stimulation in Atlantic Salmon (*Salmo salar*). *Aquacult Nutr.* 19, 47–61. doi:10.1111/anu.12094

Landis, W. G., Hughes, J. S., and Lewis, M. A. (1993). *Environmental Toxicology and Risk Assessment*. Philadelphia, PA: American Society for Testing Materials.

Leaver, M. J., Villeneuve, L. A., Obach, A., Jensen, L., Bron, J. E., Tocher, D. R., et al. (2008). Functional Genomics Reveals Increases in Cholesterol Biosynthetic Genes and Highly Unsaturated Fatty Acid Biosynthesis after Dietary Substitution of Fish Oil with Vegetable Oils in Atlantic Salmon (*Salmo salar*). *BMC Genomics* 9, 299. doi:10.1186/1471-2164-9-299

Lernoud, J., and Willer, H. (2018). *Organic Agriculture Worldwide: Current Statistics Research Institute of Organic Agriculture (FiBL), Frick, and*. Bonn: IFOAM-Organics International.

Li, P., and Gatlin, D. M., III (2006). Nucleotide Nutrition in Fish: Current Knowledge and Future Applications. *Aquaculture* 251, 141–152. doi:10.1016/j.aquaculture.2005.01.009

Lindsay, G. J. H., Walton, M. J., Adron, J. W., Fletcher, T. C., Cho, C. Y., Cowey, C. B., et al. (1984). The Growth of Rainbow Trout (*Salmo Gairdneri*) Given Diets Containing Chitin and its Relationship to Chitinolytic Enzymes and Chitin Digestibility. *Aquaculture* 37, 315–334. doi:10.1016/0044-8486(84)90297-7

Liu, P., Lu, X., Li, N., Zheng, Z., Zhao, R., Tang, X., et al. (2019). Effects and Mechanism of Free Amino Acids on Browning in the Processing of Black Garlic. *J. Sci. Food Agric.* 99, 4670–4676. Epub 2019 May 3. PMID: 30906992. doi:10.1002/jsfa.9707

Lock, E. R., Arsiwalla, T., and Waagbø, R. (2016). Insect Larvae Meal as an Alternative Source of Nutrients in the Diet of Atlantic Salmon (*Salmo salar*) Postsmolt. *Aquacult Nutr.* 22, 1202–1213. doi:10.1111/anu.12343

Lu, Y., Mu, D., Xue, Z., Xu, P., Li, Y., Xiang, W., et al. (2021). Life Cycle Assessment of Industrial Production of Microalgal Oil from Heterotrophic Fermentation. *Algal Res.* 58, 102404. doi:10.1016/j.algal.2021.102404

Maisey, J. G. (1994). Predator-prey Relationships and Trophic Level Reconstruction in a Fossil Fish Community. *Environ. Biol. Fish.* 40, 1–22. doi:10.1007/bf00002179

Martin-Creuzburg, D., and Merkel, P. (2016). Sterols of Freshwater Microalgae: Potential Implications for Zooplankton Nutrition. *J. Plankton Res.* 38, 865–877. doi:10.1093/plankt/fbw034

Martin-Creuzburg, D., Oexle, S., and Wacker, A. (2014). Thresholds for Sterol-Limited Growth of Daphnia Magna: a Comparative Approach Using 10 Different Sterols. *J. Chem. Ecol.* 40, 1039–1050. doi:10.1007/s10886-014-0486-1

Mathew, A. P., and Dufresne, A. (2002). Morphological Investigation of Nanocomposites from Sorbitol Plasticized Starch and Tunicin Whiskers. *Biomacromolecules* 3, 609–617. doi:10.1021/bm0101769

Matsumiya, M., Arakane, Y., Haga, A., Muthukrishnan, S., and Kramer, K. J. (2006). Substrate Specificity of Chitinases from Two Species of Fish, Greenling,*Hexagrammos otakii*, and Common Mackerel,Scomber Japonicus, and the Insect, Tobacco Hornworm,Manduca Sexta. *Biosci. Biotechnol. Biochem.* 70, 971–979. doi:10.1271/bbb.70.971

Miller, M. R., Nichols, P. D., and Carter, C. G. (20072007). Replacement of Fish Oil with Thraustochytrid Schizochytrium Sp. L Oil in Atlantic Salmon Parr (*Salmo salar* L) Diets. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 148, 382–392. doi:10.1016/j.cbpa.2007.05.018

Nakano, T., Sato, M., and Takeuchi, M. (1993). Superoxide Dismutasc Activity in Lite Skin of Fish. *J. Fish Biol.* 43, 492–496. doi:10.1111/j.1095-8649.1993.tb00585.x

Nickelson, R., and Finne, G. (1992). "Fish, Crustaceans, and Precooked Seafoods. Ch. 47," in *Compendium of Methods for the Microbiological Examination of Foods*. Editors C. Vanderzant and D. F. Splittstoesser. 3rd ed. (Washington, DC: American Public Health Association), 875–895.

Nogales-Merida, S., Gobbi, P., Jozefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., et al. (2019). Insect Meals in Fish Nutrition. *Rev. Aquac.* 11, 1080–1103. doi:10.1111/raq.12281

Norambuena, F., Lewis, M., Hamid, N. K., Hermon, K., Donald, J. A., and Turchini, G. M. (2013). Fish Oil Replacement in Current Aquaculture Feed: is Cholesterol a Hidden Treasure for Fish Nutrition?. *PloS one* 8 (12), e81705. doi:10.1371/journal.pone.0081705

Nøstbakken, O. J., Duinker, A., Rasinger, J. D., Nilsen, B. M., Sanden, M., Frantzen, S., et al. (2018). Factors Influencing Risk Assessments of Brominated Flame-Retardants; Evidence Based on Seafood from the North East Atlantic Ocean. *Environ. Int.* 119, 544–557. doi:10.1016/j.envint.2018.04.044

Opstvedt, J., Mundheim, H., Nygård, E., Aase, H., and Pike, I. H. (2000). Reduced Growth and Feed Consumption of Atlantic Salmon (*Salmo salar* L.) Fed Fish Meal Made from Stale Fish Is Not Due to Increased Content of Biogenic Amines. *Aquaculture* 188, 323–337. doi:10.1016/s0044-8486(00)00343-4

Orlov, A. V., Gerasimov, Y. V., and Lapshin, O. M. (2006). The Feeding Behaviour of Cultured and Wild Atlantic Salmon, *Salmo salar* L., in the Louvenga River, Kola Peninsula, Russia. *ICES J. Mar. Sci.* 63, 1297–1303. doi:10.1016/j.icesjms.2006.05.004

PhI, C. P. V., Walraven, M., Bézagu, M., Lefranc, M., and Ray, C. (2020). Industrial Symbiosis in Insect Production-A Sustainable Eco-Efficient and Circular Business Model. *Sustainability* 12, 10333. doi:10.3390/su122410333

Polymeros, K., Kaimakoudi, E., Mitsoura, A., Nikouli, E., and Mente, E. (2014). The Determinants of Consumption for Organic Aquaculture Products-Evidence from Greece. *Aquac. Econ. Manag.* 18 (1), 45–59. doi:10.1080/13657305.2014.855954

Prabhakaran, K., Ghosh, D., Chapman, G. D., and Gunasekar, P. G. (2008). Molecular Mechanism of Manganese Exposure-Induced Dopaminergic Toxicity. *Brain Res. Bull.* 76, 361–367. doi:10.1016/j.brainresbull.2008.03.004

Putnam, G. B. (1976). "Ascorbic Acid Destruction in Moist Diets," in Proc. 27th Northwest Fish Culture Conference, Twin Falls, Idaho, Dec. 1-2, 1976.

Quang Tran, H., Van Doan, H., and Stejskal, V. (2022). Environmental Consequences of Using Insect Meal as an Ingredient in Aquafeeds: A Systematic View. *Rev. Aquacult* 14, 237–251. doi:10.1111/raq.12595

Ramos-Elorduy, J., Pino, J. M., and González, O. (1981). Digestibilidad *In Vitro* de algunos insectos comestibles de México. *Folia Entomol. Mex.* 49, 141e152.

Refstie, S., Svihus, B., Shearer, K. D., and Storebakken, T. (1999). Nutrient Digestibility in Atlantic Salmon and Broiler Chickens Related to Viscosity and Non-starch Polysaccharide Content in Different Soyabean Products. *Animal Feed Sci. Technol.* 79, 331–345. doi:10.1016/s0377-8401(99)00026-7

Rojas, V., Morales-Lange, B., Avendaño-Herrera, R., Poblete-Morales, M., Tapia-Cammas, D., and Guzmán, F. (2018). Favorable Energy Conversion Efficiency of Coupling Dark Fermentation and Microalgae Production from Food Wastes. *Energy Convers. Manag.* 166, 156–162. doi:10.1016/j.enconman.2018.04.032

Rojas, V., Sánchez, D., Gallardo, J. A., and Mercado, L. (2018). Histopathological Changes Induced by Caligus Rogercresseyi in Rainbow Trout (Oncorhynchus mykiss). *Lat. Am. J. Aquat. Res.* 46, 843–848. doi:10.3856/vol46-issue4-fulltext-23

Rumsey, G. L., Winfree, R. A., and Hughes, S. G. (1992). Nutritional Value of Dietary Nucleic Acids and Purine Bases to Rainbow Trout (Oncorhynchus mykiss). *Aquaculture* 108, 97–110. doi:10.1016/0044-8486(92)90321-B

Safi, C., Charton, M., Pignolet, O., Silvestre, F., Vaca-Garcia, C., and Pontalier, P.-Y. (2013). Influence of Microalgae Cell Wall Characteristics on Protein Extractability and Determination of Nitrogen-To-Protein Conversion Factors. *J. Appl. Phycol.* 25, 523–529. doi:10.1007/s10811-012-9886-1

Samuelsen, T. A., Haustveit, G., and Kousoulaki, K. (2022). The Use of Tunicate (*Ciona intestinalis*) as a Sustainable Protein Source in Fish Feed - Effects on the Extrusion Process, Physical Pellet Quality and Microstructure. *Animal Feed Sci. Technol.* 284, 115193. doi:10.1016/j.anifeedsci.2021.115193

Samuelsen, T. A., Mjøs, S. A., and Oterhals, Å. (2013). Impact of Variability in Fishmeal Physicochemical Properties on the Extrusion Process, Starch Gelatinization and Pellet Durability and Hardness. *Animal Feed Sci. Technol.* 179, 77–84. doi:10.1016/j.anifeedsci.2012.10.009

Samuelsen, T. A., Mjøs, S. A., and Oterhals, Å. (2014). Influence of Type of Raw Material on Fishmeal Physicochemical Properties, the Extrusion Process, Starch Gelatinization and Physical Quality of Fish Feed. *Aquacult Nutr.* 20, 410–420. doi:10.1111/anu.12093

Samuelsen, T. A., Oterhals, Å., and Kousoulaki, K. (2018). High Lipid Microalgae (*Schizochytrium Sp.*) Inclusion as a Sustainable Source of N-3 Long-Chain PUFA in Fish Feed—Effects on the Extrusion Process and Physical Pellet Quality. *Animal Feed Sci. Technol.* 236, 14–28. doi:10.1016/j.anifeedsci.2017.11.020

Samuelsen, T. A., and Oterhals, Å. (2016). Water-soluble Protein Level in Fishmeal Affects Extrusion Behaviour, Phase Transitions and Physical Quality of Feed. *Aquacult Nutr.* 22, 120–133. doi:10.1111/anu.12235

Sandnes, K., Torrissen, O., and Waagbø, R. (1992). The Minimum Dietary Requirement of Vitamin C in Atlantic Salmon (*Salmo salar*) Fry Using Ca Ascorbate-2-Monophosphate as Dietary Source. *Fish. Physiol. Biochem.* 10, 315–319. doi:10.1007/bf00004480

Sørensen, M. (2012). A Review of the Effects of Ingredient Composition and Processing Conditions on the Physical Qualities of Extruded High-Energy Fish Feed as Measured by Prevailing Methods. *Aquac. Nutr.* 18, 233–248. doi:10.1111/j.1365-2095.2011.00924.x

Sørensen, M., Berge, G. M., Reitan, K. I., and Ruyter, B. (2016). Microalga *Phaeodactylum Tricornutum* in Feed for Atlantic Salmon (*Salmo salar*) - Effect on Nutrient Digestibility, Growth and Utilization of Feed. *Aquaculture* 460, 116–123. doi:10.1016/j.aquaculture.2016.04.010

Sveen, L., Timmerhaus, G., Johansen, L.-H., and Ytteborg, E. (2021). Deep Neural Network Analysis - a Paradigm Shift for Histological Examination of Health and Welfare of Farmed Fish. *Aquaculture* 532, 736024. doi:10.1016/j.aquaculture.2020.736024

Thedinga, J. F., Johnson, S. W., and Mortensen, D. G. (2006). Habitat, Age, and Diet of a Forage Fish in Southeastern Alaska: Pacific Sandfish (*Trichodon Trichodon*). *Fish. Bull.* 104, 631–637.

Tibbetts, S. M. (2018). *The Potential for 'next-Generation', Microalgae-Based Feed Ingredients for Salmonid Aquaculture in Context of the Blue Revolution*. London: Microalgal Biotechnology, 151–175. IntechOpen.

Tocher, D., Betancor, M., Sprague, M., Olsen, R., and Napier, J. (2019). Omega-3 Long-Chain Polyunsaturated Fatty Acids, EPA and DHA: Bridging the Gap between Supply and Demand. *Nutrients* 11, 89. doi:10.3390/nu11010089

Toppe, J., Aksnes, A., Hope, B., and Albrektsen, S. (2006). Inclusion of Fish Bone and Crab By-Products in Diets for Atlantic Cod, *Gadus morhua*. *Aquaculture* 253, 636–645. doi:10.1016/j.aquaculture.2005.09.015

Turon, V., Trably, E., Fouilland, E., and Steyer, J.-P. (2016) Potentialities of Dark Fermentation Effluents as Substrates for Microalgae Growth: A Review, 1843–1854. doi:10.1016/j.procbio.2016.03.018

Uauy, R., Stringel, G., Thomas, R., and Quan, R. (1990). Effect of Dietary Nucleotides on Growth and Maturation of the Developing Gut in the Rat. *Process Biochem.* 51, 497–503.

Van den Berg, M., Birnbaum, L. S., Denison, M., De Vito, M., Farland, W., Feeley, M., et al. (2006). The 2005 World Health Organization Reevaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-like Compounds. *Toxicol. Sci.* 93, 223–241. doi:10.1093/toxsci/kfl055

Vernon, B., Dauguet, J. C., and Billard, C. (1998). Sterolic Biomarkers in Marine Phytoplankton. II. Free and Conjugated Sterols of Seven Species Used in Mariculture. *J. Phycol.* 34, 273–279.

Volkman, J. K., Jeffrey, S. W., Nichols, P. D., and Rogers, G. I. (1989). Fatty Acid And Lipid Composition Of 10 Species Of Microalgae Used In Mariculture. *J. Experimental Marine Biol. Eco.* 128, 219–240. doi:10.1016/0022-0981(89)90029-4

Waagbo, R., Glette, J., Raa-Nilsen, E., and Sandnes, K. (1993). Dietary Vitamin C, Immunity and Disease Resistance in Atlantic Salmon (*Salmo salar*). *Fish. Physiol. Biochem.* 12, 61–73. doi:10.1007/BF00004323

Wang, M., Cheng, H., Chen, S., Wen, S., Wu, X., Zhang, D., et al. (2018). Microalgal Cell Disruption via Extrusion for the Production of Intracellular Valuables. *Energy* 142, 339–345. doi:10.1016/j.energy.2017.10.061

Weththasinghe, P., Lagos, L., Øvrum Hansen, J., Mydland, L. T., and Øverland, M. (2019). "The Effect of Dietary Black Soldier Fly Larvae *Hermetia Illucens* Meal and Paste on Growth Performances and Immune Parameters in Atlantic Salmon *Salmo salar*," in Aquaculture Europe 2019, Berlin, Germany, 7-10 October 2019.

Wu, T., Yu, X., Hu, A., Zhang, L., Jin, Y., and Abid, M. (2015). Ultrasonic Disruption of Yeast Cells: Underlying Mechanism and Effects of Processing Parameters. *Innovative Food Sci. Emerg. Technol.* 28, 59–65. doi:10.1016/j.ifset.2015.01.005

Yancheva, V. S., Georgieva, E. S., Velcheva, I. G., Iliev, I. N., Vasileva, T. A., Petrova, S. T., et al. (2014). Biomarkers in European Perch (*Perca fluviatilis*) Liver from a Metal-Contaminated Dam Lake. *Biologia* 69, 1615–1624. doi:10.2478/s11756-014-0460-y

Yilmaz, E. (2005). The Effects of Two Chemo-Attractants and Different First Feeds on the Growth Performances of African Catfish (*Clarias gariepinus*, Burchell, 1822) at Different Larval Stages. *Turkish J. Veterinary & Animal Sci.* 29, 309–314. doi:10.3906/vet-0305-30

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Intracellular trafficking of fatty acids in the fish intestinal epithelial cell line RTgutGC

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The shift towards higher inclusion of vegetable oils (VOs) in aquafeeds has resulted in major changes in dietary fatty acid composition, especially increased amounts of monounsaturated fatty acids (MUFAs) and decreased polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs). However, little is known about how this change in fatty acid (FA) profile affects the intracellular fate of these fatty acids in the intestinal cells. To investigate this topic, we used the rainbow trout intestinal epithelial cell line (RTgutGC) as an *in vitro* model. The cells were incubated with either palmitic acid (16:0, PA), oleic acid (18:1n-9, OA), or arachidonic acid (20:4n-6, ARA), to represent the SFA, MUFA, and PUFA, respectively. In all experiments, the RTgutGC were incubated with either non-labeled or radiolabeled FA (PA, OA, or ARA) for 16 h at 19^{0}C. The cells were then analyzed for the occurrence of cytosolic lipid droplets (CLD) with confocal microscopy, transcriptomic analysis (non-labeled FA experiments) and lipid class composition in the cells and serosal media from the basolateral side of the cells (radiolabeled FA experiments). CLD accumulation was higher in RTgutGC exposed to OA compared to cells given PA or ARA. This was coupled with increased volume, diameter, and surface area of CLDs in OA treated cells than with other FAs (PA, ARA). The results from radiolabeled FAs performed on permeable transwell inserts showed that OA increased the triacylglycerides (TAG) synthesis and was primarily stored in the cells in CLDs; whereas a significant amount of ARA was transported as TAG to the basolateral compartment. A significant proportion of free FAs was found to be excreted to the serosal basolateral side by the cells, which was significantly higher for PA and OA than ARA. Although there were clear clusters in differentially expressed genes (DEGs) for each treatment group, results from transcriptomics did not correlate to lipid transport and CLD analysis. Overall, the accumulation of TAG in CLDs was higher for oleic acid (OA) compared to arachidonic acid (ARA) and palmitic acid (PA). To conclude, carbon chain length and saturation level of FA differently regulate their intracellular fate during fatty acid absorption.

KEYWORDS

RTgutGC, fatty acids transport, lipid accumulation, cytosolic lipid droplets, triacylglycerides

Introduction

As opposed to most vertebrates, dietary triacylglycerides (TAG) are completely hydrolyzed in most teleost fish (Bogevik et al., 2008). The absence of monoacylglycerol as a digestive product in these fish is a consequence of the evolutionary loss of colipase (Sæle et al., 2018). Digested dietary fat is taken up by enterocytes, re-esterified into complex lipids in the endoplasmic reticulum and subsequently directed to synthesis of lipoproteins for transport or stored in cytosolic lipid droplets (CLDs) (Sire et al., 1981; Sheridan, 1988; Sigurgisladottir et al., 1992; Bogevik et al., 2008). CLDs are temporary lipid storage molecules, consisting of a core of neutral lipids, mostly TAG and esters, surrounded by monolayer phospholipids. It has been shown that high lipid diets increase the number and size of CLDs. Moreover, replacing fish oil with vegetable oils (VOs) could also influence the CLD accumulation (Deplano et al., 1989; Olsen et al., 1999; Olsen et al., 2000; Caballero et al., 2002; Caballero et al., 2003). Although CLDs are considered as temporary lipid storage organelles, they serve several crucial physiological functions, including sequestering toxic lipid molecules and preventing lipotoxicity, maintenance of endoplasmic reticulum membrane homeostasis, regulation of fatty acid (FA) storage, and transport (Walther and Farese, 2012; Olzmann and Carvalho, 2019). However, excessive accumulation of CLDs may also cause damage to the cells and create pathogenicity (Schaffer, 2003). For instance, studies in Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) have demonstrated extensive damage to enterocytes due to excessive accumulation of CLDs caused by VO diets high in monounsaturated fatty acids (MUFA) (Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003).

Due to the loss of colipase in fish, dietary TAG is completely hydrolyzed to single fatty acids (FAs). This leaves the monoacylglycerol pathway without substrate and all absorbed FAs are resynthesized into TAG *via de novo* synthesis and ultimately transported into CLDs within the enterocytes. We hypothesize that chylomicrons/VLDL (very low-density lipoprotein) can be generated for export only after the synthesis and storage of TAG within lipid droplets. Further, we hypothesize that the intracellular trafficking of FAs in the enterocytes is affected by carbon chain length and saturation, hence there will be consequences of changing the dietary FA composition. Therefore, the present work is aimed to study the effects of carbon length and unsaturation levels on intracellular lipid trafficking and CLDs formation *in vitro* using RTgutGC (Rainbow trout gut cell). The current study used three different approaches: *(i)* live-cell imaging for lipid droplets formation, *(ii)* radiolabeled FAs for lipid class analysis, and *(iii)* whole cell transcriptomic analysis. The FAs chosen for the current study were palmitic acid (PA), oleic acid (OA), and arachidonic acid (ARA), to represent the saturated fatty acid (SFA), MUFA, and polyunsaturated fatty acid (PUFA), respectively. These FAs were selected based on their relevance to current feeding practices in salmonids aquaculture. In particular, increased use of vegetable oils in fish feed has resulted in decreased dietary n-3 PUFA and SFA (PA); and increased n-9 (MUFA) and n-6 PUFA.

