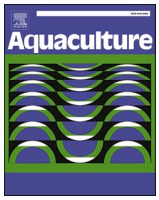
Aquaculture 598 (2025) 741957

Contents lists available at ScienceDirect

Aquaculture

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



Benefits of dietary krill meal inclusion towards better utilization of nutrients, and response to oxidative stress in gilthead seabream (*Sparus aurata*) juveniles

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ARTICLE INFO

Keywords:
Gilthead sea bream
Krill meal
Omega-3
stress

ABSTRACT

Krill meal (KM) emerges as a promising sustainable marine ingredient in aquafeeds, providing a rich source of protein, amino acids, phospholipids, omega-3 fatty acids, and bioactive compounds. This study aimed to investigate the effects of including KM (3, 5 and 7 % of the diet) on growth performance, nutrient utilization, and antioxidant defenses in juvenile gilthead sea bream (*Sparus aurata*) exposed to a crowding stress challenge. The dietary inclusion of 7 % KM could effectively replace up to 47 % FM in the diet (8 % FM in 7 % KM in comparison to 15 % FM in control diet), without compromising growth or feed conversion. Although not statistically different, dietary supplementation with 5 and 7 % KM showed a tendency to further optimize feed conversion ratio and nutrient efficiency ratios compared to the control FM diet. Under stressful conditions, a significant interaction between diet and time was observed in fish blood omega-3 index (O3I). At 24 h after the stress challenge, all dietary treatments except KM3 presented a significant increase in n-3 PUFA, EPA, DHA as well as OI3, whereas a decrease in MUFA. At 7d (168 h) post-stress, fish fed the control diet presented a significant reduction in O3I down to the basal levels. On the contrary, those fish fed KM5 and KM7 diets kept increased O3I levels as well as n-3 PUFA content to the end of the stress challenge. Indeed, 24 h after stress, fish fed KM5 and KM7 showed a lower increase of *cat* and *sod* gene expression in head kidney, which was further inversely correlated with fish blood OI3. Therefore, these results show that KM modulates red blood cells fatty acid profile by increasing fish OI3 after stress as well as potentially functioning as an antioxidant modulator in fish feeds for mitigating stressful conditions. Hence, KM is a valuable functional ingredient in aquafeeds, aiming to expand the basket of raw materials with functional properties to be used in aquafeed formulation to enhance fish robustness.

1. Introduction

The nutritional benefits and the general health and robustness of fish depend mainly on the nutrients provided by aquafeeds, which have traditionally been based mainly on finite marine resources, such as fish meal (FM) and fish oil (FO). Due to fluctuations in the supply and prices of FM and FO, the aquaculture industry has tended towards plant-based diets (Colombo and Turchini, 2021). However, very often plant-based diets may result in a deficient and unbalanced supply of essential nutrients or be associated with undesirable effects on palatability, and the presence of antinutritional factors, which may also have other negative effects on fish health, especially on marine fish which have a reduced ability to synthetize LC-PUFAs from their C18 precursors and are then considered essential nutrients for marine species (Turchini et al., 2009; Montero and Izquierdo, 2010; Torrecillas et al., 2017a, 2017b). Hence, to meet the nutritional requirements of fish without compromising fish performance and health, and at the same time utilizing aquafeed ingredients effectively, one of the possible strategies to be followed is to produce aquafeeds with sustainable functional raw materials that could enhance the bioavailability and utilization of nutrients.

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https://doi.org/10.1016/j.aquaculture.2024.741957
Received 26 June 2024; Received in revised form 25 November 2024; Accepted 26 November 2024
Available online 2 December 2024

In this sense, krill meal (KM) is positioned as a potential functional and certified sustainable marine ingredient to be included in aquafeeds. KM has gained attention in the aquaculture industry as a potential source of several important nutrients in fish feeds. For instance, KM typically contains a high-quality protein, often exceeding 56 % on a dry weight basis, as well as a balanced amino acid profile (Hertrampf et al., 2000; Tou et al., 2007; Kaur et al., 2022). Additionally, krill-derived products are also a valuable source of omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known to play vital roles in fish metabolism, as essential nutrients to be supplied in the diets of marine carnivorous species, like gilthead sea bream (*Sparus aurata*) (Izquierdo, 2005; Tocher, 2015). Noteworthy, krill lipids are mainly in the phospholipid form, particularly constituted by phosphatidylcholine, which are suspected to be more bioavailable than the triglycerides found in the traditional FM and FO, playing a role in maintaining cell membrane integrity (Köhler et al., 2015; Kaur et al., 2022). Thus, based on the nutrient composition of KM, it is a highly digestible and palatable nutrient source, potentially improving both feed conversion and nutrient efficiency in fish, ultimately improving fish growth and health. Indeed, previous studies have reported that the dietary inclusion of KM (at 5 and 7.5 % of the diet) promotes European sea bass (*Dicentrarchus labrax*) juveniles' growth and feed utilization (Torrecillas et al., 2021). In Atlantic salmon (*Salmo salar*), dietary KM at 12 %, improved fillet quality (Kaur et al., 2022, Mørkøre et al., 2020). In gilthead sea bream juveniles, the dietary inclusion of KM at 9 % also improved growth and reduced the feed conversion ratio when replacing 20 % of the dietary FM (Saleh et al., 2018). Given this high omega-3 FA content of KM, its inclusion in aquafeeds may also influence the lipid composition of fish tissues, particularly the fillet, which holds significance for meeting consumer expectations and human health.

In addition, KM also contains bioactive antioxidant compounds, including choline, selenium as well as astaxanthin and vitamin E, theoretically improving fish overall health, including fish antioxidant status and defenses (Tou et al., 2007). Oxidative stress is defined as a disturbance in the pro-oxidant/antioxidant balance that leads to potential damage and plays a key role in determining fish responses to environmental changes (Halliwell, 2007). Fish farming conditions include abiotic and biotic stressors, such as handling, high stocking densities, temperature fluctuations, salinity variations and/or pathogens, which may induce physiological changes related to oxidative stress (Sahin et al., 2014; Yang et al., 2019). Few studies have reported the beneficial effect of KM dietary supplementation in reducing the oxidative stress status or improving the antioxidant capacity of aquatic species (Saleh et al., 2015; Gao et al., 2020; Ambasankar et al., 2022; Wang et al., 2024). However, for gilthead sea bream, despite the previous described effects of dietary KM supplementation as a growth promoter and feed stimulant, to our knowledge, role of KM as a potential reducer of oxidative stress have been poorly explored in this species, especially in regard with fish facing stressful conditions, such as high stocking densities or overcrowding in sea cages and/or tanks in farms.

Therefore, the present trial aimed to evaluate the effect of krill meal (*Euphausia superba*) inclusion in diets for gilthead seabream juveniles on growth, feed utilization, fillet fatty acid profile as well as on fish response to an increase in oxidative stress induced by crowding.

2. Materials and methods

2.1. Ethical statement

The animal experiments comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. The Bioethical Committee of the University of Las Palmas de Gran Canaria approved all the protocols used in the present study (approval n° OEBA -ULPGC 19/2022).

2.2. Experimental diets

A control diet was formulated with 15 % FM. Three experimental diets were then formulated to progressively include KM in the diet. For that, KM AQUA (provided by Aker BioMarine Antarctic AS, Norway) was added to the diet at 3 (KM3), 5 (KM5), and 7 % (KM7) and FM was reduced to 20, 33 %, and 47 %, respectively. The four extruded

Table 1
Diet formulation and proximate composition (% dry weight).

