fishes

MDPI

Article

Oxidative Status of the Pyloric Caeca and Proximal Intestine in Gilthead Sea Bream Fed Diets Including Different Vegetable Oil Blends from Palm, Rapeseed and Linseed

Irene García-Meilán [1,*], Ramon Fontanillas [2], Joaquim Gutiérrez [1], Encarnación Capilla [1], Isabel Navarro [1] and Ángeles Gallardo [1]

[1] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain; jgutierrez@ub.edu (J.G.); ecapilla@ub.edu (E.C.); mnavarro@ub.edu (I.N.); mgallardo@ub.edu (Á.G.)

[2] Skretting Aquaculture Research Centre (ARC), Sjøhagen 3, 4016 Stavanger, Norway; ramon.fontanillas@skretting.com

* Correspondence: irene.garcia@ub.edu; Tel.: +34-93-403-96-35

Abstract: Nowadays, including vegetable ingredients in fish diets without growth effects is common; however, their intestinal oxidative status under these conditions is less known. Five isonitrogenous and isolipidic diets with 75% vegetable oil (VO) inclusion were formulated for juvenile gilthead sea bream (*Sparus aurata*). As VO, one diet contained palm oil (diet P), another rapeseed oil (diet R), and the other three included linseed oil (L) combined with the above-mentioned VOs (named PL, RL and RPL diets). After 18 weeks, pyloric caeca (PC) and proximal intestine (PI) were analyzed for oxidative stress biomarkers, lipid peroxidation (LPO), and gene expression. Dietary linseed oil diminished the superoxide dismutase activity in both intestinal regions, catalase in PC and glutathione reductase in PI; rapeseed oil reduced the glutathione peroxidase (GPx) and glutathione-S-transferase activities in PC, and palm oil upregulated GPx activity in PI. The PL diet triggered LPO levels in the PI, and RPL-fed fish showed the highest levels of LPO in the PC due to lower antioxidant activities, while RL-fed fish presented the best oxidative status. The results suggest that the dietary amount of n-6 and the unsaturated/saturated fatty acids ratio are factors to be considered in aquafeed formulation, including VOs, to improve the intestinal oxidative status in fish.

Keywords: lipid peroxidation; superoxide dismutase; catalase; glutathione peroxidase; glutathione reductase; glutathione-S-transferase

Key Contribution: The study demonstrates that feeding gilthead sea bream a diet including a blend of linseed and rapeseed oils improves their intestinal oxidative status due to the regulation of antioxidant enzyme activities. The ratio of unsaturated/saturated fatty acids and the amount of n-6 in the diet are key factors in aquafeed formulation, due to their effect on the intestinal oxidative status.

check for updates

Citation: García-Meilán, I.; Fontanillas, R.; Gutiérrez, J.; Capilla, E.; Navarro, I.; Gallardo, Á. Oxidative Status of the Pyloric Caeca and Proximal Intestine in Gilthead Sea Bream Fed Diets Including Different Vegetable Oil Blends from Palm, Rapeseed and Linseed. *Fishes* **2024**, *9*, 228. https://doi.org/10.3390/fishes9060228

Academic Editor: Marina Paolucci

Received: 6 May 2024
Revised: 8 June 2024
Accepted: 11 June 2024
Published: 14 June 2024

1. Introduction

Cellular metabolism and its environmental interactions led to a natural formation of reactive oxygen species (ROS), but an imbalance in ROS production and removal negatively affects fish tissues and welfare [1–4]. ROS levels promote DNA damage, enzyme inactivation, protein oxidation, and lipid peroxidation (LPO), with the latter considered the hallmark of oxidative stress [5]. At the intestinal level, LPO can compromise the physical barrier function by negatively affecting membrane structure, fluidity, and permeability [6]. Consequently, digestive and absorptive processes could be directly affected, specifically membrane digestive enzymes, amino acids and glucose transporters, and diffusion rates [7]. Cells have developed antioxidant mechanisms, both enzymatic and non-enzymatic ones, to fight radicals and maintain physiological status [5]. Among enzymatic antioxidants, which

detoxify ROS, organisms developed a primary line of defense constituted by the superoxide dismutase (SOD), which has two isoforms, SOD1-cytoplasmatic and SOD2-mitochondrial. SOD generates hydrogen peroxide, which, in turn, is removed by catalase (CAT) or glutathione peroxidase (GPx), and that also presents different isoforms, among which are GPx1 and GPx4. The xenobiotics and glutathione (GSH) conjugation were catalyzed by glutathione S transferase (GST) [5]. GPx and GST are coupled with glutathione reductase (GR), which recycles oxidized glutathione (GSSG). In addition, vitamins, carotenes, and GSH are some of the low molecular weight non-enzymatic antioxidants that confer organisms the capacity to quench ROS directly [5,8].

Nowadays, for the sustainability of aquaculture, fish oil (FO) replacement in aquafeeds with alternative ingredients is needed. Furthermore, FO substitution would reduce production costs, since FO is used in nutraceutical and agricultural industries, which makes its price more expensive [9]. However, this must be carried out without forgetting the quality of the final product, its nutritional properties, and the welfare of the fish. Moreover, in terms of oxidative stress, the high contents of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), essentials for the development and growth of fish [10–13], make fatty acids of FO prone to be highly oxidized. Despite this, some studies have demonstrated that appropriate levels of n-3 long chain-polyunsaturated fatty acids (LC-PUFA) could improve antioxidant capacities against oxidative stress in rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) [2,14,15] and would also be necessary to meet the requirements, especially for marine species, like gilthead sea bream (*Sparus aurata*) [16–18].

Vegetable oils (VOs) are used to replace FO, but unlike FO are deficient in LC-PUFA (EPA and DHA, among others), and to achieve high levels of inclusion it is therefore necessary to blend different VOs to reduce or avoid the undesirable effects [19]. Among the VOs most used in oil blending for fish diet formulation were palm oil, rapeseed oil, and linseed oil. Palm oil is rich in saturated (SFA) and monounsaturated (MUFA) fatty acids (50 and 40% of total fatty acids, respectively) [10]. Rapeseed oil is rich in MUFA (57%), mainly oleic [10]. Finally, linseed oil is one of the vegetable sources richer in n-3 fatty acids with 75% of PUFA, mainly linoleic acid (ALA). Also, it presents a moderate content of MUFA (16%) and low SFA content (9%), but its use in fish feeds is limited because of its high market price [10,20].

Considering that MUFA or SFA are less susceptible to peroxidation than PUFA [21], VO inclusion modifies fish lipid composition at whole levels and affects membrane composition, and therefore its structure, integrity, and functions are able to affect LPO formation. However, studies on oxidative status at intestinal level are scarce despite the multiple external and internal factors that trigger intestinal oxidative stress [1,6,22–27]. To our knowledge, existing studies on the effect of dietary lipids on oxidative status at intestinal level in gilthead sea bream, an important species for Mediterranean aquaculture, are limited [1,4,25,28]. Most of the above-mentioned studies analyzed the oxidative status only in proximal intestine (PI). Previous studies in our group showed that fish pyloric caeca (PC) presented different digestion and absorption dynamics [29–34]. Moreover, García-Meilán et al. [4] reported that in PC, the antioxidant activities differ from those found in PI, and this difference could be related to its unlikely functions. The PC, where pancreatic enzymes and bile are released throught the pancreatic and bile ducts [35–37], are considered an adaptation to increase gut surface area [38]. Furthermore, PC can also play a role in fermentation [39], perform relatively short food-retention [40], and play a role in lipid absorption [41,42]. Instead, the main function of PI is the completion of the digestive processes and nutrient absorption with the help of peristaltic movements that contribute to the mixing of chyme with pancreatic juices and bile. The final steps of digestion take place through the brush border membrane enzymes (i.e., peptidases, saccharidases, etc.), and then nutrients are absorbed by specific transporters [43,44]. In addition to this and related to dietary VO inclusion, in García-Meilán et al. [4]'s study, it was demonstrated that gilthead sea bream feeding, with a diet where 75% of the oil came from a blend of soybean

and linseed oils, negatively affects the intestinal oxidative status, triggering LPO, which cannot be counteracted by antioxidant activities. Instead, less intestinal LPO is produced when rapeseed or palm oils were combined with the blend of soybean and linseed oils. Among these diets, the one containing palm oil allows intestinal lipid peroxidation levels to remain lower, leading sea bream to have better intestinal health.