The RTgutGC cell derived from rainbow trout intestine exhibits apical and basolateral characteristics of intestinal epithelial cells (Kawano et al., 2011). It has been proposed as a physiologically adequate fish intestinal epithelial model, which is equivalent to human intestinal epithelial cells (Caco2 cells) (Kawano et al., 2011; Minghetti et al., 2017). In addition, RTgutGC contains functionally active polarized absorptive cells (enterocytes) that play a central role in lipid metabolism. In general, enterocytes are responsible for assimilation of luminal FAs and also support the *de novo* synthesis of FAs and cholesterol and serve as a site for production of major lipid transport proteins such as apolipoproteins. Recently, the RTgutGC cell line has been used as a model to study nutrient uptake (Antony Jesu Prabhu et al., 2018; Kim et al., 2018; Pumputis et al., 2018), test functional ingredients and gut immune function (Wang et al., 2019; Holen et al., 2021) and toxicity (Langan et al., 2017; Schug et al., 2020). To our best knowledge, most studies of FA uptake and trafficking by intestinal epithelial cells were conducted in immortalized cell lines such as Caco2 and IEC-6 as enterocyte models. Furthermore, FA uptake and transport system in fish were largely studied *in vivo*, where dietary lipids are more complex, and finding the fate of individual FA is more onerous. Thus, the current study would be the first trial using fish enterocyte cell model (RTgutGC) to study the intracellular lipid trafficking and CLDs formation in fish intestinal epithelial cells.

Materials and methods

Routine RTgutGC cell culture

The intestinal epithelial cell line from rainbow trout (RTgutGC) was obtained from the Swiss Federal Institute of Aquatic Science and Technology (Eawag), Switzerland, through a material transfer agreement. RTgutGC cells were routinely cultured as per the methods described by Kawano et al. (2011). In brief, cells were grown in a 75 cm^2 culture flask with Leibovitz' L-15 complete medium (21083027, Gibco Thermofisher), supplemented with 10% fetal bovine serum (F7524, Sigma Aldrich) and 1% Antibiotic Antimycotic Solution (A5955, Sigma Aldrich) maintained at 19°C under normal atmosphere. After reaching confluency in 7-10 days, cells were sub-cultured for routine maintenance as per the method described by Kawano et al. (2011), or harvested to be used in experiments.

Preparation of FA - BSA complexes

The non-radio labeled palmitic acid (PA, P0500, Merk), oleic acid (OA, O1008, Merk), or arachidonic acid (ARA, 10931,

Merk) were conjugated to FA-free bovine serum albumin (BSA, A6003, Sigma Aldrich), as per the method previously described by Nøstbakken et al. (2012). In brief, an appropriate amount of FA was weighed in a dark glass container to yield 3.46 mM concentration, and 0.04 ml chloroform per mg FA was added to completely dissolve the FA. After evaporating chloroform under the N_2 stream, the residue was dissolved in 0.124 M KOH at the ratio of 1:3 and vortexed continuously for 10 min. FA free-BSA (1.5 mM) dissolved in serum-free culture media (Leibovitz' L-15) was added in a 2.5:1 molar ratio to the FA and stirred continuously in water bath for 45 min at 37°C. The final concentration of FA was 3.46 mM, filter sterilized and stored in -20°C until use.

Lipotoxicity test using xCELLigence system

The lipotoxicity effects of FA in RTgutGC were carried out according to the methods described by (Berger et al., 2017). In brief, the E96 xCELLigence plate was prepared by adding 50 µl of culture media to each well and incubating in xCELLigence for 30 minutes with background corrections. The cells were seeded at a density of 20,000 cells/well in 100 µl culture media. The cell adhesion and proliferation were monitored every 15 min by the xCELLigence system. Approximately 24 h after seeding, when the cells were in the log growth phase, the cells were exposed to 50 µL of medium containing the BSA conjugated FA (PA, OA, or ARA) at concentrations 50-1000 uM in triplicate, and the experiments were continued for another 24 h. Controls received either medium only or medium + BSA.

Visualization of intracellular lipid droplets and image analysis

RTgutGC cells were seeded on µ-slide 8 well plates with a chambered coverslip (Ibidi GmbH, 80826) at a density of 1.5 x 10^5 cells/ml and incubated at 19°C for 48 h prior to FAs treatment. Cells were then washed twice with PBS and incubated with 200 µMFAs (PA, OA, or ARA) conjugated with BSA for 16 h at 19°C. BSA in serum-free cell culture media but no FA was used as a control. After the incubation, cells were washed twice with PBS and incubated with LipidSpot™ 488 (green) lipid droplet stain (1:1000) (Biotium, 70065-T) in complete cell culture medium at 19°C for 30 min as per the manufacturer's instructions, protected from light prior to imaging. Visualization of lipid droplets was achieved by Ti-E inverted microscope (Nikon, Japan) with a CFI Super Plan Fluor ELWD ADM 20 X C PH-1 objective, numerical aperture 0.45 (Nikon, Japan), and a C2 + confocal scanner (Nikon, Japan). Images were acquired using an oil immersion 60× objective, and Z-stacks were taken with a defined Z-step size of 0.93µm and 1024 x 1027 pixels. All the acquired 3D constructions were background-subtracted and analyzed using NIS Elements AR v.4.51 (Nikon, Tokyo, Japan) software to obtain data on CLDs. The total number of CLDs per cell, and the volume, diameter, surface area, and sphericity of individual CLDs were obtained. In addition, the percentage of cells that accumulated CLDs was also analyzed manually, using a minimum threshold of 5 CLDs per cell. Three independent experiments were conducted, representing 3 replicates (n=3). Each experiment consisted of three wells for each FA (PA, OA, or ARA) and the control group.

Lipid class analysis in RTgutGC exposed to radiolabeled FAs

Preparation of uptake medium

The radiolabeled ^{3}H-Palmitic acid (9,10-^{3}H(N), NET043001MC, 1mCi, PerkinElmer), ^{3}H-Oleic acid (9,10-^{3}H (N), NET289001MC, 1mCi, PerkinElmer) and^{3}H-Arachidonic acid (5,6,8,9,11,12,14,15-^{3}H(N), NET298Z050UC, 50µCi, PerkinElmer) were conjugated to non-radiolabeled FA-BSA stock solution as described earlier. An appropriate amount of radiolabeled FAs [^{3}H] was taken in a dark glass tube and dried under N_2 gas, and BSA conjugated FA (3.46 mM) was added, followed by sonication in a water bath sonicator for 1 hr. In all the cases, the final working media contained ~200 µM FAs with radioactive concentration 1µCi/ml (~37 kBq/ml) and specific activities of 0.33 mCi/mmol for each FA.

Cell culture on permeable membranes

RTgutGC cells from routine culture flasks were trypsinized, counted and were seeded at a density of 75,000 cells/cm^2 onto apical compartment of 6 wells in commercially available permeable transwell-membrane inserts (ThinCert® cell culture inserts, pore size = 0.4 µm; polyethylene terephthalate [PET] from Greiner Bio-One, Germany) as described previously (Geppert et al., 2016; Minghetti et al., 2017). The apical and basolateral compartments were filled with 2 and 3 ml of complete L-15 culture media, respectively, and the culture media from both the apical and basolateral compartments were changed once every 3 days. The cells were grown for 18 days at 19°C before the experiments. The transepithelial electric resistance (TEER) was measured to assess the tightness of the cell monolayers by using the epithelial volt-ohm meter (EVOM) with dual "chopstick" electrodes (World Precision Instruments, New Haven, CT), and TEER values were calculated as per the method described by Geppert et al. (2016).

Incubation with radiolabeled FAs

RTgutGC cells grown on permeable transwell-membrane inserts were taken out of the incubator, and the medium from apical and basolateral sides was removed. The cells (apical side) were washed twice with PBS and then cells were incubated with FA

by replacing apical medium with 2 ml of medium containing FA conjugated with BSA with a final concentration of about 200 µM (PA, OA or ARA), containing 1 µCi/ml labeled FAs [^{3}H]; specific activities 0.33 mCi/mmol). The basolateral medium was replaced with serum-free medium containing only BSA. The cells were incubated for 16 h at 19°C. After the incubation period, samples (medium from the apical compartment, basolateral compartment, and the cells along with membrane) were collected in chloroform: methanol solution (1:1) for lipid extraction.

Lipid extraction and thin-layer chromatography

Thin-layer chromatography (TLC) was used to determine the type of lipids being secreted basolaterally from the cells following apical exposure to [^{3}H] FAs. Firstly, lipids from each sample were extracted by Folch's liquid-liquid extraction method using chloroform solvent. After phase separation, the aqueous phase was removed and discarded, and the remaining total organic extract was evaporated under a gentle stream of N_2 prior to TLC analysis. Extracted lipid was spotted on thin-layer chromatography plates (20 cm x 10 cm, Silica Gel) and developed in hexane-ethyl ether-acetic acid (80:20:1). Lipid standards consisting of triacylglycerides (TAG), free-fatty acids, and cholesterol were included in the analysis. The plates were developed with iodine vapor and the spots corresponding to TAG and FFA were scraped off into 20 ml scintillation vials and dissolved in 3 ml dichloromethane plus 15 ml ultima gold scintillation liquid. The amount of radioactivity [^{3}H] was measured in a scintillation counter (Packard Tri-Carb Liquid scintillation counter, Model 1900TR). The final concentration of each FA was calculated as follows,

$$\text{FA concentration (pmol)} = \frac{\left(\frac{DPM}{a} \times b \times 10^{12}\right)}{SA}$$

Where,
DPM, Disintegrations per minute
a= 60, conversion factor for DPM to becquerel (Bq)
b= $2.7\text{x}10^{-11}$, conversion factor for becquerel to curie
SA, radioactive specificity of FA (mCi/mmol)

Transcriptomic analysis of RTgutGC cell exposed to different FAs

The RTgutGC cells were seeded in 6-well conventional culture plates at a density of 1.5 x 10^5 cells/ml in complete L15/FBS medium and incubated at 19°C for 3 days to reach the confluency (~80%). Subsequently, the medium was removed from the cells and then the cells were washed twice with PBS before treating with FAs. The FA (PA, OA, or ARA) conjugated with BSA (as described earlier) was added to the cells at a concentration of 200 µM and incubated at 19°C for 16 h. After exposure to the respective FAs, the medium was removed, and 0.4 mL of RTL lysis buffer (Qiagen, Germany) was added for 30s. The cell lysates were transferred to 2 ml tubes and stored in −80°C freezer until required for RNA isolation. Three independent parallel experiments were conducted, representing 3 replicates and n=9 for each FA (PA, OA, and ARA) and the control group.

RNA extraction

RNA isolation was performed with the RNeasy Plus Mini Kit including genomic DNA eliminator columns (Qiagen, Germany). Three independent parallel experiments were conducted, representing 3 replicates and n=9 for each FA (PA, OA, and ARA) and control group. RNA concentration and purity were measured using a Nanodrop Spectrophotometer (NanoDrop®, ND-1000, Thermo Fischer, USA). Integrity of RNA was checked for all the samples using bioanalyzer as per the method provided by manufacturer (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). All the samples except one sample from OA treatment group, had RIN values above 8.0 and were used for cDNA library preparation.

Library preparation and sequencing for RNA-seq

Library preparation and RNA-sequencing (RNA-seq) were performed by Novogene's UK Sequencing Centre, Cambridge, UK. The messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After purified RNA was fragmented, the first strand cDNA was synthesized using random hexamer primers followed by end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. RNA-seq libraries were sequenced on Illumina Novaseq 6000 platform, where 150-bp paired-end reads were obtained. The raw RNA-Seq data were deposited and released in the sequence read archive database, with the BioProject accession number of PRJNA763330.

Rainbow trout genome and genomic annotation

The reference sequence data (Omyk_1.0) were downloaded from the NCBI assembly site (https://www.ncbi.nlm.nih.gov/assembly/GCF_002163495.1).

Quality trimming, alignment and quantification of RNA-seq reads

Adaptors, low-quality bases, low-quality reads (phred scores< Q30) and reads less than 20 bases in length were trimmed from

sequence reads using cutadapt (Martin, 2011). Quality trimmed reads were aligned to the Rainbow trout RefSeq reference genome (Omyk_1.0). STAR (Dobin et al., 2013) was used with the default parameters to index the reference genome and align reads to the indexed genome. The number of reads per gene was quantified using featureCounts (Liao et al., 2014), based on genomic coordinates provided by the general feature format (GFF) file from the Omyk_1.0 reference genome. The number of the reads that uniquely mapped on protein-coding genes was 68.7% in average (Table S1). The precomp function of R (https://CRAN.R-project.org) was used to perform principal component analysis (PCA) prior to differential expression analysis.

Differential gene expression analysis

Differential expression analysis was performed in a pair-wise manner by comparing three different FAs (PA, OA, or ARA) against the control group using the DESeq2 package (Love et al., 2014). The minimum log fold change (lfc) was set to log2(1.5) using the lfcThreshold option in the results function to ensure that all DEGs were >log2(1.5) and<-log2(1.5). After p-values were adjusted by the Benjamini-Hochberg procedure, genes were identified as differentially expressed genes (DEGs) when adjusted p-values were less than 0.1. Heatmaps of overlapped DEGs were generated with the Complex Heatmap package (Gu et al., 2016). The in-house RNA-seq workflow was coordinated in a pipeline by Snakemake (Koster and Rahmann, 2012) with various R and Python scripts along with multiple bioinformatics tools.

Statistical analysis

Statistical analysis was performed using the software Statistica 13.4 (Statsoft Inc., Tulsa, OK, USA) and GraphPad Prism version 8.0 (Graphpad Software Inc., San Diago, CA, USA). Data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov test and Shapiro-Wilk test. Data were analyzed by one-way ANOVA, and comparisons between FAs were performed using Tukey's *post-hoc* analysis. Three independent experiments were conducted for CLDs analysis and transcriptomics studies, and two experiments were done for radiolabeled lipid class analysis. All experiments were performed in triplicate and values are expressed as mean ± SEM.

Results

Lipotoxicity test

The lipotoxicity test was performed for the FAs (PA, OA, and ARA) conjugated with BSA in the range of 50-1000 μM (Figure 1). The strength of cell adhesion is represented as the Cell Index (CI) which is a unit-less measurement. The cell index of cells beginning from 20,000 cells/well increased as the cell number increased. The CI was normalized at the time point before FA was added. The RTgutGC exposed to different concentration of FA (PA, OA, or ARA) grew continuously irrespective of FA concentration (50, 100, 200, 300, 400, 600, 800 and 1000 μM). However, there was significant reduction in cell index for cells exposed to higher concentration (800 and 1000 μM; $P<0.05$). Further, cell peak growth was observed between 200-300uM and 200uM was chosen for all the FA exposure experiments.

Intracellular cytosolic lipids droplets

The intracellular CLDs formation was significantly influenced by FAs supplementation in culture media (Figure 2A). The percentage of cells that accumulated CLDs was significantly different between treatments ($P<0.0001$), and the highest percentage was found for OA (97 ± 0.9%) followed by ARA (52 ± 3.6%), PA (34 ± 3.6%), and then control (13 ± 3.0%) (Figure 3A). The number of CLDs accumulated per cell was significantly ($P< 0.0001$) higher for OA (117.8 ± 7.7) compared to PA (21.10 ± 1.3), ARA (26.6 ± 2), and control (11.6 ± 1.0) and no significant differences were found between PA vs ARA ($P=0.77$) (Figure 3B). Subsequently, the mean volume of the CLDs was also significantly ($P< 0.0001$) higher for OA (2.8 ± 0.2 compared to PA (0.62 ± 0.03), ARA (0.76 ± 0.06), and the control (0.52 ± 0.02) (Figure 3C). The surface area of the CLDs (Figure 3D) was significantly ($P= 0.0001$) higher for cells supplemented with OA (12.8 ± 0.5) compared to PA (4.7 ± 0.2), ARA (4.7 ± 0.2) and the control (3.9 ± 0.14). Likewise, the diameter of the CLDs (Figure 3E) was also significantly ($P=0.0001$) higher for cell treated with OA (1.2 ± 0.01) than compared to other treatment groups (PA, 0.86 ± 0.01; ARA, 0.80 ± 0.001; Control, 0.79 ± 0.0001). The sphericity of the CLDs (~0.7) was similar for all treatment groups (Figure 3F).

Lipid classes during FA transport

TEER levels increased and stabilized at an average of 33 Ω cm^{-2} after 18 days of culture with significant difference observed over time (Figure 4). TEER values reached a stable plateau at approximately 8-10 days. Cells seemed to stop proliferating but remained viable. The total amount of [^{3}H] FFA and [^{3}H] TAG was measured from the three fractions, i.e., apical, basolateral, and as well as cells on the permeable membrane (Figures 5, 6). The percentage of [^{3}H] labeled FAs recovered from FFA and TAG fractions were 91.3 ± 2.1, 58.7 ± 7.0, and 56 ± 7.6% for OA, PA, and ARA respectively (Figure 6). The saturation level of the added FA significantly influenced the uptake of FA from the apical medium by the cells. The amount of recovered [^{3}H] labelled FFA from the apical medium was similar for ARA

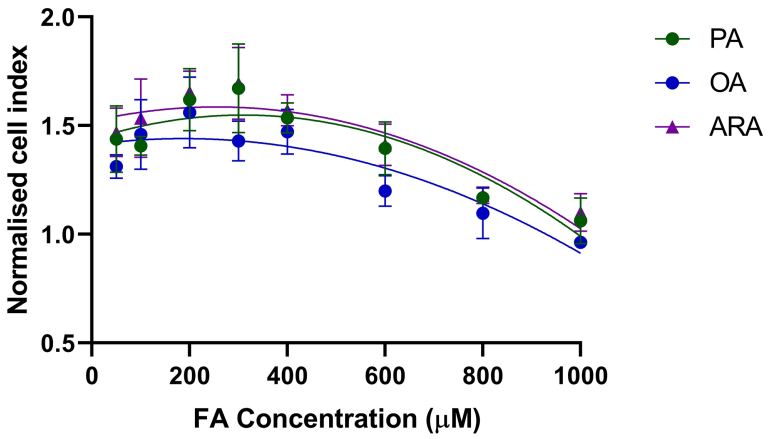

FIGURE 1
Normalized cell index of RTgutGC cells exposed to FA (PA, OA, or ARA). Cells were seeded at a density of 20,000 cells/well in a 96-well E-plate allowing the cells to adhere and proliferate for 24 h. Cells were then exposed to FA (PA, OA, or ARA) conjugated with BSA complex in the range of 50 – 1000 μM and monitored for 24 h using an xCELLigence system. Data shown here are cell adhesion as mean normalized cell index at 24 h exposure. PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid.

(347.9 ± 7.3 pmol; 50.4%) and PA (346.2 ± 4.7 pmol; 51.1%), while a numerically higher amount was observed for OA (512.4 ± 14.7 pmol; 73.5%); however, no significant difference was observed between them due to variation between the trials (P=0.3) (Figures 5A, 6). The amount of recovered [^{3}H] FFA from the basolateral medium was significantly higher for PA (19.3 ± 1.4 pmol, P=0.02; 3.5%) and OA (17.9 ± 1.0 pmol, P=0.03; 3%) compared to ARA (5.3± 0.3 pmol; 1.1%), with no significant difference found between PA and OA (P=0.8) (Figures 5B, 6). Although similar trends were seen for the [^{3}H] FFA recovered from the cell fraction, no significant difference was observed among FAs treatments (P=0.29) (Figure 5C). Similarly, the amount of [^{3}H] recovered as TAG was measured for the medium from the apical and basolateral compartments,

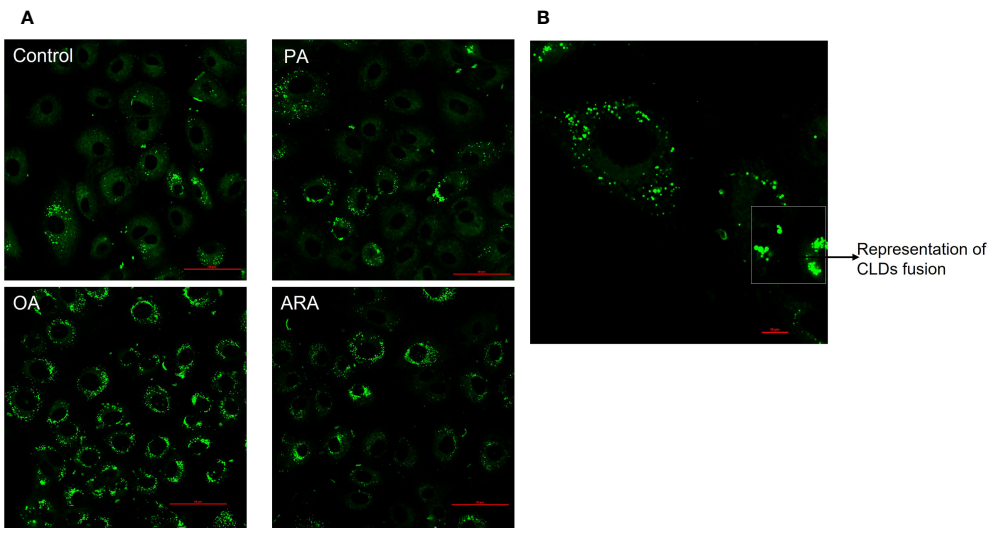

FIGURE 2
FAs differentially regulate lipid droplets accumulation in RTgutGC cells. **(A)** Representative confocal imaging of RTgutGC cells treated with 200 μM of FAs (PA, OA or ARA) control for16 h at 19°C and stained with LipidSpot^{TM} 488 (green); Scale bar: 50μm. **(B)** Representative image of CLDs fusion in RTgutGC cells treated with 200 μM of oleic acid; scale bar: 10μm. Scale bar: 10μm. All images were acquired using an oil immersion 60× objective, and Z-stacks were taken with a defined Z-step size of 0.93μm and 1024 X 1027 pixels. PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid.