	Control	KM3	KM5	KM7
Fishmeal Super Prime[1]	7.0	4.0	2.0	0.0
Fishmeal 60	8.0	8.0	8.0	8.0
Krill meal	0.0	3.0	5.0	7.0
Poultry meal[2]	12.0	12.0	12.0	12.0
Soy protein concentrate[3]	10.0	10.0	10.0	10.0
Wheat gluten[4]	8.1	8.6	8.9	9.2
Corn gluten meal[5]	10.0	10.0	10.0	10.0
Soybean meal 44[6]	14.0	14.0	14.0	14.0
Wheat meal[7]	11.0	10.6	10.4	10.2
Faba beans (low tannins)[8]	3.1	3.1	3.1	3.1
Vitamin and mineral premix[9]	1.3	1.3	1.3	1.3
Choline chloride 50 %	0.2	0.2	0.2	0.2
Antioxidant[10]	0.2	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1	0.1
MCP (Monocalcium phosphate)[11]	1.0	1.0	1.0	1.0
DL-Methionine	0.2	0.2	0.2	0.2
Fish oil[12]	5.5	5.5	5.5	5.5
Soybean oil	3.6	3.6	3.6	3.6
Rapeseed oil[13]	3.4	3.4	3.4	3.4
Palm oil	1.3	1.3	1.3	1.3
Proximate composition (% dry weight)				
Crude Protein	48.8	49.2	48.9	48.9
Crude Lipids	24.2	22.3	22.6	22.6
Ash	7.8	7.7	7.6	7.8
Moisture	9.2	11.9	5.4	4.5

1: Diamante, Pesquera diamante, Peru. 2: SAVINOR UTS, Portugal; 3: Soycomil P, ADM, the Netherlends; 4: Vital, Roquette, France; 5: COPAM, Portugal; 6: Ribeiro & Sousa Lda, Portugal; 7: Molisur, Spain; 8: Ribeiro e Sousa Lda, Portugal; 9: Premix Lda, Portugal; 10: VERDILOX, Kemin Europe NV, Belgium; 11: ALIPHOS MONOCAL, ALIPHOS, Belgium; 12: Sopropêche, France; 13: JC Coimbra, Portugal

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

	Control	KM3	KM5	KM7
14:0	2.43	2.31	2.52	2.53
14:1n-7	0.02	0.02	0.02	0.02
14:1n-5	0.09	0.08	0.09	0.07
15:0	0.21	0.19	0.17	0.18
15:1n-5	0.04	0.02	0.02	0.03
16:0ISO	0.04	0.04	0.03	0.04
16:0	16.34	16.23	16.11	15.80
16:1n-7	3.15	3.24	3.26	3.26
16:1n-5	0.14	0.14	0.12	0.14
16:2n-6	0.02	0.02	0.01	0.01
16:2n-4	0.32	0.34	0.30	0.37
17:0	0.37	0.37	0.34	0.35
16:3n-4	0.15	0.15	0.17	0.15
16:3n-3	0.10	0.09	0.09	0.10
16:3n-1	0.04	0.04	0.04	0.04
16:4n-3	0.59	0.63	0.64	0.64
16:4n-1	0.01	0.02	0.01	0.11
18:0	3.84	3.86	3.63	3.85
18:1n-9	29.40	29.16	29.00	29.06
18:1n-7	2.32	2.22	2.43	2.49
18:1n-5	0.48	0.06	0.04	0.06
18:2n-9	0.10	0.03	0.02	0.03
18:2n-6	22.00	22.18	22.26	21.91
18:2n-4	0.16	0.13	0.10	0.13
18:3n-6	0.13	0.09	0.08	0.09
18:3n-4	0.04	0.04	0.02	0.02
18:3n-3	3.79	3.94	3.86	3.85
18:3n-1	0.09	0.11	0.07	0.07
18:4n-3	0.81	0.96	0.89	0.91
18:4n-1	0.08	0.10	0.05	0.07
20:0	0.35	0.34	0.30	0.36
20:1n-9	0.13	0.10	0.05	0.06
20:1n-7	0.91	0.93	0.86	0.95
20:1n-5	0.18	0.20	0.14	0.13
20:2n-9	0.05	0.06	0.05	0.05
20:2n-6	0.13	0.14	0.11	0.13
20:3n-9	0.03	0.01	0.03	0.03
20:3n-6	0.07	0.06	0.08	0.07
20:4n-6 (ARA)	0.41	0.41	0.41	0.40
20:3n-3	0.06	0.04	0.06	0.05
20:4n-3	0.22	0.22	0.22	0.22
20:5n-3 (EPA)	4.76	5.35	5.74	5.62
22:1n-11	0.43	0.42	0.38	0.42
22:1n-9	0.21	0.23	0.25	0.28
22:4n-6	0.07	0.05	0.04	0.05
22:5n-6	0.14	0.13	0.12	0.12
22:5n-3	0.54	0.52	0.55	0.56
22:6n-3 (DHA)	3.99	4.01	4.23	4.14
SFA	23.53	23.29	23.07	23.07
MUFA	37.52	36.82	36.64	36.96
n-9	29.92	29.59	29.41	29.51
n-6	22.98	23.07	23.11	22.78
n-3	14.86	15.78	16.28	16.09
n-3/n-6	0.65	0.68	0.70	0.71
EPA/DHA	1.19	1.34	1.36	1.36
EPA/ARA	11.68	12.94	13.98	14.01
EPA + DHA	8.75	9.36	9.97	9.76

KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

experimental diets (Sparos LTD, Faro, Portugal) were isoproteic and isoenergetic. Formulation and proximate composition are presented in Table 1, and dietary fatty profiles in Table 2.

2.3. Experimental fish and conditions

The nutritional trial was carried out at the experimental facilities of the ECOAQUA-UI, at the University of Las Palmas de Gran Canaria (Spain). Gilthead sea bream juveniles with an initial body weight of 8.41 ± 0.03 g (mean ± SD), were randomly distributed in triplicate groups (3 tanks/diet) of 12 experimental tanks of 500 L that were supplied with filtered seawater in an open-flow system. Fish were reared at a density of 55 fish/tank. Experimental conditions were maintained under a natural photoperiod (12 h light: 12 h dark), dissolved oxygen was maintained between 6.8 and 7.6 ppm, and water temperature during the trial was 24.05 ± 0.31 °C. Fish were manually fed until apparent satiation with one of the four experimental diets 3 times a day (9:00, 12:00 and 16:00), 6 days per week, for 12 weeks. Uneaten feed pellets were daily recovered, dried in an oven for 24 h, and weighed to estimate feed intake (FI) and feed conversion ratio. Furthermore, fish performance was monitored at 6th and 12th week of feeding. For the sampling, fish were subjected to a 24-h fasting period, anesthetised with clove oil (0.2 mL/L; Guinama S.L; Spain, Ref. Mg83168) and individually weighed and measured. At the end of the trial, fish were euthanised with an excess of clove oil (5 mL/L) and fillets (without skin) from 6 fish per tank were also collected for determining fatty acid profile. Water quality was monitored daily to unsure high quality parameters.

2.4. Crowding stress challenge

At the end of the nutritional trial, and after 8 days of the weight sampling to ensure fish stress levels from handling were restored, 60 fish by treatment (20 per tank) were subjected to a crowding stress challenge by confining 20 fish from the same experimental treatment in submerged small cages (56.6 × 24.5 × 39.5 cm;17 g/cm^3; 3 cages/diet) for 7 days following internal common protocols (Serradell et al., 2020). During the stress challenge period, fish were manually fed with their respective experimental diets. Only two fish mortalities, unrelated to dietary treatment, were recorded throughout the stress challenge period of 7 days. In each sampling point (0 h-pre-stress, 24 h and 7 days), one cage was sampled to avoid stressing the remaining fish. In each sampling point, blood as well as head kidney samples from 15 fish per treatment were collected for Omega-3 index (OI3) determination and antioxidant defence-related gene expression. Blood was collected by caudal vein punction in heparinized syringes and conserved at −80 °C until analysis. Fish were fasted 24 h previuously to blood extraction. Head kidney samples were collected in three pools of 5 fish per treatment and conserved in RNA Later at −80 °C until analyses.