The aim of the present research, conducted on gilthead sea bream fed diets containing different palm, rapeseed, and linseed oils blends, was to test whether PC and PI exhibit a differential antioxidant response and whether the inclusion of the different VO blends affects their antioxidant status and LPO.

2. Materials and Methods

2.1. Experimental Diets

Five experimental diets, with 46% protein, 22% lipid, and where 75% of the oil included came from VO and the remaining 25% were FO, were formulated and produced by Skretting ARC (Norway). Diets contained fish meal (15%), soya concentrate (30%), corn gluten (15%), wheat (7%), fava beans (6%), wheat gluten (3.8%), and sunflower meal (3%); thus, 88% of the protein was plant protein. Two diets contained a single VO, either palm oil (P) or rapeseed oil (R). The other three diets contained a blend of linseed oil with palm (PL) or rapeseed oil (RL) or a combination of the three (RPL); these three diets showed intermediate values for the main fatty acids' classes, compared to P and R, except for n-3, which levels were higher to achieve the optimum levels of n-3 PUFA, and similar between PL, RL and RPL diets (Table 1A,B).

2.2. Fish, Feeding Trial and Sampling

Two hundred and eighty-six gilthead sea bream (81.8 ± 0.19 g) were randomly distributed in a semi-recirculating saltwater system of 13 fiberglass tanks (500 L; 22 fish per tank) and acclimatized for 11 days at the Institut de Recerca i Tecnologia Agroalimentàries facilities (IRTA, La Ràpita, Spain). During the experimental trial (October–February) the photoperiod was natural (11:24 to 10:29 h of daylight) and the temperature remained at 21.9 ± 0.85 °C. PL, RL and RPL sea bream grew in triplicate tanks and P and R groups in duplicate tanks. Fish were fed ad libitum the corresponding diet twice daily (at 8 a.m. and 2 p.m.) during the 18-week trial.

At the end of the growth trial and 24 h post-feeding, three fish per tank were anesthetized (MS-222, Sigma, Madrid, Spain), measured, weighed, and sacrificed. Fish growth did not show significant differences between groups as previously reported [19]. Independent samples were collected for both PC and PI to analyze oxidative stress markers and antioxidant enzymes expression. They were rapidly frozen in liquid nitrogen and stored at −80 °C. At the sampling time used, neither PC nor PI had intestinal content.

All procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union's assigned principles and legislation (permit number DAAM 8982).

2.3. Oxidative Stress Markers Analysis

PC and PI samples were thawed on ice, weighed individually, and a buffer solution was added (Tris-HCl, 50 mM, pH 7.5). Samples were then homogenized using rapid vibration (6500 rpm; 3 × 20 s with three breaks of 20 s; 4 °C) in a Precellys Evolution® Homogenizer combined with Cryolys® as a cooling system (Bertin Technologies, Montigny-le-Bretonneux, France). Next, homogenates were centrifuged for 15 min (2400 rpm; 4 °C; Eppendorf, 5418R), and supernatants were stored at −80 °C until the oxidative stress markers analysis.

Table 1. (A). Vegetable oil and vitamin/mineral (Vit/Min) premix composition of the experimental diets. (B). Dietary fatty acid profile of the five different experimental diets.

(**A**)					
	Diets				
Ingredients (%)	**P**	**R**	**PL**	**RL**	**RPL**
Palm oil	13.88	-	9.87	-	5.03
Linseed oil	-	-	3.94	2.02	3.00
Rapeseed oil	-	13.58	-	11.57	5.67
Vit/Min premix	1.53	1.83	1.60	1.82	1.71
(**B**)					
	Diets				
Fatty acid (%)	**P**	**R**	**PL**	**RL**	**RPL**
C14:0	2.63	2.20	2.47	2.21	2.34
C16:0	30.81	9.32	24.83	9.36	17.41
C16:1n-7	2.31	2.50	2.30	2.48	2.36
C16:2n-6	0.27	0.31	0.27	0.30	0.28
C18:0	3.73	2.13	3.82	2.47	3.19
C18:1n-9	30.57	38.67	26.79	36.19	31.73
C18:1n-7	1.43	2.84	1.41	2.67	2.04
C18:2n-6 LA	10.89	17.47	12.08	16.52	14.12
C18:3n-3 ALA	0.98	6.52	9.75	10.31	10.07
C18:4n-3	0.70	0.75	0.72	0.74	0.73
C20:1 sum. isomers	1.72	2.59	1.72	2.47	2.03
C20:4n-6 ARA	0.24	0.25	0.24	0.23	0.21
C20:4n-3	0.23	0.24	0.24	0.24	0.24
C20:5n-3 EPA	3.04	3.27	3.07	3.24	3.12
C22:1 sum. isomers	2.10	2.40	2.07	2.32	2.12
C22:5n-3	0.45	0.48	0.46	0.52	0.43
C22:6n-3 DHA	2.89	3.07	2.95	2.98	2.90
C24:1n-9	0.24	0.34	0.24	0.33	0.29
SFA not listed	0.81	1.08	0.76	1.01	0.90
Monoenes not listed	0.11	0.13	0.11	0.11	0.11
n-6 FA not listed	0.22	0.29	0.27	0.29	0.24
n-3 FA not listed	0.16	0.20	0.16	0.20	0.18
Others	0.36	0.37	0.33	0.38	0.37
Sum. SFA	37.98	14.73	31.88	15.05	23.84
Sum. MUFA	38.48	49.47	34.64	46.57	40.68
Sum. n-6 FA	11.62	18.32	12.86	17.34	14.85
Sum. n-3 FA	8.45	14.53	17.35	18.23	17.67
UFA/SFA	1.54	5.59	2.03	5.46	3.07
MUFA/PUFA	1.92	1.51	1.15	1.31	1.25
n-6/n-3	1.38	1.26	0.74	0.95	0.84
Unknown	3.10	2.60	3.0	2.40	2.60

LA: Linoleic acid; ALA: Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; FA: Fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; MUFA/PUFA: Monounsaturated fatty acids/Polyunsaturated fatty acids; UFA/SFA: Unsaturated fatty acids/Saturated fatty acids.

All enzymatic analyses were determined spectrophotometrically using a Tecan M200 spectrophotometer (Tecan Trading AG, Männedorf, Switzerland) at 25 ± 0.5 °C according to García-Meilán et al. [4]. Briefly, total SOD (EC 1.15.1.1) activity was determined at 550 nm according to Mccord and Fridovich [45] with some modifications. The method measured the rate of inhibition, by SOD, of the reduction in cytochrome C by free superoxide radicals generated by the enzymatic system xanthine–xanthine oxidase. CAT (EC 1.11.1.6) activity was determined by measuring the disappearance of H_2O_2 at 240 nm according to the method described by Aebi [46] with some modifications. GPx (EC 1.11.1.9) activity was measured according to Bell et al. [47] with some modifications, in a reaction that uses

hydrogen peroxide as a substrate and produces GSSG. The latter is regenerated by the GR, and NADPH oxidation rate is followed at 340 nm. GST (EC 2.5.1.18) was evaluated, with some modifications, as previously described by Habig et al. [48]. The increase in OD at 340 nm measured the formation of an adduct between GSH and the oxidant agent 1-chloro-2,4-dinitrobenzene (CDNB). GR (EC 1.6.4.2) activity was determined as previously described by Carlberg and Mannervik [49] with some modifications, measuring the decrease in absorbance at 340 nm produced by the NADPH oxidation used by GR to reduce GSSG. The enzymatic activities are reported as U per mg of protein. One unit of SOD activity is defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate, and one unit of CAT, GPx, GR and GST are defined as the amount of enzyme required to transform 1 μmol of the substrate per minute, under the assay conditions.