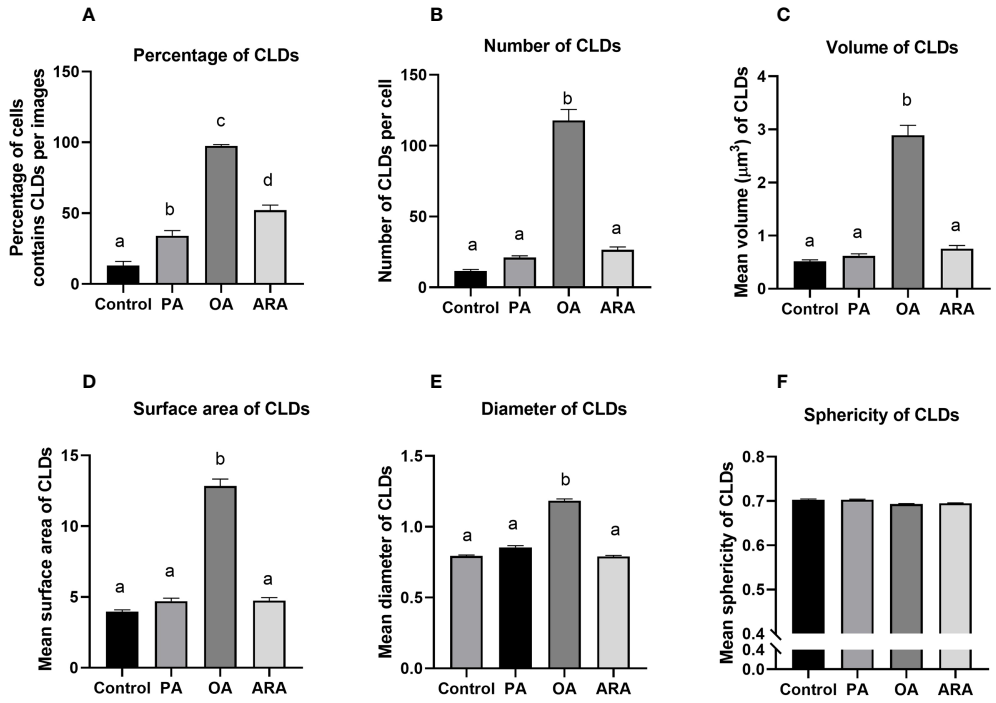

FIGURE 3
Quantification of CLDs accumulated in RTgutGC cells treated with 200 µM of FAs (PA, OA or ARA) and control for16 h at 19°C and stained with LipidSpot™ 488 (green). The Percentage of cells contained CLDs per images **(A)**, number of CLDs per cell **(B)**, mean individual volume **(C)**, surface area **(D)**, diameter **(E)**, and sphericity **(F)** of CLDs in RTgutGC cells. Different superscript (small letters) indicates statistical significance as obtained through one-way ANOVA followed by Tukey's multiple comparisons. Data are from N = 18 images analyzed from three independent experiments and are shown as mean ± SEM. PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid.

and the cells. The cells supplemented with ARA had a significantly higher amount of [^{3}H] TAG in the basolateral compartment (4.1 ± 0.3 pmol, P=0.006; 1.1%) compared to PA (0.12 ± 0.03; 0.03%) and OA (P=0.34 ± 0.12; 0.05%), with no differences between the latter two groups (P=0.9) (Figures 5E, 6). Conversely, the cell fraction had a significantly higher amount of [^{3}H] TAG for the group supplemented with OA (72.7 ± 10.3 pmol, P=0.005; 13.8%) compared to PA (20.6 ± 1.2 pmol; 3.3%) and ARA (13.1 ± 0.2 pmol; 2.8%), and no differences were found between the latter two groups (P=0.56) (Figures 5F, 6). There was no difference in [^{3}H] TAG content found in the apical fraction between the FAs (P=0.57; Figure 5D).

Transcriptomics

A summary of total RNA-seq reads generated for each sample, the corresponding STAR alignment results, and the alignment count data can be found in supplementary file 1. In general, transcriptomics analysis was performed on 35 samples of RTgutGC cells incubated for 16 h with their respective FAs conjugated with BSA (control, PA, OA, or ARA). Approximately, 21.2 to 30 million paired-end reads were obtained per sample. The results of the principal component analysis (PCA) with the normalized counts (Figure 7A) showed that PA group was distinctly separated to control group with OA and ARA being intermediary and overlapping with each other. Further, the differentially expressed genes (DEGs) were calculated as log fold changes [lfc ≥log2(1.5)] along with the P-values (adjusted P-value of 0.1) by comparing FAs (PA, OA, or ARA) against control. The DEGs analysis revealed a total of 1294, 459, and 249 DEGs for PA, ARA, and OA, respectively. Out of that, 114, 26, and 55 DEGs were shared by every combination of two groups, namely PA and ARA, PA and OA, OA and ARA, respectively. Concurrently, 1003, 139, and 17 were explicitly expressed in PA, ARA, and OA, respectively (Figure 7B).

A total of 489 target genes involved in FAs absorption, transport, lipid droplet synthesis, FA synthesis and oxidation, TAG synthesis and hydrolysis, phospholipid synthesis, etc., were selected by using the rainbow trout gene database (NCBI, Ensemble) (See supplementary file 2 for a full list of genes analyzed). Out of 489 analyzed target genes, more DEGs were observed in the ARA treatment group (6.5%; 32 DEGs), followed by OA (4.1%; 20 DEGs) and PA (3.7%; 18 DEGs) (Figure 8),

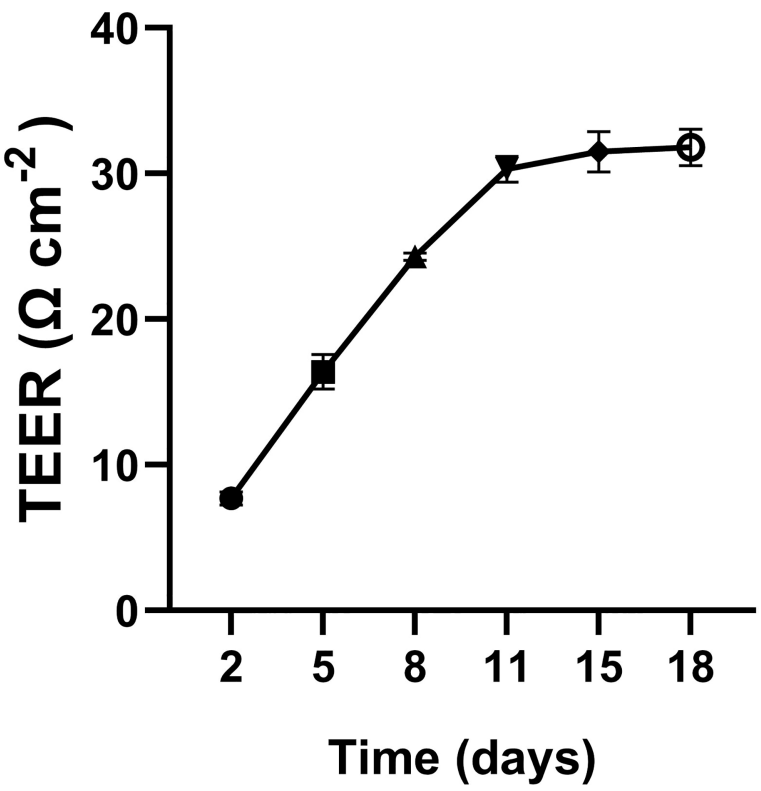

FIGURE 4
Transepithelial electrical resistance (TEER) of RTgutGC cells. Cells were seeded at a density of 75,000 cells/cm^2 on transwell membrane inserts (0.4 µm pore size) and grown for up to 18 days.

when compared to control group. A heatmap was constructed for selected target genes that were identified as DEGs (Figure 8). Several transcripts involved in FA uptake and activation (lfc ≥1.5, *fatp1-like-1, fatp1-like-2* and *abcf2a9*) were significantly upregulated in all FA supplement groups. Among the transcripts related to the TAG synthesis pathway that were analyzed, more DEGs (10) were identified in the PA group, and most of them were upregulated. On the other hand, the TAG synthesis pathway was downregulated (5 out of 6 DEGs) in OA and ARA. However, transcripts involved in TAG hydrolysis (*lpl-like-1, lpl-like-2, and pnpla2-like1*) were more downregulated in ARA followed by OA and PA. Further, the expression of *mttp*, a key protein involved in chylomicron assembly, was significantly upregulated in all FA groups compared to control group, however, this expression was significantly higher in PA (lfc, 1.49) than ARA (lfc, 1.12) and OA (lfc, 1.03). Surprisingly, no other transcripts involved in lipoprotein synthesis were differentially expressed except *sar1b* upregulation in ARA and OA. Associated with lipid droplet formation, out of 7 analyzed *plin* transcripts (*plin 1*, *plin 2*, *plin 3*, and *plin 6*), two transcripts from *plin 2* (*plin-2like1*; *plin-2 like2*) had differential expression and were significantly upregulated in all groups. Furthermore, the upregulated *plin 2* was higher in PA (*plin-2like1*, lfc 6.1; *plin-2like1*, lfc 3.5) than in ARA (*plin-2like1*, lfc 5.1; *plin-2like1*, lfc 3.1) and OA (*plin-2like1*, lfc 4.2; *plin-2like1*, lfc 3.1) groups. However, none of the transcripts in apolipoprotein synthesis were differentially expressed. In particular, very low expression was observed for Apo B. On the other hand, expression of *apoa I-IV*, *apoc*, *apod* and *apoe* was prominent, but no DEGs. More downregulated transcripts involved in FA synthesis (*acsl4a*, *acsbg2*, *acs like-1*, *acs like-2*, *fasn-like-1*, *fasn-like-1*, *acac*) were observed in ARA and OA, and their downregulation was higher in ARA compared to OA. Whereas transcripts involved in FA synthesis *(acsl4a*, lfc 0.5*; acsbg2*, lfc 0.9*; scd*, lfc 0.6, *acot1-like*, lfc 2.1) were upregulated in PA. In respect to the β-oxidation pathway, more upregulated DEGs were identified in all FAs groups, and the upregulation was significantly higher in the ARA group than compared to OA and PA, and the lowest expression was observed in PA. Interestingly no analyzed transcripts involved in endoplasmic reticulum (ER) stress (*edem*, ER degradation enhancer, mannosidase; *hsp70*, Heat shock protein 70) were significantly affected by FAs supplements.

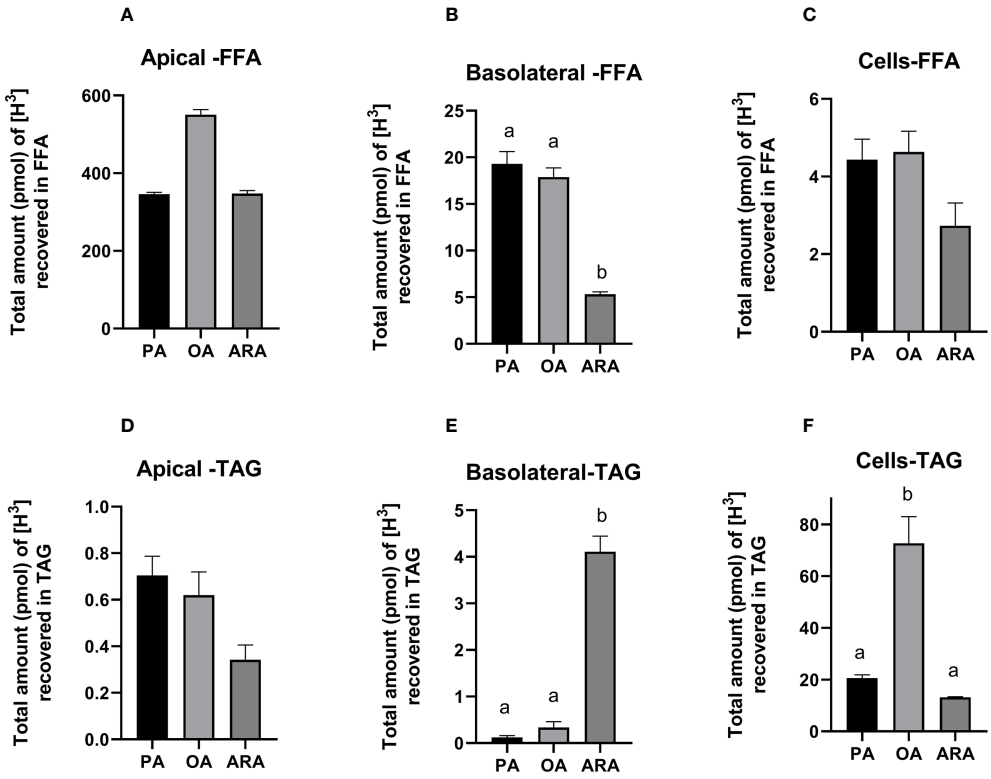

FIGURE 5
Lipid class analysis of RTgutGC cells grown on transwell membrane inserts for 18 days and then exposed to [^{3}H] labelled FAs (PA, OA or ARA) for 16 h at 19°C. FFA from apical **(A)**, basolateral **(B)** and the cells fractions **(C)**; TAG from apical **(D)**, basolateral **(E)** and the cell fractions **(F)** measured as the total amount (pmol) of [^{3}H] recovered after scintillation counting. Different superscript (small letters) indicates statistical significance as obtained through one-way ANOVA followed by Tukey's multiple comparisons. Data from two independent experiments in triplicate (N=2; n=3) and are shown as mean ± SEM. PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid; TAG, Triacylglycerides.

Discussion

In the current study, we demonstrated how the absorption and intracellular trafficking of FAs vary according to their carbon chain length and saturation level in the RTgutGC cell line. To the best of our knowledge, the present study is the first of its kind using a fish enterocyte cell line as a model to investigate the intracellular fates of different FAs. However, a human intestinal Caco2 cell line, derived from colorectal adenocarcinoma, has been extensively used as an enterocyte cell model to study the mechanism of FAs absorption and intracellular transport (Ranheim et al., 1994; Van Greevenbroek et al., 1995; Trotter et al., 1996; Nauli and Whittimore, 2015). The majority of the studies in Caco2 cells have demonstrated that MUFA (OA) induced a higher rate of TAG synthesis and CLDs than SFA and PUFA (Field et al., 1988; Dashti et al., 1990; Levin et al., 1992; Bateman et al., 2007), thus explaining that each FA has its own fate inside the enterocyte according to their chain length and degree of saturation (Yonezawa et al., 2004b; Vargas-Bello-Pérez et al., 2019). Similarly, OA-induced CLD accumulation has also been observed in other cell types such as HepG2 hepatocytes (Eynaudi et al., 2021), pancreatic β-cells (Cnop et al., 2001), bovine mammary epithelial cells (Yonezawa et al., 2004a) and H9C2 cardiomyoblasts (Akoumi et al., 2017). *In vitro* studies in adipocytes isolated from Atlantic salmon have demonstrated the increased CLDs in response to OA treatment (Todorcević et al., 2008; Bou et al., 2020). In addition to *in vitro* studies, several *in vivo* studies in fish have shown similar mechanisms of excessive LD accumulation in intestinal tissue in response to diets containing vegetable oils high in MUFA (Fontagné et al., 1998; Olsen et al., 1999; Olsen et al., 2000; Caballero et al., 2002; Caballero et al., 2003; Olsen et al., 2003). In accordance with these reports, in the present study we also observed a higher rate of TAG synthesis and subsequent CLD accumulation in OA treated cells. Hence, the RTgutGC cells respond similarly as other well-tested cell lines and *in vivo* studies, and it is deemed to be a suitable enterocyte model to investigate the intracellular fate of individual FAs.

Long chain fatty acid (LCFA) transported into cells are acylated into long-chain acyl-CoA by the action of acyl-CoA synthetases and are then destined for either TAG synthesis or β-

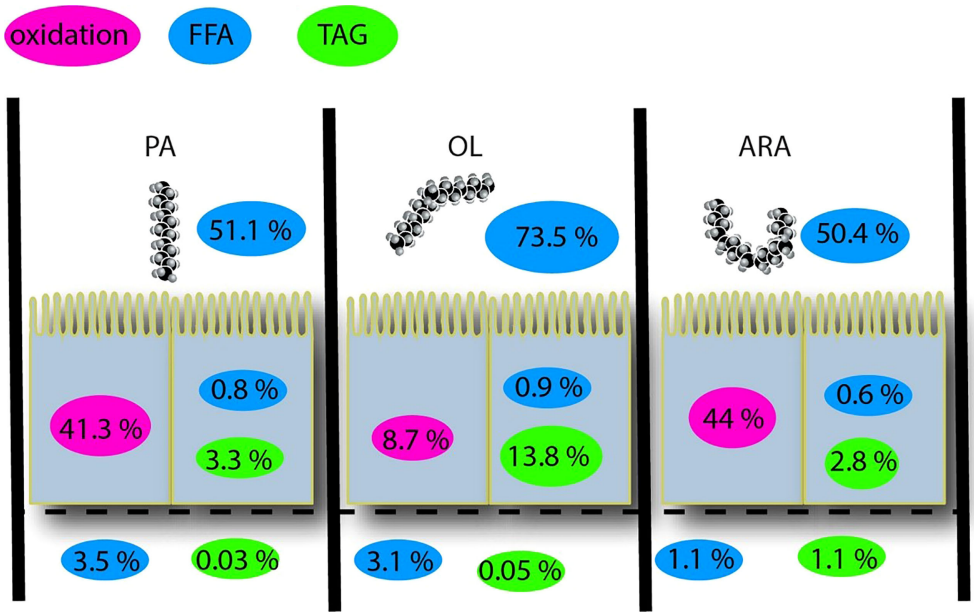

FIGURE 6
Summary of percentage of FFA and TAG in different compartment of RTgutGC cells grown on transwell membrane inserts for 18 days and then exposed to [^{3}H] labelled FAs (PA, OA or ARA) for 16 h at 19°C. FA, Fatty acid; PA, Palmitic acid; OA, Oleic acid; ARA, Arachidonic acid; TAG, Triacylglycerides; CLDs, cytosolic lipid droplets. Ox, β-Oxidation.

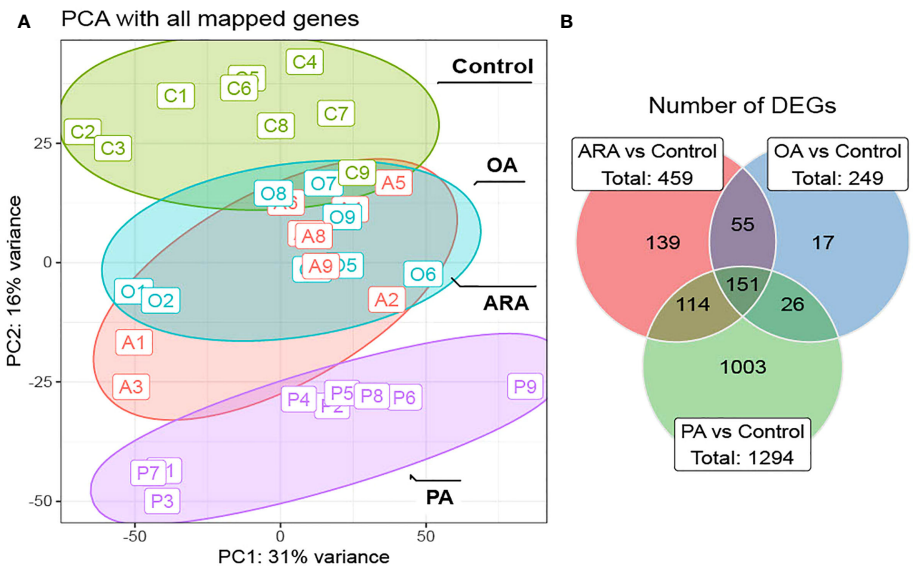

FIGURE 7
Transcriptomics analysis of RTgutGC cells treated with 200 μM of FAs (PA, OA or ARA) control for16 h at 19°C. **(A)** PCA biplot of differentially expressed genes of RNAseq counts. **(B)** Venn diagram depicting the number of common and unique genes showing differential expression in PA, OA and ARA treatment groups. 1.5- log fold change (adjusted P-value of 0.01). Differential expression analysis was performed in a pair-wise manner by comparing three different FAs (PA, OA, or ARA) against the control group using the DESeq2 package. Data are from three independent experiments in triplicate (N=3; n=3). PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid.

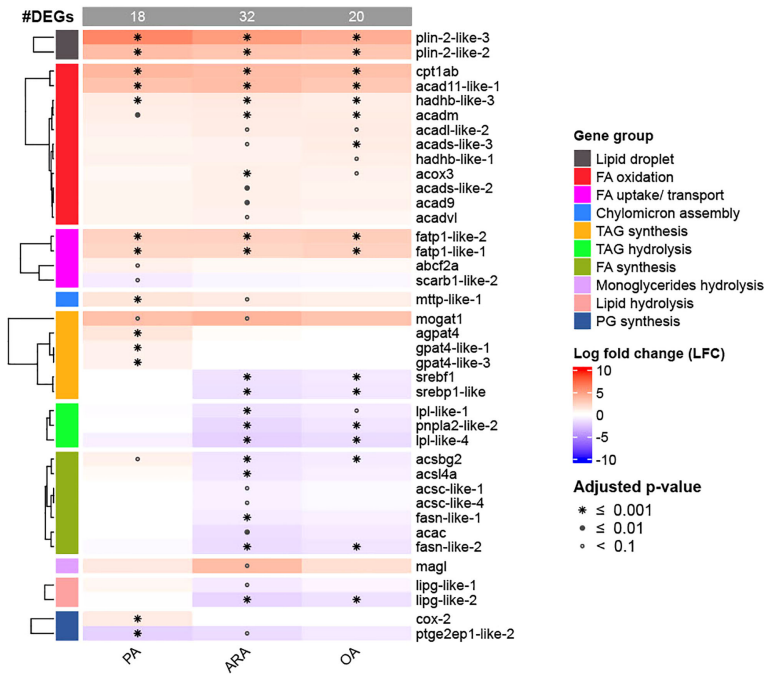

FIGURE 8
Heatmap of DEGs from selected target genes with 1.5- log fold change (adjusted P-value:<0.1). Data are from three independent experiments in triplicate (N=3; n=3). Differential expression analysis was performed in a pair-wise manner by comparing three different FAs (PA, OA, or ARA) against the control group using the DESeq2 package. The number of differentially expressed genes (DEGs) identified was 18, 32 and 20 for PA, ARA, and OA, respectively. PA, Palmitic acid; ARA, Arachidonic acid; OA, Oleic acid.

oxidation (Listenberger et al., 2003). We hypothesized that the fish enterocyte metabolizes PA, OA and ARA differently and that they are stored temporarily as CLDs before being exported to circulation as chylomicrons/VLDL (very low-density lipoprotein). This appears to be true for OA, where we found increased CLD accumulation, although the possible underlying mechanisms remain unclear. Phospholipids and lipoproteins are important components of chylomicrons/VLDL, thus enabling the transport of lipids. Recently, an *in vivo* study in fish has demonstrated that diets deficient in phospholipids leads to accumulation of CLDs, hypothesized to be caused by insufficient lipoprotein synthesis (Gu et al., 2014; Sæle et al., 2018). Furthermore, an earlier study in gilthead seabream (*Sparus aurata*) fed a rapeseed diet containing high amount of OA (46% of total FAs) observed reduced lipoprotein synthesis rates compared to diets lower in OA. They also found lower re-acylation of OA in the phospholipid fraction, suggested to be the reason behind reduced lipoprotein synthesis (Caballero et al., 2003). Hence, one could easily speculate that insufficient lipoprotein synthesis might be one possible reason for the increased CLD accumulation. However, these studies are conducted *in vivo*, where dietary lipid sources are complex mixtures of FAs and lipid classes. In contrast, in the current *in vitro* study, cells were exposed to individual FAs (PA, OA, or ARA) complexed with BSA, meaning that phospholipid level must be the same for all groups. This leads us to question why OA would require more phospholipids than other FAs (PA and ARA) to form chylomicrons/VLDL. The mechanisms behind this remain unclear and need to be addressed in future studies.