2.5. Key performance parameters calculations

Fish productive parameters related with growth performance, feed and nutrient utilization were calculated according to the following equations:

$$\text{SGR, Specific Growth Rate } (\text{SGR, \%day}^{-1}) = \left[\left(\text{Ln (final weight} - \text{Ln (initial weight)}\right) \text{ x } 100/\text{number of days}\right]$$

$$\text{Feed intake } (\text{g fish}^{-1}\ \text{day}^{-1}) = \text{feed given/feed recovered (uneaten)}$$

$$\text{Feed Conversion Ratio (FCR)} = \text{total feed fed/weight gain}$$

$$\text{Protein efficiency ratio (PER)} = \text{weight gained /weight of protein consumed}$$

$$\text{LER lipid efficiency ratio)} = \text{weight gained/weight of lipid consumed}$$

2.6. Proximate composition and fatty acid analyses

Proximate composition analyses of feeds were carried out accordingly with the standardized procedures described by AOAC (1975). Crude protein content (Nx6.25) was analysed following the Kjeldahl method. Ash content was determined by incineration at 600 °C for 12 h in a muffle furnace, whereas moisture content was determined after drying samples in an oven at 110 °C until constant weight. Total lipids of feeds and fillets were extracted with chloroform/methanol (2:1 *v*/v)

(Folch et al., 1957). The neutral (NL) and polar lipid (PL) fractions of fish flesh was separated to analyse the fatty acid profile of each fraction. For that, the total lipids underwent a filtration using a Sep-Pack NH2 cartridge, where NL were eluted with 30 ml of chloroform and 20 ml of chloroform/methanol (49:1 v/v), followed by the elution of PL with 30 ml of methanol, in 10 ml series (Juaneda and Rocquelin, 1985). Lipid fractions were *trans*-methylated for fatty acid methyl esters obtention (Christie et al., 1989), which were separated by gas chromatography following the conditions described by Izquierdo (1989). Fatty acid methyl esters were quantified (in % of total fatty acids) by a flame ionization detector and identified by comparison with external and well-characterized FO standards (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.7. Omega-3 index in red blood cells

Fish blood samples were centrifuged at 1000*g* for 20 min to pellet red blood cells (RBC). After removing plasma and white cells, 30 µl of RBC was spotted by duplicate on a blood card, let do dry, and then stored at −80 °C until analysis and shipped to University of Stirling for RBC fatty acid determination and subsequent omega-3 index (O3I) calculation (McBurney et al., 2022a, 2022b). RBC fatty acids were methylated as described by Christie et al. (1989), and then analysed using GLC as previously described (Bell et al., 2011). O3I calculation corresponds to the sum of total EPA and DHA content.

2.8. Gene expression of antioxidant defence system-related genes

Head kidney RNA was extracted in TRI-reagent (Sigma-Aldrich, Sant Louis, MO, USA) with a RNeasy Mini Kit (Qiagen, Hilden, Germany) following commercial procedure. Subsequently, complementary DNA (cDNA) was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions and employing an iCycler thermal cycler (Bio-Rad).

The relative expression of three different antioxidant enzymes, including *catalase (cat)*, *superoxide dismutase (sod)* and *glutathione peroxidase (gpx)*, was assessed by *mRNA* level quantification using RT-PCR on an iQ5 Multicolour Real-Time PCR detection system from Bio-Rad. Elongation factor 1 alpha (*ef1a*) was used as housekeeping gene. All primer sequences are detailed in Table 3. The RT-PCR conditions involved an initial step of 3 min 30 s at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at the annealing temperature of 58 °C, 30 s at 72 °C, and a final step of 1 min at 95 °C. Reactions were conducted in a final volume of 15 µl, comprising 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad, Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution), and 1.3 µl of MilliQ water. The relative expression of each gene was then estimated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analyses

All the statistical analyses were performed with R Project for Statistical Computing. Means and standard deviations (SD) were calculated for each parameter measured. Before performing the statistical analysis, all data tested were analysed for outlier detection defining the outside cut-offs as 1.5 times the Inter-Quantile Range (IQR) below the first and above the third quantiles (Hoaglin and Iglewicz, 1987; Feng et al., 2008). A one-way ANOVA analysis was performed to analyse the differences on growth parameters and fish blood OI3 indexes increment from the basal experimental point ($t = 0$ h pre-stress challenge) to the posterior sampling points ($t = 24$ h and 7d post-stress challenge) between the different experimental dietary treatments. A two-way ANOVA analysis was performed to analyse the differences in fish blood O3I between the different dietary treatments and the sampling point (t = 0 h pre-stress challenge and t = 24 h and 7d post-stress challenge). All data analysed were tested for normality and homogeneity standards, with an alpha value set a 0.05. When significant differences were found ($p < 0.05$), a Tukey post-hoc test was performed for multiple means comparison (Tukey, 1953; Benjamini and Braun, 2002). When applicable, data were subjected to the best fit correlations, which were checked for significance at $p < 0.05$, and Pearson's coefficients were determined. A principal component analysis (PCA) was performed to analyse the effects of the different dietary treatments on fish blood fatty acid profiles, displaying the patterns of similarity between the observed variables and experimental treatments as points in a bi-dimensional map (Ringnér, 2008; Abdi and Williams, 2010). PCA representation was assessed by explained variation fraction, calculated as the sum of the two principal component of explained variance (Bro and Smilde, 2014).

3. Results

3.1. Growth performance, feed, and nutrient utilization

After 12 weeks of feeding the experimental diets, no significant differences in any of the productive parameters evaluated were observed (Table 4). However, a tendency towards improved FCR by 4 and 7 % was observed in fish fed KM7 and KM5 diets, respectively, compared to those fed control diet ($r = -0.467$; $p = 0.126$; Table 4). Furthermore, this trend aligned with an indication of improved growth performance by 5 % in fish fed the KM7 diet as compared to the control group, in agreement with improved LER in fish fed with KM diets ($r = 0.715$; $p = 0.009$), irrespective of the dietary level (8 % for KM3 and KM5, and 10 % for KM7, respectively). A similar tendency was observed for PER in fish fed KM7 diet (5 %) compared to control fish ($r = 0.451$; $p = 0.141$).

3.2. Fatty acid profile of fish fillets

Fatty acid profile of fish fillets was similar among fish fed Control diet and those fed KM diets, in both neutral and polar lipids (Tables 5 and 6, respectively). Despite no significant differences were observed among FA profiles, DHA content in NL ($r = 0.441$, $p = 0.151$;Table 5) and EPA in PL ($r = 0.357$, $p = 0.254$) as well as for n-3 PUFA ($r = 0.557$, $p = 0.059$) and n-3 LC-PUFA ($r = 0.0.515$, $p = 0.086$) in NL (Tables 5 and 6) were correlated to KM dietary levels.

Table 3
Primer sequences of the different genes analysed and their RT-PCR conditions.