LPO levels were determined based on the concentration of malondialdehyde (MDA) [50]. Samples were thawed on ice and mixed with HCl 0.024 N and thiobarbituric acid 0.06 M solution at pH 7.0. After heating the mixture for 10 min at 95 °C, samples were kept in darkness and ice for 5 min, followed by the addition of cold butanol and, finally, the samples were centrifuged for 10 min at 2000 rpm at 4 °C (Eppendorf, 5418R). MDA fluorescence was recorded using a Tecan infinite 200 spectrofluorometer (Tecan Trading AG, Männedorf, Switzerland) with a 515/548 nm (excitation/emission) filter. MDA concentration was calculated using a calibration curve (range of 0–10 μm MDA) and expressed as nmol MDA per mg of protein.

The Bradford method [51] was used to determine homogenates' protein concentration using bovine serum albumin as a standard.

All coenzymes, purified enzymes, substrates, and reagents were purchased from Merck and Bio-Rad Laboratories, Inc.

2.4. RNA Extraction, cDNA Synthesis and Real-Time Quantitative-PCR (qPCR)

Total RNA extraction was performed from 30 mg PC or PI in 1 mL TRIzol® reagent solution (Applied Biosystems, Alcobendas, Spain), following the manufacturer's instructions. A Nanodrop 2000 (Thermo Scientific, Alcobendas, Spain) was used to determine RNA concentration and purity, and its integrity was checked with SYBR-Safe DNA gel stain (Life Technologies, Alcobendas, Spain) in a 1% agarose gel. Before cDNA synthesis, 1 μg of total RNA was treated with DNase I (Invitrogen, Alcobendas, Spain), to eliminate all genomic DNA, following the manufacturer's recommendations. Finally, anchored-oligo(dT)15 and random hexamer primers were used to carried out reverse transcription using the Transcriptor First Strand cDNA synthesis kit (Roche, Sant Cugat, Spain) following the manufacturer's instructions.

Gene expression (mRNA) analyses were performed by qPCR using a CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain), according to the requirements of the MIQE guidelines [52]. The antioxidant genes examined in PC and PI, whose expression was previously validated for gilthead sea bream [4], were the following: *sod1* and *sod2*, cat, *gpx1*, and *gpx4*, *gr*, and *gst*. While *β-actin*, elongation factor 1 alpha, *ef1α*, and ribosomal protein S18, *rps18* were used as reference genes. The analyses were performed in triplicate using 2.5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 2), and 1 μL of diluted cDNA for each sample in a final volume of 5 μL. The reactions comprised an initial denaturation step of 3 min at 95 °C, 40 cycles of 10 s at 95 °C, 30 s at 60 °C, followed by an amplicon dissociation analysis from 55 to 95 °C at 0.5 °C increase each 30 s. Reaction specificity, the absence of primer-dimers, and a dilution curve with a pool of samples was performed to find out suitable cDNA dilution for each gene, prior to analyses. The expression levels of each of the genes were calculated according to the Pfaffl method [53], relative to the gene expression or geometric mean expression of the most stable reference genes analyzed, using the Bio-Rad CFX Manager 3.1 software.

Table 2. Primers used for real-time quantitative PCR: sequence, annealing temperature (Ta) and GenBank accession numbers. F: forward, R: reverse.

Gene		Sequence (5′-3′)	Ta (°C)	Accession Number
β-actin	F	TCCTGCGGAATCCATGAGA	60	X89920
	R	GACGTCGCACTTCATGATGCT		
ef1α	F	CTTCAACGCTCAGGTCATCAT	60	AF184170
	R	GCACAGCGAAACGACCAAGGGGA		
rps18	F	GGGTGTTGGCAGACGTTAC	60	AM490061.1
	R	CTTCTGCCTGTTGAGGAACCA		
cat	F	TTCCCGTCCTTCCATTCACTC	60	FG264808
	R	CTCCAGAAGTCCCACACCAT		
gpx1	F	GAAGGTGGATGTGAATGGAAAAGATG	60	DQ524992
	R	CTGACGGGACTCCAAATGATGG		
gpx4	F	TGCGTCTGATAGGGTCCACTGTC	60	AM977818
	R	GTCTGCCAGTCCTCTGTCGG		
gr	F	CAAAGCGCAGTGTGATTGTGG	60	AJ937873
	R	CCACTCCGGAGTTTTGCATTTC		
gst3	F	CCAGATGATCAGTACGTGAAGACCGTC	60	JQ308828
	R	TGCTGATGTGAGGAATGTACCGTAAC		
sod1	F	CCATGGTAAGAATCATGGCGG	60	AJ937872
	R	CGTGGATCACCATGGTTCTG		
sod2	F	CCTGACCTGACCTACGACTATGG	60	JQ308832
	R	AGTGCCTCCTGATATTTCTCCTCTG		

β-actin: beta actin; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18; *cat*: catalase, *gpx1*: mitochondrial glutathione peroxidase 1; *gpx4*; cytosolic glutathione peroxidase, *gr*: glutathione reductase; *gst*: glutathione-S-transferase; *sod1*: superoxide dismutase 1 and *sod2*: superoxide dismutase 2.

2.5. Statistical Analyses

Shapiro–Wilk and Levene's tests were used to test data normality and homogeneity, respectively. Data following normality was tested by a one-way analysis of variance (ANOVA), and a post hoc Tukey's multiple range test was used to determine significant differences among means. The Kruskal–Wallis non-parametric test was used when normality failed, followed by the Mann–Whitney U test. The significance level was established at $p < 0.05$. The sums of squares were partitioned by orthogonal contrasts to analyze differences due to a determined oil inclusion. The contrasts were distributed as follows: C1, fish fed with diets including palm oil, with respect to the diets that do not contain it; C2, fish fed with diets including rapeseed oil, with respect to the diets that do not contain it; and C3, fish fed with diets including linseed oil, with respect to the diets that do not contain it. Statistical analysis was performed by SPSS software (IBM-SPSS Statistics v.25.0, SPSS Inc., Chicago, IL, USA), and GraphPad version 7.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for graphs.

3. Results

When comparing antioxidant enzyme activities from PC and PI, no differences in SOD and GPx were found, except for RPL-fed fish, which showed higher SOD activity and lower GPx in PC versus PI. By contrast, CAT, GST and GR activities presented significantly higher activities in PI for all experimental conditions (Table 3). Furthermore, antioxidant enzyme activities in all experimental groups and in both intestinal regions followed a similar pattern, with CAT being higher than GST and even 10^3 times more than GPx (Table 3).

Table 3. Antioxidant activities (U × mg prot^{-1}) 24 h post-feeding in pyloric caeca and proximal intestine in gilthead sea bream fed the five different experimental diets.

Pyloric Caeca					
Dietary Treatment	**SOD**	**CAT**	**GPx**	**GST**	**GR**
P	525.2 ± 67.3 **a**	168.0 ± 9.7 **a**	0.482 ± 0.078 **a**	1.81 ± 0.10 **a**	1.24 ± 0.07
R	516.4 ± 54.8 **a**	159.8 ± 7.3 **ab**	0.251 ± 0.037 **b**	1.43 ± 0.10 **b**	1.23 ± 0.09
PL	372.5 ± 26.6 **ab**	141.6 ± 6.7 **b**	0.376 ± 0.048 **a**	1.79 ± 0.16 **ab**	1.32 ± 0.12
RL	309.9 ± 36.0 **b**	142.0 ± 7.8 **ab**	0.276 ± 0.037 **b**	1.65 ± 0.15 **ab**	1.33 ± 0.13
RPL	327.3 ± 50.0 **b** *	148.9 ± 11.3 **ab**	0.195 ± 0.010 **c**	1.69 ± 0.06 **ab**	1.00 ± 0.10
Proximal Intestine					
Dietary Treatment	**SOD**	**CAT**	**GPx**	**GST**	**GR**
P	477.9 ± 42.8 **m**	366.6 ± 17.7 **n** *	0.372 ± 0.035 **mn**	3.39 ± 0.24 **m** *	4.49 ± 0.44 **mn** *
R	461.3 ± 50.2 **m**	352.3 ± 21.0 **no** *	0.305 ± 0.032 **n**	2.51 ± 0.14 **n** *	4.88 ± 0.49 **m** *
PL	335.9 ± 26.7 **n**	420.0 ± 25.5 **m** *	0.399 ± 0.034 **m**	3.51 ± 0.20 **m** *	3.91 ± 0.37 **mn** *
RL	411.5 ± 43.7 **mn**	416.0 ± 14.1 **m** *	0.327 ± 0.040 **mn**	3.38 ± 0.15 **m** *	3.65 ± 0.31 **n** *
RPL	135.0 ± 16.9 **o**	317.0 ± 18.1 **o** *	0.397 ± 0.039 **m** *	3.20 ± 0.13 **m** *	3.50 ± 0.48 **n** *

Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Differences between intestinal segments for the same dietary condition are shown by * ($p < 0.05$) in the segment with the highest activity. Significant differences between dietary conditions in the same intestinal segment are shown by letters in PC (a–c) and PI (m–o) ($p < 0.05$).

