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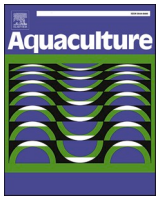

Strategy combining mammalian fats with supplementation of pungent spices in aquafeeds, to mitigate negative impacts of fish oil replacement in fish performance, fillet quality and hepatic condition of gilthead seabream (*Sparus aurata*)

Alberto Ruiz [a], Enric Gisbert [a], Alicia Estevez [a], Felipe E. Reyes-López [b], Eva Vallejos-Vidal [b,c], Lluís Tort [d], Jorge Dias [e], Sofia Engrola [f], Sara Magalhães [g], Tiago Aires [g], Sofia Morais [h,*]

[a] *Aquaculture Program, Institut de Recerca i Tecnologia Agroaliment'aries (IRTA), Centre de La Ràpita, La Ràpita, Spain*
[b] *Fish Health and Integrative Physiogenomics Research Team, Centro de Biotecnología Acuícola, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile*
[c] *Núcleo de Investigaciones Aplicadas en Ciencias Veterinarias y Agronómicas, Facultad de Medicina Veterinaria y Agronomía, Universidad De Las Américas, La Florida, Santiago, Chile*
[d] *Dpt. Cell Biology, Physiology and Immunology, Universitat Autonoma de Barcelona, Spain*
[e] *Sparos Lda., Área Empresarial de Marim, Olhão, Portugal*
[f] *Centre of Marine Sciences (CCMAR/CIMAR LA), Campus de Gambelas, Universidade do Algarve, Faro, Portugal*
[g] *Sorgal S.A., Sociedade de Óleos e Rações, EN109 Pardala, 3880-728 São João Ovar, Portugal*
[h] *Lucta S.A., Innovation Division, Animal Science Unit, Bellaterra, Spain*

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A B S T R A C T

The replacement of fish oil (FO) in aquafeeds usually leads to imbalances in the dietary content of n-3 and n-6 polyunsaturated fatty acids (PUFA), with negative consequences for fish performance, health, and fillet quality. Animal-rendered fats are an alternative lipid source high in saturated fatty acids, potentially sparing n-3 PUFA from oxidation, and with lower n-6 PUFA contents than plant-based oils, especially in the case of mammalian fats (MF). Hence, this work assessed the effect of replacing 45% FO by MF (negative control, NC) compared to a diet containing only FO (positive control, PC) in gilthead seabream (*Sparus aurata*, initial body weight: 85 ± 4 g, mean ± standard deviation) at low water temperature. In addition, we studied the effect of supplementing the NC diet with a combination of pungent spices with hypolipidemic and anti-inflammatory properties at three inclusion levels: 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$). At the end of the trial (112 days), FO substitution by MF led to poorer fish performance in terms of body weight (BW), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER). Supplementation of the NC diet with spices numerically improved SGR, FCR and PER at all inclusion levels (non-significantly different from the PC group), being FCR and PER significantly different from the NC group in the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ treatments. A remarkable increase in lipid level was observed in fillets of fish fed the NC diet, but was fully counteracted by spice supplementation, especially in fish fed the $SPICY_{0.15\%}$ diet. A lower fat accumulation was also found in the liver of fish fed the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets, with respect to the PC and NC groups. The fillets' fatty acid profile mostly reflected the diet composition, but the $SPICY_{0.15\%}$ diet modified it in an inverse direction to that observed in the NC, to more closely resemble the profile of fish fed the PC diet. In particular, the $SPICY_{0.15\%}$ diet reduced fillet levels of MUFA, linoleic and linolenic acids, and increased n-3 LC-PUFA (including EPA and DHA), compared to the NC. A microarray-based transcriptomic analysis revealed a better hepatic health status, as indicated by different biological processes associated to immunity. Overall, supplementation with the combination of pungent spices at 0.15% enabled the incorporation of alternative lipid sources, such as MF, in aquafeeds without significantly compromising growth and feeding performance, liver health, and quality of the edible product.

* Corresponding author.
E-mail address: sofia.morais@lucta.com (S. Morais).

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1. Introduction

Traditionally, aquafeeds were formulated using fish oil (FO) as the main lipid source, as it is an important source of energy and n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) (Dobrzański et al., 2002). These n-3 PUFA, in particular, docosahexaenoic (C22:6 n-3, DHA) and eicosapentaenoic (C20:5 n-3, EPA) acids, have well-known growth- and health-promoting effects in fish (Sargent et al., 2002; Peng et al., 2014). Since the fish body composition is directly related to the feed composition, maintaining high dietary levels of n-3 PUFA (*i.e.*, EPA and DHA) is also essential for supporting a healthy diet from the consumer's point of view (Ballester-Lozano et al., 2011). However, the availability of FO has become increasingly compromised during the last decades due to its high demand associated to the growth of the aquaculture industry and to human consumption as a nutraceutical supplement (Shepherd and Bachis, 2014). Its limited availability, increasing prices and sustainability concerns, have led to the search for other functional ingredients to be used as lipid sources in the aquafeed industry (Klinger and Naylor, 2012). Recent research has focused on the incorporation of other n-3 LC-PUFA-rich oils derived from microalgae, zooplankton, mesopelagic fish, single-cell organisms, and/or genetically modified terrestrial plant seeds and oilseeds in aquafeeds (Turchini et al., 2009; Tocher, 2015; Alhazzaa et al., 2019). Nevertheless, there is still a long way to go to have these novel ingredients widely available to feed formulators, considering their low supply volumes and high production costs, as well as the widespread public rejection of genetically modified products (Tocher, 2015; Turchini et al., 2009). Thus, other nutritional strategies need to be explored to face the challenge of guaranteeing a reliable supply of good quality fats for aquafeeds without compromising fish health and nutritional quality. The amount of FO included in aquafeeds has already been substantially diminishing during the last decades, being replaced by other lipid sources, mostly plant-based oils (Naylor et al., 2021). Although partial substitution of FO by plant-based oils does not usually jeopardize fish growth and feeding efficiency, it results in decreased levels of n-3 LC-PUFA and increased contents of n-6 LC-PUFA in the whole-body and fillet proximate composition (Trushenski et al., 2009; Kenari et al., 2011). Such imbalances in the fatty acid profile can entail physiological disorders, such as dysregulations in energy and lipid metabolism, promoting lipogenesis (Panserat et al., 2009; Morais et al., 2012a; Xu et al., 2022), accumulation of fat deposits in digestive organs (Olsen et al., 2000; Ballester-Lozano et al., 2015) and inflammation (Ballester-Lozano et al., 2015; Milián-Sorribes et al., 2021; Xu et al., 2022). In this sense, inflammation can potentially be induced by the production of pro-inflammatory eicosanoid lipid mediators derived from n-6 PUFA and decreased generation of anti-inflammatory eicosanoids derived from n-3 PUFA (Saini and Keum, 2018).

An alternative to plant oils in replacement of FO in aquafeeds is the incorporation of animal-rendered fats. Rendered fats are affordable by-products, widely produced with a much lower carbon footprint than plant-based ingredients grown specifically for animal feeds (EFPRA, 2021), so their valorization could be a further step towards an economically and environmentally sustainable development of the aquaculture industry. In terms of fish health and quality, the two main advantages of the use of animal-rendered fats are: i) their content of n-6 LC-PUFA is markedly lower than in plant-based oil, especially in the case of mammalian fats (MF), which barely contain n-6 LC-PUFA (Moretti and Corino, 2008; Marques et al., 2022), and ii) rendered fats have high levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (Turchini et al., 2009). In this sense, the preferential β-oxidation of SFA and MUFA has been shown to spare n-3 LC-PUFA from catabolism, helping to better conserve DHA and EPA in fish tissues (Fonseca-Madrigal et al., 2005; Trushenski and Lochmann, 2009). However, the low levels of n-3 PUFA provided by animal-rendered fats can also cause some of the above-mentioned physiological effects attributed to FO reduction, such as imbalances in the fatty acid profile or increase in fat accumulation (Monteiro et al., 2018; Campos et al., 2019). In addition, SFA have a lower digestibility (Trushenski et al., 2009), especially at low water temperatures (Ng et al., 2003, 2004). Consequently, other complementary strategies need to be explored in order to mitigate the negative impacts of the reduction of FO and their replacement by alternative lipid sources, including animal-rendered fats.

In a previous nutritional trial with gilthead seabream (*Sparus aurata*), we tested the effect of supplementing diets containing high levels of poultry fat and reduced levels of FO and soybean oil with a combination of pungent spices (Ruiz et al., 2023). As a result, we observed an enhanced growth and feeding efficiency, an increase in bile acid secretion and bile salt-activated lipase activity in the intestine, coupled with a regulation of lipid metabolism and reduction of perivisceral fat and lipid deposits in the digestive organs. In the present trial, our purpose was to further evaluate the previously tested combination of pungent spices in a diet where 45% of FO was replaced by MF, in gilthead seabream juveniles. Moreover, the trial was conducted during the winter-spring (January to May) grow-out period, when low water temperature (16.2 ± 1.9 °C) can be seen as a constraining factor in terms of lipid digestibility and fish condition.

2. Material and methods

2.1. Ethics statement

All procedures involving animal care, manipulation, and sampling were carried out by trained scientists (following the Federation of European Laboratory Animal Science Associations – FELASA category C recommendations) and were conducted according to the European (Directive 2010/63/EU of European Parliament and the Council of the European Union) and Portuguese (Decreto-Lei n° 113/2013 de 7 de Agosto) legislation on the protection of animals used for scientific purposes.

2.2. Diets

Diets (3 mm pellet size) were manufactured by Sparos Lda. (Portugal) as described in Ruiz et al. (2024). The five diets manufactured for this trial were isoproteic (49% crude protein), isolipidic (17% crude fat), and isoenergetic (21.8 MJ kg^{-1}) (Table 1). In brief, all powder ingredients, including the encapsulated combination of oleoresins from pungent spices, were mixed (TGC Extrusion model 500 L, France) and ground below 400 μm in a micropulverizer hammer mill (Hosokawa-Alpine model SH1, Germany). Extrusion conditions were: feeder rate (83 kg h^{-1}), screw speed (247 rpm), water addition (approximately 290 mL min^{-1}), temperature barrel 1 (24–29 °C), temperature barrel 2 (59–62 °C), and temperature barrel 3 (105–109 °C). The resulting pellets were dried in a vibrating fluid bed dryer (TGC Extrusion model DR100, France) and then coated with oils using a vacuum coater (Dinnissen model PG-10VCLAB, The Netherlands). One diet was used as the positive control (PC), with FO as the main lipid source (11.5%, Savinor UTS), and a second formulation was prepared and used as the negative control (NC), with 45% FO being replaced by MF (a mixture of pork lard and beef tallow, Savinor UTS). The NC diet contained higher levels of palmitic acid (C16:0, PA; 24% of total fatty acids, TFA) and stearic acid (C18:0, SA; 9% TFA), oleic acid (C18:1n-9, OA; 25% TFA) and linoleic acid (C18:2n-6, LA; 9% TFA), and lower levels of n-3 PUFA compared to the PC diet (13% TFA; Table 2). The other three diets contained the same ingredient formulation and proximate composition as the NC diet but were supplemented with increasing levels of an encapsulated combination of pungent spices at 0.05, 0.1 and 0.15% ($SPICY_{0.05\%}$, $SPICY_{0.1\%}$, and $SPICY_{0.15\%}$, respectively). The encapsulated (fat matrix) combination of spices contained a mixture of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde (Lucta S.A., Spain). During the feed manufacturing process, the mixture of spices was added into the pre-extruded mash with the rest of the powdered ingredients. Their

Table 1
Ingredient formulation (%) and proximate composition (% in dry basis) of the experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets				
Ingredients (%)	PC	NC	$SPICY_{0.05\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Fishmeal Super Prime	10.00	10.00	10.00	10.00	10.00
Fishmeal 60	7.00	7.00	7.00	7.00	7.00
Fish protein concentrate	2.00	2.00	2.00	2.00	2.00
Feather meal hydrolysate	5.00	5.00	5.00	5.00	5.00
Porcine blood meal	2.50	2.50	2.50	2.50	2.50
Poultry meal	15.00	15.00	15.00	15.00	15.00
AminoPRO 70	4.00	4.00	4.00	4.00	4.00
Corn gluten meal	5.00	5.00	5.00	5.00	5.00
Soybean meal 48	10.00	10.00	10.00	10.00	10.00
Sunflower meal	5.00	5.00	5.00	5.00	5.00
Wheat meal	13.26	13.26	13.21	13.16	13.11
Whole peas	5.00	5.00	5.00	5.00	5.00
Pea starch	2.50	2.50	2.50	2.50	2.50
Fish oil	11.50	6.33	6.33	6.33	6.33
Mammalian-rendered fat	0.00	5.18	5.18	5.18	5.18
Vitamin and mineral premix	1.00	1.00	1.00	1.00	1.00
Vitamin C35	0.05	0.05	0.05	0.05	0.05
Vitamin E50	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.10	0.10	0.10	0.10	0.10
Betaine HCl	0.20	0.20	0.20	0.20	0.20
Antioxidant	0.20	0.20	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10	0.10	0.10
Monoammonium phosphate	0.35	0.35	0.35	0.35	0.35
DL-Methionine	0.20	0.20	0.20	0.20	0.20
Combination of spices (Lucta)	0.00	0.00	0.05	0.10	0.15
Yttrium oxide	0.02	0.02	0.02	0.02	0.02
Proximate composition in dry basis					
Dry matter (%)	94.2 ± 0.03	94.4 ± 0.02	94.0 ± 0.04	94.9 ± 0.01	94.8 ± 0.04
Crude protein (%)	48.8 ± 0.06	48.7 ± 0.07	48.8 ± 0.13	48.4 ± 0.01	48.5 ± 0.02
Crude fat (%)	17.2 ± 0.09	17.3 ± 0.14	17.4 ± 0.08	17.2 ± 0.04	17.1 ± 0.02
Ash (%)	8.3 ± 0.08	8.3 ± 0.01	8.3 ± 0.02	8.3 ± 0.08	8.4 ± 0.02
Gross energy (MJ kg^{-1})	21.8 ± 0.06	21.8 ± 0.05	21.9 ± 0.08	21.7 ± 0.04	21.8 ± 0.07

The proximate composition of the diet was analyzed in duplicate; values are represented as mean ± standard deviation (SD).

encapsulation enabled spices' manipulation during feed preparation since pungent substances can be irritable at high concentrations. In addition, yttrium oxide (Y_2O_3, at 0.2 g kg^{-1}) was incorporated as an inert tracer to measure the apparent digestibility coefficients (ADCs) of macronutrients and energy from the feed.