	Primer nucleotide sequence 5′-3′	Access number	Annealing Temperature (°C)	Transcription lenght (pair of bases)	Reference
ef1α	R:TCCTGCACGACCATTCATTTC F: CATGGTTGTGGAGCCCTTCT	AF184170	58.1	174	Dominguez et al. (2021)
cat	R:AGTGGAACTTGCAGTAGAAAC F: ATGGTGTGGGACTTCTGGAG	JQ308823	58.1	166	Dominguez et al. (2020)
gpx	R:CTGACGGGACTCCAAATGATGG F: GAAGGTGGATGTGAATGGAAAAGATG	DQ524992	58.0	129	Teles et al. (2016)
sod	R:CCTGACCTGACCTACGACTATGG F: AGTGCCTCCTGATATTTCTCCTCTG	JQ308833	58.0	134	–

Table 4
Growth parameters of gilthead sea bream (*Sparus aurata*) fed the experimental diets.

Diet	Growth parameters						
	IBW	FBW	SGR	FCR	FI	PER	LER
Control	8.4 ± 0.0	47.1 ± 2.4	1.9 ± 0.1	1.23 ± 0.1	0.52 ± 0.01	1.68 ± 0.12	3.37 ± 0.25
KM3	8.4 ± 0.0	47.3 ± 1.2	1.9 ± 0.0	1.22 ± 0.0	0.53 ± 0.02	1.67 ± 0.04	3.68 ± 0.09
KM5	8.4 ± 0.1	46.7 ± 0.9	1.9 ± 0.0	1.18 ± 0.1	0.50 ± 0.02	1.73 ± 0.09	3.69 ± 0.19
KM7	8.4 ± 0.1	48.6 ± 2.2	2.0 ± 0.1	1.15 ± 0.1	0.51 ± 0.01	1.78 ± 0.08	3.84 ± 0.18

One-way ANOVA: Diet; Data expressed in mean ± SD ($n = 3$). IBW, initial bodyb weight; FBW, final body weight (g); SGR, specific growth rate (% day^{-1}); FCR, feed conversion ratio; FI, feed intake (g fish^{-1} day^{-1}); PER, protein efficiency ratio; LER, lipid efficiency ratio. KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

3.3. Fatty acid profile and omega-3 indices of red blood cells in response to stress

In general, the contents of 20:4n-6, 20:5n-3, 22:6n-3, n-3 PUFA and total PUFA were increased at 24 h and 7 days of crowding stress compared with basal levels ($p < 0.05$; Table S1). The contrary tendency was observed for 18:1n-9, 18:2n-6, 18:3n-3, as well as total MUFA, whose contents decreased after crowding stress ($p < 0.05$; Table S1). Diet affected the contents of SFA at 7 days of crowding with fish fed the control diet presenting higher content of SFA compared with those fed on KM5 (Table S1). Furthermore, n-6 PUFA content of RBC was also higher in fish fed the control diet compared with those fed KM diets, especially KM5 and KM7, both at basal and 24 h of crowding stress (Table S1). Concerning n-3 PUFA, only KM diets affected those levels after 7 days of crowding stress, particularly, KM5 showing higher n-3 PUFA content compared with Control. EPA was significantly higher in RBC of fish fed KM5 and KM7 before crowding stress, and it was maintained throughout the whole time of stress challenge ($t = 24$ h and $t = 7$ days) (Table S1).

The dietary treatments did not induce significant differences in fish basal ($t = 0$ h pre-stress challenge) blood O3I, although higher absolute values observed in those fish fed with KM5 and KM7 diets (Table 7).

Table 5
Fillet fatty acid composition (% total fatty acids) of neutral lipids from gilthead sea bream (*Sparus aurata*) fed the experimental diets.

Fatty acid (% total fatty acids)	Diets			
	Control	KM3	KM5	KM7
14:0	2.10 ± 1.14	1.73 ± 0.59	1.48 ± 0.57	2.25 ± 1.17
15:0	0.25 ± 0.08	0.20 ± 0.02	0.20 ± 0.01	0.23 ± 0.11
16:0	21.32 ± 7.34	15.01 ± 1.54	16.23 ± 1.78	15.40 ± 5.94
16:1n-7	3.76 ± 1.63	3.89 ± 0.61	4.03 ± 0.49	4.22 ± 0.83
17:0	0.16 ± 0.09	0.20 ± 0.05	0.18 ± 0.09	0.20 ± 0.06
16:3n-4	0.19 ± 0.05	0.23 ± 0.01	0.30 ± 0.11	0.22 ± 0.06
16:3n-3	0.10 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.05
18:0	6.33 ± 4.10	4.30 ± 0.83	4.69 ± 0.76	4.01 ± 1.19
18:1n-9	28.44 ± 6.98	32.89 ± 4.05	33.00 ± 0.91	27.62 ± 4.86
18:1n-7	5.15 ± 1.70	4.23 ± 1.95	5.15 ± 2.58	7.97 ± 7.56
18:1n-5	0.81 ± 0.47	0.38 ± 0.45	0.51 ± 0.34	1.40 ± 2.19
18:2n-9	0.34 ± 0.11	0.30 ± 0.06	0.28 ± 0.12	0.43 ± 0.35
18:2n-6	18.18 ± 2.43	20.34 ± 1.03	17.75 ± 3.20	19.04 ± 2.18
18:2n-4	0.46 ± 0.33	0.15 ± 0.10	0.13 ± 0.04	0.43 ± 0.42
18:3n-6	0.38 ± 0.24	0.28 ± 0.12	0.24 ± 0.08	0.28 ± 0.13
18:3n-3	2.10 ± 1.12	2.92 ± 0.52	2.65 ± 0.60	3.09 ± 0.55
18:4n-3	0.35 ± 0.23	0.61 ± 0.20	0.55 ± 0.20	0.67 ± 0.08
20:0	0.28 ± 0.02	0.38 ± 0.12	0.32 ± 0.06	0.25 ± 0.09
20:1n-9	0.19 ± 0.14	0.12 ± 0.02	0.11 ± 0.00	0.09 ± 0.05
20:1n-7	0.76 ± 0.17	1.08 ± 0.35	1.39 ± 0.63	0.93 ± 0.15
20:1n-5	0.14 ± 0.01	0.21 ± 0.03	0.24 ± 0.08	0.18 ± 0.05
20:2n-9	0.13 ± 0.03	0.26 ± 0.02	0.21 ± 0.03	0.19 ± 0.04
20:2n-6	0.28 ± 0.06	0.36 ± 0.10	0.35 ± 0.06	0.28 ± 0.03
20:3n-6	0.18 ± 0.05	0.25 ± 0.04	0.17 ± 0.04	0.21 ± 0.03
20:4n-6	0.50 ± 0.50	0.27 ± 0.05	0.43 ± 0.24	0.25 ± 0.08
20:4n-3	0.20 ± 0.10	0.35 ± 0.06	0.27 ± 0.11	0.31 ± 0.02
20:5n-3	2.32 ± 0.60	2.91 ± 0.76	2.43 ± 1.07	3.40 ± 0.51
22:1n-11	0.18 ± 0.11	0.37 ± 0.32	0.39 ± 0.10	0.21 ± 0.06
22:1n-9	0.24 ± 0.16	0.25 ± 0.17	0.57 ± 0.31	0.37 ± 0.11
22:5n-3	0.52 ± 0.22	1.03 ± 0.28	0.88 ± 0.29	1.05 ± 0.05
22:6n-3	2.39 ± 0.75	3.14 ± 0.98	3.44 ± 1.20	3.38 ± 0.70
SFA	30.44 ± 10.83	21.82 ± 1.94	23.10 ± 1.70	22.34 ± 7.98
MUFA	39.90 ± 7.53	43.62 ± 2.67	45.61 ± 4.20	43.21 ± 3.95
n-9	29.34 ± 7.34	33.83 ± 3.99	34.18 ± 0.95	28.71 ± 4.67
n-6	19.69 ± 2.18	21.81 ± 1.19	19.27 ± 2.76	20.24 ± 2.15
n-3	8.22 ± 1.45	11.35 ± 2.84	10.58 ± 2.99	12.29 ± 1.39
n-3 PUFA	7.95 ± 1.48	11.05 ± 2.76	10.29 ± 2.91	11.97 ± 1.48
n-6 PUFA	19.69 ± 2.18	21.73 ± 1.05	19.27 ± 2.75	20.24 ± 2.15
n-3 LC-PUFA	5.50 ± 1.37	7.52 ± 2.05	7.09 ± 2.24	8.22 ± 1.12
EPA + DHA	4.71 ± 1.35	6.06 ± 1.71	5.87 ± 1.87	6.77 ± 1.10
EPA/DHA	0.99 ± 0.07	0.94 ± 0.09	0.74 ± 0.28	1.02 ± 0.15
EPA/ARA	7.21 ± 4.00	10.92 ± 3.09	7.09 ± 4.95	14.68 ± 6.15

One-way ANOVA: Diet; Data expressed in mean ± SD (n = 3). KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

Table 6
Fillet fatty acid composition (% total fatty acids) of polar lipids from gilthead sea bream (*Sparus aurata*) fed the experimental diets.