A radial chart has been used to better show the comparison of the antioxidant activities in the PC and PI of gilthead sea bream fed the different dietary treatments (Figure 1). For this purpose, some data were scaled up to settle them in the same chart and visualize the differences between experimental conditions. In PC, lower SOD and CAT activities were measured in fish fed diets containing linseed oil (Figure 1A, Table 3), finding significant differences between RL and RPL versus P and R groups for SOD activity and between PL and P groups for CAT activity. Also, in PC, low GPx and GST activities were found in fish fed diets that included rapeseed oil, with the RPL-fed fish having the lowest GPx activity and the R group having the lowest GST activity. Both enzymes showed significant differences between the PC activity in R and P fish, being lower in the former. No significant differences in GR activity were found in PC among all treatments.

In the PI, SOD activity was lower in gilthead sea bream fed diets containing linseed oil (Figure 1B, Table 3). Instead, CAT activity was increased significantly in the PL versus P group and in RL versus R fish, but the RPL group showed the lowest CAT activity. Higher GPx activity was also observed in fish fed diets containing palm oil; whereas the fish fed the diet R presented the lowest GPx and GST activities. Regarding GR, the lowest levels were found in gilthead sea bream fed linseed oil diets, especially in the RL and RLP groups.

Generally, antioxidant gene expression at 24 h post-ingestion was not affected by dietary treatments or intestinal region (Table 4). But *gpx1* expression was significantly lower in the PC of fish fed diets containing linseed oil, and *gpx4* expression was significantly higher in the PI of R-fed gilthead sea bream comparing with the other experimental groups.

Moreover, differences in LPO among sea bream under the different dietary treatments were found (Figure 2). Fish fed with diets that included one (P and R) or two VOs (PL and RL) showed significantly higher LPO levels in the PI than in the PC, whereas RPL fish showed similar values in both intestinal segments. In the PC, the highest LPO levels were found in the RPL group, and PL-fed fish showed a significantly higher LPO level than the RL group. In the PI, dietary linseed oil inclusion triggered LPO levels when this oil was combined with palm oil (PL versus the P group), but this was not the case when combined with rapeseed oil (i.e., in the RL). Furthermore, the RPL gilthead sea bream had the lowest levels (Figure 2).

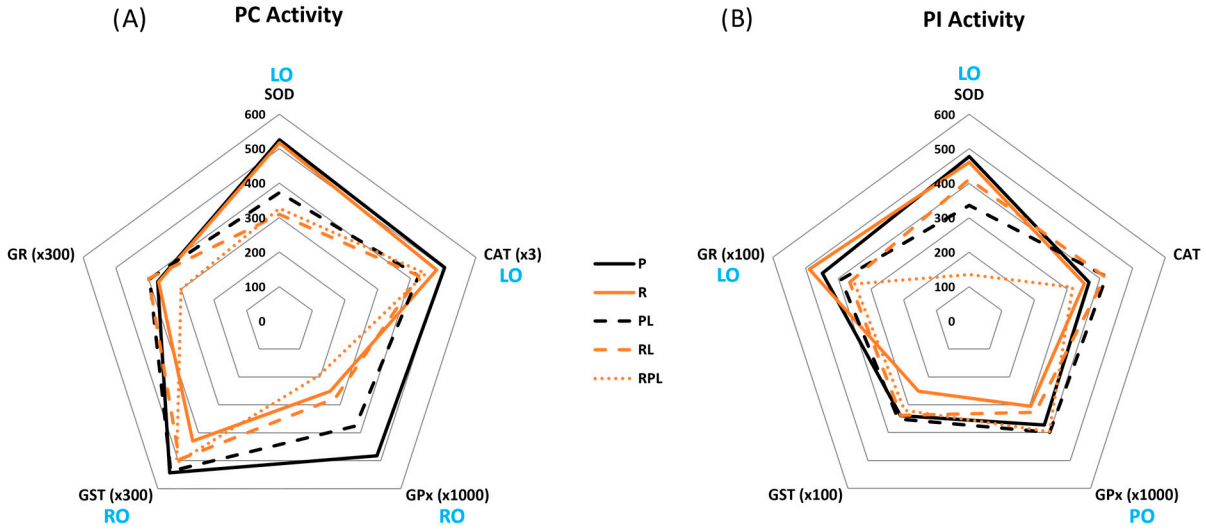

Figure 1. Radial chart showing pyloric caeca (**A**) and proximal intestine (**B**) antioxidant activities 24 h post-feeding in gilthead sea bream fed the experimental diets. Enzyme activities are expressed as U × mg prot^{-1} and values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). CAT, GPx, GST and GR were scaled up to place them in the same chart and to visualize the differences between experimental conditions. If the inclusion of a specific vegetable oil in either diet causes a significant difference ($p < 0.05$) with respect to the diets that do not contain it, the name of the oil is indicated in blue in the corners. PO: palm oil, RO: rapeseed oil, and LO: linseed oil.

Table 4. Gene expression in pyloric caeca and proximal intestine 24 h post-feeding in gilthead sea bream fed the experimental diets.

Pyloric Caeca							
Dietary Treatment	***sod1***	***sod2***	***cat***	***gpx1***	***gpx4***	***gr***	***gst***
P	1.32 ± 0.28	2.05 ± 0.53	2.19 ± 0.47	3.16 ± 0.84	1.10 ± 0.14	1.46 ± 0.44	1.36 ± 0.37
R	1.35 ± 0.29	1.49 ± 0.26	0.92 ± 0.10	1.28 ± 0.33	0.98 ± 0.26	0.60 ± 0.07	1.12 ± 0.19
PL	1.42 ± 0.23	1.73 ± 0.25	1.27 ± 0.28	1.01 ± 0.29	1.06 ± 0.18	1.06 ± 0.31	1.43 ± 0.35
RL	2.05 ± 0.49	1.89 ± 0.31	1.22 ± 0.17	1.00 ± 0.23	1.15 ± 0.17	1.56 ± 0.29	1.29 ± 0.20
RPL	1.39 ± 0.31	1.97 ± 0.39	1.20 ± 0.27	0.89 ± 0.21	0.98 ± 0.18	1.05 ± 0.32	1.31 ± 0.27
Proximal Intestine							
Dietary Treatment	***sod1***	***sod2***	***cat***	***gpx1***	***gpx4***	***gr***	***gst***
P	0.76 ± 0.13	1.35 ± 0.24	0.95 ± 0.12	1.75 ± 0.23	0.55 ± 0.07 **n**	1.92 ± 0.34	1.13 ± 0.32
R	0.83 ± 0.06	1.84 ± 0.23	0.99 ± 0.08	2.15 ± 0.23	1.07 ± 0.08 **m**	1.89 ± 0.23	1.32 ± 0.17
PL	0.83 ± 0.03	2.03 ± 0.26	1.27 ± 0.28	2.34 ± 0.49	1.35 ± 0.51 **mn**	2.00 ± 0.21	1.39 ± 0.24
RL	0.80 ± 0.13	2.02 ± 0.36	0.82 ± 0.14	1.85 ± 0.19	0.66 ± 0.09 **n**	2.11 ± 0.26	0.81 ± 0.11
RPL	0.73 ± 0.07	1.71 ± 0.17	0.81 ± 0.05	1.73 ± 0.14	0.67 ± 0.08 **n**	2.21 ± 0.26	1.02 ± 0.15

Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Significant differences between dietary conditions in the same intestinal segment are shown by letters ($p < 0.05$). *sod1*: superoxide dismutase 1; *sod2*: superoxide dismutase 2; *cat*: catalase, *gpx1*: mitochondrial glutathione peroxidase 1; *gpx4*; cytosolic glutathione peroxidase, *gr*: glutathione reductase and *gst*: glutathione-S-transferase.