2.3. Fish rearing, feeding trial and sampling procedures

Juveniles of gilthead seabream were purchased from a commercial fish farm (Atlantik Fish Lda., Portugal) and transported to the research facilities of the Center of Marine Science (CCMAR) (Faro, Portugal). Fish were stocked at the research facilities for >6 months and fed with a standard commercial feed (Standard Orange, Aquasoja, Portugal: 43% crude protein, 17% crude fat) until the beginning of the experiment.

Table 2
Fatty acid profile (% of total fatty acids) of the experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by rendered mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets				
	PC	NC	$SPICY_{0.05\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
14:0	6.91 ± 1.08	4.61 ± 0.93	4.85 ± 0.95	4.30 ± 0.91	4.55 ± 0.92
16:0	21.47 ± 1.72	23.79 ± 1.79	24.89 ± 1.83	21.89 ± 1.73	22.86 ± 1.76
18:0	4.63 ± 0.93	8.98 ± 1.20	9.46 ± 1.22	8.77 ± 1.19	9.20 ± 1.21
Total SFA	35.34 ± 2.13	39.49 ± 2.24	41.38 ± 2.28	36.90 ± 2.17	38.57 ± 2.21
16:1n-7	7.57 ± 1.12	4.77 ± 0.94	4.82 ± 0.94	5.11 ± 0.96	5.26 ± 0.98
18:1n-7	2.91 ± 0.79	2.36 ± 0.74	2.66 ± 0.77	2.47 ± 0.75	2.50 ± 0.75
18:1n-9	15.80 ± 1.51	25.33 ± 1.84	25.09 ± 1.83	25.64 ± 1.85	25.55 ± 1.85
20:1n-9	1.81 ± 0.68	1.17 ± 0.59	1.15 ± 0.59	1.25 ± 0.60	1.24 ± 0.60
22:1n-11	1.74 ± 0.67	1.14 ± 0.59	0.89 ± 0.55	1.26 ± 0.60	1.05 ± 0.57
Total MUFA	30.62 ± 2.00	35.33 ± 2.13	35.15 ± 2.12	36.29 ± 2.15	36.16 ± 2.15
18:2n-6	5.74 ± 1.01	8.54 ± 1.17	8.60 ± 1.18	8.40 ± 1.17	8.48 ± 1.17
20:4n-6 (ARA)	0.88 ± 0.55	0.55 ± 0.48	0.54 ± 0.48	0.61 ± 0.50	0.60 ± 0.49
Total n-6 PUFA	7.08 ± 1.09	9.37 ± 1.22	9.39 ± 1.22	9.29 ± 1.21	9.38 ± 1.22
18:3n-3	1.05 ± 0.57	0.88 ± 0.55	0.88 ± 0.55	0.90 ± 0.55	0.91 ± 0.55
20:5n-3 (EPA)	13.65 ± 1.42	6.22 ± 1.04	6.01 ± 1.03	7.14 ± 1.09	7.06 ± 1.09
22:5n-3	1.57 ± 0.65	0.77 ± 0.53	0.81 ± 0.53	0.84 ± 0.54	0.84 ± 0.54
22:6n-3 (DHA)	9.60 ± 1.23	4.82 ± 0.94	4.58 ± 0.93	5.41 ± 0.99	5.24 ± 0.97
Total n-3 PUFA	25.87 ± 1.86	12.69 ± 1.43	12.28 ± 1.36	14.29 ± 1.50	14.05 ± 1.44
Total PUFA	33.51 ± 2.08	22.65 ± 1.80	22.23 ± 1.74	24.18 ± 1.85	23.99 ± 1.80

Data are shown as the mean ± SD (analyzed in triplicate). Abbreviations: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

At the start of the experiment, triplicate groups of 40 gilthead seabream (initial body weight, $BW_i = 84.7 \pm 3.8$ g, mean ± SD) were randomly distributed into 15 circular tanks of 1000 L (initial density = 3.5 kg m^{-3}) under an open-flow aerated system (water-flow rate: 5.0 L min^{-1}; dissolved oxygen >6.9 mg L^{-1}). The trial took place under natural photoperiod during the Winter-Spring period (January to May). Water temperature fluctuated between 11.3 and 19.6 °C (average 16.2 ± 1.9 °C) and salinity was 35.1 ± 0.4 ppt.

Each experimental diet was randomly assigned to three replicate tanks. Fish were fed for the 112 days of the trial by hand to apparent satiety twice a day (10.00 and 16.00 h) during labor days and once a day during weekends (10.00 h), with utmost care to avoid feed losses. The staff in charge of fish feeding and care, sample collection and processing, worked under blinded conditions. Distributed feed was quantified throughout the trial, and tanks were thoroughly cleaned to remove any uneaten feed. The uneaten pellets were then dried in an oven (100 °C) and weighed to estimate feed intake (FI). Once a month (every 3–4

weeks), all fish in each tank were anesthetized with 100 mg L^{-1} of tricaine methane sulfonate (MS-222, Sigma-Aldrich), counted and bulk weighed to calculate the following key performance indicators (KPIs):

$$\text{Body weight, BW (g)} = \text{biomass per tank/number of fish per tank}$$

$$\text{Specific growth rate, SGR } (\%\text{day}^{-1}) = (ln\ \text{BW} – ln\ \text{BW}_i) \text{ x } 100/\text{days}$$

$$\text{Feed intake, FI } (\%\text{BW day}^{-1}) = (\text{total FI}/((\text{BW}_i + \text{BW})/2/\text{days}) \text{ x } 100$$

$$\text{Feed conversion ratio, FCR} = \text{total FI/biomass increase}$$

$$\text{Protein efficiency ratio, PER} = \text{weight gain/crude protein intake}$$

At the end of the trial, nine fish from each tank were euthanized with an overdose of MS-222 (300 mg L^{-1}). From those, six fish from each tank were stored together at −20 °C for subsequent whole-body composition analysis, and the following tissues of each fish were separately collected from the remaining three fish: skinless fillets were stored at −20 °C for studying the composition of total lipids and fatty acids; a piece of liver was preserved in 10% buffered formalin solution (pH = 7.2) at 4 °C for histological examination; and a piece of liver was immersed in RNA-later® (Sigma-Aldrich) and stored at −80 °C until RNA extraction.

For evaluation of ADCs of protein, fat, and energy, at the end of the trial, triplicate groups of 8 fish per diet (final body weight, $BW_f = 150 \pm 23$ g) were transferred into 60 L volume-tanks supplied with partially recirculated seawater (water-flow rate: 3.9 L min^{-1}; dissolved oxygen above 6.2 mg L^{-1}; thermo-regulated at 17 ± 1 °C), where they were acclimated for 10 days. After removing all feed residues, faeces were collected daily for the next 8 days using a Guelph system-decantation column (Pereira et al., 2020) and frozen at −20 °C for further analysis.

2.4. Biochemical analysis

Pools from six whole-body samples from each tank were minced, mixed, and a representative sample was freeze-dried and homogenized with a laboratory mill before analysis. Each pool of samples was analyzed in duplicate. Faeces were freeze-dried and ground before analysis. The chemical composition analysis of diets, whole fish and faeces was performed following the standard procedures of the Association of Official Analytical Chemists (AOAC, 2006). Dry matter content was determined after drying at 105 °C for 24 h (method 934.01). Total ash content was measured by combustion in a muffle furnace at 550 °C for 12 h (method 942.05). A flash combustion technique, followed by gas chromatographic separation and thermal conductivity detection (Leco Nitrogen Protein Analyzer, Model FP428, Leco Corporation, USA), was used to analyze crude protein (N × 6.25) (method 990.03). Crude fat was determined by the dichloromethane extraction of Soxhlet (method 920.39). Gross energy was measured in an adiabatic bomb calorimeter (Model Werke C2000 basic, IKA-Werke GmbH & Co. KG, Germany).

The diet and fish fillet composition of total lipids and fatty acids was quantified as described in Ruiz et al. (2023). In brief, lipids were extracted in chloroform/methanol (2:1) and the solvent was evaporated under a stream of nitrogen followed by vacuum desiccation overnight, in order to gravimetrically quantify total crude fat (Folch et al., 1957). After transesterification, fatty acids from the lipid fraction of the diet and fillets were extracted, purified, and quantified by gas-liquid chromatography on a Thermo Trace GC (Thermo Fisher) coupled to a TRACE™ TR-FAME GC Column (Thermo Scientific), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}, helium (1.2 mL min^{-1} at constant flow rate) as the carrier gas and on column injection and flame ionization detection at 250 °C. Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well-characterized fish oil (Marinol D40, Loders Croklaan, Malaysia), based on the retention time of the fatty acids they contained, and quantified by using a known amount of henecosanoic acid (21:0) as internal standard (Skalli et al., 2020). Fatty acids were expressed as % of identified fatty acids methyl esters (FAME).

Based on the fillets' fatty acid composition, the following indices described by Chen and Liu (2020) were calculated to estimate the nutritional quality of the edible flesh lipid fraction:

$$\text{Index of atherogenicity (IA)} = [(\text{C12}:0 + (4 \text{ x C14}:0) + \text{C16}:0)] / (\Sigma\text{MUFA} + \Sigma\text{n} - 6\ \text{PUFA} + \Sigma\text{n} - 3\ \text{PUFA})$$

$$\text{Index of thrombogenicity (IT)} = (\text{C14}:0 + \text{C16}:0 + \text{C18}:0) / [(0.5 \text{ x } \Sigma\text{MUFA}) + (0.5 \text{ x } \Sigma\text{n} - 6\ \text{PUFA}) + (3 \text{ x } \Sigma\text{n} - 3\ \text{PUFA}) + (\Sigma\text{n} - 3\ \text{PUFA}/\Sigma\text{n} - 6\ \text{PUFA})]$$

$$\text{Hypocholesterolemic/hypercholesterolemic fatty acids ratio (h/H)} = (\text{C18}:1\text{n}-9 + \text{C18}:3\text{n}-6 + \text{C18}:3\text{n}-3 + \text{C20}:5\text{n}-3 + \text{C22}:6\text{n}-3)/(\text{C12}:0 + \text{C14}:0 + \text{C16}:0)$$

Yttrium concentration in feeds and faeces was determined by atomic absorption spectrometry (SpectrAA 220 FS, Varian) (Reis et al., 2008). Apparent digestibility coefficients (ADCs) of dietary nutrients and energy in the experimental diets were calculated according to NRC (2011):

$$\text{ADC of nutrient (\%)} = 100 \times [1 – (\%\text{Y}_2\text{O}_3 \text{ in diet}/\%\text{Y}_2\text{O}_3 \text{ in faeces}) \times (\%\text{nutrient in faeces}/\%\text{nutrient in diet})]$$

2.5. Histological analysis

The fixed livers of three fish per tank ($n = 9$ per dietary treatment) were segmented into pieces of 0.5–1.0 cm^2, dehydrated in solutions with graded ethanol concentration, cleared with xylene, and embedded in paraffin. Then, serial sections were taken and stained with hematoxylin-eosin (H&E) using standard histological techniques. Histological cuts were examined for hepatic morphology under a light microscope (DP72, Olympus). The electronic images were further analyzed using ImageJ software (NIH, Bethesda, USA; Schneider et al., 2012) to measure fat deposit accumulation using a semiquantitative scoring as follows. Score 1: small hepatocytes with central nuclei and small vacuoles in the cytoplasm (very low lipid accumulation); Score 2: slightly enlarged hepatocytes with central nuclei and many vacuoles (low lipid accumulation); Score 3: enlarged hepatocytes with nuclei displaced towards the periphery and lots of vacuoles or one large vacuole (moderate lipid accumulation); Score 4: almost all hepatocytes are significantly enlarged, and the cytoplasm seems empty due to large vacuoles (large lipid accumulation); Score 5: significantly enlarged hepatocytes, some of them broken and or with pyknotic nuclei, the cytoplasm seems empty due to large vacuoles, there are areas of necrosis and lymphocytes in vascular tissue, inflammation in the parenchyma and signs of steatosis

(very large lipid accumulation).