Conversely, the lower accumulation of CLDs for PA and ARA might indicate that they are preferentially used for β-oxidation instead of TAG synthesis and LD storage or phospholipid synthesis. This is further supported by quantitative data, where 91% of FAs was recovered for OA, but only 58% of PA and 56% were recovered for PA and ARA, respectively. PA is an important component of phosphatidylcholine (PC) and is required for lipoprotein synthesis (Bell et al., 1985; Olsen et al., 2000). Similarly, PUFAs such as EPA, DHA and ARA are preferentially esterified into phospholipids than less unsaturated FA like OA. This is probably due to their essential function to maintain membrane integrity and functionality of the cells biomembrane (Bell et al., 1985). Therefore, some amount of PA and ARA might be acylated into the phospholipid fractions. Unfortunately, we did not measure the recovered radioactivity in β-oxidation products or the phospholipid fraction and hence

can only speculate that this was the fate of these FA. However, in support of our speculation, an *in vitro* study in the HepG2 cell line demonstrated that PA preferably stimulates mitochondrial oxidative metabolism, while OA results in abundant CLDs with a slower mitochondrial oxidation (Eynaudi et al., 2021). Similarly, a study on isolated hepatocytes from Atlantic salmon reported that ARA, followed by PA were the preferential substrates for β-oxidation compared to OA (Stubhaug et al., 2005). In the present study, significantly lower recovery of [^{3}H] FA for PA and ARA compared to OA, might indicate enhanced β-oxidation of these FA. However, the expression of genes related to β-oxidation does not show any significant difference between FA. Furthermore, the cells that are exposed to high FFA concentrations cause ER stress which may lead to cell dysfunction, and apoptosis (Cui et al., 2013). However, in the present study we did not observe upregulation of transcripts involved in ER stress (*edem, hsp70*).

Additionally, our study showed that OA not only increases the CLDs accumulation, but it also promotes CLDs with larger volume, surface and diameter. This is probably due to fusion of CLDs, assumed to be mediated through members of the SNARE protein family (Boström et al., 2007). Although it is generally accepted that formation of large CLDs protects the cell from lipotoxic effects from excess FFA (Bateman et al., 2007; Ricchi et al., 2009; Eynaudi et al., 2021). However, the formation of large CLDs along with excessive accumulation in intracellular space may also lead to cell dysfunction or cell death (Schaffer, 2003). Accordingly, several *in vivo* studies in fish have demonstrated extensive damage to enterocytes caused by high lipid accumulation (Deplano et al., 1989; Olsen et al., 1999; Olsen et al., 2000). Our study demonstrated that OA contributes to lipid accumulation in enterocytes through increased number and size of CLDs, suggesting that dietary inclusion of OA rich oils should be limited.

The perilipins, encoded by the *plin* genes, are the major proteins associated to CLD surface that regulate lipid droplet stability and turnover (Ko et al., 2020). Among reported *plin* (*plin1*, *plin2, plin3*, and *plin6*) in fish, including rainbow trout, *plin2* is ubiquitously expressed in all tissue and is associated with increased CLDs accumulations. In addition, the microsomal triglyceride transfer protein (*mtp*), primarily involved in lipoprotein assembly and also reported to be present in CLDs, has been found to increase as a response to excessive CLD accumulation (Love et al., 2015). In the current study, all three FA types triggered the overexpression of *plin2* and *mtp* transcripts compared to the control. However, the expression was significantly higher for PA followed by ARA and OA, which is not correlated with the CLDs observed from image analysis. Similar patterns were also observed for the transcripts involved in the TAG pathway (*mogat, agpat4*). A recent study by Etayo et al. (2021) in ballan wrasse (*Labrus bergylta*) fed lipid rich meal, reported the initial increase in gene expression involved in serotonin synthesis and then declined over time post-prandially. Simultaneously, serotonin (5-HT) levels increased in the same tissues. A highly abundant protein will usually have a highly expressed mRNA. Nevertheless, there are factors involved in the process between gene transcription and translation into protein, which can result in a mismatch between gene expression and protein level. For example, the half-life of different proteins can vary from minutes to days, whereas mRNA degrades within hours (Hargrove and Schmidt, 1989). Other possible factors include the lower rate of mRNA transcription than protein translation and possible negative feedback mechanisms, which might also have contributed to the currently observed lack of correlation between mRNA transcripts and CLDs accumulation. A further study with time series samplings could provide additional information in regulations at transcriptional levels.

FA chain length and degree of saturation influence the uptake by intestinal cells (Wang et al., 2013). However, the way it is regulated also depends on several other factors including the number of monomers delivered, membrane solubility, permeability of monolayer, and membrane carrier protein activity (*fabp*, scavenger *cd36, fatp*). In the present study, the mean uptake of labelled OA from the apical compartment was significantly lower than the other FAs, which is concurrent with the increased number and size of CLDs observed. Thus, this result might indicate that excessive accumulation of CLDs in the cytosol may present a physical barrier and cause the lower cellular uptake for this OA (Morais et al., 2007). On the other hand, the uptake of PA and ARA from the apical compartment by cells was quite efficient. The greater uptake of PA may be due to lower water solubility and the greater membrane solubility of PA compared to OA or could also be caused by a greater efficiency of carrier protein (*fabp* and scavenger *cd36, fatp*) in transporting PA compared to OA, despite similar affinity for PA and OA (Trotter et al., 1996). Furthermore, the higher affinity of cytosolic FABP and higher esterification rate for PUFA (Sire et al., 1981; Pérez et al., 1999) could possibly explain the better uptake of complex FAs like ARA.

To transport re-esterified FA (TAG) from enterocytes to systemic circulation and to peripheral tissues, TAG must be incorporated into lipoproteins (*apob, apoa1, apoa4, apoc, apoe*, and *apod*). The *apob* is a major secretory lipoprotein, and its role in transporting TAG from enterocytes to circulation is well recognized. Previous studies in caco2 cells have clearly demonstrated the expression of *apob* both in the presence or absence of FAs supplements (Liao and Chan, 2000; Bateman et al., 2007). Similarly, the induction of *apob* expression in response to diets has also been demonstrated in the fish intestine, including rainbow trout (Kamalam et al., 2013a; Kamalam et al., 2013b). However, in the current study, we observed very low expression of *apob*, and although other apolipoproteins (*apoa1, apoa4, apoc, apoe*, and *apod*) were expressed, no DEGs for any of these apolipoproteins in all treatment groups, including control. This might be related to

the time points at which the cells were sampled after FAs exposure as described in Etayo et al. (2021).

While it has been widely accepted that LCFAs are transported mainly as esterified form (TAG), in the present study, we observed a significant amount of fatty acids as FFA in the basolateral region in the PA and OA treatments, but also some in the ARA group. A possible explanation for this could be that the high amount of FA load in the apical region might cause transport of a certain amount of these FFA directly into the basolateral chamber (and into circulation). Similar results have been observed *in vivo* in Atlantic salmon (Denstadli et al., 2011), where they reported the transport of OA both as esterified lipids (TAG) as well as FFA form in portal blood. Similarly, an earlier study by Kayama and Iijima (1976) in carp fed radiolabeled PA found significant amount of labelled FFA in the circulation and suggested that the FFA plays an important role in fish lipid transport system. Similarly, in the current study, for the more complex FA like ARA, the amount of recovered FFA in the basolateral fraction was quite low compared to PA and OA. On the other hand, the TAG amount in the basolateral fraction was significantly higher for ARA than others. These results might indicate a better regulated intracellular transport of complex FAs like ARA.

Conclusion

The present study demonstrated that RTgutGC cells have the characteristics of absorbing and transporting the FA, which is comparable to similar mammalian cell lines as well as *in vivo* studies in fish or mammals. Thus, RTgutGC could serve as a suitable *in vitro* model to study the intracellular trafficking of fatty acids and their metabolism. As hypothesized, carbon-chain length and saturation level of FA differently regulate the TAG synthesis and subsequent CLDs accumulation, where the accumulation of TAG in CLDs was higher for oleic acid (OA) compared to arachidonic acid (ARA) and palmitic acid (PA). Accumulation of CLDs negatively affected the absorption of FA into the cells, such that PA and ARA are better absorbed by the cells than OA. The relatively higher amount of FFA being transported to the serosal basolateral side for PA and OA is backed up by findings *in vivo* in fish. The lower recovery of radiolabeled FA for PA might suggest that PA and ARA are preferred substrates for β-oxidation over OA, warranting further understanding of intestinal lipid transport in teleost.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

CS- the main author of the manuscript, conducted the experiments, data analysis, and wrote the manuscript. TS- carried out Transcriptomics analysis. NS- conceived and planned the experiments AP- conceived and planned the experiments. ØS - conceived, designed, and coordinated the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.954773/full#supplementary-material

References

Akoumi, A., Haffar, T., Mousterji, M., Kiss, R. S., and Bousette, N. (2017). Palmitate mediated diacylglycerol accumulation causes endoplasmic reticulum stress, Plin2 degradation, and cell death in H9C2 cardiomyoblasts. *Exp. Cell Res.* 354, 85–94. doi: 10.1016/j.yexcr.2017.03.032

Antony Jesu Prabhu, P., Stewart, T., Silva, M., Amlund, H., Ornsrud, R., Lock, E. J., et al. (2018). Zinc uptake in fish intestinal epithelial model RTgutGC: Impact of media ion composition and methionine chelation. *J. Trace Elem Med. Biol.* 50, 377–383. doi: 10.1016/j.jtemb.2018.07.025

Bateman, P. A., Jackson, K. G., Maitin, V., Yaqoob, P., and Williams, C. M. (2007). Differences in cell morphology, lipid and apo b secretory capacity in caco-2 cells following long term treatment with saturated and monounsaturated fatty acids. *Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids* 1771, 475–485. doi: 10.1016/j.bbalip.2007.02.001

Bell, M. V., Henderson, R. J., and Sargent, J. R. (1985). Changes in the fatty acid composition of phospholipids from turbot (*Scophthalmus maximus*) in relation to dietary polyunsaturated fatty acid deficiencies. *Comp. Biochem. Physiol. Part B: Comp. Biochem.* 81, 193–198. doi: 10.1016/0305-0491(85)90182-8

Berger, E., Nassra, M., Atgié, C., Plaisancié, P., and Géloën, A. (2017). Oleic acid uptake reveals the rescued enterocyte phenotype of colon cancer caco-2 by HT29-MTX cells in Co-culture mode. *Int. J. Mol. Sci.* 18, 1573. doi: 10.3390/ijms18071573

Bogevik, A. S., Oxley, A., and Olsen, R. E. (2008). Hydrolysis of acyl-homogeneous and fish oil triacylglycerols using desalted midgut extract from Atlantic salmon, *Salmo salar*. *Lipids* 43, 655–662. doi: 10.1007/s11745-008-3185-2

Boström, P., Andersson, L., Rutberg, M., Perman, J., Lidberg, U., Johansson, B. R., et al. (2007). SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nat. Cell Biol.* 9, 1286–1293. doi: 10.1038/ncb1648

Bou, M., Wang, X., Todorčević, M., Østbye, T.-K. K., Torgersen, J., and Ruyter, B. (2020). Lipid deposition and mobilisation in Atlantic salmon adipocytes. *Int. J. Mol. Sci.* 21, 2332. doi: 10.3390/ijms21072332

Caballero, M. J., Izquierdo, M. S., Kjørsvik, E., Montero, D., Socorro, J., Fernández, A. J., et al. (2003). Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* 225, 325–340. doi: 10.1016/S0044-8486(03)00299-0

Caballero, M. J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., and Izquierdo, M. S. (2002). Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 214, 253–271. doi: 10.1016/S0044-8486(01)00852-3

Cnop, M., Hannaert, J. C., Hoorens, A., Eizirik, D. L., and Pipeleers, D. G. (2001). Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 50, 1771–1777. doi: 10.2337/diabetes.50.8.1771

Cui, W., Ma, J., Wang, X., Yang, W., Zhang, J., and Ji, Q. (2013). Free fatty acid induces endoplasmic reticulum stress and apoptosis of β-cells by Ca2+/Calpain-2 pathways. *PloS One* 8, e59921. doi: 10.1371/journal.pone.0059921

Dashti, N., Smith, E. A., and Alaupovic, P. (1990). Increased production of apolipoprotein b and its lipoproteins by oleic acid in caco-2 cells. *J. Lipid Res.* 31, 113–123. doi: 10.1016/S0022-2275(20)42765-8

Denstadli, V., Bakke, A. M., Berge, G. M., Krogdahl, Å., Hillestad, M., Holm, H., et al. (2011). Medium-chain and long-chain fatty acids have different postabsorptive fates in Atlantic salmon. *J. Nutr.* 141, 1618–1628. doi: 10.3945/jn.111.141820

Deplano, M., Connes, R., Diaz, J. P., and Paris, J. (1989). Intestinal steatosis in the farm-reared sea bass dicentrarchus labrax. *Dis. Aquat. organinsm* 6, 121–130. doi: 10.3354/dao006121

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

Etayo, A., Le, H. T. M. D., Araujo, P., Lie, K. K., and Sæle, Ø. (2021). Dietary lipid modulation of intestinal serotonin in ballan wrasse (*Labrus bergylta*)–*In vitro*. *Analyses. Front. Endocrinol.* 12, 560055. doi: 10.3389/fendo.2021.560055

Eynaudi, A., Díaz-Castro, F., Bórquez, J. C., Bravo-Sagua, R., Parra, V., and Troncoso, R. (2021). Differential effects of oleic and palmitic acids on lipid droplet-mitochondria interaction in the hepatic cell line HepG2. *Front. Nutr.* 8, 775382. doi: 10.3389/fnut.2021.775382

Field, F. J., Albright, E., and Mathur, S. N. (1988). Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCo-2 cells. *J. Lipid Res.* 29, 1427–1437. doi: 10.1016/S0022-2275(20)38423-6

Fontagné, S., Geurden, I., Escaffre, A.-M., and Bergot, P. (1998). Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. *Aquaculture* 161, 213–223. doi: 10.1016/S0044-8486(97)00271-8

Geppert, M., Sigg, L., and Schirmer, K. (2016). A novel two-compartment barrier model for investigating nanoparticle transport in fish intestinal epithelial cells. *Environ. Sci: Nano* 3, 388–395. doi: 10.1039/C5EN00226E

Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32, 2847–2849. doi: 10.1093/bioinformatics/btw313

Gu, M., Kortner, T. M., Penn, M., Hansen, A. K., and Krogdahl, Å. (2014). Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). *Br. J. Nutr.* 111, 432–444. doi: 10.1017/S0007114513002717

Hargrove, J. L., and Schmidt, F. H. (1989). The role of mRNA and protein stability in gene expression. *FASEB J.* 3, 2360–2370. doi: 10.1096/fasebj.3.12.2676679

Holen, E., Austgulen, M. H., and Espe, M. (2021). RNA Form baker's yeast cultured with and without lipopolysaccharide (LPS) modulates gene transcription in an intestinal epithelial cell model, RTgutGC from rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 119, 397–408. doi: 10.1016/j.fsi.2021.10.018

Kamalam, B. S., Médale, F., Larroquet, L., Corraze, G., and Panserat, S. (2013a). Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PloS One* 8, e76570–e76570. doi: 10.1371/journal.pone.0076570

Kamalam, B. S., Panserat, S., Aguirre, P., Geurden, I., Fontagné-Dicharry, S., and Médale, F. (2013b). Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 164, 417–427. doi: 10.1016/j.cbpa.2012.11.020

Kawano, A., Haiduk, C., Schirmer, K., Hanner, R., Lee, L. E. J., Dixon, B., et al. (2011). Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide. *Aquacult Nutr.* 17, e241–e252. doi: 10.1111/j.1365-2095.2010.00757.x

Kayama, M., and Iijima, N. (1976). Studies on lipid transport mechanism in the fish. *Bull. Japanese Soc. Sci. Fish* 42, 987–996. doi: 10.2331/suisan.42.987

Kim, J. J., Pham, P. H., Hamilton, M. E., Lee, L. E. J., and Bols, N. C. (2018). Effect of selenomethionine on cell viability and heat shock protein 70 levels in rainbow trout intestinal epithelial cells at hypo-, normo-, and hyper-thermic temperatures. *J. Thermal Biol.* 76, 107–114. doi: 10.1016/j.jtherbio.2018.07.011

Ko, C. W., Qu, J., Black, D. D., and Tso, P. (2020). Regulation of intestinal lipid metabolism: current concepts and relevance to disease. *Nat. Rev. Gastroenterol. Hepatol.* 17, 169–183. doi: 10.1038/s41575-019-0250-7

Koster, J., and Rahmann, S. (2012). Snakemake–a scalable bioinformatics workflow engine. *Bioinformatics* 28, 2520–2522. doi: 10.1093/bioinformatics/bts480

Langan, L. M., Harper, G. M., Owen, S. F., Purcell, W. M., Jackson, S. K., and Jha, A. N. (2017). Application of the rainbow trout derived intestinal cell line (RTgutGC) for ecotoxicological studies: Molecular and cellular responses following exposure to copper. *Ecotoxicology* 26, 1117–1133. doi: 10.1007/s10646-017-1838-8

Levin, M. S., Talkad, V. D., Gordon, J. I., and Stenson, W. F. (1992). Trafficking of exogenous fatty acids within caco-2 cells. *J. Lipid Res.* 33, 9–19. doi: 10.1016/S0022-2275(20)41878-4

Liao, W., and Chan, L. (2000). Apolipoprotein b, a paradigm for proteins regulated by intracellular degradation, does not undergo intracellular degradation in CaCo2 cells. *J. Biol. Chem.* 275, 3950–3956. doi: 10.1074/jbc.275.6.3950

Liao, Y., Smyth, G. K., and Shi, W. (2014). featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656

Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Ory, D. S., et al. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3077–3082. doi: 10.1073/pnas.0630588100

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. doi: 10.1186/s13059-014-0550-8

Love, J. D., Suzuki, T., Robinson, D. B., Harris, C. M., Johnson, J. E., Mohler, P. J., et al. (2015). Microsomal triglyceride transfer protein (MTP) associates with cytosolic lipid droplets in 3T3-L1 adipocytes. *PloS One* 10, e0135598–e0135598. doi: 10.1371/journal.pone.0135598

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17, 10–12. doi: 10.14806/ej.17.1.200

Minghetti, M., Drieschner, C., Bramaz, N., Schug, H., and Schirmer, K. (2017). A fish intestinal epithelial barrier model established from the rainbow trout (*Oncorhynchus mykiss*) cell line, RTgutGC. *Cell Biol. Toxicol.* 33, 539–555. doi: 10.1007/s10565-017-9385-x

Morais, S., Conceição, L. E. C., Rønnestad, I., Koven, W., Cahu, C., Zambonino Infante, J. L., et al. (2007). Dietary neutral lipid level and source in marine fish larvae: Effects on digestive physiology and food intake. *Aquaculture* 268, 106–122. doi: 10.1016/j.aquaculture.2007.04.033

Nøstbakken, O. J., Goksøyr, A., Martin, S., Cash, P., and Torstensen, B. E. (2012). Marine n–3 fatty acids alter the proteomic response to methylmercury in Atlantic salmon kidney (ASK) cells. *Aquat. Toxicol.* 106-107, 65–75. doi: 10.1016/j.aquatox.2011.10.008

Nauli, A. M., and Whittimore, J. D. (2015). Using caco-2 cells to study lipid transport by the intestine. *J. visualized experiments JoVE.* 102, e53086. doi: 10.3791/53086

Olsen, R. E., Myklebust, R., Kaino, T., and Ringø, E. (1999). Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin. *Fish Physiol. Biochem.* 21, 35–44. doi: 10.1023/A:1007726615889

Olsen, R. E., Myklebust, R., Ringø, E., and Mayhew, T. M. (2000). The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): A quantitative ultrastructural study. *Fish Physiol. Biochem.* 22, 207–216. doi: 10.1023/A:1007879127182

Olsen, R. E., Tore Dragnes, B., Myklebust, R., and Ringø, E. (2003). Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *oncorhynchus mykiss* Walbaum. *Fish Physiol. Biochem.* 29, 181–192. doi: 10.1023/B:FISH.0000045708.67760.43

Olzmann, J. A., and Carvalho, P. (2019). Dynamics and functions of lipid droplets. *Nat. Rev. Mol. Cell Biol.* 20, 137–155. doi: 10.1038/s41580-018-0085-z

Pérez, J. A., Rodríguez, C., and Henderson, R. J. (1999). The uptake and esterification of radiolabelled fatty acids by enterocytes isolated from rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* 20, 125–134. doi: 10.1023/A:1007795516689

Pumputis, P. G., Dayeh, V. R., Lee, L. E. J., Pham, P. H., Liu, Z., Viththiyapaskaran, S., et al. (2018). Responses of rainbow trout intestinal epithelial cells to different kinds of nutritional deprivation. *Fish Physiol. Biochem.* 44, 1197–1214. doi: 10.1007/s10695-018-0511-3

Ranheim, T., Gedde-Dahl, A., Rustan, A. C., and Drevon, C. A. (1994). Fatty acid uptake and metabolism in CaCo-2 cells: Eicosapentaenoic acid (20:5(n-3)) and oleic acid (18:1(n-9)) presented in association with micelles or albumin. *Biochim. Biophys. Acta* 1212, 295–304. doi: 10.1016/0005-2760(94)90203-8

Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., et al. (2009). Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J. Gastroenterol. Hepatol.* 24, 830–840. doi: 10.1111/j.1440-1746.2008.05733.x

Sæle, Ø., Rød, K. E. L., Quinlivan, V. H., Li, S., and Farber, S. A. (2018). A novel system to quantify intestinal lipid digestion and transport. *Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids* 1863, 948–957. doi: 10.1016/j.bbalip.2018.05.006

Schaffer, J. E. (2003). Lipotoxicity: when tissues overeat. *Curr. Opin. Lipidol.* 14 (3), 281–287. doi: 10.1097/00041433-200306000-00008

Schug, H., Maner, J., Hulskamp, M., Begnaud, F., Debonneville, C., Berthaud, F., et al. (2020). Extending the concept of predicting fish acute toxicity *in vitro* to the intestinal cell line RTgutGC. *ALTEX* 37, 37–46. doi: 10.14573/altex.1905032

Sheridan, M. A. (1988). Lipid dynamics in fish: Aspects of absorption, transportation, deposition and mobilization. *Comp. Biochem. Physiol. Part B: Comp. Biochem.* 90, 679–690. doi: 10.1016/0305-0491(88)90322-7