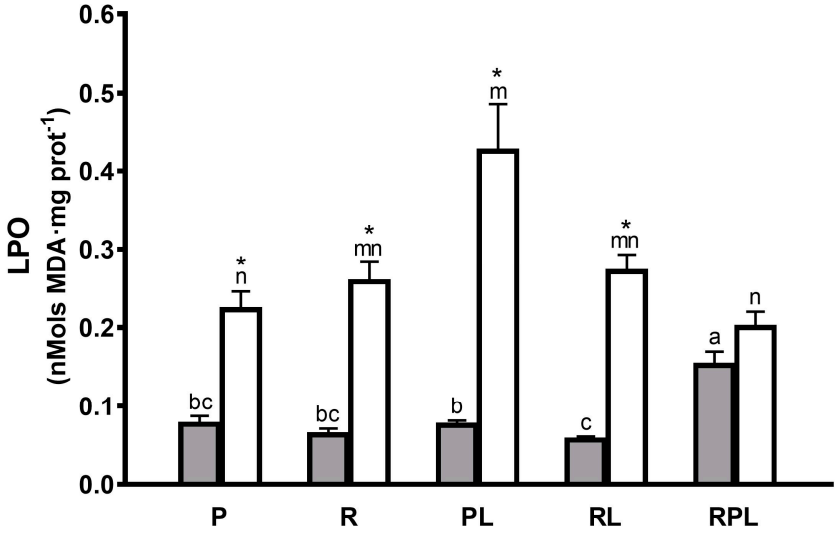

Figure 2. Lipid peroxidation (LPO) levels in pyloric caeca (grey bars) and proximal intestine (white bars) 24 h post-feeding in gilthead sea bream fed the experimental diets. Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Significant differences between dietary conditions in the same intestinal segment are shown by letters in PC (a–c) and PI (m, n). Differences between intestinal segments for the same dietary condition are shown by * ($p < 0.05$).

Table 5 shows the normalization of SOD, CAT, GPx and GST activities by LPO levels in the two intestinal segments studied. PC presented proportionally more antioxidant enzyme activities to deal with LPO than PI, except the fish fed the RPL diet, as the antioxidant/LPO ratios from the P, R, PL, and RL gilthead sea bream show. Moreover, the higher LPO in PC of the PL fish in comparison to the RL group could be related to its comparatively lower SOD/LPO, CAT/LPO and GST/LPO ratios. Regarding PI, the RPL fish showed more antioxidant activity proportionally to their LPO levels, except for SOD, and the PL versus P fish presented the lowest CAT/LPO ratio. Differentially, the RPL gilthead sea bream presented significantly higher GPx/LPO and GST/LPO ratios when compared with the other treatments.

Table 5. SOD/LPO, CAT/LPO, GPx × 1000/LPO and GST × 100/LPO ratios in pyloric caeca and proximal intestine 24 h post-feeding in gilthead sea bream fed the experimental diets.

Pyloric Caeca				
Dietary Treatment	**SOD/LPO**	**CAT/LPO**	**GPx × 1000/LPO**	**GST × 100/LPO**
P	6.48 ± 0.95 **a** *	2.49 ± 0.25 **ab** *	4.60 ± 0.49 **a** *	2.38 ± 0.28 **a** *
R	7.89 ± 1.26 **a** *	2.61 ± 0.41 **ab** *	3.84 ± 1.10 **a** *	2.58 ± 0.43 **a** *
PL	5.00 ± 0.38 **a** *	2.18 ± 0.15 **b** *	5.78 ± 0.87 **a** *	2.60 ± 0.25 **a** *
RL	5.70 ± 0.58 **a** *	2.86 ± 0.17 **a** *	5.13 ± 0.66 **a** *	2.84 ± 0.21 **a** *
RPL	2.70 ± 0.25 **b** *	1.11 ± 0.09 **c**	1.41 ± 0.14 **b**	1.23 ± 0.12 **b**
Proximal Intestine				
Dietary Treatment	**SOD/LPO**	**CAT/LPO**	**GPx × 1000/LPO**	**GST × 100/LPO**
P	2.12 ± 0.36 **m**	1.76 ± 0.21 **m**	1.66 ± 0.20 **mn**	1.19 ± 0.07 **n**
R	1.99 ± 0.29 **m**	1.60 ± 0.16 **mn**	1.39 ± 0.24 **no**	1.07 ± 0.07 **n**
PL	1.32 ± 0.21 **m**	1.26 ± 0.13 **n**	1.30 ± 0.23 **no**	1.14 ± 0.14 **n**
RL	1.68 ± 0.19 **m**	1.54 ± 0.11 **mn**	1.17 ± 0.18 **o**	1.31 ± 0.12 **n**
RPL	0.552 ± 0.05 **n**	1.60 ± 0.12 **mn** *	1.95 ± 0.14 **m** *	1.70 ± 0.14 **m** *

Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Differences between intestinal segments for the same dietary condition are shown by * ($p < 0.05$) in the segment with the highest ratio. Significant differences between dietary conditions in the same intestinal segment are shown by letters in PC (a–c) and PI (m–o) ($p < 0.05$).

The relationship between some antioxidant enzymes was also calculated (Figures 3 and 4). For all experimental conditions, the (CAT+GPx)/SOD ratio was significantly higher in the PI than in the PC; in contrast, in the PC the ratio (GST+GPx)/GR was significantly higher. Both intestinal segments showed higher (CAT+GPx)/SOD ratios in the fish fed diets containing linseed oil, being highest in the RPL gilthead sea bream (Figure 3A). In PC, the (GST+GPx)/GR ratio was more elevated in fish fed diets containing palm oil; instead, in PI, this ratio was higher in the fish fed diets containing linseed oil (Figure 3B).

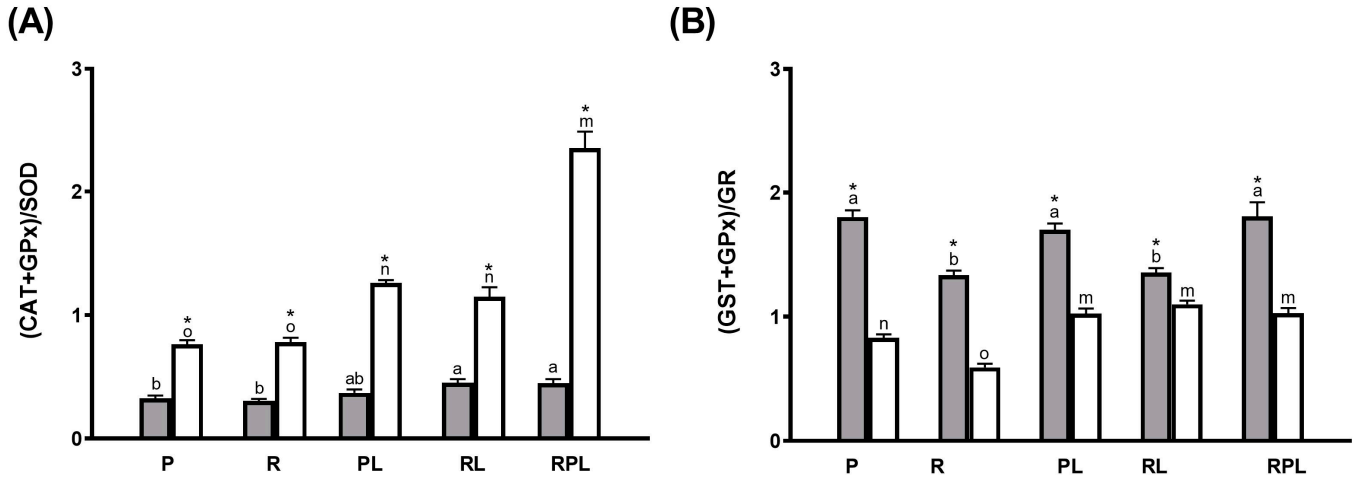

Figure 3. (**A**) (CAT+GPx)/SOD and (**B**) (GST+GPx)/GR ratios in pyloric caeca (grey bars) and proximal intestine (white bars) 24 h post-feeding in gilthead sea bream fed the experimental diets. Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Significant differences between dietary conditions in the same intestinal segment are shown by letters in PC (a,b) and PI (m–o). Differences between intestinal segments for the same dietary condition are shown by * ($p < 0.05$).