2.6. RNA isolation and quality control

Total RNA from the liver of three fish per tank (n = 9 per dietary treatment) was extracted using TRI Reagent® (ref. T9424, Sigma-Aldrich, Sant Louis, MO, USA) according to the manufacturer's instructions. This protocol consisted in an initial homogenization of the tissue in TRI Reagent®, separation in 3 phases using 1-bromo-3-chloropropane: the organic phase containing protein, the interphase with DNA, and an upper aqueous phase containing RNA, followed by RNA isolation by means of 2-propanol and ethanol (75%). The RNA pellet was dissolved in nuclease-free water and immediately stored at −80 °C until use. Total RNA concentration was quantified using a NanoDrop ND-2000 (Thermo Scientific), and RNA integrity and quality were checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Ceder Creek, TX, USA). Only samples with an RNA integrity number (RIN) > 7.0 were chosen for further analysis.

2.7. Microarray-based transcriptional analysis

The AquaGenomic *S. aurata* oligonucleotide microarray (SAQ) platform was used for transcriptional analysis. The microarrays (4x44K; 4 arrays with 44,000 probes in the one-single slide) contain 43,398 oligonucleotide probes for 7285 transcripts with annotated sequences. Information on this platform can be found in the public repository of Gene Expression Omnibus (GEO) at the United States National Center for Biotechnology Information (NCBI), under accession number GPL13442. One-color microarray was carried out according to the manufacturer's protocols (Agilent Technologies, Santa Clara, CA, USA). Briefly, 200 ng of total RNA were reversed transcribed along with spike-in (Agilent One-Color RNA Spike-In kit, Agilent Technologies). For each dietary group, total RNA samples were pooled using the same final concentration (133 ng μL^{-1} each pool). Then, RNA was used as a template for the synthesis and amplification of Cyanine 3 (Cy3)-labeled cRNA, using the Quick Amp Labeling kit (Agilent Technologies). The RNeasy micro kit (Qiagen) was used to purify cRNA samples. Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000 Spectrophotometer. Then, labeled cRNA (1.5 mL) with a specific activity higher than 6.0 pmol Cy3 mg $cRNA^{-1}$ was kept at 60 °C for 30 min for fragmentation and hybridized to the arrays in the presence of the hybridization buffer (Gene expression hybridization kit, Agilent Technologies) at 65 °C for 17 h. Washes were conducted as recommended by the manufacturer, using gene expression wash buffers and a stabilization and drying solution (Agilent Technologies). Microarray slides were scanned with Agilent Technologies Scanner model G2505B, and the extracted raw data were analyzed with GeneSpring (version 14.5 GX software, Agilent Technologies).

2.8. Statistical analysis

After verifying the normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of data, a one-way ANOVA followed by Tukey's *posthoc* test was performed for testing significant differences between dietary groups ($P < 0.05$). For microarray data, the 75% percentile normalization was used to standardize the arrays for comparisons, and data were filtered by expression (Firmino et al., 2020). Differentially expressed genes (DEGs) between diets were identified with an unpaired *t*-test ($P < 0.05$) and grouped according to their fold change (FC). The principal component analysis (PCA) and the hierarchical heatmap analysis based on Pearson distances regarding gene expression values (log2-expression ratios) were obtained with Genespring version 14.5 GX. In order to determine the interactions that take place between genes in the gilthead seabream liver, a functional network analysis (interactome) was carried out. Using the Search Tool for the Retrieval of Interacting Genes (STRING, version 12.0), protein-protein interaction networks based on the DEGs were obtained, establishing a confidence interaction score of 0.9 from a comparative analysis based on *Homo sapiens* (Reyes-López et al., 2021). To increase the interaction among DEGs ten maximum additional interactors were included (ten most associated, but not differentially expressed genes with the DEGs dataset). Gene ontology (GO) pathway enrichment analysis for biological processes (GO_BiologicalProcess-EBI-UniProt-GOA-ACAP-ARAP_10.11.2020) was obtained using ClueGO v2.5.7 app through Cytoscape 3.9.0 platform from all the DEGs and the ten maximum interactors. The Statistical analysis used was Enrichment/Depletion (two-sided hypergeometric test) with a *P* cut-off of 0.05 and corrected by Bonferroni step down. A GO Fusion was performed to avoid redundant terms with a Kappa Score Threshold of 0.4 to propose more stringent GO terms associated with the mechanism of response for the experimental diet. In addition, grouping of the GO terms was conducted when the sharing groups' percentage was above 50, and $P < 0.05$ was considered as significant. The statistically significant GOs obtained from the enrichment analysis were assigned to each one of the nodes represented in the functional network. The nodes classified in different clusters according to their functionality were represented with ClueGo v2.5.7.

3. Results

3.1. Fish performance

No differences were found in growth performance among dietary groups at 29 and 59 days ($P > 0.05$; Table 3). However, at 92 and 112 days there was a significant decrease in BW and SGR values in gilthead seabream fed the NC diet compared to the PC diet ($P < 0.05$). When supplementing the NC diet with the combination of pungent spices at 0.1 and 0.15%, intermediate values of SGR between the PC and NC diets were obtained at 92 days ($P > 0.05$), increasing in a dose-responsive manner. At the end of the trial, BW of the fish fed the $SPICY_{0.15\%}$ diet was not significantly different from fish fed the PC and NC diets ($P > 0.05$). In addition, all groups fed the NC basal diet supplemented with different levels of spices ($SPICY_{0.05\%}$, $SPICY_{0.1\%}$, and $SPICY_{0.15\%}$) showed an intermediate SGR compared to the PC and NC diets ($P > 0.05$).

Regarding feeding performance, no differences were observed in FI when replacing FO by MF at any sampling time ($P > 0.05$; Table 3). However, a reduction in FI was observed at 92 and 112 days in gilthead seabream fed the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets compared to the NC group ($P < 0.05$), while no significant differences were found in relation to the PC diet ($P > 0.05$). On the other hand, a significant increase in FCR and reduced values of PER were observed in fish fed the NC diet with respect to their congeners fed the PC diet along the entire trial (at 29, 92 and 112 days; $P < 0.05$). When supplementing the NC diet with spices, at all inclusion levels, values of FCR and PER were improved in a dose-response manner, being non-significantly different from the PC group ($P > 0.05$) Additionally, fish fed the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets showed a significantly lower FCR than the NC group ($P < 0.05$). Similarly, a higher PER than the NC group was observed for fish fed the $SPICY_{0.15\%}$ diet at 29 and 112 days, and for the group fed the $SPICY_{0.1\%}$ diet at 92 and 112 days ($P < 0.05$).

3.2. Apparent digestibility coefficients

The five groups of fish did not show significant differences regarding dry matter, protein, lipid, and energy digestibility ($P > 0.05$; Table 4). However, the mean values of ADCs from the NC group were numerically lower than those from the PC group. A non-significant ($P > 0.05$) but numerical increase was also observed when increasing the levels of the combination of spices in the diet at ≥0.1%, and particularly in the $SPICY_{0.15\%}$ diet, reaching values closer to the PC group.

Table 3
Growth and feed performance parameters in gilthead seabream (*Sparus aurata*) fed the experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets					
	PC	NC	$SPICY_{0.05\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$	*P*-value
BW_i (g)	85.2 ± 0.3	84.7 ± 1.3	84.7 ± 1.0	84.2 ± 0.8	85.2 ± 1.0	0.675
29 days						
BW (g)	100.8 ± 0.3	98.4 ± 0.5	98.6 ± 0.8	99.3 ± 2.6	99.9 ± 1.9	0.364
SGR (% day^{-1})	0.58 ± 0.01	0.52 ± 0.04	0.52 ± 0.03	0.57 ± 0.09	0.55 ± 0.05	0.553
FI (% BW day^{-1})	0.76 ± 0.02	0.81 ± 0.04	0.79 ± 0.02	0.76 ± 0.06	0.72 ± 0.02	0.093
FCR	1.31 ± 0.05^{a}	1.56 ± 0.05^{b}	1.50 ± 0.06^{ab}	1.34 ± 0.11^{a}	1.32 ± 0.09^{a}	0.005
PER	1.66 ± 0.06^{b}	1.40 ± 0.04^{a}	1.45 ± 0.06^{ab}	1.63 ± 0.13^{ab}	1.65 ± 0.11^{b}	0.010
59 days						
BW (g)	115.8 ± 2.6	110.3 ± 1.5	111.5 ± 1.9	113.3 ± 1.3	114.6 ± 2.8	0.055
SGR (% day^{-1})	0.52 ± 0.04	0.45 ± 0.04	0.47 ± 0.05	0.50 ± 0.03	0.50 ± 0.06	0.327
FI (% BW day^{-1})	0.66 ± 0.03	0.65 ± 0.04	0.68 ± 0.02	0.66 ± 0.04	0.66 ± 0.03	0.847
FCR	1.29 ± 0.05	1.46 ± 0.10	1.47 ± 0.12	1.33 ± 0.04	1.33 ± 0.12	0.099
PER	1.70 ± 0.07	1.50 ± 0.11	1.49 ± 0.12	1.64 ± 0.05	1.64 ± 0.14	0.107
92 days						
BW (g)	144.3 ± 1.4^{b}	133.8 ± 2.8^{a}	135.2 ± 1.9^{a}	137.2 ± 2.0^{a}	138.4 ± 1.7^{a}	<0.001
SGR (% day^{-1})	0.57 ± 0.01^{b}	0.50 ± 0.01^{a}	0.51 ± 0.03^{a}	0.53 ± 0.03^{ab}	0.53 ± 0.03^{ab}	0.015
FI (% BW day^{-1})	0.69 ± 0.02^{ab}	0.72 ± 0.00^{b}	0.69 ± 0.01^{ab}	0.67 ± 0.01^{a}	0.67 ± 0.01^{a}	0.003
FCR	1.23 ± 0.06^{a}	1.48 ± 0.02^{b}	1.39 ± 0.06^{ab}	1.28 ± 0.06^{a}	1.31 ± 0.07^{a}	0.003
PER	1.77 ± 0.09^{b}	1.47 ± 0.02^{a}	1.57 ± 0.06^{ab}	1.70 ± 0.09^{b}	1.67 ± 0.09^{ab}	0.006
112 days						
BW_f (g)	158.0 ± 3.4^{b}	147.1 ± 3.3^{a}	147.5 ± 1.6^{a}	150.4 ± 2.3^{a}	153.9 ± 2.6^{ab}	0.003
SGR (% day^{-1})	0.55 ± 0.02^{b}	0.49 ± 0.03^{a}	0.50 ± 0.02^{ab}	0.52 ± 0.01^{ab}	0.53 ± 0.02^{ab}	0.044
FI (% BW day^{-1})	0.66 ± 0.02^{ab}	0.69 ± 0.03^{b}	0.64 ± 0.01^{ab}	0.65 ± 0.01^{ab}	0.63 ± 0.01^{a}	0.036
FCR	1.24 ± 0.04^{a}	1.43 ± 0.04^{b}	1.34 ± 0.08^{ab}	1.30 ± 0.04^{a}	1.24 ± 0.06^{a}	0.008
PER	1.76 ± 0.06^{b}	1.53 ± 0.04^{a}	1.64 ± 0.10^{ab}	1.68 ± 0.05^{b}	1.76 ± 0.09^{b}	0.011

Data are shown as the mean ± SD ($n = 3$ tanks per dietary group). Different superscript letters denote significant differences among groups (ANOVA and Tukey's *posthoc* test, $P < 0.05$). Abbreviations: BW_i: initial body weight; BW: body weight; SGR: specific growth rate; FI: feed intake; FCR: feed conversion ratio; PER: protein efficiency ratio.