Sigurgisladottir, S., Lall, S. P., Parrish, C. C., and Ackman, R. G. (1992). Cholestane as a digestibility marker in the absorption of polyunsaturated fatty acid ethyl esters in Atlantic salmon. *Lipids* 27, 418. doi: 10.1007/BF02536382

Sire, M. F., Lutton, C., and Vernier, J. M. (1981). New views on intestinal absorption of lipids in teleostean fishes: An ultrastructural and biochemical study in the rainbow trout. *J. Lipid Res.* 22, 81–94. doi: 10.1016/S0022-2275(20)34743-X

Stubhaug, I., Tocher, D. R., Bell, J. G., Dick, J. R., and Torstensen, B. E. (2005). Fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) hepatocytes and influence of dietary vegetable oil. *Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids* 1734, 277–288. doi: 10.1016/j.bbalip.2005.04.003

Todorcević, M., Vegusdal, A., Gjøen, T., Sundvold, H., Torstensen, B. E., Kjaer, M. A., et al. (2008). Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; effects of n-3 and n-9 fatty acids. *Biochim. Biophys. Acta* 1781, 326–335. doi: 10.1016/j.bbalip.2008.04.014

Trotter, P. J., Ho, S. Y., and Storch, J. (1996). Fatty acid uptake by caco-2 human intestinal cells. *J. Lipid Res.* 37, 336–346. doi: 10.1016/S0022-2275(20)37620-3

Van Greevenbroek, M. M., Voorhout, W. F., Erkelens, D. W., Van Meer, G., and De Bruin, T. W. (1995). Palmitic acid and linoleic acid metabolism in caco-2 cells: different triglyceride synthesis and lipoprotein secretion. *J. Lipid Res.* 36, 13–24. doi: 10.1016/S0022-2275(20)39750-9

Vargas-Bello-Pérez, E., Loor, J. J., and Garnsworthy, P. C. (2019). Effect of different exogenous fatty acids on the cytosolic triacylglycerol content in bovine mammary cells. *Anim. Nutr.* 5, 202–208. doi: 10.1016/j.aninu.2018.09.002

Walther, T. C., and Farese, R. V.Jr. (2012). Lipid droplets and cellular lipid metabolism. *Annu. Rev. Biochem.* 81, 687–714. doi: 10.1146/annurev-biochem-061009-102430

Wang, J., Lei, P., Gamil, A., Lagos, L., Yue, Y., Schirmer, K., et al. (2019). Rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cells as a model for studying gut immune function and effects of functional feed ingredients. *Front. Immunol.* 10, 152. doi: 10.3389/fimmu.2019.00152

Wang, T. Y., Liu, M., Portincasa, P., and Wang, D. Q. H. (2013). New insights into the molecular mechanism of intestinal fatty acid absorption. *Eur. J. Clin. Invest.* 43, 1203–1223. doi: 10.1111/eci.12161

Yonezawa, T., Yonekura, S., Kobayashi, Y., Hagino, A., Katoh, K., and Obara, Y. (2004a). Effects of long-chain fatty acids on cytosolic triacylglycerol accumulation and lipid droplet formation in primary cultured bovine mammary epithelial cells. *J. Dairy Sci.* 87, 2527–2534. doi: 10.3168/jds.S0022-0302(04)73377-9

Yonezawa, T., Yonekura, S., Sanosaka, M., Hagino, A., Katoh, K., and Obara, Y. (2004b). Octanoate stimulates cytosolic triacylglycerol accumulation and CD36 mRNA expression but inhibits acetyl coenzyme a carboxylase activity in primary cultured bovine mammary epithelial cells. *J. Dairy Res.* 71 398 – 404. doi: 10.1017/S0022029904000408

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Hydroxytyrosol-rich extract from olive juice as an additive in gilthead sea bream juveniles fed a high-fat diet: Regulation of somatic growth

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The dietary inclusion of plant-based products in fish feeds formulation is required for the sustainable development of aquaculture. Moreover, considering functional diets, hydroxytyrosol, one of the major phenolic compounds found in olives (*Olea europaea*), has been identified as a potential candidate to be used in the aquafeeds industry due to its health promoting abilities. The aim of this study was to evaluate the effects of the inclusion of an olive juice extract rich in hydroxytyrosol as an additive (0.52 g HT/kg feed) in a high-fat (24% lipids) diet in gilthead sea bream (*Sparus aurata*) juveniles. Moreover, the experimental diets, with or without the extract, were administered daily at a standard (3% of total biomass in the tank) or restricted ration (40% reduction) for 8–9 weeks. Growth and biometric parameters, insulin-like growth factor 1 (IGF-1) plasma levels and growth hormone/IGF axis-, myogenic- and osteogenic-related genes expression in liver, white muscle and/or bone were analyzed. Moreover, *in vitro* cultures of vertebra bone-derived cells from fish fed the diets at a standard ration were performed at weeks 3 and 9 to explore the effects of hydroxytyrosol on osteoblasts development. Although neither body weight or any other biometric parameter were affected by diet composition after 4 or 8 weeks, the addition of the hydroxytyrosol-rich extract to the diet increased IGF-1 plasma levels, regardless of the ration regime, suggesting an anabolic condition. In muscle, the higher mRNA levels of the binding protein *igfbp-5b* and the myoblast fusion marker *dock5* in fish fed with the hydroxytyrosol-rich diet suggested that this compound may have a role in muscle, inducing development and a better muscular condition. Furthermore in bone, increased osteogenic potential while delayed matrix mineralization after addition to the diet of the olive juice extract was supported by the upregulated expression of *igf-1* and *bmp4* and reduced transcript levels of *osteopontin*. Overall, this study provides new insights into the beneficial use of hydroxytyrosol as a dietary additive in gilthead sea bream functional diets to improve muscle-skeletal condition and, the aquaculture industry.

KEYWORDS

olive polyphenols, aquafeeds, endocrine regulation, GH-IGF system, myogenesis, osteogenesis

1 Introduction

Olive (*Olea europaea*) oil is the major source of fats in the Mediterranean diet, and it is considered one of the main factors in the reduced occurrence of different chronic diseases, certain cancers, cardiovascular risk, and in the prolonged longevity associated to this diet (Sofi et al., 2010; Warleta et al., 2011; Marković et al., 2019). Its human health benefits are attributed to the presence of a high content of monounsaturated fatty acids (MUFAs), as well as many minor components that are functionally bioactive. These include tocopherols, phytosterols, carotenoids, triterpenic alcohols, pentacyclic triterpenes and phenolic compounds, which constitute the unsaponifiable fraction of the oil (Ghanbari et al., 2012). Nevertheless, in terms of health promoting abilities, polyphenols appear to be in the center of research interest (Marković et al., 2019). The main phenolic components in olive oil are hydroxytyrosol (3,4-dihydroxyphenylethanol), oleuropein and tyrosol (Tuck and Hayball, 2002) and, among them, hydroxytyrosol has shown the strongest antioxidant activity (Aydar et al., 2017). Besides, in mammals, hydroxytyrosol has been demonstrated to have anti-inflammatory (Gong et al., 2009), anti-obesogenic (Stefanon and Colitti, 2016; Illesca et al., 2019), antimicrobial (Medina et al., 2006) and anticancer (Corona et al., 2009; Fabiani et al., 2012) properties. In addition, olive polyphenols, especially hydroxytyrosol, exhibit a protective role in the prevention of osteoporosis, mainly due to stimulation of osteoblasts proliferation and differentiation while reducing the formation of osteoclasts (Chin and Ima-Nirwana, 2016; García-Martínez et al., 2016).

In the context of improving aquaculture sustainability, in addition to the shift toward the use of plant-derived ingredients in diets formulation instead of marine feedstuffs, the inclusion of phytocompounds in functional diets is nowadays uptrend. In this sense, olive oil inclusion in partially substituted fish oil diets has been evaluated in several fish species, including gilthead sea bream (*Sparus aurata*) (Torstensen et al., 2004; Mourente and Bell, 2006; Seno-o et al., 2008; Nasopoulou et al., 2011; Turchini et al., 2011). However, information about the potential benefits of using the extracts of olive pulp, oil or leaves or its chemical components as additives in aquafeeds is still scarce. Only recently, a triterpene-enriched olive extract in black sea bream (*Acanthopagrus schlegelii*) and an olive leaf extract in rainbow trout (*Oncorhynchus mykiss*) have shown immune potentiating properties (Baba et al., 2018; Rong et al., 2020). Furthermore, in gilthead sea bream, an olive oil bioactive extract added to the diet was demonstrated to improve gut health and functionality, as well as to enhance somatic growth (Gisbert et al., 2017).

In vertebrates, the main promoter of growth is the growth hormone (GH)-insulin-like growth factor (IGF) system (Reindl and Sheridan, 2012; Fuentes et al., 2013). The IGFs (IGF-1 and IGF-2) are produced in the liver in response to GH stimulation, are transported in circulation by the IGF binding proteins (IGFBPs), which modulate their half-life and actions, and exert their effects through the IGF-1 receptor (IGF-1R). Locally in skeletal muscle, hyperplastic growth is also controlled by myogenic regulatory factors (MRFs), with some being crucial for commitment of satellite cells [myogenic factor 5 (Myf5) and myogenic determination protein (MyoD)], while others contribute to myocytes differentiation [myogenic regulatory factor 4 (Mrf4) and myogenin] (Johnston, 2006; Vélez et al., 2014). Hydroxytyrosol and an olive leaf extract have been demonstrated, using *in vitro* and *in vivo* mammalian models, to have beneficial effects in muscle mass, thus protecting from sarcopenia (Salucci and Falcieri, 2020; González-Hedström et al., 2021). Indeed, some of these positive effects in rats consisted of reverting the aging-induced changes caused in expression of both, IGF system members (*igf-1r* and *igfbp5*) and myogenic markers (*myod* and *myogenin*). In fish, dietary supplementation with an olive leaf extract to red sea bream (*Pagrus major*) has been also reported to exert growth-promoting effects (i.e., hypertrophy) by increasing myofibril and collagen content in dorsal muscle (Arsyad et al., 2018).

Besides, coordinated growth between muscle and bone is required for proper musculoskeletal development. This has received increasing attention in farmed teleost species because skeletal anomalies are a major problem in the aquaculture industry (Witten et al., 2006). Runt-related transcription factor 2 (Runx2) is one of the key factors involved in the early stages of osteogenesis (Infante and Rodríguez, 2018). Afterwards, osteoblasts are known to produce different components of the extracellular matrix (ECM), including fibronectin (Fib1a) and collagen type 1 (Col1A1), and also non-collagenous proteins such as osteonectin (ON), osteopontin (OP) and osteocalcin (OCN), which are essential for osteoblasts maturation and matrix mineralization (Witten and Huysseune, 2009; Vieira et al., 2013). In fact, the bone protecting effects of olive oil phenolic compounds seem to be mediated by the modulation of osteoblasts markers expression (e.g., *runx2* and *ocn*) in mammalian cell models (Anter et al., 2016; Melguizo-Rodríguez et al., 2019). However, there is no information up to date about the specific effects of such polyphenols in the fish skeleton.

Furthermore, hydroxytyrosol has been demonstrated to have anti-obesogenic properties not only in mammals, but also in fish. In this sense, in a recent study by our group, hydroxytyrosol significantly decreased head and viscera adiposity of zebrafish larvae (*Danio rerio*), and it was able to counteract the obesogenic effects produced by rosiglitazone in primary cultured rainbow trout adipocytes (Lutfi et al., 2017). In terms

of aquafeeds formulation, it is well known that within a certain range, increasing dietary lipids can promote fish development, spare dietary protein and reduce production costs (Zhang et al., 2017), whereas excessive dietary fat can cause metabolic alterations, fat deposition and inflammation, as reported in different fish species (Wang et al., 2015; Cao et al., 2019; Jin et al., 2020). Indeed, high-fat diets have been used as a model of obesity and related disorders in both mammals and fish (Hariri and Thibault, 2010; Seth et al., 2013; Salmerón et al., 2015; Ka et al., 2020). Specifically, in relation with the musculoskeletal system, long-term high-fat diet feeding can cause a decrease in swimming capacity and skeletal muscle fiber area, as well as muscular atrophy in zebrafish, generating a model of sarcopenic obesity (Zou et al., 2022). Besides, an imbalance of fat metabolism caused by a high-fat diet in the same species induced an increase in osteoclastic activity in the exoskeleton, generating an osteoporosis-like phenotype (Carnovali et al., 2018). Nevertheless, further investigation about the role of hydroxytyrosol modulating adiposity in fish is beyond the scope of the present manuscript, since this is part of a larger study. Therefore, since hydroxytyrosol has demonstrated multiple positive effects at different tissues, we considered when setting the trial, to evaluate fish fed a high-fat diet, and administered at two rations, standard (*ad libitum*) or restricted, to establish two different conditions in terms of energy intake and to test whether the effects of hydroxytyrosol on muscle and bone homeostasis may vary in the two nutritional situations. Thus, our hypothesis in this first study, considering the beneficial properties of hydroxytyrosol, is that the dietary administration of an extract rich in this polyphenol to gilthead sea bream could prevent the possible alterations associated to a high-fat diet feeding by improving the muscular and skeletal growth capacity of the fish.

In this framework, the objective of this study was to evaluate whether the inclusion of an olive juice extract rich in hydroxytyrosol in a high-lipid content diet could improve musculoskeletal growth of gilthead sea bream juveniles fed at standard or reduced ration. To this end, changes in growth and somatic parameters, IGF-1 plasma levels, as well as in the mRNA levels of members of the GH-IGF axis, and myogenic and osteogenic markers in muscle and/or bone were analyzed. Moreover, modulation of proliferation and differentiation of bone-derived cells from the same fish was also assessed to evaluate if the *in vivo* feeding history of the fish could affect the osteogenic development of bone-derived cells *in vitro*.

2 Materials and methods

2.1 Animals and experimental design

Gilthead sea bream (*Sparus aurata*) juveniles were obtained from the commercial fishery Piscimar (Burriana, Spain) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona (Spain). Animals were kept in a seawater recirculation system at 23 ± 1°C, under a 12 h light/12 h dark photoperiod, and fed *ad libitum* two times a day with a commercial diet (Optibream Skretting, Burgos, Spain). After 1 month of acclimation, fish were weighed and distributed between eight 200 L (n = 15 fish for each tank) and four 400 L (n = 30 fish per tank) tanks. Before starting the trial, fish were fed *ad libitum* with the experimental diet without the hydroxytyrosol-rich extract for 1 week, in order to have the fish habituated to it, but also, to identify the ration to which the visual satiation level corresponded to set it in the experimental trial as the standard one. The initial body weight (BW) and biomass density of fish were 80.81 ± 1.43 g and 6.06 kg/m^3, respectively.

The experimental high-fat diet (24% lipids of the dry matter) was formulated and produced by Skretting ARC (Stavanger, Norway) with 50% of total oils being rapeseed oil, the other 50% fish oil, and 23 MJ/kg of digestible energy (Table 1). Moreover, this diet was formulated in the absence (HF) or presence (HF + HT) of a hydroxytyrosol-rich extract (0.52 g HT/kg feed), and was administered daily at a standard ration (3% of total biomass in the tank) (ST) or at restricted conditions (40%

TABLE 1 Composition of the experimental high-fat diets without (HF) or with hydroxytyrosol (HF + HT). The hydroxytyrosol included in the formulation (0.52 g HT/kg feed) was provided by the commercial olive juice extract HIDROX® (Oliphenol LLC., Hayward, CA, United States) that contains a >12% of simple and total polyphenols from which 3.136% is hydroxytyrosol.

	HF	HF + HT
Ingredients (%)		
Corn gluten	3.80	3.80
Wheat gluten	20.00	20.00
Fava beans	8.00	8.00
Soya concentrate	25.00	25.00
Fish oil	9.98	9.98
Fish meal	15.00	15.00
Rapeseed oil	10.14	10.14
Yttrium premix	0.10	0.10
Phosphate	1.04	1.04
Vitamin mineral premix	0.44	0.44
Wheat	6.50	4.85
HIDROX®	0.00	1.66
Composition (%)		
Dry matter	93.0	93.0
Moisture	7.0	7.0
Crude protein	46.8	46.7
Crude fat	24.0	24.2
Ash	5.4	5.6
Crude fiber	1.9	1.8
Starch	8.8	7.8

reduction) (RE) for 8–9 weeks (from the end of August to the end of October). Each of the four experimental groups of fish was distributed in triplicate tanks (one of 400 L and two of 200 L due to the particular tanks distribution at the fish facility). The olive juice extract rich in hydroxytyrosol used in the formulation (HIDROX® > 12% simple and total polyphenols: 3.136% HT, 0.216% oleuropein and 0.408% tyrosol; according to the certificate of analysis 12-190403-000) was provided by Oliphenol LLC., (Hayward, CA, United States). The dose of 0.52 g HT/kg feed was selected according to data presented from an experiment performed in the yellowtail kingfish (*Seriola lalandi*) at an international conference (Bas et al., World Aquaculture Society Congress 2019). The groups that received the standard ration (HF_ST and HF + HT_ST) were fed two times a day, with 60% of the feed in the morning ration and 40% in the afternoon, whereas the ones with the reduced regime (HF_RE and HF + HT_RE) were fed only once a day in the morning with 60% of the total standard ration. Daily ration relative to the BW was adjusted every 2 weeks. The main trial lasted 8 weeks, but some fish from the groups HF_ST and HF + HT_ST were maintained for an extra week in order to perform the *in vitro* experiments. Those were performed on weeks 3 and 9 to avoid overlapping with the fish samplings.

At sampling, fish were fasted for 24 h in order to deplete the gastrointestinal tract and avoid contamination of the tissues. Ten animals per condition (four and three from the 400-L and 200-L tanks, respectively) were anesthetized with MS-222 (150 mg/L) (E10521, Sigma-Aldrich, Tres-Cantos, Spain), measured, weighed, and blood was taken from the caudal vessels. Samples of blood were immediately placed on ice and centrifuged at 5,000 rpm for 5 min at room temperature to obtain the plasma. After sacrifice by sectioning the spinal cord, samples of white skeletal muscle, vertebrae bone and liver were obtained and directly frozen in liquid nitrogen. All samples were stored at −80°C until further analysis. All animal handling procedures carried out in this study complied with the Guidelines of the European Union Council directive (EU 2010/63) and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 34/20 and DAAM 11251), following the regulations and procedures established by the Spanish and Catalan governments.

2.2 Biometric parameters

BW was monitored at times 0, 2, 4, 6 and 8 weeks. The rest of the biometric parameters were measured at the middle (4 weeks) and end (8 weeks) of the experimental trial. BW and standard length (BL) were used to determine the condition factor (CF) [$BW/BL^3 \times 100$] individually, although the average values for each tank ($n = 3$) were used for statistical analysis. Similarly, weight of the liver was obtained, and the hepatosomatic index (HSI) [(liver weight/BW) × 100] calculated and averaged per each tank ($n = 3$). Total biomass from triplicate tanks ($n = 3$) was obtained to calculate feed conversion ratio (FCR), as grams of total feed intake/(final BW- initial BW).

2.3 Insulin-like growth factor 1 plasma levels

IGF-1 plasma levels were determined in 10 fish per group at the middle (4 weeks) and end (8 weeks) of the experimental trial after sample extraction by the acid-ethanol method (Shimizu et al., 2000). The concentration of IGF-1 was measured using the Fish IGF-1 ELISA kit (CSB-E12122Fh, CUSABIO, Wuhan, China), according to the manufacturer's instructions, as previously done with plasma samples of the same species by Rodríguez-Viera et al. (2022).

2.4 Gene expression analyses

2.4.1 RNA extraction and cDNA synthesis

White skeletal muscle (~100 mg) and vertebral bone tissues (~100 mg) were homogenized with 1 ml of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using the Precellys® Evolution Homogenizer coupled to a Cryolys cooling unit (Bertin Instruments, Montigny-le-Bretonneux, France). Next, RNA extraction was performed according to the manufacturer's recommendations and resuspended in DEPC-treated water (RNase-free). RNA concentration of each sample was measured in a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain), and its integrity was confirmed in a 1% agarose gel (w/v) stained with 3% SYBR®-Safe Gel Stainer (Bio-Rad, El Prat de Llobregat, Spain). Afterwards, 1,500 ng of RNA were treated with DNase I (Life Technologies, Alcobendas, Spain), to remove any residual genomic DNA, and retrotranscribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain).

2.4.2 Real-time quantitative polymerase chain reaction

Gene expression analyses were performed by quantitative polymerase chain reaction (qPCR) in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain), according to the requirements of the MIQE guidelines (Bustin et al., 2009). The primers sequences and GenBank accession numbers used for each reaction are listed in Supplementary Table S1. All the analyses were performed in triplicate wells using 384-well plates with 2.5 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers and 1 µL of diluted cDNA for each sample, in a final volume of 5 µL. The qPCR program was performed as previously described (Balbuena-Pecino et al., 2019). The level of

expression of each target gene was calculated relative to the geometric mean of the two most stable reference genes from the three determined for each tissue [ribosomal protein s18 (*rps18*) and elongation factor 1 alpha (*ef1α*) in all three tissues], according to the Pfaffl method (Pfaffl, 2001). Reference genes stability and relative expression of the target genes were determined using the Bio-Rad CFX Manager Software v. 3.1.

2.5 Primary culture of bone-derived cells

Primary cultures of gilthead sea bream bone-derived cells were performed following the protocol of Capilla et al. (2011). After 3 and 9 weeks of the experimental trial, vertebral columns of 8 fish per dietary condition (HF and HF + HT at standard ration) were used per culture, each one considered an independent replicate. After cleaning the vertebral column of all adherent tissues, small fragments (<1 mm) were obtained by mechanical disruption. After that, two digestions of 30 and 90 min were done with 0.125% type II collagenase in Hank's balanced salt solution with gentle agitation. Next, bone fragments were washed twice with Dulbecco's Modified Eagle Medium (DMEM) and plated in growth medium composed of DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution in 12-well plates, and incubated at 23°C with 2.5% CO_2. The bone fragments were removed at day 7 of culture development in order to perform the corresponding assays with the attached cells. Medium was changed every 2 days. All plastic materials were obtained from Nunc (LabClinics, Barcelona, Spain).

2.6 Proliferation assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to assess cell proliferation, as previously done with the same cellular model and species (Balbuena-Pecino et al., 2019). Briefly, on days 8 and 15, bone-derived cell samples of two-duplicate wells from the 12-well plates were incubated for 3 h in DMEM with a final concentration of 0.5 mg/ml of MTT (M5655, Sigma-Aldrich, Tres-Cantos, Spain). Then, cells were washed with phosphate buffered saline and the blue formazan crystals formed were allowed to resuspend in dimethyl sulfoxide. The normalized proliferation values (n = 8) were obtained from the absorbance measured at 570 nm in duplicate 96-wells, subtracting the background read at 650 nm, using a microplate reader (Tecan Infinite M200, Männedorf, Switzerland).