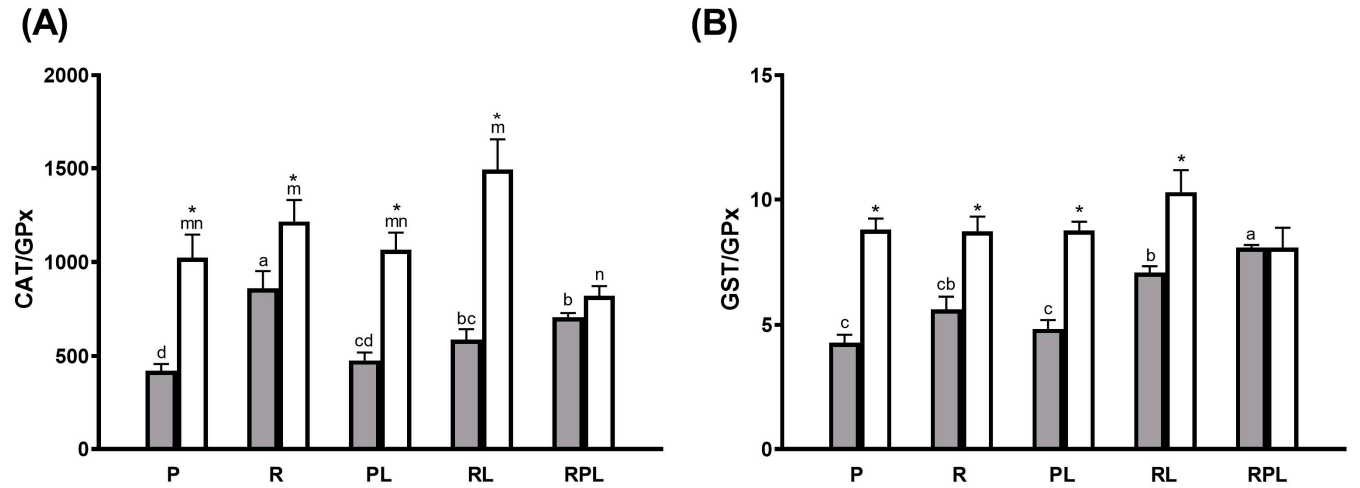

Figure 4. (**A**) CAT/GPx and (**B**) GST/GPx ratios in pyloric caeca (grey bars) and proximal intestine (white bars) 24 h post-feeding in gilthead sea bream fed the experimental diets. Values are the mean ± SEM (n = 6 in P and R, n = 9 in PL, RL, RPL). Significant differences between dietary conditions in the same intestinal segment are shown by letters in PC (a–d) and PI (m,n). Differences between intestinal segments for the same dietary condition are shown by * ($p < 0.05$).

The P, R, PL, and RL groups had higher CAT/GPx and GST/GPx ratios in PI than in PC; instead, the RPL sea bream showed similar values for these ratios in both intestinal segments (Figure 4). The fish fed diets containing rapeseed oil presented higher CAT/GPx

and GST/GPx ratios in PC. Instead, the sea bream fed diets that contained palm oil presented a lower CAT/GPx ratio in PI, a trend that was not found for the GST/GPx ratio.

4. Discussion

Lipid dietary composition is an essential factor that can trigger ROS production, generating different peroxidation levels depending on the fatty acid profile, since PUFA, mainly highly UFA, are more susceptible to LPO than MUFA or SFA [21]. Most of the oxidative status studies had focused on the liver due to its significant involvement in energy metabolism, also being the largest producer and exporter of GSH [54]. In this sense, an increase in LPO, SOD and CAT, and lower GPx activities were found in the liver of sea bass fed a diet containing 70% of plant protein versus those fish fed with a diet based on fish meal, pointing to a higher superoxide anion production [55]. Despite this, in that study, both diets presented cod liver oil as a sole lipid source, suggesting that the differences in oxidative status could be due to the presence of phenolic compounds, flavonoids, vitamins, or carotenoids in the vegetable ingredients [55]. However, several other studies have shown that the intestine was more susceptible to oxidative stress due to its high turnover and higher exposure to the toxins or allergens present in diets than the liver [56–58]. To protect cells from the deleterious effects of endogenous ROS, there must be a balance between ROS production and antioxidant mechanisms, thus maintaining the physiological status regulated through different metabolic pathways in each tissue [1,59].

In the present study, the origin and amount of dietary plant proteins were the same in all experimental groups. However, among the VOs used and according to their susceptibility to LPO, palm oil has greater oxidative stability than linseed oil [60], whereas rapeseed oil has intermediate values [21]. Therefore, as fish flesh reflects the composition of the given diet, LPO must be considered in these types of studies [21]. In this sense, in a recent study on gilthead sea bream, it was demonstrated that the inclusion of a soybean and linseed oil blend in the diet could negatively affect the intestinal oxidative status, triggering exacerbated LPO levels that antioxidant enzymes cannot counteract [4]. Despite this, it was also observed that the oxidative status improved if palm and/or rapeseed oil were also added to the previous blend. In the present study, dietary linseed oil inclusion led to a depletion in SOD activity in both intestinal segments, especially in the PI of RPL-fed gilthead sea bream. Similar results were obtained in the liver of Japanese sea bass fed diets containing linseed and soybean oils [61], or in the intestine of European sea bass fed diets containing microalgae blends rich in PUFA, like *Nannochloropsis* or *Chlorella* [56]. In addition, CAT activity also diminished in PC, while for GR it diminished in PI in groups fed linseed oil. SOD, CAT and GR reduction could be due to an impairment in ROS production caused by the low n-6/n-3 ratios (<1) of the diets containing linseed oil, since higher LPO levels were found in PL and RPL groups, while the levels were lower in the case of the RL group. Magalhaes et al. [25,28] found similar results in LPO in gilthead sea bream fed diets supplemented with different ARA, EPA, and DHA levels. Moreover, it is known that CAT activity is enhanced by high levels of H_2O_2 responding to an oxidative stress situation, while in the basal antioxidant situation the low H_2O_2 levels induced GPx activity [5,62]. In the present study, CAT activity was higher in the PI of PL- and RL-fed gilthead sea bream, according to the increase in ROS production, suggesting that this enzyme contributes to fighting against the rise in oxidative stress, particularly in this intestinal region. This idea was reinforced by the antioxidants/LPO ratios, where only a significant depletion in the CAT/LPO ratio was found in both intestinal segments for PL fish and in PC for RPL sea bream. Overall, this indicates the great importance of this enzyme activity in situations of high oxidative stress and points to its upregulation by high LPO.