Table 4
Apparent digestibility coefficients (ADCs) in gilthead seabream (*Sparus aurata*) fed the experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets					
	PC	NC	$SPICY_{0.05\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$	*P*-value
Dry matter (%)	64.7 ± 2.8	63.5 ± 3.8	62.6 ± 2.5	63.3 ± 2.7	64.2 ± 3.3	0.931
Protein (%)	87.3 ± 0.7	86.4 ± 0.9	86.3 ± 1.6	86.7 ± 1.1	87.0 ± 1.3	0.828
Lipid (%)	87.7 ± 1.5	86.2 ± 0.7	86.5 ± 2.7	87.5 ± 2.1	87.8 ± 2.1	0.793
Energy (%)	75.6 ± 3.1	75.1 ± 1.6	74.2 ± 1.9	75.5 ± 2.6	76.1 ± 2.8	0.903

Data are shown as the mean ± SD (n = 3 tanks per dietary group).

Table 5
Proximate composition in a fresh matter basis of the whole body from gilthead seabream (*Sparus aurata*) fed the experimental diets: the positive control (PC) with FO as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.05 ($SPICY_{0.05\%}$), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets					
	PC	NC	$SPICY_{0.05\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$	*P*-value
Moisture (%)	66.7 ± 1.1	67.1 ± 1.1	68.3 ± 1.3	66.7 ± 1.2	67.4 ± 0.8	0.159
Protein (%)	16.7 ± 0.8	17.2 ± 0.6	17.3 ± 0.4	17.4 ± 0.4	17.2 ± 0.4	0.267
Lipid (%)	12.0 ± 0.8^{b}	11.2 ± 1.2^{ab}	10.0 ± 1.5^{a}	11.9 ± 0.7^{b}	10.6 ± 0.6^{ab}	0.007
Ash (%)	4.2 ± 0.4	3.9 ± 0.2	4.2 ± 0.5	3.7 ± 0.5	4.3 ± 0.4	0.097
Energy (MJ kg^{-1})	8.2 ± 0.4	8.1 ± 0.6	7.7 ± 0.6	8.4 ± 0.3	7.9 ± 0.2	0.127

Data are shown as the mean ± SD (n = 3 tanks per dietary group).

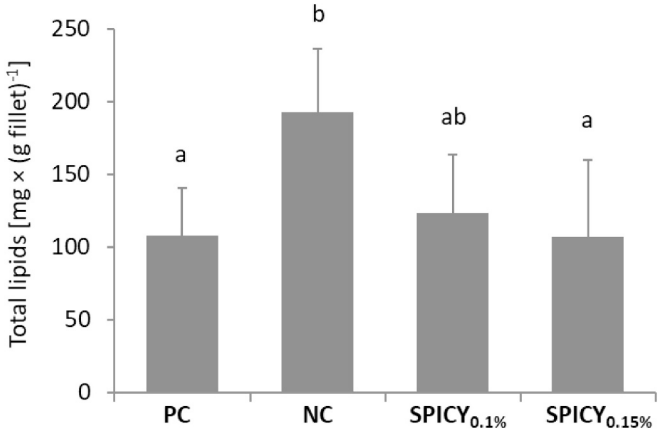

Fig. 1. Levels of total lipids (mg/g of dry weight) in the fillet of gilthead seabream (*Sparus aurata*; n = 3 tanks per dietary group) fed the following experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$). Different letters denote significant differences among groups (ANOVA and Tukey's *posthoc* test, $P < 0.05$).

3.3. Whole-body proximate composition

No significant differences were found in the whole-body proximate composition of dry matter, protein, ash and energy among the control groups, or groups fed the diets supplemented with the combination of pungent spices ($P > 0.05$; Table 5). On the other hand, fish fed the $SPICY_{0.05\%}$ diet showed a lower lipid content than those fed the PC and $SPICY_{0.1\%}$ diets, with no differences being observed among the remaining treatments.

3.4. Fillet's lipid levels and fatty acid profile

Considering the lack of effects of the $SPICY_{0.05\%}$ treatment on fish performance, and apparent digestibility, only the groups fed with the PC, NC, $SPICY_{0.1\%}$, and $SPICY_{0.15\%}$ diets were selected for further analysis of the fillet's lipid composition and of the accumulation of fat deposits in the liver.

The replacement of 45% FO by MF resulted in an increased level (almost double) of total lipids (mg/g of dry weight) in gilthead seabream fillets ($P < 0.05$; Fig. 1). The supplementation of this diet with the combination of pungent spices caused a reduction in fillet total lipid levels, non-significantly at an inclusion level of 0.1% ($P > 0.05$), or significantly at 0.15% ($P < 0.05$), where levels were similar to those from fish fed the diet without FO substitution.

The fillet fatty acid profile of gilthead seabream fed the different

Table 6
Fillet fatty acid profile (% of total FAs), total fatty acids (mg/g lipids) and total lipids (mg/g of fillet, in dry or wet basis) in gilthead seabream (*Sparus aurata*) fed the following experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets				
	PC	NC	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$	*P*-value
14:0	2.54 ± 0.48^{b}	1.99 ± 0.14^{ab}	1.92 ± 0.40^{a}	2.08 ± 0.41^{ab}	0.035
15:0	0.28 ± 0.02^{c}	0.26 ± 0.02^{bc}	0.22 ± 0.02^{a}	0.23 ± 0.03^{ab}	<0.001
16:0	17.22 ± 0.40	16.85 ± 0.62	17.81 ± 1.01	17.48 ± 1.09	0.302
18:0	4.65 ± 0.34^{a}	4.70 ± 0.19^{ab}	5.18 ± 0.45^{ab}	5.26 ± 0.40^{b}	0.009
20:0	0.25 ± 0.03	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.02	0.964
22:0	0.16 ± 0.02^{b}	0.16 ± 0.02^{ab}	0.13 ± 0.02^{a}	0.14 ± 0.01^{ab}	0.013
Total SFA	25.12 ± 0.74	24.20 ± 0.81	25.50 ± 1.49	25.44 ± 1.47	0.269
14:1n-5	0.09 ± 0.06	0.12 ± 0.01	0.11 ± 0.02	0.08 ± 0.06	0.632
16:1n-9	5.49 ± 0.53	4.95 ± 0.28	5.04 ± 0.50	4.77 ± 0.72	0.106
18:1n-7	3.24 ± 0.19	3.13 ± 0.06	3.07 ± 0.15	3.15 ± 0.29	0.544
18:1n-9	22.97 ± 1.87^{a}	30.69 ± 2.12^{b}	29.49 ± 2.64^{b}	27.53 ± 2.26^{b}	<0.001
20:1n-9	1.06 ± 0.13	0.89 ± 0.16	0.98 ± 0.16	1.03 ± 0.12	0.213
24:1n-9	0.48 ± 0.05^{b}	0.38 ± 0.06^{a}	0.40 ± 0.04^{a}	0.44 ± 0.03^{ab}	0.003
Total MUFA	33.26 ± 2.12^{a}	40.07 ± 2.50^{b}	39.02 ± 3.34^{b}	36.97 ± 3.03^{ab}	<0.001
18:2n-6	14.45 ± 1.87^{a}	19.75 ± 3.25^{b}	16.48 ± 2.48^{ab}	16.73 ± 2.84^{ab}	0.016
18:3n-6	0.38 ± 0.04	0.44 ± 0.05	0.37 ± 0.11	0.39 ± 0.08	0.411
20:4n-6 (ARA)	1.02 ± 0.21^{b}	0.62 ± 0.07^{a}	0.83 ± 0.13^{ab}	0.96 ± 0.26^{b}	0.010
Total n-6 PUFA	15.85 ± 1.76^{a}	20.81 ± 3.24^{b}	17.68 ± 2.48^{ab}	18.09 ± 2.78^{ab}	0.022
18:3n-3	1.77 ± 0.22^{ab}	2.11 ± 0.11^{b}	1.79 ± 0.27^{ab}	1.72 ± 0.21^{a}	0.025
20:3n-3	0.17 ± 0.05^{ab}	0.09 ± 0.09^{a}	0.23 ± 0.06^{b}	0.20 ± 0.02^{b}	0.003
20:5n-3 (EPA)	7.76 ± 0.71^{c}	3.97 ± 0.27^{a}	4.89 ± 0.42^{ab}	5.21 ± 0.68^{b}	<0.001
21:5n-3	0.48 ± 0.16	0.40 ± 0.03	0.57 ± 0.27	0.47 ± 0.19	0.550
22:5n-3	3.04 ± 0.11^{c}	2.00 ± 0.19^{a}	2.29 ± 0.14^{a}	2.59 ± 0.24^{b}	<0.001
22:6n-3 (DHA)	11.32 ± 2.42^{b}	6.28 ± 0.94^{a}	7.96 ± 1.15^{a}	9.25 ± 2.12^{ab}	0.001
Total n-3 PUFA	25.69 ± 2.87^{c}	14.84 ± 1.42^{a}	17.73 ± 1.63^{ab}	19.44 ± 2.52^{b}	<0.001
Total PUFA	41.54 ± 2.46^{b}	35.65 ± 3.00^{a}	35.41 ± 3.84^{a}	37.53 ± 3.34^{ab}	0.006
ARA/EPA	0.13 ± 0.02^{a}	0.16 ± 0.01^{ab}	0.17 ± 0.02^{ab}	0.18 ± 0.04^{b}	0.004
DHA/EPA	1.45 ± 0.23	1.58 ± 0.14	1.62 ± 0.11	1.77 ± 0.29	0.070
n-3/n-6	1.65 ± 0.31^{b}	0.73 ± 0.16^{a}	1.01 ± 0.09^{a}	1.10 ± 0.24^{a}	<0.001
Total fatty acids (mg/g lipids)	712.7 ± 26.7^{ab}	752.0 ± 15.7^{b}	737.6 ± 31.8^{ab}	706.9 ± 30.5^{a}	0.032
Total lipids (mg/g dry weight)	107.4 ± 33.1^{a}	192.6 ± 43.9^{b}	123.2 ± 40.3^{ab}	106.8 ± 53.2^{a}	0.009
Total lipids (mg/g wet weight)	28.6 ± 10.0^{a}	57.6 ± 11.8^{b}	33.9 ± 12.7^{ab}	28.8 ± 16.6^{a}	0.007
IA	0.37 ± 0.03	0.33 ± 0.02	0.34 ± 0.03	0.35 ± 0.04	0.197
IT	0.24 ± 0.02^{a}	0.31 ± 0.02^{b}	0.30 ± 0.03^{b}	0.29 ± 0.02^{b}	<0.001
h/H	2.24 ± 0.14	2.31 ± 0.14	2.26 ± 0.12	2.26 ± 0.15	0.847

Data are shown as the mean ± SD (n = 3 tanks per dietary group). Different superscript letters denote significant differences among groups (ANOVA and Tukey's *posthoc* test, $P < 0.05$). Abbreviations: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; IA: index of atherogenicity; IT: index of thrombogenicity; h/H: hypocholesterolemic/hypercholesterolemic ratio.

diets is shown in Table 6. Gilthead seabream fed the NC diet showed increased levels of total MUFA and n-6 PUFA with respect to the PC group, particularly 11-eicosenoic acid (C20:1 n-9) ($P < 0.05$). There was also a decrease in the levels of nervonic (C24:1 n-9, NA) and arachidonic (C20:4 n-6, ARA) acids ($P < 0.05$). In addition, a reduction in the content of total n-3 PUFA was found and, in particular, in EPA (C20:5 n-3), docosapentaenoic acid (C22:5 n-3, DPA) and DHA (C22:6 n-3), which led to lower levels of total PUFA and lower n-3/n-6 ratio in the fillet of the NC group, and higher IT values than in the PC treatment ($P < 0.05$).

The levels of LA and ARA were re-established with respect to the PC values when supplementing the NC diet with pungent spices at both 0.1% and 0.15% ($P > 0.05$; Fig. 2). Furthermore, the supplementation of the NC diet with pungent spices at 0.1% led to a reduction in the levels of pentadecanoic (C15:0) acid and increase in eicosatrienoic acid (C20:3 n-3, EET), and at 0.15% to increased levels of EET, EPA, and total n-3 PUFA with respect to the NC group ($P < 0.05$), even though still not reaching values similar to those of the fish fed the PC diet ($P < 0.05$). Moreover, the $SPICY_{0.15\%}$ diet was able to restore the levels of NA, DHA, total MUFA and PUFA to similar levels as the PC group ($P > 0.05$). The content of alpha-linolenic acid (C18:3 n-3, ALA) significantly increased in the $SPICY_{0.15\%}$ group ($P < 0.05$), and fish fed both the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets showed similar levels of ALA as in fillets of fish fed the PC diet ($P > 0.05$). On the other hand, gilthead seabream fed the $SPICY_{0.15\%}$ diet showed a higher ARA/EPA ratio than the PC group ($P < 0.05$). Albeit none of the supplemented diets was able to restore the n-3/n-6 ratio ($P < 0.05$), the ratio was ≥ 1 in both treatments, in contrast with the NC (having a value of 0.73).