	Diets			
Fatty acids (% total fatty acids)	**C**	**KM3**	**KM5**	**KM7**
14:0	1.16 ± 1.40	0.55 ± 0.16	0.53 ± 0.27	0.60 ± 0.17
15:0	0.33 ± 0.37	0.16 ± 0.01	0.15 ± 0.04	0.16 ± 0.03
16:0	23.39 ± 7.64	21.49 ± 0.94	21.28 ± 5.94	24.13 ± 1.19
16:1n-7	1.75 ± 0.73	1.22 ± 0.21	1.28 ± 0.47	1.35 ± 0.15
17:0	0.18 ± 0.19	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
16:3n-1	0.54 ± 0.09	0.98 ± 0.23	0.63 ± 0.27	0.98 ± 0.28
16:4n-3	0.24 ± 0.07	0.42 ± 0.13	0.30 ± 0.15	0.40 ± 0.07
18:00	12.16 ± 2.37	11.71 ± 2.50	10.15 ± 1.92	11.34 ± 1.95
18:1n-9	22.24 ± 0.80	21.08 ± 0.94	19.39 ± 2.71	20.58 ± 1.30
18:1n-7	4.52 ± 1.17	4.26 ± 1.00	4.21 ± 2.06	4.28 ± 0.94
18:1n-5	0.21 ± 0.07	0.20 ± 0.08	0.20 ± 0.16	0.28 ± 0.27
18:2n-9	0.15 ± 0.01	0.19 ± 0.05	0.17 ± 0.00	0.18 ± 0.05
18:2n-6	15.13 ± 6.80	16.24 ± 1.34	15.96 ± 1.01	15.65 ± 1.30
18:2n-4	0.14 ± 0.06	0.13 ± 0.10	0.17 ± 0.07	0.15 ± 0.06
18:3n-6	0.13 ± 0.06	0.15 ± 0.07	0.15 ± 0.02	0.11 ± 0.03
18:3n-4	0.05 ± 0.02	0.04 ± 0.01	0.06 ± 0.03	0.04 ± 0.02
18:3n-3	1.09 ± 0.40	1.05 ± 0.13	1.13 ± 0.20	1.04 ± 0.17
20:0	0.44 ± 0.18	0.31 ± 0.09	0.27 ± 0.06	0.29 ± 0.09
20:1n-9	0.17 ± 0.13	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.01
20:1n-7	1.06 ± 0.40	0.77 ± 0.16	0.71 ± 0.15	0.73 ± 0.18
20:2n-6	0.43 ± 0.16	0.53 ± 0.06	0.46 ± 0.05	0.47 ± 0.04
20:3n-6	0.39 ± 0.15	0.58 ± 0.03	0.46 ± 0.15	0.51 ± 0.02
20:4n-6	1.30 ± 0.61	1.60 ± 0.24	1.67 ± 0.58	1.49 ± 0.25
20:4n-3	0.18 ± 0.07	0.25 ± 0.02	0.24 ± 0.11	0.22 ± 0.04
20:5n-3	3.99 ± 2.07	5.00 ± 0.87	6.41 ± 3.18	5.45 ± 1.38
22:1n-11	0.11 ± 0.06	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.04
22:5n-6	0.27 ± 0.10	0.36 ± 0.06	0.44 ± 0.29	0.30 ± 0.05
22:5n-3	0.84 ± 0.34	1.17 ± 0.21	1.49 ± 1.06	1.08 ± 0.31
22:6n-3	6.00 ± 2.67	7.89 ± 1.84	6.02 ± 2.76	6.85 ± 2.17
SFA	37.66 ± 12.07	34.28 ± 2.30	32.44 ± 8.18	36.56 ± 2.56
MUFA	30.58 ± 1.86	28.02 ± 1.87	26.20 ± 5.56	27.65 ± 2.81
n-9	22.78 ± 0.86	21.58 ± 0.94	19.78 ± 2.73	21.01 ± 1.43
n-6	17.78 ± 7.89	19.62 ± 1.40	19.31 ± 1.15	18.64 ± 1.59
n-3	12.68 ± 5.48	16.08 ± 3.07	20.55 ± 12.98	15.28 ± 4.01
n-3 PUFA	12.30 ± 5.50	15.55 ± 2.96	20.15 ± 12.90	14.77 ± 4.09
n-6 PUFA	17.78 ± 7.89	19.61 ± 1.41	19.30 ± 1.13	18.64 ± 1.59
n-3 LC-PUFA	11.07 ± 5.14	14.39 ± 2.90	18.92 ± 12.72	13.66 ± 3.90
EPA + DHA	10.00 ± 4.74	12.90 ± 2.67	17.13 ± 11.53	12.30 ± 3.55
EPA/DHA	0.65 ± 0.06	0.64 ± 0.06	0.70 ± 0.19	0.81 ± 0.06
EPA/ARA	3.09 ± 0.54	3.12 ± 0.09	3.71 ± 0.59	3.62 ± 0.33

One-way ANOVA: Diet; Data expressed in mean ± SD (n = 3). KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

Table 7
Blood omega-3 index (% total fatty acids) of gilthead sea bream (*Sparus aurata*) fed the experimental diets over the stress challenge.

Diet	**Time**			**Two-Way ANOVA**		
	0 h	**24 h**	**7 days**	**Diet**	**Time**	**Diet x Time**
Control	18.76 ± 3.40^{1}	22.56 ± 3.76^{2}	19.92 ± 4.59^{1a}			
KM3	17.88 ± 2.52^{1}	20.46 ± 4.18^{12}	22.14 ± 3.93^{2ab}	$p < 0.05$	$p < 0.05$	*n.s*
KM5	19.15 ± 2.97^{1}	22.31 ± 3.85^{2}	23.85 ± 2.67^{2b}	KM3 < KM7	0 h < 24 h, 7d	
KM7	19.52 ± 3.48^{1}	23.72 ± 3.26^{2}	23.12 ± 2.56^{2ab}			

Different numbers denote significant differences ($p < 0.05$) between experimental sampling points (Two-way ANOVA: Diet x Time x Diet*Time; Tukey post-hoc test); Different letters denote significant differences ($p < 0.05$) between experimental diets (Two-way ANOVA: Diet x Time x Diet*Time; Tukey post-hoc test); Data expressed in mean ± SD ($n = 15$ x dietary treatment x sampling time). KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

Meanwhile, when fish were subjected to the stress challenge, a significant interaction between the dietary treatment fed and time was observed affecting fish blood O3I ($p < 0.05$; Table 7). At 24 h after the stress challenge, all dietary treatments except KM3 presented a significant increase in OI3 ($p < 0.05$; Tables 7 and 8). At 7d post-stress challenge, fish fed the control diet presented a significant reduction in O3I down to the basal levels ($t = 0$ h post-stress challenge) ($p < 0.05$; Table 7). On the contrary, fish fed with KM5 and KM7 diets kept the higher O3I until the end of the stress challenge, with O3I significantly higher in fish fed KM5 than in those fed the control diet (Table 7). Regarding the increase in O3I from the basal sampling point (t = 0 h pre-stress challenge) to the different post-stress challenge points ($t = 24$ h

Table 8
Blood omega-3 index increase (in % total fatty acids) of gilthead sea bream (*Sparus aurata*) fed the experimental diets over the stress challenge.