2.7 Differentiation assay

Cell differentiation was evaluated according to mineralization of the ECM. The deposition of minerals was analyzed by alizarin red S (ARS) staining, as previously described in Capilla et al. (2011). Briefly, at day 20, cell samples of two-duplicate wells from 12-well plates were fixed with 10% formalin, rinsed three times with distilled water and stained with 2% ARS (pH 4.1-4.3) for 20 min. Next, cells were washed three times with distilled water, and a 10% acetic acid solution was used to elute the ARS dye with the help of a scrapper. Then, cells were heated at 85°C, cooled on ice and centrifuged at 16,000 g for 15 min. At this point, 10% ammonium hydroxide was added to the supernatant to neutralize the acid and aliquots of the different samples were read in triplicate 96-wells at 405 nm, using a microplate reader (Tecan Infinite M200, Männedorf, Switzerland). Data are presented as optical density arbitrary units (n = 8).

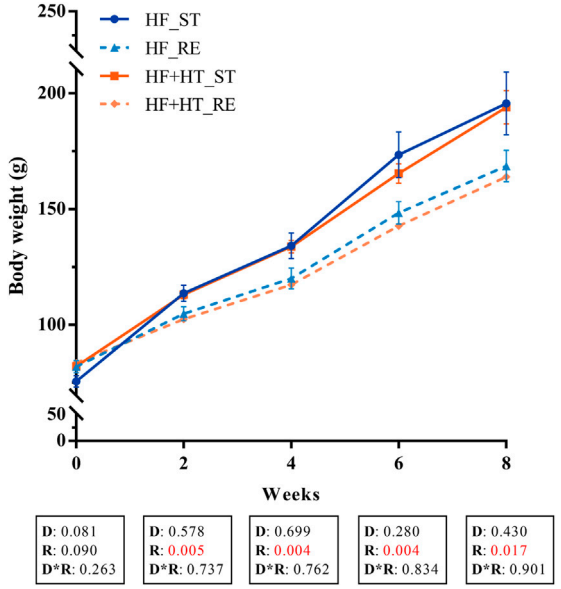

FIGURE 1
Mean body weight of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction), for 0, 2, 4, 6 and 8 weeks. Values are presented as Mean ± SEM (n = 3 tanks). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$, showed in red).

2.8 Statistical analyses

Data were analyzed using IBM SPSS Statistics v. 22 (IBM Corp., Armonk, NY, United States) and plotted as Mean + Standard Error of the Mean (SEM) using GraphPad Prism v. 7 (GraphPad Software, La Jolla, CA, United States). As a biological replicate, tank was used for biometric parameters and individual fish for IGF-1 plasma levels, gene expression

TABLE 2 Somatic indices and IGF-1 plasma levels of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 4 weeks.

	HF_ST	HF_RE	HF + HT_ST	HF + HT_RE	D	R	D*R
BW (g)	134.14 ± 5.54	120.04 ± 4.47	133.81 ± 2.78	117.30 ± 0.52	0.699	0.004	0.762
BL (cm)	16.96 ± 0.14	16.59 ± 0.27	17.19 ± 0.13	16.68 ± 0.21	0.444	0.057	0.730
CF	2.85 ± 0.05	2.57 ± 0.04	2.80 ± 0.09	2.64 ± 0.05	0.266	<0.001	0.569
FCR	1.53 ± 0.34	0.97 ± 0.09	1.53 ± 0.11	1.05 ± 0.04	0.841	0.024	0.827
Liver (g)	1.91 ± 0.11	1.43 ± 0.09	2.03 ± 0.17	1.51 ± 0.04	0.393	0.002	0.868
HSI (%)	1.39 ± 0.06	1.21 ± 0.08	1.34 ± 0.03	1.25 ± 0.05	0.976	0.049	0.442
IGF-1 (ng/ml)	3.27 ± 0.16	3.19 ± 0.18	3.88 ± 0.09	3.72 ± 0.10	<0.001	0.417	0.773

Data are shown as Mean ± SEM (n = 3 tanks, except for IGF-1 plasma levels n = 10 fish). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$). BW, body weight; BL, body length; CF, condition factor (BW/BL^3) × 100; FCR, feed conversion ratio (g total feed intake/(BW- initial BW); HSI, hepatosomatic index (liver weight/BW) × 100.

TABLE 3 Somatic indices and IGF-1 plasma levels of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 8 weeks.

	HF_ST	HF_RE	HF + HT_ST	HF + HT_RE	D	R	D*R
FBW (g)	195.63 ± 13.54	168.65 ± 6.81	194.00 ± 7.24	163.96 ± 1.40	0.430	0.017	0.901
BL (cm)	19.04 ± 0.40	18.34 ± 0.09	19.31 ± 0.17	18.09 ± 0.34	0.977	0.009	0.381
CF	2.85 ± 0.04	2.75 ± 0.06	2.78 ± 0.05	2.66 ± 0.06	0.203	0.085	0.794
FCR	1.66 ± 0.25	0.99 ± 0.06	1.68 ± 0.15	1.08 ± 0.10	0.744	0.004	0.822
Liver (g)	2.42 ± 0.14	2.08 ± 0.20	2.56 ± 0.21	1.96 ± 0.20	0.973	0.036	0.523
HSI (%)	1.23 ± 0.05	1.20 ± 0.09	1.29 ± 0.003	1.23 ± 0.06	0.466	0.475	0,859
IGF-1 (ng/ml)	3.88 ± 0.13^a	4.70 ± 0.12^{b,c}	4.97 ± 0.10^c	4.44 ± 0.11^b	0.001	0.225	<0.001

Data are shown as Mean ± SEM (n = 3 tanks, except for IGF-1 plasma levels n = 10 fish). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$). When the interaction between the two factors (D*R) was significant, comparisons among groups were analyzed by a Tukey's post-hoc test and, significant differences are indicated by different letters ($p < 0.05$). FBW, final body weight; BL, body length; CF, condition factor (FBW/BL^3) × 100; FCR, feed conversion ratio (g total feed intake/(FBW- initial BW); HSI, hepatosomatic index (liver weight/FBW) × 100.

and *in vitro* results. Data normality and homoscedasticity were checked with Shapiro-Wilk and Levene's tests, respectively. Next, statistical differences were assessed by two-way analysis of variance (two-way ANOVA) with diet and ration set as independent factors, and their interaction. When the interaction between the two factors was significant, comparisons among groups were analyzed by a Tukey's *post-hoc* test. With regards to *in vitro* assays, statistical significance between dietary groups was performed with unpaired student t-tests. Statistical differences were considered significant for all analyses when $p < 0.05$.

3 Results

3.1 Growth and somatic parameters

BW was measured every 2 weeks along the experimental trial and values are presented in Figure 1. Two-way ANOVA analysis showed that BW was significantly affected by ration regime but not by the diet formulation from week 2 to the end of the experiment. During this period, fish fed with the standard ration (HF_ST and HF + HT_ST) presented higher BW values compared to those fish fed with the restricted ration (HF_RE and HF + HT_RE) (Figure 1). Besides BW, other somatic indices together with the IGF-1 plasma levels, evaluated at weeks 4 and 8, are shown in Tables 2, 3, respectively. At week 4, under restricted feeding, fish had significantly lower values of CF, FCR, liver weight and HSI regardless of the diet. Moreover, the circulating IGF-1 plasma levels were affected by the diet composition, and were significantly higher in fish fed with the diet containing the hydroxytyrosol-rich extract in comparison to fish fed with HF diet at both feeding regimes. On the other hand, BL was not affected by any factor (Table 2).

At the end of the experimental trial (week 8), the ration factor was found to significantly affect BL, FCR and liver weight. Both groups of fish fed with a restricted regime had lower values in comparison with their respective fish groups fed at a standard ration. Concerning IGF-1, a diet effect and an interaction between the two

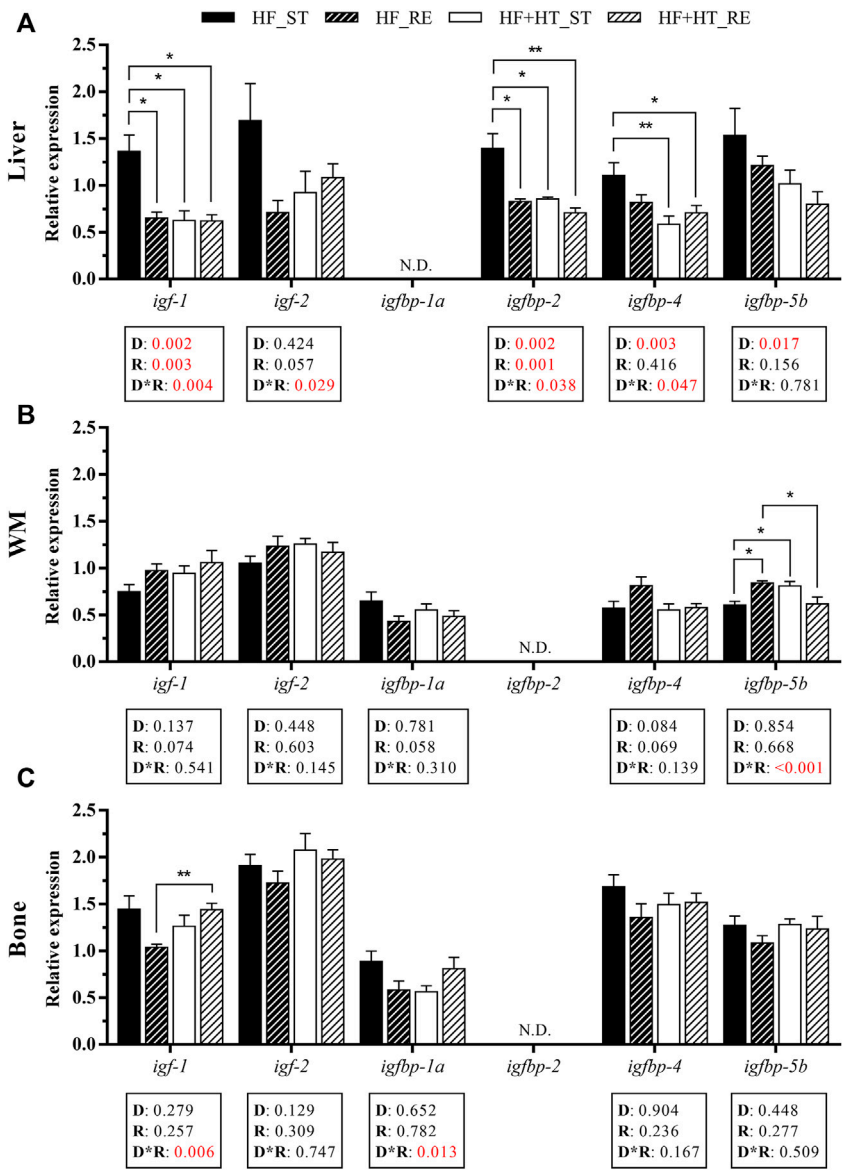

FIGURE 2
Relative gene expression of insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) in **(A)** liver, **(B)** white muscle (WM) and **(C)** bone of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 8 weeks. Data are shown as Mean + SEM (n = 10). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$, showed in red). When the interaction between the two factors (D*R) was significant, comparisons among groups were analyzed by a Tukey's post-hoc test and, significant differences are indicated by asterisks ($p < 0.05$ shown as *; $p < 0.01$ **). N.D., non-detectable.

factors was observed. In this sense, the highest value of circulating IGF-1 was found in the group fed the HF + HT diet and the lowest in the HF-fed fish when both groups received a standard ration. Animals fed with the HF + HT diet presented higher values of circulating IGF-1 when fed the standard ration compared to the restricted one, but the situation was the opposite in the animals fed with the HF diet. Finally, CF and HSI parameters were unaffected either by diet or ration (Table 3).

3.2 Gene expression related to GH-IGFs axis

In the liver, the transcript levels of *igf-1* and the IGF binding protein *igfbp-2* were affected by the diet composition, the feeding ration, and by the interaction between both factors. Hence, multiple comparisons analysis revealed that the expression of these two genes was significantly higher in HF_ST fish respect to

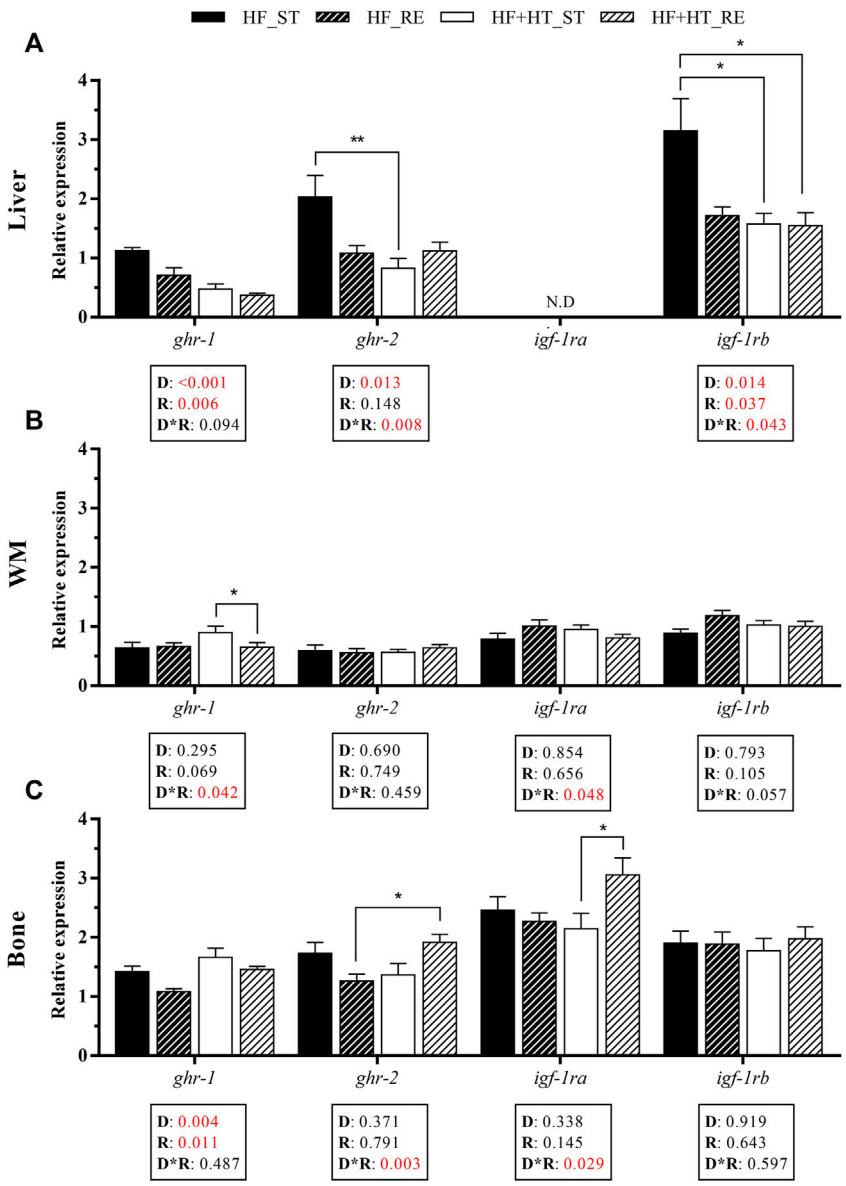

FIGURE 3
Relative gene expression of growth hormone (GH) and insulin-like growth factor (IGF-1) receptors in **(A)** liver, **(B)** white muscle (WM) and **(C)** bone of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 8 weeks. Data are shown as Mean + SEM (n = 10). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$, showed in red). When the interaction between the two factors (D*R) was significant, comparisons among groups were analyzed by a Tukey's post-hoc test and, significant differences are indicated by asterisks ($p < 0.05$ shown as *; $p < 0.01$ **). N.D., non-detectable.

the other three groups. Moreover, a diet effect was also found in *igfbp-4* and *igfbp-5b*, presenting both genes higher expression levels in the animals fed the HF diet compared to those fed with the HF + HT diet regardless of the feeding regime. In addition, *igf-2* and *igfbp-4* showed interaction of both variables. Particularly, fish fed with the HF diet at the standard ration had significantly increased mRNA levels of *igfbp-4* when compared with both groups of HF + HT diet-fed fish. Finally, *igfbp-1a* levels could not be detected on this tissue (Figure 2A).

With regards to white muscle, a significant interaction between diet and ration regime was observed in the mRNA levels of *igfbp-5b*, which were significantly higher in the HF_RE and HF + HT_ST groups compared to fish fed with the HF diet at standard ration, and also, with HF + HT_RE group in the case of

the HF_RE group. Apart from this, differences were not found in the case of *igf-1*, *igf-2*, *igfbp-1a* and *igfbp-4*, whereas *igfbp-2* was undetectable in this tissue (Figure 2B).

In bone, there was a significant interaction between diet and feeding ration in the gene expression of *igf-1* and *igfbp-1a*; thus, transcript levels of the former were higher in the HF + HT_RE group compared to HF_RE fed fish. However, the mRNA levels of *igf-2*, *igfbp-4* and *igfbp-5b* were not affected by any of the factors, and *igfbp-2* gene expression was again undetectable (Figure 2C).

With respect to the transcriptional profile of the receptors from the GH-IGF axis in liver, the mRNA levels of the GH receptor *ghr-1* were modulated by the diet composition and the feeding ration. An upregulation of this gene was observed in the HF group compared to the HF + HT one, with these levels being reduced by restriction regime (HF_RE and HF + HT_RE). Similarly, a diet effect and an interaction between the two factors was found for *ghr-2* gene expression. In this sense, an upregulation of *ghr-2* mRNA levels was detected in animals fed with HF diet at a standard ration compared to those in fish fed with HF + HT diet at the same ration. Moreover, the transcript levels of the IGF-1 receptor *igf-1rb* were altered by the diet composition, the feeding regime and, by the interaction of both factors. *igf-1rb* mRNA levels were higher in fish that received the HF diet at a standard ration in comparison with both groups of HF + HT diet. On the other hand, *igf-1ra* levels could not be detected on this tissue (Figure 3A).

In the case of white muscle, the interaction between diet and feeding ration affected *ghr-1* and *igf-1ra* gene expression and, in the case of *ghr-1*, higher levels were found in HF + HT-fed fish at a standard ration compared with those in fish fed with the same diet but at the restricted regime. The mRNA levels of the other receptors were not modified (Figure 3B).

In bone, diet composition and feeding ration had significant effects on *ghr-1*, and an interaction effect was observed in *ghr-2* and *igf-1ra* gene expression. The transcript levels of *ghr*-1 were significantly upregulated in the HF + HT-fed fish with respect to the HF-fed ones, and their levels were lower in the fish fed either diet under restricted feeding conditions. Moreover, *ghr-2* and *igf-1ra* mRNA levels were higher in those animals that received HF + HT diet at a restricted feeding regime compared to HF_RE and HF + HT_ST groups, respectively (Figure 3C).

3.3 Gene expression related to myogenesis and osteogenesis

Concerning MRFs, the transcript levels of the dedicator of cytokinesis *dock5* were significantly upregulated in animals fed with the HF + HT diet respect to those fed with the HF diet. On the other hand, the remaining myogenesis-related genes were not altered by either diet composition or feeding regime (Figure 4).

Gene expression of bone ECM components, and markers of bone turnover in vertebra bone is presented in Figure 5. Regarding the mRNA levels of the matrix components, the interaction between diet and regime conditions significantly affected the gene expression of *fib1a*. Fish fed with the HF diet at the restricted ration showed a downregulated expression of *fib1a* compared to those fish fed with the same diet but at their corresponding standard ration. In addition, the mRNA levels of *op* were modulated by the diet factor, and were significantly higher in fish fed with HF diet respect to the HF + HT-fed group. Besides, the remaining ECM components evaluated did not change in response to any factor (Figure 5A).

Furthermore, the transcript levels of the osteogenic genes *runx2* and the bone morphogenetic protein *bmp4* responded to ration and factors interaction, and only diet factor, respectively. The gene expression of *runx2* was downregulated in the animals fed with the HF diet at the restricted ration in comparison with those fed at the standard one. Moreover, the fish fed the HF + HT diet showed increased mRNA levels of *bmp4* when compared to HF groups (Figure 5B). Concerning the osteoclasts markers, the gene expression of cathepsin k (*ctsk*) and the matrix metalloproteinase *mmp9* was modulated by the ration factor and the interaction between both factors, respectively. The mRNA levels of *ctsk* were lower in the fish fed with both diets under the restricted feeding regime, whereas in the case of *mmp9* the highest transcript levels were observed in the HF-fed fish at standard ration. In contrast, the tartrate-resistant acid phosphatase (*trap*) mRNA levels remained unaltered (Figure 5C).

3.4 Proliferation and differentiation in bone-derived cells

To further investigate the effects of hydroxytyrosol on bone tissue, cell proliferation and differentiation of cultured bone-derived cells obtained from fish fed with the HF and HF + HT diets at a standard ration were analyzed after 3 and 9 weeks of feeding trial. The proliferation data revealed higher rates at day 15 than at day 8, but no differences between groups, regardless of the day of culture development or week of experiment (Figure 6A). In parallel to this, cells coming from fish fed with the HF + HT_ST diet did not present differences in terms of ECM mineralization after 20 days in culture in comparison with cells obtained from animals fed with the HF_ST diet, independently of the experimental week (Figure 6B).

4 Discussion

The beneficial properties of hydroxytyrosol or hydroxytyrosol-rich extracts have been widely studied in mammals; however, the effects of this polyphenol in fish functional feeds are less known. In the current study, we evaluated the inclusion of a hydroxytyrosol-rich extract in a

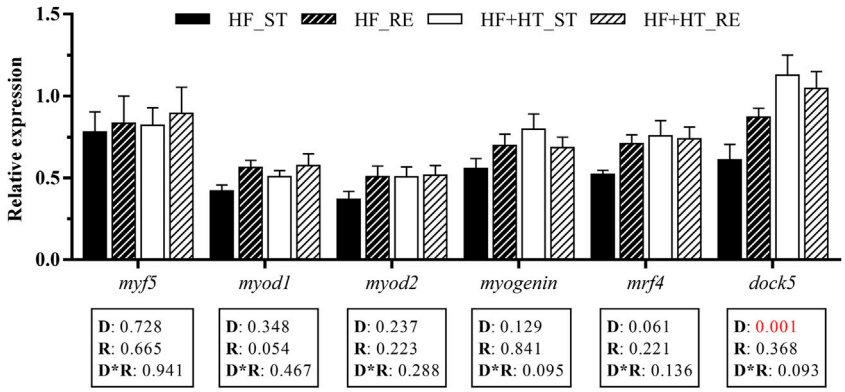

FIGURE 4
Relative gene expression of myogenic markers in white muscle of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 8 weeks. Data are shown as Mean + SEM (n = 10). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$, showed in red).