3.5. Accumulation of fat deposits in the liver

Under histological examination, hepatocytes were polyhedral, with varying degrees of vacuolization depending on the diet, and were arranged in anastomosing plates separated by sinusoidal capillaries leading to central veins. No signs of inflammation or infiltration of lymphocytes were observed, and neither very low (score 1) nor excessive (score 5) accumulation of fat deposits were found in all dietary groups. The substitution of 45% FO by MF reduced the percentage of individuals with a large accumulation of fat deposits in the liver (score 4), while increasing those with a low and moderate lipid accumulation (scores 2 and 3; Fig. 3). The administration of $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets reduced much more the accumulation of fat deposits, especially in the group fed the $SPICY_{0.15\%}$ diet. In particular, in most individuals the size of the hepatocytes was smaller, and the nuclei were less displaced towards the periphery than in the case of fish from the PC and NC groups.

3.6. Hepatic microarray-based transcriptomic profile

Based on the above-described results, the transcriptomic response in the liver of gilthead seabream was only analyzed in fish fed the NC and the $SPICY_{0.15\%}$ diets, to assess the effect of the spicy supplementation. When comparing both groups, a total of 159 DEGs were found, of which 82 genes are known, while the other 77 genes are not annotated ($P < 0.05$; Supplementary Table 1). Among DEGS, a total of 55 genes were up-regulated, while 104 were down-regulated in fish fed the $SPICY_{0.15\%}$ diet relative to the NC (Fig. 4A). The PCA results showed a clear clustering among replicates from each dietary group (Fig. 4B), which was consistent with the heatmap hierarchical clustering, showing a differential expression profile of DEGs between both the NC and the $SPICY_{0.15\%}$ diets (Fig. 4C).

3.7. Functional enrichment analysis

The transcripteractome revealed five different clusters (Fig. 5). Among them, three single-node clusters were identified including defense response to fungus (GO:0050832), cellular response to interleukin 6 (GO:0071354), and regulation of protein processing (GO:0070613). Another cluster was composed by the following biological processes: stimulatory C-type lectin receptor signaling pathway (GO:0002223), antigen processing and presentation of exogenous peptide antigen *via* MHC class I, TAP dependent (GO:0002479), and negative regulation of G2/M transition of mitotic cell cycle (GO:0010972). The fifth cluster showed the following biological processes: response to interleukin-1 (GO:0070555), cellular response to hypoxia (GO:0071456), regulation of transcription from RNA polymerase II promoter in response to hypoxia (GO:0061418), TRIF-dependent Toll-like receptor signaling pathway (GO:0061418), intracellular response of virus (GO:0075733), nucleotide-excision repair (GO:0006289), nucleotide-excision repair, DNA incision, 5′-to lesion (GO:0006296), nucleotide-binding oligomerization domain containing signaling pathway nucleotide-excision repair (GO:0070423), error-free translesion synthesis (GO:0070987), error-prone translesion synthesis (GO:0042276), and protein targeting to perixosome (GO:0006625).

Belonging to the single-node cluster "regulation of protein processing", the gene transmembrane protein 59 (*tmem59*) was up-regulated in the $SPICY_{0.15\%}$ treatment, while plasminogen receptor with a C-terminal lysine (*plgrkt*) was down-regulated (Supplementary Table 2). From the cluster "cellular response to interleukin-6", signal transducer and activator of transcription 1 (*stat1*) was up-regulated and hepcidin antimicrobial peptide (*hamp*), down-regulated. Two down-regulated genes [*hamp*, jagunal homolog 1 (*jagn1*)] were identified in the cluster "defense response to fungus", whereas in the node "stimulatory C-type lectin receptor signaling pathway", two genes were up-regulated [proteasome subunit beta type 7 precursor (*psmb7*), 26S protease regulatory subunit S10B (*psmc6*)], and another three were down-regulated [HRAS proto-oncogene, GTPase (*hras*), proteasome subunit, beta type 8 (*psmb8*), Raf-1 proto-oncogene, serine/threonine kinase (*raf1*)]. From the node "antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent", we identified two up-regulated genes (*psmb7*, *psmc6*), and two that were down-regulated [major histocompatibility complex, class I, A (*hla-a*), *psmb8*]. In the case of "negative regulation of G2/M transition of mitotic cell cycle", "cellular response to hypoxia" and "regulation of transcription from RNA polymerase II promoter in response to hypoxia", all these nodes showed a common up-regulation of *psmb7* and *psmc6*, and down-regulation of *psmb8*. The same up- and down-regulated genes were found in the group "response to interleukin-1", but in this case C—C motif chemokine 20 (*ccl20*) was also down-regulated. Considering the node "protein targeting to peroxisome", three down-regulated genes were identified [glutathione S-transferase kappa 1 (*gstk1*), hydroxyacid oxidase 2 (*hao2*), peroxisomal trans-2-enoyl-CoA reductase (*pecr*)].

4. Discussion

4.1. Growth performance and feeding efficiency

Replacement of FO by different lipid sources may not compromise growth performance, as long as essential fatty acid requirements are met (Trushenski and Lochmann, 2009). However, some studies have shown a reduction in growth performance when different MF were used as dietary lipid sources (Huang et al., 1998; Yildirim-Aksoy et al., 2007; Han et al., 2013). Similarly, we observed a significant reduction in growth of gilthead seabream fed the NC diet containing a lower level of FO (6.33%) and 5.18% of MF, compared to the PC diet containing only FO (11.5%). On the other hand, the supplementation of the NC diet with the combination of spices had a positive effect, numerically increasing SGR values in a dose-response manner (intermediate values, non-significantly different from the NC and PC groups). Similar results in terms of enhancement of growth performance were observed when supplementing the same combination of spices (at 0.1% and 0.15%) in diets containing poultry fat (8.0%) as the main lipid source (Ruiz et al., 2023). There are few reports evaluating the combination of spices herein

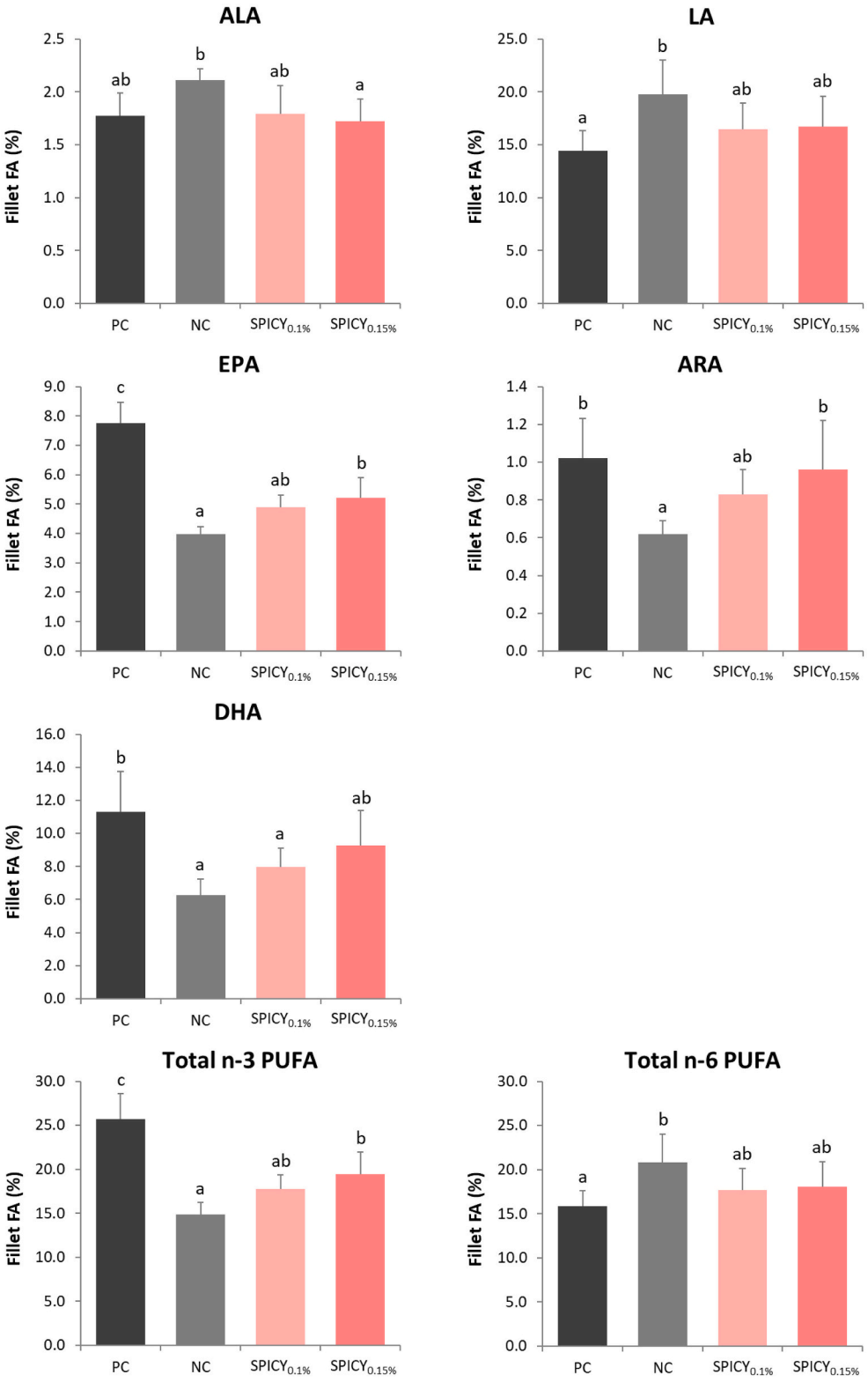

Fig. 2. Fillet fatty acid content (% of total fatty acids, FA) of the main polyunsaturated fatty acids (PUFA) of the n-3 series – alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) – and of the n-6 series – linoleic acid (LA), arachidonic acid (ARA) – and total n-3 and n-6 PUFA, in gilthead seabream (*Sparus aurata*; n = 3 tanks per dietary group) fed the following experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

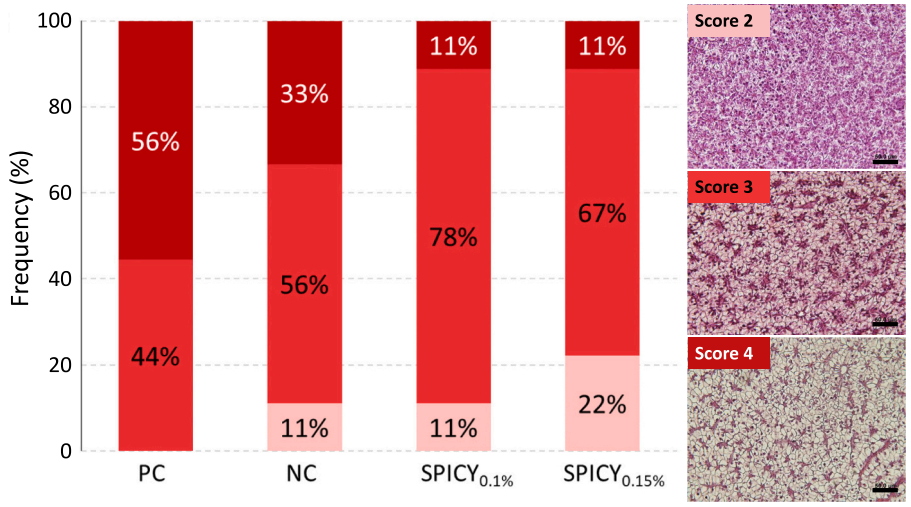

Fig. 3. Semi-quantitative scoring results (frequency, in %, of specimens of each score) of fat deposit accumulation in the liver of gilthead seabream (*Sparus aurata*; n = 3 tanks per dietary group) fed the following experimental diets: the positive control (PC) with fish oil as the main lipid source, the negative control (NC) in which 45% of fish oil was replaced by mammalian-rendered fat, and the NC formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$). The photographs in the right are examples of histological slides from the liver of individuals with low lipid accumulation (score 2), moderate lipid accumulation (score 3), and high lipid accumulation (score 4). The black line in the photographs corresponds to 50.0 μm.

tested in fish (Ruiz et al., 2023), although their effects on growth performance have been individually assessed. However, the use of a mixture of spices may have several advantages over the use of a single one, since synergistic and complementary effects have been described that may enhance the overall effectiveness of the supplement, and/or enable a broader range of effects on different tissues and physiological mechanisms (Platel and Srinivasan, 2004). When considered as a single spice, controversial results have been found for capsicum and black pepper, and their active principles, capsaicin and piperine. In particular, while some studies have shown that they do not affect fish growth (Wassef et al., 2010; Yilmaz et al., 2013; El-Houseiny et al., 2019; Wojno et al., 2021), other recent investigations have demonstrated an improved BW and SGR when supplementing fish diets with capsicum (Ibrahim et al., 2023; Yilmaz et al., 2023) and black pepper or piperine (Giri et al., 2023; Malintha et al., 2023). On the other hand, improved growth performance has been shown in the vast majority of studies in which aquafeeds were supplemented with ginger (Fazelan et al., 2020; Mohammadi et al., 2020; Aqmasjed et al., 2023; Ashry et al., 2023) and cinnamaldehyde (Zhou et al., 2020; Abd El-Hamid et al., 2021; Wang et al., 2021; Gu et al., 2022). Inconsistencies between studies may be due to the different sources and purity of tested spices, mode of dietary inclusion, basal diet formulation, and experimental conditions, among others (Firmino et al., 2021).