Diet	**Δ Omega 3 index (0 h to 24 h)**	**Δ Omega 3 index (0 h to 7d)**
Control	5.19 ± 2.9	3.75 ± 2.1
KM3	2.73 ± 1.3	3.85 ± 1.6
KM5	2.73 ± 1.7	5.7 ± 3
KM7	6.15 ± 3.93	5.02 ± 3.5

One-way ANOVA: Diet; No significant differences were observed. Data expressed in mean ± SD (n = 15 x dietary treatment x sampling time). KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

and 7d), no significant differences were found between dietary treatments (Table 8).

At $t = 0$ h, KM5, and KM7 were the most influenced diets by all the omega-3 related variables, presenting similar values of these fatty acids general classes according to the PCA. Meanwhile, the Control diet was mostly influenced by the total saturated fatty acids presence (Fig. 1).

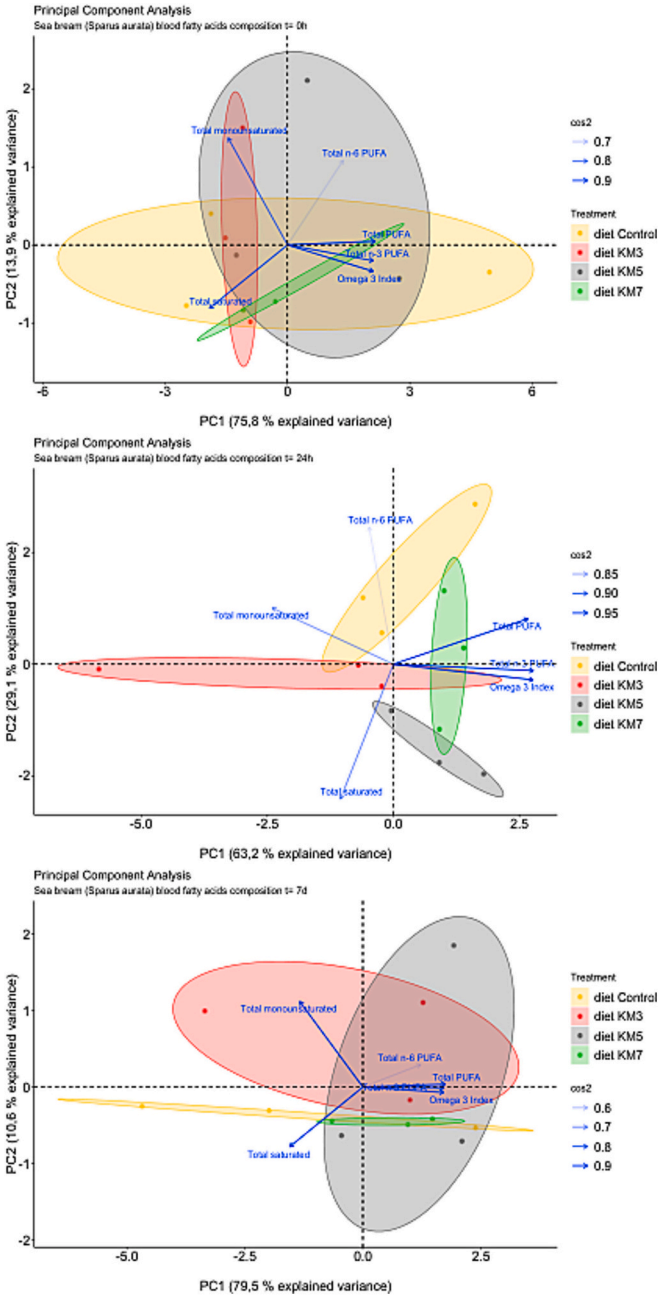


Fig. 1. Principal component analysis of red blood cells fatty acid composition at the three different points of the stress challenge ($t = 0$, $t = 24$ h, $t = 7$ days). The percentage of total fatty acids is represented as cos*2* function by an intensity scale, and confidence ellipses are generated around mean group points. The points correspond to the replicates (tanks) and are coloured according to the diet fed (KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %). The fatty acids are plotted in the PCA as arrows indicating the level of each fatty acid contribution to the formation of PC1 and PC2. The stronger the correlation of a fatty acid to PC1 or PC2, the closer its arrowhead to the circle plotted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At t = 24 h, KM7 was the most influenced diet by omega-3-related variables. At this sampling point, the Control diet O3I won importance on its distribution in the plot, meanwhile, the saturated fatty acids contents lost their influence (Fig. 1).

At $t = 7$d, the experimental diets grouped around the omega-3 indicators pointing to the importance of this variable on fish blood fatty acids profile. On the contrary, similarly to $t = 0$ h, the omega-3 indicators lost their influence on Control diet fish fatty acid contents, indicating a reduction of these groups on fish blood (Fig. 1).

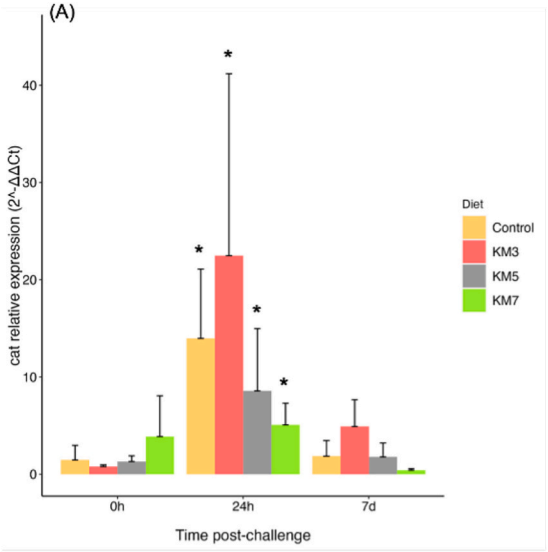
3.4. Relative expression of antioxidant defence system-related genes

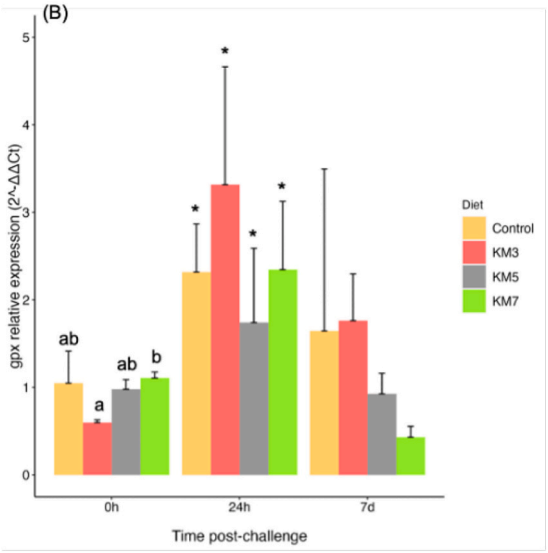
The relative gene expression of *cat*, *gpx,* and *sod* of head kidney in gilthead sea bream was significantly increased at 24 h post-stress, compared with basal levels (t = 0) and irrespective of the dietary treatment ($p < 0.05$; Fig. 2; Table S2). After 7 days of crowding stress, gene expression levels returned to basal levels in fish fed all the diets. Overall, differences between diets were only observed in *sod* expression levels, with fish fed KM3 showing an up-regulation compared to fish fed KM7 diet, while fish fed Control and KM5 diets presented intermediate expression levels (Fig. 2; Table S2). Indeed, fish fed with KM7 diet showed a tendency to present higher basal expression levels for *sod* as well as for *cat* compared with those fed Control diet, albeit not significant. Despite the generally similar pattern of the three antioxidant gene expressions across time of crowding in fish fed the four dietary treatments, there was a slight non-significant tendency for maintaining lower expression levels during the stress challenge, in fish fed KM5 and/or KM7 compared with the other diets, with those fish showing a reduced rate of up-regulation of the genes, resulting in significantly lower expression levels 7 days after crowding stress, although non significantly different from fish fed Control diet (Fig. 2).