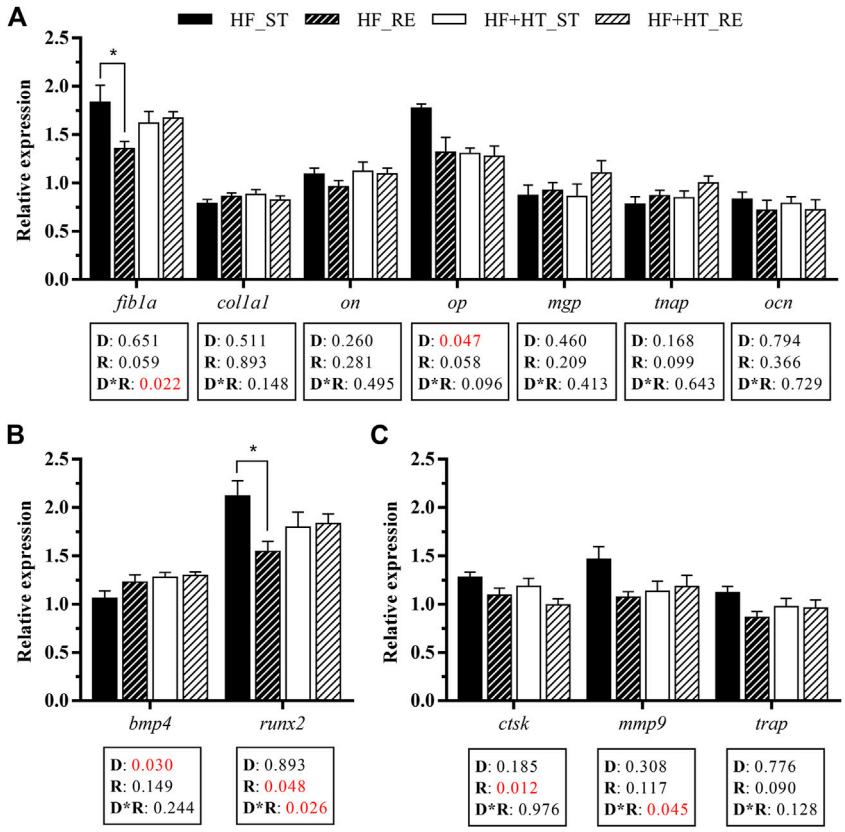

FIGURE 5
Relative gene expression of **(A)** extracellular matrix components, **(B)** osteogenic and **(C)** osteoclastic markers in bone of gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard (ST) (3% biomass/tank) or restricted ration (RE) (40% daily reduction) for 8 weeks. Data are shown as Mean +SEM (n = 10). Statistical differences are indicated in 3 components: diet (D), ration (R) and interaction (D*R), using two-way ANOVA ($p < 0.05$, showed in red). When the interaction between the two factors (D*R) was significant, comparisons among groups were analyzed by a Tukey's post-hoc test and, significant differences among groups are indicated by asterisks ($p < 0.05$).

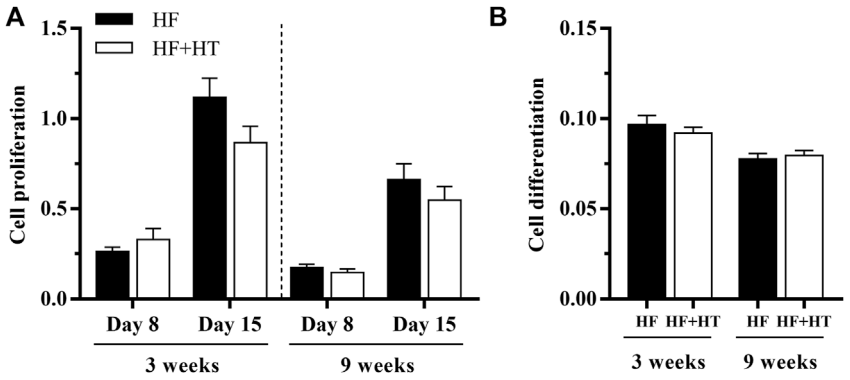

FIGURE 6
(A) Proliferation and **(B)** differentiation of bone-derived cells at days 8 and 15, or 20 respectively, of culture development, extracted from gilthead sea bream juveniles fed with a high-fat diet in the absence (HF) or presence (HF + HT) of hydroxytyrosol (0.52 g HT/kg feed), at a standard ration (3% biomass/tank) for 3 and 9 weeks. Data are shown as Mean + SEM (n = 8). No differences were observed between diets, assessed by student-unpaired t-tests ($p < 0.05$).

high-fat diet on the somatic growth regulation of gilthead sea bream juveniles fed at a standard or reduced ration.

The addition of the hydroxytyrosol-rich extract to the diet did not change BW along the trial, neither any other biometric parameter of the fish after 4 or 8 weeks, regardless of the ration regime. Nonetheless, although IGF-1 plasma levels were relatively low, probably following their seasonal profile (Escobar-Aguirre et al., 2020), dietary hydroxytyrosol was found to significantly affect them, being the levels higher in HF + HT groups, suggesting an anabolic condition (Picha et al., 2008). IGFs are known to be the primary mediators of the growth-promoting effects of GH in fish (Reindl and Sheridan, 2012). In this sense, contrary to GH levels, circulating IGF-1 levels are considered to be one of the most reliable markers of growth performance in a wide variety of fish species (Pérez-Sánchez et al., 2018). A positive correlation between circulating IGF-1 and growth rate was observed in gilthead sea bream after sustained exercise (Vélez et al., 2016) or a recombinant bovine GH injection (Vélez et al., 2018). In fact, the applicability of this parameter as a growth marker is based not only on its relationship to current growth status, but also on its ability to predict future BW gain, as long as feeding and other conditions are unaltered (Pierce et al., 2001; Picha et al., 2008; Izutsu et al., 2022). According to this, it cannot be discarded that an experiment with younger animals (e.g., less than 10 g initial BW) or a prolonged trial could have shown increased growth performance of the animals fed with the HF + HT diet in comparison to the fish fed the same diet but in the absence of hydroxytyrosol. In this context, hydroxytyrosol supplementation (100 mg/kg) on a high-fat diet (15% fat) led to a significant improvement of the BW gain, specific growth rate, FCR and protein efficiency ratios in blunt snout bream (*Megalobrama amblycephala*) juveniles after 10 weeks, together with an alleviation of the excessive lipid accumulation induced by the high-fat diet (Dong et al., 2020). As far as we know, apart from that research work, other *in vivo* studies including hydroxytyrosol *per se* in fish diets have not been reported to date. The fact that the authors include only this polyphenol and not a natural olive extract containing other compounds may explain the high dose used in their study.

Nevertheless, the effects of the dietary inclusion of olive oil products in aquafeeds have been evaluated in several fish species (reviewed by Hazreen-Nita et al., 2022). Likewise, it has been shown that a diet supplemented with an olive oil bioactive extract (rich in polyphenols) promoted growth in a dose-dependent manner in gilthead sea bream juveniles, without affecting feed efficiency parameters (Gisbert et al., 2017). However, the authors proposed that these results could be partially attributed to polyphenols, but also related to the improved intestinal health condition. Indeed, this was also found in rainbow trout (*Onchorynchus mykiss*) after a 6-week feeding trial with an olive waste cake (Hoseinifar et al., 2020). A similar response in terms of growth was also reported in common carp (*Cyprinus carpio*) fed with an olive leaf extract-supplemented diet (Zemheri-Navruz et al., 2020; Sokooti et al., 2021). In contrast, growth performance values, such as BW gain and standard growth rate, or feed utilization were not affected in rainbow trout fed with a diet containing different levels of olive leaf extract for 60 days (Baba et al., 2018). Taken together, these data suggest that a direct link between the polyphenols and somatic growth exists, although differences from various studies may be related to fish species, concentrations of the extracts or the enriched compounds within, the diverse intestinal microbiota and/or experimental conditions (Hazreen-Nita et al., 2022). Overall, the promising effects of the present study should be taken into consideration in the aquaculture sector, since in

addition to the positive effects of hydroxytyrosol, gilthead sea bream appears to be more tolerant to high levels of polyphenols in the feed than other fish species (Sicuro et al., 2010).

On the other hand, in our study, fish subjected to 40% feed restriction were unable to maintain the same growth rate as the ones that received the standard ration, regardless of the diet, therefore BW, BL, CF, liver weight, or HSI parameters were significantly lower in that group. However, the feed conversion efficiency in the fish under the restricted regime was improved, indicated by the lower FCR values obtained, which is consistent with previous studies in the same species (Eroldoğan et al., 2008; Bonaldo et al., 2010). In fact, fish tend to compensate the restricted rations optimizing the digestion process, which allows a more efficient use of the nutrients (Bonaldo et al., 2010).

Parallel to this, the transcriptional regulation of the GH-IGFs-related genes was highly affected by the addition of hydroxytyrosol to the diet, but also by the interaction of diet and feeding regime. The liver showed the most significant differences, compared to the other two tissues studied, given its key role within this endocrine axis and, the benefits of the supplementation with hydroxytyrosol in this tissue, were mostly observed in the fish fed the standard ration; while the restricted conditions, were apparently able to mitigate some of the negative effects induced by the elevated input of lipids received by the animals feeding the high-fat diet. The decrease in the hepatic mRNA levels of most of the genes of the axis (mainly *igf-1*, *igfbp-2* and *igfbp-4*) in the HF + HT dietary groups, but also in the HF_RE group, could be the result of a negative feedback inhibition from circulating IGF-1 after continued signaling by this hormone, as a mechanism to sustain GH/IGF system homeostasis. Also in the liver, the receptors *ghr-1*, *ghr-2* and *igf-1rb* showed the same gene expression pattern as the other members of the axis, indicating a possible GH and IGF-1 desensitization. A similar downregulation in response to high circulating levels of hormones has been previously described in terms of receptor binding in isolated hepatocytes from both, Atlantic salmon (*Salmo salar*) and rainbow trout (Plisetskaya et al., 1993), as well as from lamprey (*Lampetra fluviatilis*) (Leibush and Lappova, 1995). Besides, a downregulation of some hepatic elements of the system (*igf-1, igfbp-1a* and *ifgbp-2)* has been also described when the GH/IGF axis becomes overstimulated after GH administration in gilthead sea bream (Vélez et al., 2019). Interestingly, the contrary effect (i.e., receptors upregulation) has been observed in fish white muscle in response to high insulin levels (Párrizas et al., 1994). This agrees with the highest *ghr-1* mRNA levels found in the HF + HT_ST group in both, white muscle and bone. Apart from this, expression of GH-IGFs-related genes in white muscle was not markedly affected by dietary inclusion of the hydroxytyrosol-rich extract, as reflected by the similar BW observed along the trial between the fish groups fed the two experimental diets, regardless of the ration regime.

Notwithstanding, in white muscle, an interaction between diet composition and feeding ration was found for the paralog *igfbp-5b*, and the highest gene expression values were those in HF_RE and HF + HT_ST groups. In several species, it has been shown that pro-growth stimuli such as amino acids, IGFs or sustained exercise increase the expression of this gene (Salem et al., 2010; Azizi et al., 2016). Hence, this is in accordance with the results of the present study, where the highest IGF-1 plasma levels (at week 8) were found in the same experimental groups with upregulated *igfbp-5b*. In addition, this binding protein is induced during myogenic differentiation and its importance in such a process in teleost fish has been well established (Duan et al., 2010; Garcia de la serrana and Macqueen, 2018). In line with this, the relative expression of the cytoplasmic protein *dock5*, a regulator of the myoblast/myocyte fusion process (Moore et al., 2007; Chen et al., 2020), was also modulated by diet composition. Thus, the increase observed in the HF + HT groups compared to the HF groups in *dock5*, together with the upregulation of *igfbp-5b*, suggest that hydroxytyrosol can have a role in inducing this process of muscle growth. Thus, although histological analysis had been performed to better discern if this could have resulted in an increase in the number (hyperplasia) and/or size (hypertrophy) of multinucleated myofibers in the last term, due to technical problems we could not examine muscle sections as it was designed. In any case, Szychlinska et al. (2019) found a significant increase in muscle hypertrophy in a osteoarthritis rat model fed with an extra virgin olive oil-enriched diet, supporting this hypothesis and the fact that hydroxytyrosol could counteract in muscle the negative changes potentially caused by feeding a high fat diet. Only when applying the 40% feed restriction in the HF + HT group, it seems that some of the beneficial effects of hydroxytyrosol were lost, as it happened for instance with the gene expression of *igfbp-5b* in white muscle; however, this could be explained by the lower absolute quantity of the extract that this experimental group would take in, with respect to the HF + HT_ST fish.

In the bone tissue, the addition of hydroxytyrosol to the diet upregulated the signaling molecule *bmp4* in parallel to decreasing the gene expression of the non-collagenous protein *op*. A positive relationship between *bmp4* expression and ossification has been established in different groups of vertebrates including fish (Chen et al., 2002; Albertson et al., 2005). *bmp4* is expressed in osteoblasts prior to formation of mineralized bone nodules being indicative of new bone formation, although high levels of expression have been also reported in response to elevated temperatures, and linked to increased jaw malformations (Ytteborg et al., 2010; Ma et al., 2016). Indeed, *bmp4* has been shown to play a role in jaw development (Albertson et al., 2005) and more recently, in the determination of intermuscular bone distribution (Su and Dong, 2018). Contrarily, *op* is highly expressed in mineralized tissues but not in soft tissues, and its transcript levels increase during embryonic and larval development (Fonseca et al., 2007; Riera-Heredia et al., 2018); therefore, it could be involved in the control of mineral

deposition in fish as well as in mammals. In fact, in MC3T3-E1 murine cells, *op* has been demonstrated to negatively regulate proliferation and differentiation and more interestingly, to do it through interaction with the osteoinductive bone morphogenetic protein, *bmp2* (Huang et al., 2004). Thus overall our data, could suggest that feeding the diet HF + HT induces in fish osteoblasts proliferation while it decreases the advancement towards the maturation step (i.e., mineralization), therefore causing a major bone growth potential.

With regards to feeding regime, the decrease in the mRNA levels of *runx2* and *fib1a*, early markers of osteogenesis in this species (Riera-Heredia et al., 2018), in the animals fed with HF diet at the restricted ration compared to the standard one, suggested a reduced capacity of the cells to proliferate when fish are subjected to this level of feed restriction. However, these genes' downregulation did not occur in the fish fed with the diet containing hydroxytyrosol, supporting for this phytocompound a potential osteoinductor role in fish, as well as in mammals (García-Martínez et al., 2016). This observation is in agreement with the changes in expression found in bone in the present study concerning the members of the GH-IGF axis namely *igf-1*, *ghr-2* and *igf-1ra*, which presented the highest mRNA levels in the HF + HT group, specially under restricted conditions. Nevertheless, it has to be considered that the positive effect of hydroxytyrosol stimulating osteoblasts proliferation was mostly determined using *in vitro* treatments (García-Martínez et al., 2016) and not all cellular models respond equally, since absence (Hagiwara et al., 2011) or even contrary effects (Anter et al., 2016) have also been reported. Besides, synergistic effects among phenolic compounds present in olives and its derivatives have been suggested to be responsible for osteoblasts proliferation (Chin and Ima-Nirwana, 2016). For this reason, we aimed to evaluate if the *in vivo* administration of the hydroxytyrosol-rich extract could affect osteoblasts development *in vitro*, but neither cell proliferation nor differentiation were significantly changed, although in previous studies it has been demonstrated that the history of the fish could influence the osteogenic process *in vitro* (Balbuena-Pecino et al., 2019).

Finally, in our experimental conditions, the bone-resorbing process by osteoclasts seems not to be modulated at a transcriptional level by the dietary inclusion of hydroxytyrosol-rich extract, since *ctsk* was the only osteoclast gene marker affected by the feeding regime, being its expression reduced in those fish fed with the restricted ration. In mammalian studies, this polyphenol has been reported to inhibit the formation of osteoclasts in a dose dependent manner *in vitro* (Hagiwara et al., 2011) and to prevent bone loss in ovariectomized (osteoporosis model) rats (Puel et al., 2008). Therefore, an *in vitro* system of fish osteoclasts would be key to further evaluate the potential of hydroxytyrosol and other polyphenols in regulating bone turnover, and specifically, resorption.

Summarizing, the bone results may indicate that hydroxytyrosol does not markedly affect bone metabolism in gilthead sea bream, and only somewhat induces growth parameters in the conditions evaluated.

In conclusion, although the fish fed the hydroxytyrosol-rich extract-containing diet (HF + HT) did not show increased somatic growth in comparison to the fish fed the diet without hydroxytyrosol (HF) after 8 weeks, the fish presented elevated IGF-1 plasma levels, as well as increased expression of *igfbp-5b*, *ghr-1* and *dock5* in muscle and *igf-1*, *ghr-1* and *bmp4* in bone together with reduced *op*, suggesting improved growth potential. Therefore, even though further studies should be performed, especially in terms of exploring the specific effects of hydroxytyrosol on muscle and bone structure and remodeling, the present data supports the beneficial use of hydroxytyrosol-rich extracts in functional diets for the optimization of gilthead sea bream aquaculture since the growth capacity of the musculoskeletal system of the fish was improved even if feeding a high-fat diet.

Data availability statement

The data presented in the study are deposited in the CORA repository and can be accessed at: https://doi.org/10.34810/data217.

Ethics statement

The animal study was reviewed and approved by Ethics and Animal Care Committee of the University of Barcelona (Comitè Ètic d'Experimentació Animal—CEEA).

Author contributions

Conceptualization, IG-M, RF, AG, IN and EC; methodology, SB-P and MM; software, SB-P and MM; validation, IN and EC; formal analysis, SB-P and MM; investigation, SB-P, IG-M, AG, IN and EC; data curation, SB-P; writing—original draft preparation, SB-P, IN, and EC; writing—review and editing, SB-P, IG-M, AG, JG, IN and EC; supervision, IN and EC; funding acquisition, JG, IN and EC. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.966175/full#supplementary-material

References

Albertson, R. C., Streelman, J. T., Kocher, T. D., and Yelick, P. C. (2005). Integration and evolution of the cichlid mandible: The molecular basis of alternate feeding strategies. *Proc. Natl. Acad. Sci. U. S. A.* 102, 16287–16292. doi:10.1073/pnas.0506649102

Anter, J., Quesada-Gómez, J. M., Dorado, G., and Casado-Díaz, A. (2016). Effect of hydroxytyrosol on human mesenchymal stromal/stem cell differentiation into adipocytes and osteoblasts. *Arch. Med. Res.* 47, 162–171. doi:10.1016/j.arcmed.2016.06.006

Arsyad, M. A., Akazawa, T., Nozaki, C., Yoshida, M., Oyama, K., Mukai, T., et al. (2018). Effects of olive leaf powder supplemented to fish feed on muscle protein of red sea bream. *Fish. Physiol. Biochem.* 44, 1299–1308. doi:10.1007/s10695-018-0521-1

Aydar, A. Y. (2017). The Effect of Ultrasound on Extraction Yield and Quality Characteristics of Olive Oil PhD Thesis. Manisa Celal Bayar University, Department of Food Engineering.