In the current study, partial FO substitution by MF also compromised feeding efficiency, with increased FCR and decreased PER values with respect to fish fed the PC diet. The worsening of FCR values may be caused, at least partly, by the increased levels of SFA and MUFA, which have lower digestibility (Trushenski et al., 2009), especially at low water temperatures as those in the current study. Given that the diets were isolipidic in crude terms, and fish are known to regulate their feed intake based on dietary digestible energy level (Bendiksen et al., 2002; Lekva et al., 2010), fish fed the NC diet tended to consume slightly more feed ($P > 0.05$), even though this was not enough to achieve the same SGR. However, the supplementation of the NC diet with the combination of pungent spices significantly improved FCR and PER in gilthead seabream groups fed the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets, with no differences being observed, statistically or numerically, between the PC and $SPICY_{0.15\%}$ diets at the end of the trial. In our previous study, the same spices' combination also led to an improved feed utilization (Ruiz et al., 2023). Furthermore, the potential of ginger and cinnamaldehyde to improve FCR has been well-demonstrated in several fish species (Fazelan et al., 2020; Mohammadi et al., 2020; Abd El-Hamid et al., 2021; Wang et al., 2021; Gu et al., 2022; Ashry et al., 2023), while few studies have shown an improved conversion efficiency in presence of capsicum (Ibrahim et al., 2023; Yilmaz et al., 2023) and black pepper or piperine (Stoev and Zhelyazkov, 2021; Giri et al., 2023). Improvements in PER have also been observed when supplementing the spices individually in diets of different fish species (Zhou et al., 2020; Wang et al., 2021; Aqmasjed et al., 2023; Ashry et al., 2023; Ibrahim et al., 2023; Malintha et al., 2023). Hence, the convergent or synergetic effect of all spices combined might have contributed to the full recovery of feeding efficiency when 45% of FO was replaced by MF, at the 0.15% supplementation level.

Spices have been described to stimulate fat digestion in mammals through different mechanisms, such as increased bile acid synthesis and stimulation of digestive enzyme activity or secretion (Platel and Srinivasan, 2004), which was also suggested in our previous studies in gilthead seabream (Ruiz et al., 2023; Ruiz et al., 2024). This might be particularly relevant when FO is replaced by fats with lower digestibility, such as MF containing high levels of SFA, and especially during winter, at low water temperatures, and could be one of the factors underlying the improved FCR in the spicy-supplemented treatments. Nevertheless, the digestibility assay performed at the end of the growth trial did not indicate a difference in the digestibility of any of the diets. It should be kept in mind, however, that the assay was conducted in thermoregulated and higher temperature conditions (17 ± 1 °C) than during the growth trial performed in a flow-through system (16.2 ± 1.9 °C in average, varying between 11.3 and 19.6 °C). Furthermore, feed intake can also be suggested to indicate a potentially improved digestibility of dietary fat in the $SPICY_{0.15\%}$ group, considering how fish commonly adjust their feed intake rate to the digestible or metabolizable energy level of the feed, as previously discussed, and seeing how it was significantly reduced compared to the NC group (and similar to the PC), in fish fed this diet.

4.2. *Proximate composition and fillets' fatty acid profile*

The whole-body proximate composition of gilthead seabream was generally maintained among dietary treatments, except for a reduction in the lipid content of the $SPICY_{0.05\%}$ treatment compared to the PC and

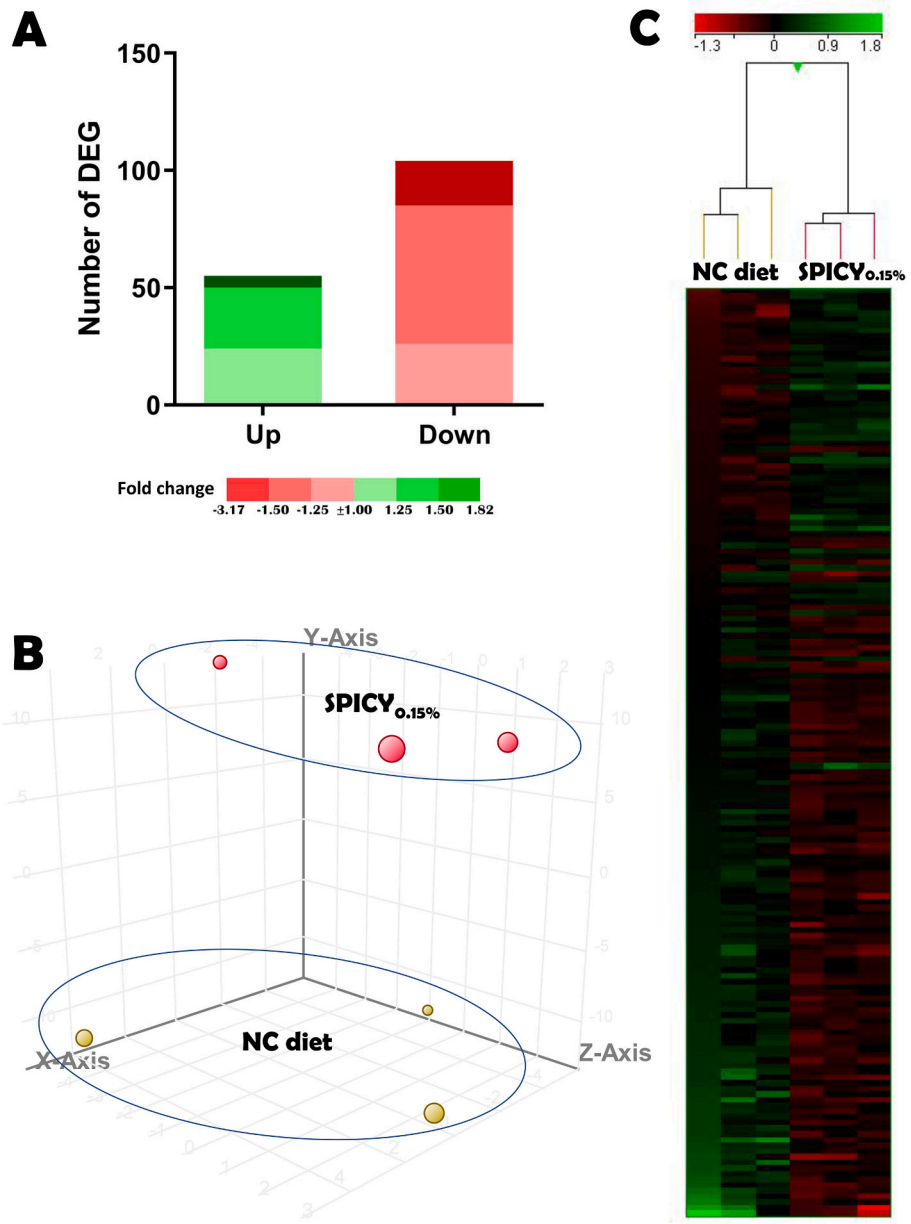

Fig. 4. Differential expression response in the liver of gilthead seabream (*Sparus aurata*) fed the negative control (NC) diet in which 45% of fish oil was replaced by mammalian-rendered fat, or the same formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.15% ($SPICY_{0.15\%}$). **(A)** Number of differentially expressed genes (DEGs) in fish fed the $SPICY_{0.15\%}$ diet compared to those fed the NC diet. **(B)** Principal component analysis (PCA) of the triplicates from fish fed the NC and $SPICY_{0.15\%}$ diets based on the expression of the DEGs. **(C)** Hierarchical clustering representing the expression of the DEGs from the liver of fish fed the NC and $SPICY_{0.15\%}$ diets.

$SPICY_{0.1\%}$ groups, which is difficult to ascribe to either the dietary fat composition or spicy supplementation. This is consistent with the lack of differences found in other studies using MF, such as pork lard and beef tallow, as an alternative or complementary lipid source (Dosanjh et al., 1984, 1988; Xue et al., 2006; Yun et al., 2013). However, in the edible product there was a significant increase in fillet lipid levels of seabream fed the NC diet compared to those fed the PC. This change can have a significant negative impact in the end-product quality, due to its potential to affect the texture, organoleptic qualities and even shelf-life (higher propensity for rancidity) of the fillet (Grigorakis, 2007). The increased lipid deposition in fish muscle is likely associated to the reduction in dietary levels of n-3 LC-PUFA in the NC diet. The hypolipidemic and cardioprotective effects of these fatty acids are well known in vertebrates, being that EPA and DHA are natural ligands of several nuclear receptors and transcription factors, including PPARs, LXR, HNF-4, NFkappaB, and SREBPs (Jump, 2004; Shibabaw, 2021). These, in turn, coordinately regulate the expression of lipid metabolism genes that enhance hepatic and skeletal muscle β-oxidation, while repressing lipogenic, glycolytic and cholesterogenic pathways, and regulating lipid transport and mobilization. These molecular mechanisms are overall well conserved in fish, and similar consequences of FO reductions, in terms of lipid metabolism changes, have been described (Morais et al., 2012b; Carmona-Antoñanzas et al., 2014; Houston et al., 2017). On the other hand, supplementation of the NC diet with 0.1% or 0.15% of the spicy combination substantially reduced the fillets' levels of crude fat with respect to the NC group, with seabream fed the $SPICY_{0.15\%}$ diet having an equal lipid content as those fed the PC diet. Albeit through different mechanisms from n-3 LC-PUFA (further discussed below), spices are known to also promote lipid catabolism, equally having hypolipidemic and hypocholesterolemic effects in mammals