In addition, correlations (Pearson's correlations) between the expression levels of antioxidant genes and fatty acid profile of fish RBC were noted. For instance, the expression of the *cat* and *sod* at 24 h of stress were inversely correlated with RBC omega-3 index ($r = -0.931$, $p = 0.069$ and $r = -0.961$, $p = 0.039$, respectively) as well as total n-3 PUFA ($r = -0.908$, $p = 0.092$ and $r = -0.939$, $p = 0.061$ respectively), EPA ($r = -0.899$, $p = 0.101$ for *cat*), or DHA ($r = -0.876$, $p = 0.124$ for *sod*).

4. Discussion and conclusions

KM is rich in phospholipids, n-3 LC-PUFA as well as several bioactive compounds that might play a role in improving the antioxidant system of fish under conditions that may favour the increase of oxidative stress (Köhler et al., 2015). All these characteristics could position KM as a good candidate in aquafeeds, especially to marine species not able to synthetize LC-PUFA *de novo* (Izquierdo, 2005; Tocher, 2015) and for which the replacement of marine-derived raw materials is not devoid of negative consequences on fish performance, health and/or resistance to stressful events (Montero and Izquierdo, 2010). In the present study, it was possible to decrease the dietary FM, included at 15 % of the diet in Control diet by the dietary supplementation of 7 % of the diet with KM (meaning a 47 % of replacement; 8 % FM in 7 % KM in comparison to 15 % FM in control diet), without compromising fish growth and feed conversion. These effects are in line to what was observed in a previous study with European sea bass (Torrecillas et al., 2021). Interestingly, although not statistically different, the higher levels of KM in the diet (5 and 7 %) showed a tendency to further improve FCR up to 6.5 % compared with a Control diet, accordingly with the same tendency to enhance PER (5 % enhancement) and LER (11 % enhancement). These results suggest that KM could optimize the utilization of the dietary nutrients boosting feed efficiency. Similarly to the results of the present study, in previous trials, gilthead sea bream (Saleh et al., 2018) and European sea bass juveniles (Torrecillas et al., 2021) fed KM-supplemented diets up to 9 % and 7 %, respectively also displayed an





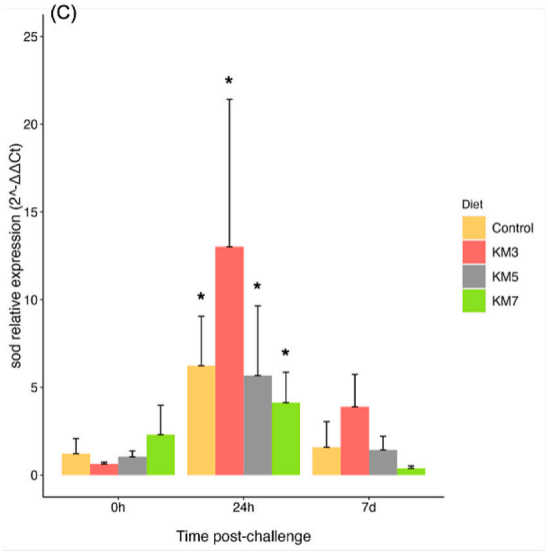


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Fig. 2. Relative expression of antioxidant genes in head kidney, including catalase (A), glutathione peroxidase (B) and super oxide dismutase (C) in gilthead sea bream fed the experimental diets at the three different points of the stress challenge (t = 0, t = 24 h, t = 7 days). Relative expression of genes, expressed in $2^{-\Delta\Delta ct}$, are pondered based on the expression values of Control diet on t = 0 to detect basal differences among fish fed the experimental diets, and pondered based on t = 0 expression values of its respective treatment on t = 24 h and t = 7d to detect differences in the response pattern among fish fed the experimental diets. *denotes significant differences ($P < 0.05$) of t = 24 h compared with t = 0 and t = 7d inside each dietary treatment. Different lowercase letters denote significant differences ($p < 0.05$) among dietary treatments between diets inside a time point. KM3- krill meal at 3 %; KM5- krill meal at 5 %; KM7- krill meal at 7 %.

improved FCR and PER. Authors related these results to the favourable availability of nutrients from krill, such as proteins, vitamins, minerals, phospholipids (PL), and astaxanthin, as well as the high bioactivity of the n-3 LC-PUFA contained in KM. Although the DHA contents were similar among the diets (only up to 5 % increase in KM diets), the inclusion of KM led to a 13–17 % increase in the dietary EPA contents, which could partially explain the better LER observed in fish fed KM diets. Indeed, EPA was shown to regulate lipid transport and metabolism in fish by increasing the activity of lipoprotein lipase (Yu et al., 2022), which hydrolyse triglycerides in circulating lipoproteins, as well as decreasing that of fatty acid synthase (Alvarez et al., 2000; Kolditz et al., 2008; Yu et al., 2022), an enzyme responsible for *de novo* lipid synthesis. Other studies also related higher EPA contents with better lipid efficiency ratios in gilthead sea bream (Carvalho et al., 2020) as well as in meagre (*Argyrosomus regius*) (Carvalho et al., 2022). Furthermore, a recent study also found that when Atlantic Salmon (*Salmo salar*) is fed with KM-supplemented diets, lipid transport was improved and an elevation of free fatty acids (FFA) in skeletal muscle could be detected, indicating a possible influence of KM on the preservation of n-3 and n-6 fatty acid series (Mørkøre et al. in 2020). In the present study, KM in the diet well-maintained fatty acid profiles of fish flesh, both in neutral and polar lipid fractions, although a slight tendency (not statistical) was observed for increasing DHA and EPA contents in flesh from fish fed the highest KM diets (KM7), which is of special interest to maintain flesh nutritional quality.

In addition to their role in fish metabolism, growth and nutrient efficiencies, n-3 LC-PUFA, like EPA and DHA, play also important roles in fish health, including the modulation of the stress response (Montero and Izquierdo, 2010). In particular, oxidative stress can be caused by lipid peroxidation, contaminants, DNA damage or by regulation of intracellular signal transduction (Yoshikawa and Naito, 2002). This also includes stressors like crowding or persecution, which are known to increase fish metabolic rate leading to increased oxygen consumption and triggering oxidative stress-related processes as a response to cellular reactive oxygen species (ROS) production (Yang et al., 2019). Furthermore, oxidative stress disrupts the structure of RBC membrane, affecting the lipids asymmetry in membranes and leading to a decrease in the flexibility of RBC, with implications on the health of individuals (Minetti et al., 2007; McBurney et al., 2022a). In this sense, fish possess an antioxidant defence line against oxidative damage, in which SOD, GPX and CAT are the most important enzymes involved and an up-regulation or higher activities of those enzymes during stressful events could be an indicator of increased oxidative damage (Ogueji et al., 2020). As expected, in the present study, an up-regulation of the genes coding for these three antioxidant enzymes (CAT, GPX and SOD) was observed 24 h post crowding stress in fish fed all the diets and no differences were observed among dietary treatments, recovering basal levels 7 days post-crowding. However, fish fed the highest levels of KM (KM5 and KM7) showed a tendency, albeit not significant, to present a lower increase compared with those fish fed Control or KM3 diets. This suggests a more discrete alteration of the antioxidant endogenous system of fish and a more attenuated response to oxidative stress which will be beneficial to

the fish health. This was also consistent with the tendency to present lower relative gene expression at the end of the stress panel in fish fed KM5 and KM7 diets, albeit non-significantly different from those fed Control diet. In this regard, although the antioxidant compounds of KM were not analysed in the present study, an attenuated response might be, at least partially, a reflection of the antioxidant potential of some bioactive compounds present in KM that confer antioxidant protection from the increase in ROS to fish under stressful conditions.