Azizi, Sh., Nematollahi, M. A., Amiri, B. M., Vélez, E. J., Lutfi, E., Navarro, I., et al. (2016). Lysine and leucine deficiencies affect myocytes development and IGF signaling in gilthead sea bream (*Sparus aurata*). *PLoS One* 11, e0147618–e0147620. doi:10.1371/journal.pone.0147618

Baba, E., Acar, Ü., Yılmaz, S., Zemheri, F., and Ergün, S. (2018). Dietary olive leaf (*Olea europea* L.) extract alters some immune gene expression levels and disease resistance to *Yersinia ruckeri* infection in rainbow trout *Oncorhynchus mykiss*. *Fish. Shellfish Immunol.* 79, 28–33. doi:10.1016/j.fsi.2018.04.063

Balbuena-Pecino, S., Riera-Heredia, N., Vélez, E. J., Gutiérrez, J., Navarro, I., Riera-Codina, M., et al. (2019). Temperature affects musculoskeletal development and muscle lipid metabolism of gilthead sea bream (*Sparus aurata*). *Front. Endocrinol.* 10, 173. doi:10.3389/fendo.2019.00173

Bonaldo, A., Isani, G., Fontanillas, R., Parma, L., Grilli, E., and Gatta, P. P. (2010). Growth and feed utilization of gilthead sea bream (*Sparus aurata*, L.) fed to satiation and restrictively at increasing dietary energy levels. *Aquac. Int.* 18, 909–919. doi:10.1007/s10499-009-9312-0

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622. doi:10.1373/clinchem.2008.112797

Cao, X. F., Dai, Y. J., Liu, M. Y., Yuan, X. Y., Wang, C. C., Huang, Y. Y., et al. (2019). High-fat diet induces aberrant hepatic lipid secretion in blunt snout bream by activating endoplasmic reticulum stress-associated IRE1/XBP1 pathway. *Biochim. Biophys. Acta. Mol. Cell Biol. Lipids* 1864, 213–223. doi:10.1016/j.bbalip.2018.12.005

Capilla, E., Teles-García, Á., Acerete, L., Navarro, I., and Gutiérrez, J. (2011). Insulin and IGF-I effects on the proliferation of an osteoblast primary culture from sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol.* 172, 107–114. doi:10.1016/j.ygcen.2011.03.020

Carnovali, M., Luzi, L., Terruzzi, I., Banfi, G., and Mariotti, M. (2018). Metabolic and bone effects of high-fat diet in adult zebrafish. *Endocrine* 61, 317–326. doi:10.1007/s12020-017-1494-z

Chen, B., You, W., Wang, Y., and Shan, T. (2020). The regulatory role of Myomaker and Myomixer–Myomerger–Minion in muscle development and regeneration. *Cell. Mol. Life Sci.* 77, 1551–1569. doi:10.1007/s00018-019-03341-9

Chen, Y., Cheung, K. M. C., Kung, H. F., Leong, J. C. Y., Lu, W. W., and Luk, K. D. K. (2002). *In vivo* new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. *Biochem. Biophys. Res. Commun.* 298, 121–127. doi:10.1016/S0006-291X(02)02394-X

Chin, K. Y., and Ima-Nirwana, S. (2016). Olives and bone: A green osteoporosis prevention option. *Int. J. Environ. Res. Public Health* 13, 755. doi:10.3390/ijerph13080755

Corona, G., Deiana, M., Incani, A., Vauzour, D., Dessì, M. A., and Spencer, J. P. E. (2009). Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. *Mol. Nutr. Food Res.* 53, 897–903. doi:10.1002/mnfr.200800269

Dong, Y. Z., Li, L., Espe, M., Lu, K. L., and Rahimnejad, S. (2020). Hydroxytyrosol attenuates hepatic fat accumulation via activating mitochondrial biogenesis and autophagy through the AMPK pathway. *J. Agric. Food Chem.* 68, 9377–9386. doi:10.1021/acs.jafc.0c03310

Duan, C., Ren, H., and Gao, S. (2010). Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins: Roles in skeletal muscle growth and differentiation. *Gen. Comp. Endocrinol.* 167, 344–351. doi:10.1016/j.ygcen.2010.04.009

Eroldoğan, O. T., Taşbozan, O., and Tabakoğlu, S. (2008). Effects of restricted feeding regimes on growth and feed utilization of juvenile gilthead sea bream. *Sparus Aurata. J. World Aquac. Soc.* 39, 267–274. doi:10.1111/j.1749-7345.2008.00157.x

Escobar-Aguirre, S., Felip, A., Mazón, M. J., Ballester-Lozano, G., Pérez-Sánchez, J., Björsson, B. T., et al. (2020). Long-term feeding of a maintenance ration affects the release of Igf-1 and leptin, and delays maturation in a male teleost fish, *Dicentrarchus labrax* L. *Dicentrarchus labrax L. Aquac.* 527, 735467. doi:10.1016/j.aquaculture.2020.735467

Fabiani, R., Sepporta, M. V., Rosignoli, P., De Bartolomeo, A., Crescimanno, M., and Morozzi, G. (2012). Anti-proliferative and pro-apoptotic activities of hydroxytyrosol on different tumour cells: The role of extracellular production of hydrogen peroxide. *Eur. J. Nutr.* 51, 455–464. doi:10.1007/s00394-011-0230-3

Fonseca, V. G., Laizé, V., Valente, M. S., and Cancela, M. L. (2007). Identification of an osteopontin-like protein in fish associated with mineral formation. *FEBS J.* 274, 4428–4439. doi:10.1111/j.1742-4658.2007.05972.x

Fuentes, E. N., Valdés, J. A., Molina, A., and Björnsson, B. T. (2013). Regulation of skeletal muscle growth in fish by the growth hormone - insulin-like growth factor system. *Gen. Comp. Endocrinol.* 192, 136–148. doi:10.1016/j.ygcen.2013.06.009

Garcia de la Serrana, D., and Macqueen, D. J. (2018). Insulin-like growth factor-binding proteins of teleost fishes. *Front. Endocrinol.* 9, 80–12. doi:10.3389/fendo.2018.00080

García-Martínez, O., De Luna-Bertos, E., Ramos-Torrecillas, J., Ruiz, C., Milia, E., Lorenzo, M. L., et al. (2016). Phenolic compounds in extra virgin olive oil stimulate

human osteoblastic cell proliferation. *PLoS One* 11, e0150045. doi:10.1371/journal.pone.0150045

Ghanbari, R., Anwar, F., Alkharfy, K. M., Gilani, A. H., and Saari, N. (2012). Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.)-A review. *Int. J. Mol. Sci.* 13, 3291–3340. doi:10.3390/ijms13033291

Gisbert, E., Andree, K. B., Quintela, J. C., Calduch-Giner, J. A., Ipharraguerre, I. R., and Pérez-Sánchez, J. (2017). Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* 117, 351–363. doi:10.1017/S0007114517000228

Gong, D., Geng, C., Jiang, L., Cao, J., Yoshimura, H., and Zhong, L. (2009). Effects of Hydroxytyrosol-20 on carrageenan-induced acute inflammation and hyperalgesia in rats. *Phytother. Res.* 23, 646–650. doi:10.1002/ptr.2686

González-Hedström, D., Priego, T., Amor, S., de la Fuente-Fernández, M., Martín, A. I., López-Calderón, A., et al. (2021). Olive leaf extract supplementation to old wistar rats attenuates aging-induced sarcopenia and increases insulin sensitivity in adipose tissue and skeletal muscle. *Antioxidants* 10, 737. doi:10.3390/antiox10050737

Hagiwara, K., Goto, T., Araki, M., Miyazaki, H., and Hagiwara, H. (2011). Olive polyphenol hydroxytyrosol prevents bone loss. *Eur. J. Pharmacol.* 662, 78–84. doi:10.1016/j.ejphar.2011.04.023

Hariri, N., and Thibault, L. (2010). High-fat diet-induced obesity in animal models. *Nutr. Res. Rev.* 23, 270–299. doi:10.1017/S0954422410000168

Hazreen-Nita, M. K., Abdul Kari, Z., Mat, K., Rusli, N. D., Mohamad Sukri, S. A., Che Harun, H., et al. (2022). Olive oil by-products in aquafeeds : Opportunities and challenges. *Aquac. Rep.* 22, 100998. doi:10.1016/j.aqrep.2021.100998

Hoseinifar, S. H., Shakouri, M., Yousefi, S., Van Doan, H., Shafiei, S., Yousefi, M., et al. (2020). Humoral and skin mucosal immune parameters, intestinal immune related genes expression and antioxidant defense in rainbow trout (*Oncorhynchus mykiss*) fed olive (*Olea europea* L.) waste. *Fish. Shellfish Immunol.* 100, 171–178. doi:10.1016/j.fsi.2020.02.067

Huang, W., Carlsen, B., Rudkin, G., Berry, M., Ishida, K., Yamaguchi, D. T., et al. (2004). Osteopontin is a negative regulator of proliferation and differentiation in MC3T3-E1 pre-osteoblastic cells. *Bone* 34, 799–808. doi:10.1016/j.bone.2003.11.027

Illesca, P., Valenzuela, R., Espinosa, A., Echeverría, F., Soto-Alarcon, S., Ortiz, M., et al. (2019). Hydroxytyrosol supplementation ameliorates the metabolic disturbances in white adipose tissue from mice fed a high-fat diet through recovery of transcription factors Nrf2, SREBP-1c, PPAR-γ and NF-κB. *Biomed. Pharmacother.* 109, 2472–2481. doi:10.1016/j.biopha.2018.11.120

Infante, A., and Rodríguez, C. I. (2018). Osteogenesis and aging: Lessons from mesenchymal stem cells. *Stem Cell Res. Ther.* 9, 244. doi:10.1186/s13287-018-0995-x

Izutsu, A., Tadokoro, D., Habara, S., Ugachi, Y., and Shimizu, M. (2022). Evaluation of circulating insulin-like growth factor (IGF)-I and IGF-binding proteins as growth indices in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 320, 114008. doi:10.1016/j.ygcen.2022.114008

Jin, M., Zhu, T., Tocher, D. R., Luo, J., Shen, Y., Li, X., et al. (2020). Dietary fenofibrate attenuated high-fat-diet-induced lipid accumulation and inflammation response partly through regulation of pparα and sirt1 in juvenile black seabream (*Acanthopagrus schlegelii*). *Dev. Comp. Immunol.* 109, 103691. doi:10.1016/j.dci.2020.103691

Johnston, I. A. (2006). Environment and plasticity of myogenesis in teleost fish. *J. Exp. Biol.* 209, 2249–2264. doi:10.1242/jeb.02153

Ka, J., Pak, B., Han, O., Lee, S., and Jin, S. W. (2020). Comparison of transcriptomic changes between zebrafish and mice upon high fat diet reveals evolutionary convergence in lipid metabolism. *Biochem. Biophys. Res. Commun.* 530, 638–643. doi:10.1016/j.bbrc.2020.07.042

Leibush, B. N., and Lappova, Y. L. (1995). Insulin receptor downregulation in isolated hepatocytes of river lamprey (*Lampetra fluviatilis*). *Gen. Comp. Endocrinol.* 100, 10–17. doi:10.1006/gcen.1995.1126

Lutfi, E., Babin, P. J., Gutiérrez, J., Capilla, E., and Navarro, I. (2017). Caffeic acid and hydroxytyrosol have anti-obesogenic properties in zebrafish and rainbow trout models. *PLoS One* 12, e0178833. doi:10.1371/journal.pone.0178833

Ma, Z., Zhang, N., Qin, J. G., Fu, M., and Jiang, S. (2016). Water temperature induces jaw deformity and bone morphogenetic proteins (BMPs) gene expression in golden pompano *Trachinotus ovatus* larvae. *Springerplus* 5, 1475. doi:10.1186/s40064-016-3142-0

Marković, A. K., Torić, J., Barbarić, M., and Brala, C. J. (2019). Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules* 24, 2001. doi:10.3390/molecules24102001

Medina, E., De Castro, A., Romero, C., and Brenes, M. (2006). Comparison of the concentrations of phenolic compounds in olive oils and other plant oils: Correlation with antimicrobial activity. *J. Agric. Food Chem.* 54, 4954–4961. doi:10.1021/jf0602267

Melguizo-Rodríguez, L., Manzano-Moreno, F. J., Illescas-Montes, R., Ramos-Torrecillas, J., Luna-Bertos, E. D., Ruiz, C., et al. (2019). Bone protective effect of extra-virgin olive oil phenolic compounds by modulating osteoblast gene expression. *Nutrients* 11, 1722. doi:10.3390/nu11081722

Moore, C. A., Parkin, C. A., Bidet, Y., and Ingham, P. W. (2007). A role for the myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion. *Development* 134, 3145–3153. doi:10.1242/dev.001214

Mourente, G., and Bell, J. G. (2006). Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax* L.) over a long term growth study: Effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 145, 389–399. doi:10.1016/j.cbpb.2006.08.012

Nasopoulou, C., Stamatakis, G., Demopoulos, C. A., and Zabetakis, I. (2011). Effects of olive pomace and olive pomace oil on growth performance, fatty acid composition and cardio protective properties of gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). *Food Chem.* 129, 1108–1113. doi:10.1016/j.foodchem.2011.05.086

Párrizas, M., Baños, N., Baró, J., Planas, J., and Gutiérrez, J. (1994). Up-regulation of insulin binding in fish skeletal muscle by high insulin levels. *Regul. Pept.* 53, 211–222. doi:10.1016/0167-0115(94)90170-8

Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha, J. A., Perera, E., Bermejo-Nogales, A., et al. (2018). Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Front. Endocrinol.* 9, 687. doi:10.3389/fendo.2018.00687

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29, e45–e2007. doi:10.1093/nar/29.9.e45

Picha, M. E., Turano, M. J., Beckman, B. R., and Borski, R. J. (2008). Endocrine biomarkers of growth and applications to aquaculture: A minireview of growth hormone, insulin-like growth factor (IGF)-I, and IGF-binding proteins as potential growth indicators in fish. *N. Am. J. Aquac.* 70, 196–211. doi:10.1577/a07-038.1

Pierce, A. L., Beckman, B. R., Shearer, K. D., Larsen, D. A., and Dickhoff, W. W. (2001). Effects of ration on somatotropic hormones and growth in coho salmon. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 128, 255–264. doi:10.1016/S1096-4959(00)00324-9

Plisetskaya, E. M., Fabbri, E., Moon, T. W., Gutiérrez, J., and Ottolenghi, C. (1993). Insulin binding to isolated hepatocytes of Atlantic salmon and rainbow trout. *Fish. Physiol. Biochem.* 11, 401–409. doi:10.1007/BF00004590

Puel, C., Mardon, J., Agalias, A., Davicco, M. J., Lebecque, P., Mazur, A., et al. (2008). Major phenolic compounds in olive oil modulate bone loss in an ovariectomy/inflammation experimental model. *J. Agric. Food Chem.* 56, 9417–9422. doi:10.1021/jf801794q

Reindl, K. M., and Sheridan, M. A. (2012). Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 163, 231–245. doi:10.1016/j.cbpa.2012.08.003

Riera-Heredia, N., Martins, R., Mateus, A. P., Costa, R. A., Gisbert, E., Navarro, I., et al. (2018). Temperature responsiveness of gilthead sea bream bone; an *in vitro* and *in vivo* approach. *Sci. Rep.* 8, 11211–11214. doi:10.1038/s41598-018-29570-9

Rodríguez-Viera, L., Martí, I., Martínez, R., Perera, E., Estrada, M. P., Mancera, J. M., et al. (2022). Feed supplementation with the GHRP-6 peptide, a ghrelin analog, improves feed intake, growth performance and aerobic metabolism in the gilthead sea bream *Sparus aurata*. *Fishes* 7, 31. doi:10.3390/fishes7010031

Rong, J., Han, Y., Zha, S., Tang, Y., Shi, W., Guan, X., et al. (2020). Triterpene-enriched olive extract as an immunopotentiator in black sea bream (*Acanthopagrus schlegelii*). *J. Ocean. Univ. China* 19, 428–438. doi:10.1007/s11802-020-4232-8

Salem, M., Kenney, P. B., Rexroad, C. E., and Yao, J. (2010). Proteomic signature of muscle atrophy in rainbow trout. *J. Proteomics* 73, 778–789. doi:10.1016/j.jprot.2009.10.014

Salmerón, C., Johansson, M., Angotzi, A. R., Rønnestad, I., Jönsson, E., Björnsson, B. T., et al. (2015). Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout. *Gen. Comp. Endocrinol.* 210, 114–123. doi:10.1016/j.ygcen.2014.10.016

Salucci, S., and Falcieri, E. (2020). Polyphenols and their potential role in preventing skeletal muscle atrophy. *Nutr. Res.* 74, 10–22. doi:10.1016/j.nutres.2019.11.004

Seno-o, A., Takakuwa, F., Hashiguchi, T., Morioka, K., Masumoto, T., and Fukada, H. (2008). Replacement of dietary fish oil with olive oil in young yellowtail *Seriola quinqueradiata*: Effects on growth, muscular fatty acid

composition and prevention of dark muscle discoloration during refrigerated storage. *Fish. Sci.* 74, 1297–1306. doi:10.1111/j.1444-2906.2008.01655.x

Seth, A., Stemple, D. L., and Barroso, I. (2013). The emerging use of zebrafish to model metabolic disease. *Dis. Model. Mech.* 6, 1080–1088. doi:10.1242/dmm.011346

Shimizu, M., Swanson, P., Fukada, H., Hara, A., and Dickhoff, W. W. (2000). Comparison of extraction methods and assay validation for salmon insulin-like Growth Factor-I using commercially available components. *Gen. Comp. Endocrinol.* 119, 26–36. doi:10.1006/gcen.2000.7498

Sicuro, B., Daprà, F., Gai, F., Palmegiano, G. B., Schiavone, R., Zilli, L., et al. (2010). Olive oil by-product as a natural antioxidant in gilthead sea bream (*Sparus aurata*) nutrition. *Aquac. Int.* 18, 511–522. doi:10.1007/s10499-009-9262-6

Sofi, F., Abbate, R., Gensini, G. F., and Casini, A. (2010). Accruing evidence on benefits of adherence to the Mediterranean diet on health: An updated systematic review and meta-analysis. *Am. J. Clin. Nutr.* 92, 1189–1196. doi:10.3945/ajcn.2010.29673

Sokooti, R., Chelemal Dezfoulnejad, M., and Javaheri baboli, M. (2021). Effects of olive leaf extract (*Olea europaea Leecino*) on growth, haematological parameters, immune system and carcass composition in common carp (*Cyprinus carpio*). *Aquac. Res.* 52, 2415–2423. doi:10.1111/are.15091

Stefanon, B., and Colitti, M. (2016). Original Research: Hydroxytyrosol, an ingredient of olive oil, reduces triglyceride accumulation and promotes lipolysis in human primary visceral adipocytes during differentiation. *Exp. Biol. Med.* 241, 1796–1802. doi:10.1177/1535370216654226

Su, S., and Dong, Z. (2018). Comparative expression analyses of bone morphogenetic protein 4 (BMP4) expressions in muscles of tilapia and common carp indicate that BMP4 plays a role in the intermuscular bone distribution in a dose-dependent manner. *Gene Expr. Patterns* 27, 106–113. doi:10.1016/j.gep.2017.11.005

Szychlinska, M. A., Castrogiovanni, P., Trovato, F. M., Nsir, H., Zarrouk, M., Lo Furno, D., et al. (2019). Physical activity and Mediterranean diet based on olive tree phenolic compounds from two different geographical areas have protective effects on early osteoarthritis, muscle atrophy and hepatic steatosis. *Eur. J. Nutr.* 58, 565–581. doi:10.1007/s00394-018-1632-2

Torstensen, B. E., Frøyland, L., and Lie, O. (2004). Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil - effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquac. Nutr.* 10, 175–192. doi:10.1111/j.1365-2095.2004.00289.x

Tuck, K. L., and Hayball, P. J. (2002). Major phenolic compounds in olive oil: Metabolism and health effects. *J. Nutr. Biochem.* 13, 636–644. doi:10.1016/S0955-2863(02)00229-2

Turchini, G. M., Francis, D. S., Senadheera, S. P. S. D., Thanuthong, T., and De Silva, S. S. (2011). Fish oil replacement with different vegetable oils in Murray cod: Evidence of an "omega-3 sparing effect" by other dietary fatty acids. *Aquaculture* 315, 250–259. doi:10.1016/j.aquaculture.2011.02.016

Vélez, E. J., Lutfi, E., Jiménez-Amilburu, V., Riera-Codina, M., Capilla, E., Navarro, I., et al. (2014). IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. *Gen. Comp. Endocrinol.* 205, 296–304. doi:10.1016/j.ygcen.2014.05.024

Vélez, E. J., Azizi, Sh., Millán-Cubillo, A., Fernández-Borràs, J., Blasco, J., Chan, S. J., et al. (2016). Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 310, R313–R322. doi:10.1152/ajpregu.00230.2015

Vélez, E. J., Perelló, M., Azizi, Sh., Moya, A., Lutfi, E., Pérez-Sánchez, J., et al. (2018). Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol.* 257, 192–202. doi:10.1016/j.ygcen.2017.06.019

Vélez, E. J., Perelló-Amorós, M., Lutfi, E., Azizi, S., Capilla, E., Navarro, I., et al. (2019). A long-term growth hormone treatment stimulates growth and lipolysis in gilthead sea bream juveniles. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 232, 67–78. doi:10.1016/j.cbpa.2019.03.012

Vieira, F. A., Thorne, M. A. S., Stueber, K., Darias, M., Reinhardt, R., Clark, M. S., et al. (2013). Comparative analysis of a teleost skeleton transcriptome provides insight into its regulation. *Gen. Comp. Endocrinol.* 191, 45–58. doi:10.1016/j.ygcen.2013.05.025

Wang, X., Li, Y., Hou, C., Gao, Y., and Wang, Y. (2015). Physiological and molecular changes in large yellow croaker (*Pseudosciaena crocea* R.) with high-fat diet-induced fatty liver disease. *Aquac. Res.* 46, 272–282. doi:10.1111/are.12176

Warleta, F., Quesada, C. S., Campos, M., Allouche, Y., Beltrán, G., and Gaforio, J. J. (2011). Hydroxytyrosol protects against oxidative DNA damage in human breast cells. *Nutrients* 3, 839–857. doi:10.3390/nu3100839

Witten, P. E., and Huysseune, A. (2009). A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. *Biol. Rev. Camb. Philos. Soc.* 84, 315–346. doi:10.1111/j.1469-185X.2009.00077.x

Witten, P. E., Obach, A., Huysseune, A., and Baeverfjord, G. (2006). Vertebrae fusion in Atlantic salmon (*Salmo salar*): Development, aggravation and pathways of containment. *Aquaculture* 258, 164–172. doi:10.1016/j.aquaculture.2006.05.005

Ytteborg, E., Vegusdal, A., Witten, P. E., Berge, G. M., Takle, H., Østbye, T. K., et al. (2010). Atlantic salmon (*Salmo salar*) muscle precursor cells differentiate into osteoblasts *in vitro*: Polyunsaturated fatty acids and hyperthermia influence gene expression and differentiation. *Biochim. Biophys. Acta* 1801, 127–137. doi:10.1016/j.bbalip.2009.10.001

Zemheri-Navruz, F., Acar, Ü., and Yılmaz, S. (2020). Dietary supplementation of olive leaf extract enhances growth performance, digestive enzyme activity and growth related genes expression in common carp *Cyprinus carpio*. *Gen. Comp. Endocrinol.* 296, 113541. doi:10.1016/j.ygcen.2020.113541

Zhang, Y., Sun, Z., Wang, A., Ye, C., and Zhu, X. (2017). Effects of dietary protein and lipid levels on growth, body and plasma biochemical composition and selective gene expression in liver of hybrid snakehead (*Channa maculata* ♀ × *Channa argus* ♂) fingerlings. *Aquaculture* 468, 1–9. doi:10.1016/j.aquaculture.2016.09.052

Zou, Y. Y., Chen, Z. L., Sun, C. C., Yang, D., Zhou, Z. Q., Xiao, Q., et al. (2022). A high-fat diet induces muscle mitochondrial dysfunction and impairs swimming capacity in zebrafish: A new model of sarcopenic obesity. *Nutrients* 14, 1975. doi:10.3390/nu14091975

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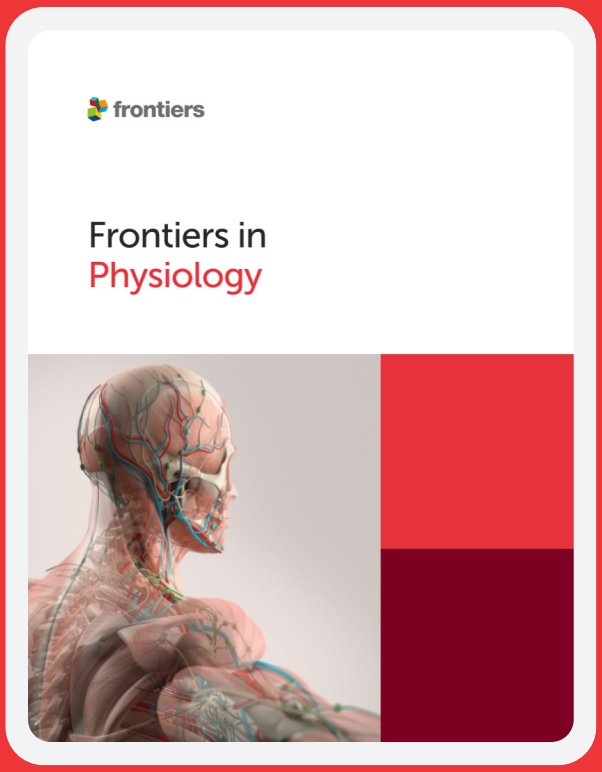

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