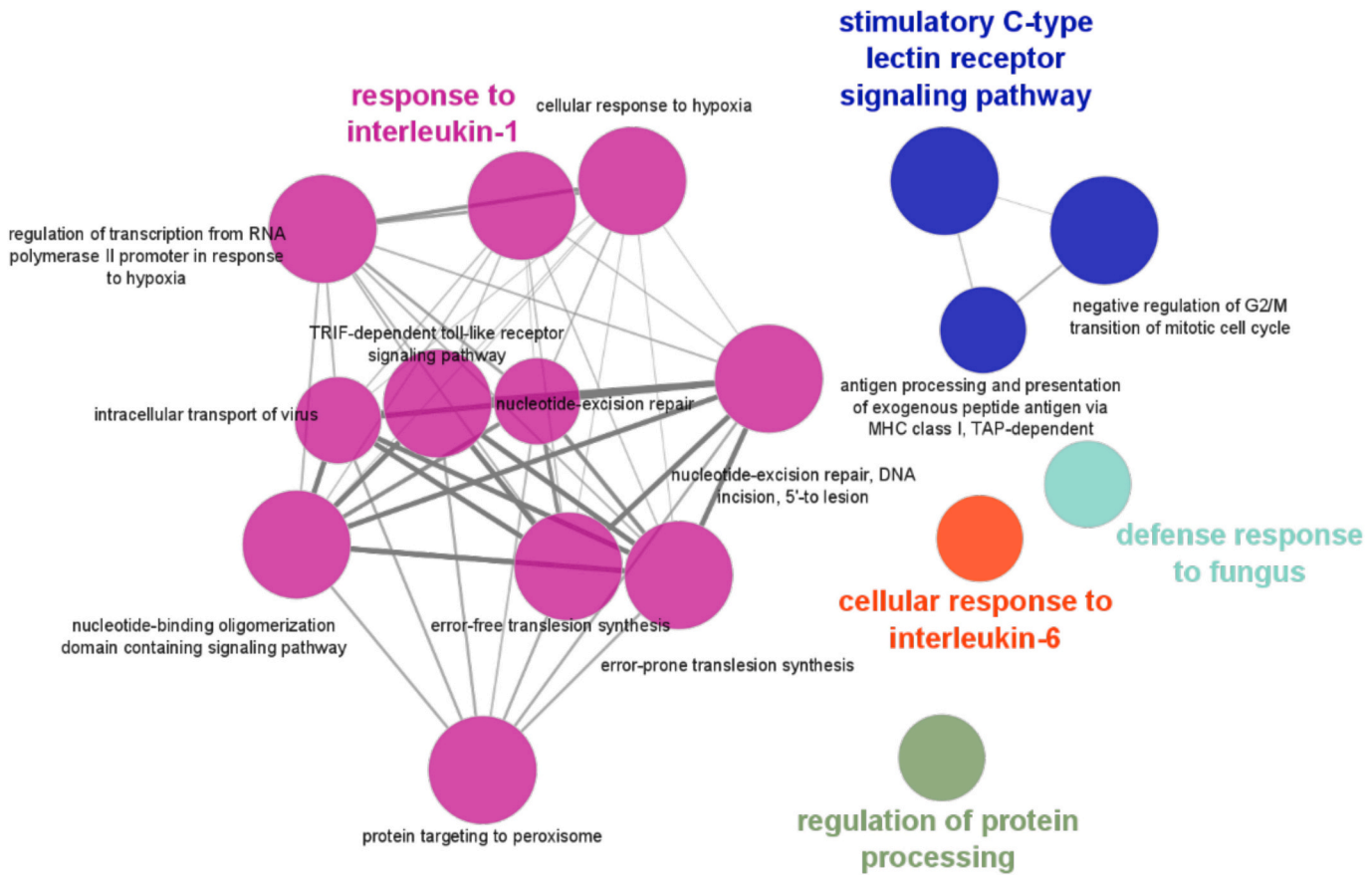

Fig. 5. Functional enrichment network analysis for biological processes based on the related functions of the differentially expressed genes (DEGs) in the liver of gilthead seabream (*Sparus aurata*) fed the negative control (NC) diet in which 45% of fish oil was replaced by mammalian-rendered fat, or the same formulation supplemented with a mixture of pungent spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) at a dietary inclusion level of 0.15% ($SPICY_{0.15\%}$). Each node represents a specific enriched biological process, and each color indicates a cluster of closely related biological processes. The node size is proportional to the significance of the biological process (Enrichment/Depletion (two-sided hypergeometric test) and corrected by Bonferroni step down; $P < 0.05$).

(Srinivasan, 2005; Duangjai et al., 2013; Magied et al., 2014), which makes them good candidates to test as supplements to mitigate some of the negative consequences of FO replacement in fish diets.

The fillet fatty acid profile usually reflects the diet's fatty acid composition (Grigorakis, 2007). Accordingly, the inclusion of MF in the diet led to an increase in the levels of total MUFA, specifically OA, and total n-6 PUFA, in particular LA, which were the dominant fatty acids in the NC diet. Reduced levels of n-3 PUFA, especially EPA and DHA, were found in the fillet of fish fed the NC diet, reflecting also the lower levels of n-3 PUFA in this diet. Nonetheless, the replacement of 45% FO with MF did not affect the fillet's DHA/EPA ratio, which was within the expected range for farmed gilthead seabream juveniles (Lenas et al., 2011). It was also noteworthy to observe that while the levels of DHA were higher in the fillet of the fish (PC group: 11.3%, NC group: 6.3%) than in the diets (PC diet: 9.6%, NC diet: 4.8%), the opposite occurred for the levels of EPA, which showed reduced levels in the fish fillets (PC group: 7.8%, NC group: 4.0%) with respect to the diets (PC diet: 13.7%, NC diet: 6.2%). This has been described in several other fish studies and is explained by the preferential tissue deposition of DHA with respect to EPA and other n-3 PUFA (Bell et al., 2001; Coccia et al., 2014; Glencross et al., 2014; Campos et al., 2019) and/or to the conversion of part of the EPA content into intermediate substrates for DHA synthesis (Coccia et al., 2014; Emery et al., 2016; Morais et al., 2020) to help meet DHA requirements. On the other hand, the levels of total SFA were quite conserved in the fillets of fish fed the two basal formulations (NC group: 24.2%, PC group: 25.1%), in spite of the higher content of this fatty acid class in the NC diet (39.5% *versus* 35.3% in the PC diet). This has been also described in previous studies (Han et al., 2013; Yun et al., 2013; Marques et al., 2022), and has been attributed to the preferential oxidation of SFA (Henderson and Sargent, 1985; Fonseca-Madrigal et al., 2005; Trushenski and Lochmann, 2009). These results are largely in agreement with previous trials in which FO was partly or totally replaced by animal-rendered fats (Yun et al., 2013; Xue et al., 2006; Monteiro et al., 2018; Campos et al., 2019). Another important consequence of the FO replacement by MF was the significant reduction in fillet n-3/n-6 ratio in fish fed the NC diet. The n-3/n-6 ratio of tissues can be used as a biomarker that correlates with the health and immune status in both fish and humans (Oliva-Teles, 2012; Carr et al., 2023), and a pronounced decrease in this ratio is associated to pro-inflammatory responses in fish (Holen et al., 2018). Nonetheless, the levels of n-6 PUFA in animal-rendered fats, and especially in MF (3–12% of total fatty acids, TFA; Moretti and Corino, 2008; Trushenski and Lochmann, 2009; Turchini et al., 2009; Campos et al., 2019; Marques et al., 2022), are typically much lower than the n-6 PUFA content of vegetable oils (*e.g.*, 50% TFA in soybean oil, 20% TFA in canola/rapeseed oil and 65% TFA in sunflower oil; Turchini et al., 2009). This makes MF a better alternative lipid source, from a nutritional point of view, than plant-based oils to minimize fish health problems and physiological disorders when decreasing dietary FO content. In addition, their use as a byproduct from the rendering industry, especially when locally available, has the potential to promote the sustainability of aquafeeds (Woodgate et al., 2022).

The supplementation of the NC diet with the combination of pungent spices at 0.1% only generated a significant decrease in the levels of pentadecanoic acid, and an increase in eicosatrienoic acid (C20:3n-3, EET) in the fillets. The differential content of these fatty acids could be

related to changes in lipid metabolism, fatty acid oxidation or lipid mobilization (Reske et al., 1985; Muhlhausler, 2018), in line with the differential results of fat accumulation in fillet and liver described in the present work. On the other hand, fish fed the $SPICY_{0.15\%}$ diet showed increased levels of total n-3 PUFA in fillet with respect to the NC group, which is in line with the increased hepatic n-3 PUFA content observed in our previous study when supplementing gilthead seabream diets with 0.15% of the same spice combination (Ruiz et al., 2023). In the current trial, the increase in n-3 PUFA was mostly caused by a significant increase in EET, DPA and EPA in the fillets of fish fed the $SPICY_{0.15\%}$ diet compared to the NC group, although still not reaching the levels of the PC treatment in the case of DPA and EPA. Furthermore, the concentration of DHA in the fillet of fish fed the $SPICY_{0.15\%}$ diet was increased and significantly approached the PC group but was not statistically different from the NC group. In relation to this, it is noteworthy that in our previous study using the same combination of spices added to a poultry fat-rich diet, we observed a significant increase in the DHA content of liver when the diet contained 0.15% of the supplement (Ruiz et al., 2023). Surprisingly, though, the fillet content of the n-3 C18 PUFA ALA, which significantly increased in fish fed the NC diet compared to the PC group, was significantly reduced (restored to the same level as the PC) in the $SPICY_{0.15\%}$ treatment. Considering the reduced ability of marine fish to synthesize n-3 LC-PUFA, and their importance for optimal growth, development, reproduction and health of marine fish, as well as health-promoting effects in human consumers (Tocher, 2015; Carr et al., 2023), the increased levels of such fatty acids in the fillet of fish fed the $SPICY_{0.15\%}$ diet with respect to the NC group can be regarded as highly beneficial. Taking into account some of the previously discussed effects of SFA-rich animal-rendered fats and those of pungent spices, the higher deposition of LC-PUFA in fish tissues could be suggested to result from the preferential oxidation of SFA followed by MUFA (supplied by MF), further stimulated by the fatty acid oxidative effect of spices (Westerterp-Plantenga et al., 2006; Ruiz et al., 2023). This could explain the conserved levels of SFA in all treatments, in spite of differences in dietary supply (being the surplus metabolized), and the significantly reduced levels of MUFA in fish fed the $SPICY_{0.15\%}$ diet compared to the NC (in which these accumulated), reaching similar values to the group fed the PC diet. On the other hand, the levels of total n-6 PUFA and of LA, which were significantly increased in the fillet of fish fed the NC diet, were reduced in both groups fed the spice-supplemented diets, having intermediate and non-significantly different values to the PC and NC groups. However, and just as described for the n-3 series, changes in the content of ARA, a LC-PUFA, were inversed to that of the C18 PUFA: ARA was significantly decreased when FO was replaced by MF in the NC, and significantly increased, reaching values similar to the PC, when supplementing the NC diet with 0.15% of the spices' combination. These results are puzzling and it's difficult to explain, based on current knowledge, the opposite effect that the combination of spices had in the fillet levels of C18 PUFA (promoting their decrease) and LC-PUFA (increased) in both n-3 and n-6 series. In any case, it is particularly relevant to note that both changes brought the fillet fatty acid profile of fish fed the $SPICY_{0.15\%}$ diet closer to that fed the PC, at least partially counteracting the effect of FO replacement by MF. Furthermore, the opposite trend of change observed in n-3 PUFA and n-6 PUFA levels in the fillets of fish fed the SPICY diets, enabled to increase the n-3/n-6 ratio from 0.73 in the NC to 1.10 in the $SPICY_{0.15\%}$ treatment, although the difference was not statistically significant, and was still far from the 1.65 ratio observed in the PC group. Nevertheless, considering that n-3 and n-6 PUFA compete for the same metabolic enzymes, and an excess of one class can cause a significant decrease in the metabolism of the other group of PUFA (Schmitz and Ecker, 2008), improving the n-3/n-6 ratio to a value $\geq$1 might be of functional relevance.

To characterize the nutritional quality of the fillet lipid fraction for the human consumer, the atherogenicity (IA) and thrombogenicity (IT) indices, as well as the hypocholesterolemic/hypercholesterolemic ratio (h/H) were calculated. While no differences were found in the IA and h/H among experimental diets, an increase from 0.24 to 0.31 was found in the IT when replacing 45% of FO by MF, which was not significantly changed by the supplementation with pungent spices. The IT indicates the amenability to clot formation in blood vessels (Chen and Liu, 2020). Nevertheless, despite the significant differences, values of IT reported in the current study were within the normal range found in gilthead seabream (Grigorakis, 2007), and far below the limits considered harmful for the consumer's health (1.0) (Marques et al., 2022).

4.3. Liver condition and accumulation of fat in hepatocytes

There were no signs of inflammation or other physiological alterations associated to dietary fatty acid unbalances in the liver of gilthead seabream (Gisbert et al., 2008). In addition, none of the observed fish showed an extremely low (score 1) or excessive (score 5) accumulation of fat deposits. A common consequence of partial or total FO substitution with vegetable oils (Caballero et al., 2004; Benedito-Palos et al., 2008) and animal-rendered fats is a higher accumulation of fat in the liver (Monteiro et al., 2018; Campos et al., 2019), although this condition is usually non-pathological and reversible (Caballero et al., 2004). This is normally explained by imbalances in the rates of oxidation, lipogenesis and lipoprotein synthesis (*i.e.*, lipid transport) related to changes in dietary fatty acid composition, associated to the lipogenic effect of LA in opposition to the hypolipidemic effect of n-3 PUFA and, to a lower extent, of MUFA (Halvorsen et al., 2001; Caballero et al., 2004; Kjær et al., 2008; Coccia et al., 2014). However, in the current study, the levels of fat deposits found in the hepatocytes of fish fed the PC and NC diet were quite similar, with even a slight decrease in the number of individuals with a high lipid accumulation and an increase in those with moderate and low lipid accumulation in the NC group. This could be related to the fact that the substitution of FO by MF resulted in a relatively moderate dietary increase in LA, coupled with increases in SFA and MUFA (which are preferentially oxidized). Dietary supplementation with spices, on the other hand, noticeably decreased the levels of fat deposits in the hepatic parenchyma of fish fed the $SPICY_{0.1\%}$ and $SPICY_{0.15\%}$ diets, in a dose-responsive manner. This result confirms the hypolipidemic effect of the pungent spices herein tested, which in mammals have the potential to enhance lipid metabolism (*e.g.*, accelerate the lipid oxidation rate) and increase energy expenditure, among other properties, leading to changes in serum lipid profile, enhancing triglyceride transport out of the liver, and reducing adipose tissue (Lee et al., 2003; Westerterp-Plantenga et al., 2006; Srinivasan, 2013; Xue et al., 2017). The most well characterized mechanism of action of several pungent active principles is through the up-regulation of hepatic cholesterol-7α-hydroxylase, which catalyzes the conversion of cholesterol to bile acids, while not affecting, or even down-regulating, the expression of 3-hydroxy-3-methylglutaryl-CoA reductase that is involved in cholesterol synthesis (Nammi et al., 2010; Liang et al., 2013; Lei et al., 2014; Lai et al., 2016; Zhang et al., 2013). Consequently, this affects the expression of hepatic LDL receptors and leads to decreased cholesterol absorption, accompanied by increased excretion of cholesterol and bile acids through the faeces (Lee et al., 2003; Liang et al., 2013; Lei et al., 2014; Xue et al., 2017). Our previous studies testing combinations of spices in gilthead seabream have shown phenotypical, physiological and molecular changes (Ruiz et al., 2023; Ruiz et al., 2024) that are consistent with the mammalian literature, suggesting that these mechanisms are conserved in vertebrates.