Dietary rapeseed oil inclusion led to a fall in GST and GPx activities in the PC region. Furthermore, the dietary fatty acid profile also affected GR activity, which showed a significant reduction in PI when rapeseed oil was blended with another VO. These results agreed with the obtained values of the (GST+GPx)/GR ratio, that acts as a marker for the recycling of GSSG to GSH. These observations could be related to a higher GSSG production

in the intestine, suggesting that the antioxidant activity in this tissue is more dependent on GSH than that in the liver [57], since GSH is used bivalently as a scavenger for H_2O_2, and indirectly, as the substrate for GPx and GST to eliminate lipid peroxides or H_2O_2 and xenobiotics, respectively [5].

The dietary presence of palm oil upregulated PI GPx activity; despite this, the intestinal LPO levels in RPL-fed fish were still high when compared with the RL group. An intestinal-enhanced GPx activity was also found in gilthead sea bream fed diets containing over 50% of soybean oil, contributing to the downregulated LPO levels [4].

In the PC of sea bream fed diets containing linseed oil, *gpx1* was downregulated. As GPx1 is involved in the membrane phospholipid turnover from the undamaged free fatty acids of the cytosolic pool [21,63], this process could be affected in the fish fed PL, RL and RPL diets. Moreover, a high expression of *gpx4* was found in the PI of fish fed R and PL diets, suggesting that those sea bream are primed to repair peroxidized membrane phospholipids in situ [21,63], as was also previously found in the intestine of sea bream fed diets containing soybean oil, among others [4]. In the same sense, Saera-Vila et al. [63] described a differential hepatic regulation of *gpx1* and *gpx4* when dietary fish oil was replaced by a mixture of vegetable oils (from rapeseed, palm and mainly linseed), describing a *gpx1* decreased gene expression and an increase in the *gpx4*. Instead, when yellowtail kingfish were fed a diet containing canola oil as a lipid source, hepatic *gpx1* expression was raised, whereas *gpx4* was unaffected [21]. The differences in hepatic antioxidant gene expression between studies may be due to the different compositions of VOs used, especially concerning their fatty acid profile. It is important to highlight that this *gpx4* gene expression and increased GPx4 activity could confer an adaptative advantage, especially in marine species that present low C18 VOs bioconversion to C22 PUFA, through the reduction in the membrane phospholipid turnover [63].

In recent studies in gilthead sea bream, the differences in oxidative status at the intestinal level have been related to different dietary n-6/n-3 LC-PUFA [25,28]. In the present study, this ratio was similar for all the experimental conditions, and data showed that not only n-6/n-3 LC-PUFA content affects the antioxidant capacity. In this sense, LPO levels found in R- and RL-fed sea bream were similar to those found in the P group and completely different from those of the PL group. The main difference between the PL diet and those containing rapeseed oil (R and RL) was its lower UFA/SFA ratio due to the higher content of SFA compared to the other lipid classes. Thus, all the changes in the activity of the above-mentioned antioxidant enzymes suggest that dietary UFA/SFA ratio could modulate their activities, as well as GSH production. This effect of the UFA/SFA ratio on antioxidant enzyme activities has also been found in the intestine of gilthead sea bream fed diets containing soybean oil [4]. Furthermore, RPL-fed sea bream, whose diet presented an intermediate UFA/SFA ratio, showed the highest LPO levels in PC, while in the PI, the levels remained low compared to the other groups.

An elevated GST activity points to the presence of substances that can be toxic in the diet at high concentrations [56,57], which was not observed in the present experiment either in PC or in PI.

In general terms, the different oxidative status, antioxidant activities, and LPO levels between intestinal regions could be related to their differences in function within the digestive process. Thus, the PC region presents a retrograde contractile activity that confers it their characteristic mixing movements [64] that could be associated with an immune-related function [38,65,66]. Moreover, this contractile activity aids in the mix of the chyme with digestion juices, and slows down intestinal transit to maximize nutrient digestion and absorption and contribute to food short retention. In addition, a study on rainbow trout [67] suggests that PC are subjected to a larger cell renewal rate than the PI, which could contribute to the lower LPO levels found in this intestinal region versus the PI. Besides, this intestinal region was commonly associated with lipid and wax absorption [68–70], which reinforces the low levels of LPO that could be found in PC in comparison with PI. Moreover, in the present study, higher levels of antioxidant activities (CAT, GPx, GST and

GR) were found in PI rather than in PC, which may be related to the attempt to preserve the intestinal epithelium, which is more mature and whose main functions are digestion and absorption capacities, unlike PC region.

Finally, among all the VO blends tested, gilthead sea bream fed the RL diet had the best intestinal oxidative status. The RL group maintained a lower LPO content versus PL-fed fish, and was able to maintain the antioxidant activities in both intestinal segments to counteract ROS production, unlike what occurs in the RPL-fed animals that present a deficiency of antioxidants in PC and of SOD in PI. When the RL diet is compared with the PL and RPL diets, they present similar levels of n-3, but RL contains a significantly higher content of n-6 and a higher UFA/SFA ratio.

5. Conclusions

Overall, the current results suggest that the dietary amount of n-6 per se and the UFA/SFA ratio of a diet are key factors to consider in aquafeed formulation that includes VOs. The consideration of these parameters would allow for the formulation of fish diets with adequate intestinal health and lipid profiles, contributing to the sustainability and improvement of aquaculture.

Author Contributions: Conceptualization, R.F., J.G., E.C., I.N. and Á.G.; methodology, all authors carried out the sampling; I.G.-M. and Á.G. performed laboratory analyses and interpreted the data; software, I.G.-M. and Á.G.; writing—original draft preparation, I.G.-M. and Á.G.; writing—review and editing, I.G.-M., J.G., E.C., I.N. and Á.G. drafted and critically reviewed the manuscript; funding acquisition, I.N. and E.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the "Ministerio de Ciencia e Innovación" MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe" of the European Union) through the projects AGL2014-57974-R, AGL2017-89436-R, PID2020-116172RB-I00 and the "Generalitat de Catalunya" (2017SGR-1574, 2021 SGR 00713). The project leading to these results has received funding from "la Caixa" Foundation (ID 100010434), under agreement (LCF/PR/PR06/51010001).

Institutional Review Board Statement: The animal study protocol was approved by the Ethics and Animal Care Committee of University of Barcelona following the European Union assigned principles and legislation (permit number DAAM 8982).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The authors would like to thank the personnel from IRTA (Magda Monllaó and Alicia Estévez) for the maintenance of the fish during the in vivo trial, and Irina Agredano, Natalia García and Anna Montserrat for their help with oxidative stress analyses.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Castro, C.; Diógenes, A.F.; Coutinho, F.; Panserat, S.; Corraze, G.; Pérez-Jiménez, A.; Peres, H.; Oliva-Teles, A. Liver and Intestine Oxidative Status of Gilthead Sea Bream Fed Vegetable Oil and Carbohydrate Rich Diets. *Aquaculture* **2016**, *464*, 665–672. [CrossRef]
2. Kiron, V.; Thawonsuwan, J.; Panigrahi, A.; Scharsack, J.P.; Satoh, S. Antioxidant and Immune Defences of Rainbow Trout (*Oncorhynchus mykiss*) Offered Plant Oils Differing in Fatty Acid Profiles from Early Stages. *Aquac. Nutr.* **2011**, *17*, 130–140. [CrossRef]
3. Kjær, M.A.; Aursnes, I.A.; Berge, G.M.; Sørensen, M.; Marchenko, Y.; Gjøen, T.; Ruyter, B. The Influence of Different Dietary Oil Qualities on Growth Rate, Feed Utilization and Oxidative Stress in Atlantic Cod. *Aquac. Nutr.* **2014**, *20*, 192–204. [CrossRef]
4. García-Meilán, I.; Fontanillas, R.; Gutiérrez, J.; Capilla, E.; Navarro, I.; Gallardo, Á. Effects of Dietary Vegetable Oil Mixtures Including Soybean Oil on Intestinal Oxidative Stress in Gilthead Sea Bream (*Sparus aurata*). *Animals* **2023**, *13*, 1069. [CrossRef] [PubMed]
5. Halliwell, B.; Gutteridge, J.M.C. *Free Radicals in Biology and Medicine*; Oxford University Press: Oxford, UK, 2015.