In addition, O3I measures the percentage of EPA and DHA in RBC membranes and it has been used as a reliable biomarker of omega-3 fatty acid intake and status in the body in humans (Harris and Von Schacky, 2004). Omega-3 fatty acids have been shown to mostly influence RBC cell membranes fluidity, integrity and flexibility (Cartwright et al., 1985; Bach et al., 1989; McBurney et al., 2022a). Furthermore, EPA and DHA possess also anti-inflammatory properties, through the production of specialized pro-resolving lipid mediators (SPMs), such as resolvins, protectins and maresins, which can help mitigate oxidative stress by reducing inflammation (McBurney et al., 2022a). For instance, in humans, EPA and DHA-derived SPMs reduced neutrophil infiltration and consequently the production of ROS, thus lowering oxidative stress status (McBurney et al., 2022b). Low O3I levels were associated with non-regular distribution of RBC sizes in humans and consequently to lower health of individuals (McBurney et al., 2022a). In the present study, there were also notable changes in FA profile of RBC following crowding stress. Specifically, there was an increase in the contents of essential fatty acids like EPA, after 24 h and 7 days of crowding stress compared to basal levels, whereas a contrary trend was observed for MUFA. These changes in FA composition of RBC during the crowding stress could be related to a shift in metabolic processes or dietary intake during crowding stress, possibly towards increased utilization or depletion of MUFA-rich lipid reserves for energy production (lipolysis) while conserving most bioactive FA with functional properties for membrane fluidity like n-3 LC-PUFA (Izquierdo, 2005), as observed in previous studies (Bell et al., 2001; Borges et al., 2014; Carvalho et al., 2019). Furthermore, O3I, total n-3 PUFA and EPA in RBC were particularly increased in fish fed the highest KM diets (KM5 and KM7) in comparison to the control group after 7 days of crowding stress, suggesting that KM can modulate RBC fatty acid composition of gilthead sea bream and may favour the accumulation of n-3 PUFA in RBC under stressful conditions. Interestingly, *mRNA* levels of the three antioxidant enzymes were further negatively correlated with O3I, total n-3 PUFA and EPA. Consequently, these results suggest that KM rich in n-3 LC-PUFA, and, probably added to the presence of other antioxidants present in KM, could potentially mitigate the oxidative stress status of fish. The determination of antioxidant coumpounds in the diets containing KM at these levels would have been interesting to corroborate this relationship.

In agreement with the present results, high dietary n-3 LC-PUFA also generated lesser alterations of oxidative stress-related genes in meagre (*Argyrosomus regius*) in response to a prolonged crowding stress similar to that of our study, and that was associated with a reduced cortisol level in fish plasma (Carvalho et al., 2019). In gilthead sea bream larvae, krill phospholipids also reduced the expression of *cat*, *gpx* and *sod* genes associated with a decrease in TBARs of whole-larvae and thus decreasing peroxidation risk (Saleh et al., 2015). Further analyses on oxidative and stress metabolites, such as malondialdehyde or cortisol, are necessary to corroborate the lower oxidative status as well as the reduced stress levels of fish fed KM diets of the present study. However, these results highlight the importance of n-3 LC-PUFA in modulating fish antioxidant defence system before and during stressful conditions, by either directly modifying RBC membranes fatty acid composition and the production of ROS or as previously suggested, generating a higher basal pro-oxidant environment that can help fish to cope better with a posterior acute increase in oxidative stress (Pérez-Sánchez et al., 2013). Indeed, it is noteworthy that fish fed KM7 showed a tendency to present higher basal relative expression for *cat* and *sod* but not for *gpx*, possibly suggesting this higher pro-oxidative environment in fish fed high KM diets, that might favour the basal antioxidant system of fish and help the animal coping with subsequent stress.

In conclusion, the inclusion of up to 7 % of KM in low fish meal diets for sea bream could lead to an increase of up to 12 % in the dietary EPA + DHA. This inclusion levels can efficiently replace FM, without negatively affecting growth performance or feed utilization in gilthead sea bream juveniles. KM also contributed to the mitigation of oxidative stress, particularly under crowding conditions by increasing omega-3 index in red blood cells during stress period, potentially modulating membrane integrity and fluidity. Finally, KM modulated antioxidant defence mechanisms under crowding stress, potentially reducing reactive oxygen species (ROS) production by presenting a more attenuated gene expression of oxidative markers (*cat, sod and gpx*) response to stress. These changes might ultimately result in a more effective strategy of fish to cope with stress related processes. Therefore, the results of the present study point to a possible role of dietary KM as antioxidant modulator within a pro-oxidative environment, and thus as a good functional marine ingredient in aquafeeds, aiming to contributing to the long-term sustainability of the aquaculture sector. However, while KM benefits fish, it is more expensive than traditional ingredients like FM and soybean meal. Therefore, consider its cost-effectiveness is important and deserves further research for weighing KM's nutritional value and benefits in fish feeds during economic analysis.

Funding

The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 871108 (AQUAEXCEL3.0). The work was performed at ULPGC-FITU (Feed Ingredients and Additives Testing Unit) research infrastructure, under grant agreement PID is 27403 (TNA programme). This output reflects only the author's view, and the European Union cannot be held responsible for any use that may be made of the information contained therein. Silvia Torrecillas is financed by a Ramón y Cajal fellowship (RYC2021-031414-I) funded by MICIU/AEI/10.13039/501100011033 and, as appropriate, by "ESF Investing in your future", by "ESF+" or by "European Union NextGenerationEU/PRTR.

Author contributions

Marta Carvalho: Investigation; Data Curation; Writing - Original Draft; **Daniel Montero**: Conceptualization; Writing - Review & Editing; Supervision; Funding acquisition; **Mónica Betancor**: Methodology; Writing - Review & Editing **Kiranpreet Kaur**: Conceptualization; Funding acquisition; Writing - Review & Editing; **Antonio Serradell**: Formal analysis; Writing - Original Draft; **Marisol Izquierdo**: Writing - Review & Editing; Rafael Ginés: conceptualization, funding adquisition; Review & Editing; **Virginie Claeyssens**: Funding acquisition; **Silvia Torrecillas**: Conceptualization; Writing - Review & Editing; Supervision; Funding acquisition.

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Marta Carvalho: Writing – original draft, Investigation, Data curation. **Daniel Montero:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Mónica Betancor:** Writing – review & editing, Methodology. **Kiranpreet Kaur:** Writing – review & editing. **Antonio Serradell:** Data curation. **Marisol Izquierdo:** Writing – review & editing. **Rafael Ginés:** Writing – review & editing. **Virginie Claeyssens:** Funding acquisition. **Silvia Torrecillas:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Kiranpreet Kaur and Virginie Claeyssens reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741957.

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