In this context, although the microarray analysis performed in this study is biased towards evaluating the expression of immune-related genes, a down-regulation of phosphatidylserine decarboxylase (*pisd*) was found in the liver of fish fed the $SPICY_{0.15\%}$ diet with respect to the NC group. This enzyme is involved in lipid droplet biogenesis by catalyzing the decarboxylation of phosphatidylserine and generation of phosphatidylethanolamine (Kumar et al., 2021). Thus, the down-regulation of *pisd* is consistent with the observed decrease in lipid deposits. In line with this, when totally replacing fish meal and FO by

plant-based ingredients in European seabass (*Dicentrarchus labrax*), increased expressions of *pisd* and other genes involved in lipogenic pathways were reported in the liver (Geay et al., 2011).

4.4. Hepatic immune response

A transcriptomic analysis with an immune-enriched genes' microarray was carried out to investigate the immune response generated by the inclusion of the combination of pungent spices at 0.15% in the NC diet. The identification of several DEGs clearly indicated an anti-inflammatory immune response in gilthead seabream fed the $SPICY_{0.15\%}$ diet. These results are consistent with previous reports showing an anti-inflammatory response induced by this combination of pungent spices in gilthead seabream (Ruiz et al., 2023), as well as by ginger and cinnamaldehyde when evaluated individually in rohu fish (*Labeo rohita*) and zebrafish (*Danio rerio*), respectively (Faikoh et al., 2014; Sukumaran et al., 2016).

Several immune-related pathways were found to be modulated by genes whose expression was significantly affected by the $SPICY_{0.15\%}$ diet with respect to the NC diet, such as the "stimulatory C-type lectin receptor signaling pathway" (GO:0002223). C-type lectin receptors are pattern recognition receptors (PRRs) expressed on multiple immune cells (dendritic cells, monocytes, macrophages, neutrophils, and B cells, among others) that recognize carbohydrate structures associated to pathogens. Ligand binding triggers signaling cascades which lead to different immune responses, such as antigen presentation and cytokine production (Geijtenbeek and Gringhuis, 2009). The Dendritic Cell-Specific ICAM-3-Grabbing nonintegrin (DC-SIGN) is a C-type lectin receptor found on the surface of dendritic cells, and expressed in many mammal and fish tissues, including the liver (Lin et al., 2009; Ojeda et al., 2020). Such C-type lectin receptor recognizes a wide range of pathogens through interaction with their mannose and fucose structures, triggering the activation of GTPase Ras proteins, and subsequent phosphorylation (activation) of the serine/threonine protein kinase RAF1, leading to the classical RAF1–MEK–ERK signaling cascade (Geijtenbeek and Gringhuis, 2009). Since previous studies have suggested that the RAF-MEK-ERK cascade is involved in the response to viral or parasitic infections in fish (Mo et al., 2020), the down-regulation of *hras* and *raf1* in gilthead seabream fed the $SPICY_{0.15\%}$ diet may indicate a better health condition of this group of fish in terms of a lower potential exposure to possible pathogens in presence of the combination of pungent spices. These findings are in agreement with the well-known antimicrobial properties of all the spices herein tested in mammals (Friedman, 2017; Beristain-Bauza et al., 2019; Takooree et al., 2019; Bhatti et al., 2022), and with the down-regulation in the expression of some PRRs which was reported in our previous studies when supplying the same (Ruiz et al., 2023) or similar (Ruiz et al., 2024) combinations of spices to gilthead seabream.

Furthermore, within GO:0002223 and in common with other immune-related GOs [*i.e.*, "antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent" (GO:0002479), "response to interleukin-1" (GO:0070555)], a differential regulation was found of genes coding for proteasomal subunits (*psmb7*, *psmb8*, *psmc6*). Ubiquitination and protein degradation are often critical steps in downstream signaling cascades initiated by PRR activation. In this sense, the 26S proteasome complex is composed of a 20S proteolytic core and by one or two 19S regulatory particles which recognize ubiquitinated substrates and translocate them into the 20S core for protein degradation. Moreover, the 20S core consists of three subunits that catalyze peptide hydrolyzation, which are PSMB5, PSMB6 and PSMB7, while PSMC6 is one of the subunits that constitute the base of the 19S complexes (Shi et al., 2020). It has been reported in mammals that, upon exposure to interferon-γ (IFN-γ), the three subunits of the 20S core are respectively substituted by their homologous PSMB8, PSMB9 and PSMB10, giving rise to the so-called immunoproteasome (Angeles et al., 2012; Shi et al., 2020). The pleiotropic cytokine IFN-γ has antibacterial and antiviral activities and is involved in the regulation of immune and inflammatory responses. In particular, many studies have shown the activation of pro-inflammatory cytokines in response to IFN-γ in different fish species (Zou and Secombes, 2016). In turn, the immunoproteasome regulates pro-inflammatory cytokine production, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α), as well as other immune functions, such as T cell differentiation and proliferation, and presentation of MHC-I antigen (Angeles et al., 2012). Consequently, the up-regulation of *psmb7* and *psmc6*, and down-regulation of *psmb8* in individuals fed the $SPICY_{0.15\%}$ diet indicated a lower activation of the 20S immunoproteasome complex than in fish fed the NC diet. This hypothesis may be in line with the suggested lower exposure to potential pathogens and with the observed modulation of other clusters identified in the enrichment analysis, as "antigen processing and presentation of exogenous peptide antigen *via* MHC class I, TAP-dependent" (GO:0002479), "response to interleukin-1" (GO:0070555)] and "cellular response to interleukin-6" (GO:0071354). Indeed, a down-regulation of the gene encoding for MHC class I antigen (*hla-a*) was observed in gilthead seabream fed the SPICY0.15% diet with respect to the NC group.

In relation to the differential modulation of GOs associated with a response to inflammatory cytokines, a down-regulation of the gene encoding for C—C motif chemokine 20 (*ccl20*) was observed when supplementing the NC diet with the combination of pungent spices. This cytokine is highly constitutively expressed in some fish tissues and plays a defensive role against pathogens, whereas under inflammatory conditions it attracts immune cells to sites of infection or inflammation (Peatman and Liu, 2007; Liu et al., 2020). In addition, previous studies have shown that the pro-inflammatory cytokine IL-1β can lead to an increase in *ccl20* expression (Brand et al., 2015). Hence, under the current nutritional conditions, the down-regulation of *ccl20* may be responding to a non-inflammatory hepatic status in the absence of any exposure to potential pathogens. This hypothesis is supported by the regulation of the "cellular response to interleukin-6" (GO:0071354), coupled with an up-regulation of the signal transducer and activator of transcription 1 (*stat1*) and a down-regulation of the hepcidin antimicrobial peptide (*hamp*) that were observed in fish fed the $SPICY_{0.15\%}$ diet. In this respect, capsaicin has been shown to inhibit the transcription of the gene encoding for the pro-inflammatory cytokine *il-6* in mammals (Shin et al., 2020). This cytokine is involved in the regulation of *hamp* expression *via* the JAK/STAT signaling pathway. In brief, when IL-6 binds to its cognate membrane-bound receptor, the protein Janus kinase 2 (JAK2) is activated and subsequently, the signal transducer and activator of transcription 3 (STAT3) is phosphorylated and relocated to the nucleus, where it induces the transcription of *hamp*, among other genes (Rah et al., 2023). Similarly, previous reports in mammals have shown that cinnamaldehyde derivatives can inhibit the JAK/STAT signaling pathway (Wang et al., 2022). In addition, previous studies in gilthead seabream have suggested a major role of HAMP in the innate immune response to bacteria and viruses (Cuesta et al., 2008), so the down-regulation of *hamp* may also be consistent with the antimicrobial effect of the tested combination of pungent spices. Furthermore, when activated by IL-6, STAT3 exerts a negative regulation on the transcription factor STAT1 by competing for docking sites of shared receptors (Wang et al., 2023), and therefore *stat1* up-regulation may also be in line with the inhibition of the JAK/STAT pathway due to the effect of capsaicin and/or cinnamaldehyde. On the other hand, a recent study has also suggested that STAT1 may additionally be involved in defense against viral and bacterial infection in fish, since an increased expression of *stat1* was found in the liver, spleen, and kidney of Japanese eel (*Anguilla japonica*) injected with pathogen-associated molecular patterns (PAMPs) or infected with *Aeromonas hydrophila* (Wang et al., 2019). However, considering the overall results, it is plausible that the up-regulation of *stat1* in this study is responding to its described competition with STAT3 and/or is a preventive defensive mechanism in case of potential pathogen infections, rather than indicating an actual higher

exposure to potential pathogens.

Overall, despite the absence of signs of hepatic inflammation in any of the individuals in the current study, these results suggested an improved health status in the liver of gilthead seabream fed with the $SPICY_{0.15\%}$ diet in terms of an induced anti-inflammatory immune response. Consequently, the herein tested combination of pungent spices may be used to help mitigate the inflammatory disorders that some of the nutritional strategies involving fish oil reduction can cause (Ballester-Lozano et al., 2015). Such results were consistent with the improved hepatic condition observed under histological examination, as well as with the enhancement in growth and feeding performance and results of proximate composition in fish fed the $SPICY_{0.15\%}$ diet compared to the control group.

5. Conclusions

In this study, we evaluated the effects associated to the replacement of 45% FO by MF in diets of gilthead seabream, and proposed the supplementation with a combination of pungent spices as a complementary dietary strategy when using alternative lipid sources in aquafeeds. The results of this work showed that the combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde, effectively compensated the growth and feeding performance decline caused by the reduction in dietary FO. In addition, supplementation of the diet with spices fully reverted the high increase in lipid content in the fillets of fish fed the FO-replaced diet, especially in the case of the $SPICY_{0.15\%}$ diet. Moreover, although the fillet fatty acid profile mostly reflected the composition of the diet being fed, supplementation with spices, notably at 0.15%, had some important effects. In particular, the fillets of fish fed the $SPICY_{0.15\%}$ diet, compared to the NC group, showed a significant decrease in the levels of total MUFA and n-6 PUFA (mainly LA), while n-6 LC-PUFA (ARA) was increased, and total n-3 PUFA were also increased, due to the rise in n-3 LC-PUFA (including EPA and DHA), whereas ALA was decreased. Consequently, the n-3/n-6 ratio was slightly elevated from the NC treatment, rising to a value >1 in the $SPICY_{0.15\%}$ treatment. These changes were inverse to those observed in the NC group, respective to the PC, and enabled to somewhat counteract the effects of substituting 45% of FO by MF, approaching the fillet profile of $SPICY_{0.15\%}$-fed fish to that of the PC group. These results are suggested to result from the beneficial combination of the pungent spices with a dietary lipid source (MF) relatively low in n-6 PUFA and rich in SFA and MUFA, the latter being preferentially oxidized and having the potential to spare PUFA, and LC-PUFA in particular, for tissue deposition. In liver, supplementation of the NC diet with the combination of pungent spices at 0.15% led to a lower accumulation of fat in enterocytes, and to a better hepatic health condition, as indicated by the transcriptomic data. Overall, these findings suggest that the properties of spices, as promotors of lipid oxidation, with hypolipidemic and hypocholesterolemic effects in mammals, appear to be equally beneficial in fish, and could be used as a strategy to mitigate some of the negative consequences of FO replacement in fish diets.

CRediT authorship contribution statement

Alberto Ruiz: Writing – original draft, Visualization, Formal analysis. **Enric Gisbert:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Alicia Estevez:** Writing – review & editing, Methodology, Formal analysis. **Felipe E. Reyes-López:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Eva Vallejos-Vidal:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lluís Tort:** Methodology. **Jorge Dias:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sara Magalhães:** Writing – review & editing, Methodology. **Tiago Aires:** Writing – review & editing, Methodology. **Sofia Morais:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Transcriptomic data can be found in the public repository of Gene Expression Omnibus (GEO, https://www.y/acncbi.nlm.nih.gov/geo/querc.cgi) at the United States National Center for Biotechnology Information (NCBI), under accession number GPL13442.

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Appendix A. Supplementary data

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