6. Xiao, W.; Jiang, W.; Feng, L.; Liu, Y.; Wu, P.; Jiang, J.; Zhang, Y.; Zhou, X. Effect of Dietary Enzyme-Treated Soy Protein on the Immunity and Antioxidant Status in the Intestine of Juvenile Jian Carp (*Cyprinus carpio var. Jian*). *Aquac. Res.* **2019**, *50*, 1411–1421. [CrossRef]
7. Morais, S.; Edvardsen, R.B.; Tocher, D.R.; Bell, J.G. Transcriptomic Analyses of Intestinal Gene Expression of Juvenile Atlantic Cod (*Gadus morhua*) Fed Diets with Camelina Oil as Replacement for Fish Oil. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2012**, *161*, 283–293. [CrossRef] [PubMed]
8. Bresciani, G.; da Cruz, I.B.M.; González-Gallego, J. Manganese Superoxide Dismutase and Oxidative Stress Modulation. *Adv Clin Chem* **2015**, *68*, 87–130. [CrossRef] [PubMed]
9. FAO. *The State of World Fisheries and Aquaculture 2022*; FAO: Rome, Italy, 2022.
10. Glencross, B.D. Exploring the Nutritional Demand for Essential Fatty Acids by Aquaculture Species. *Aquac. Res.* **2009**, *48*, 71–124. [CrossRef]
11. National Research Council. *Nutrient Requirements of Fish and Shrimp*; The Nacional Academies Press: Washington, DC, USA, 2011. [CrossRef]
12. Turchini, G.M.; Torstensen, B.E.; Ng, W.K. Fish Oil Replacement in Finfish Nutrition. *Rev. Aquac.* **2009**, *1*, 10–57. [CrossRef]
13. Tocher, D.R. Fatty Acid Requirements in Ontogeny of Marine and Freshwater Fish. *Aquac. Res.* **2010**, *41*, 717–732. [CrossRef]
14. Puangkaew, J.; Kiron, V.; Somamoto, T.; Okamoto, N.; Satoh, S.; Takeuchi, T.; Watanabe, T. Nonspecific Immune Response of Rainbow Trout (*Oncorhynchus mykiss Walbaum*) in Relation to Different Status of Vitamin E and Highly Unsaturated Fatty Acids. *Fish. Shellfish Immunol.* **2004**, *16*, 25–39. [CrossRef] [PubMed]
15. Østbye, T.K.; Kjær, M.A.; Rørå, A.M.B.; Torstensen, B.; Ruyter, B. High N-3 HUFA Levels in the Diet of Atlantic Salmon Affect Muscle and Mitochondrial Membrane Lipids and Their Susceptibility to Oxidative Stress. *Aquac. Nutr.* **2011**, *17*, 177–190. [CrossRef]
16. Seiliez, I.; Panserat, S.; Corraze, G.; Kaushik, S.; Bergot, P. Cloning and Nutritional Regulation of a Δ6-Desaturase-like Enzyme in the Marine Teleost Gilthead Seabream (*Sparus aurata*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2003**, *135*, 449–460. [CrossRef] [PubMed]
17. Morais, S.; Monroig, O.; Zheng, X.; Leaver, M.J.; Tocher, D.R. Highly Unsaturated Fatty Acid Synthesis in Atlantic Salmon: Characterization of ELOVL5- and ELOVL2-like Elongases. *Mar. Biotechnol.* **2009**, *11*, 627–639. [CrossRef]
18. Betancor, M.B.; Howarth, F.J.E.; Glencross, B.D.; Tocher, D.R. Influence of Dietary Docosahexaenoic Acid in Combination with Other Long-Chain Polyunsaturated Fatty Acids on Expression of Biosynthesis Genes and Phospholipid Fatty Acid Compositions in Tissues of Post-Smolt Atlantic Salmon (*Salmo salar*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2014**, *172–173*, 74–89. [CrossRef] [PubMed]
19. Sánchez-Moya, A.; García-Meilán, I.; Riera-Heredia, N.; Vélez, E.J.; Lutfi, E.; Fontanillas, R.; Gutiérrez, J.; Capilla, E.; Navarro, I. Effects of Different Dietary Vegetable Oils on Growth and Intestinal Performance, Lipid Metabolism and Flesh Quality in Gilthead Sea Bream. *Aquaculture* **2020**, *519*, 734881. [CrossRef]
20. Goyal, A.; Sharma, V.; Upadhyay, N.; Gill, S.; Sihag, M. Flax and Flaxseed Oil: An Ancient Medicine & Modern Functional Food. *J. Food Sci. Technol.* **2014**, *51*, 1633–1653. [CrossRef] [PubMed]
21. Bowyer, J.N.; Rout-Pitt, N.; Bain, P.A.; Stone, D.A.J.; Schuller, K.A. Dietary Fish Oil Replacement with Canola Oil Up-Regulates Glutathione Peroxidase 1 Gene Expression in Yellowtail Kingfish (*Seriola lalandi*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2012**, *162*, 100–106. [CrossRef] [PubMed]
22. Bacchetta, C.; Rossi, A.S.; Cian, R.E.; Drago, S.R.; Cazenave, J. Dietary β-Carotene Improves Growth Performance and Antioxidant Status of Juvenile *Piaractus mesopotamicus*. *Aquac. Nutr.* **2019**, *25*, 761–769. [CrossRef]
23. Jiang, J.; Shi, D.; Zhou, X.Q.; Yin, L.; Feng, L.; Liu, Y.; Jiang, W.D.; Zhao, Y. Effects of Glutamate on Growth, Antioxidant Capacity, and Antioxidant-Related Signaling Molecule Expression in Primary Cultures of Fish Enterocytes. *Fish Physiol. Biochem.* **2015**, *41*, 1143–1153. [CrossRef] [PubMed]
24. Jiang, J.; Wu, X.Y.; Zhou, X.Q.; Feng, L.; Liu, Y.; Jiang, W.D.; Wu, P.; Zhao, Y. Effects of Dietary Curcumin Supplementation on Growth Performance, Intestinal Digestive Enzyme Activities and Antioxidant Capacity of Crucian Carp *Carassius auratus*. *Aquaculture* **2016**, *463*, 174–180. [CrossRef]
25. Magalhães, R.; Guerreiro, I.; Santos, R.A.; Coutinho, F.; Couto, A.; Serra, C.R.; Olsen, R.E.; Peres, H.; Oliva-Teles, A. Oxidative Status and Intestinal Health of Gilthead Sea Bream (*Sparus aurata*) Juveniles Fed Diets with Different ARA/EPA/DHA Ratios. *Sci. Rep.* **2020**, *10*, 13824. [CrossRef] [PubMed]
26. Yang, Q.; Liang, H.; Mokrani, A.; Ji, K.; Yu, H.; Ge, X.; Ren, M.; Xie, J.; Pan, L.; Sun, A. Dietary Histidine Affects Intestinal Antioxidant Enzyme Activities, Antioxidant Gene Expressions and Inflammatory Factors in Juvenile Blunt Snout Bream (*Megalobrama amblycephala*). *Aquac. Nutr.* **2019**, *25*, 249–259. [CrossRef]
27. Zhang, Y.L.; Jiang, W.D.; Duan, X.D.; Feng, L.; Wu, P.; Liu, Y.; Jiang, J.; Kuang, S.Y.; Tang, L.; Zhou, X.Q. Soybean Glycinin Caused NADPH-Oxidase-Regulated ROS Overproduction and Decreased ROS Elimination Capacity in the Mid and Distal Intestine of Juvenile Grass Carp (*Ctenopharyngodon idella*). *Aquaculture* **2020**, *516*, 734651. [CrossRef]
28. Magalhães, R.; Martins, N.; Fontinha, F.; Couto, A.; Serra, C.R.; Santos, R.A.; Olsen, R.E.; Peres, H.; Oliva-Teles, A. Effects of Dietary ARA, DHA, and Carbohydrates Levels on Gilthead Sea Bream Liver and Intestine Oxidative Stress, Tissue Histomorphology, and Gut Microbiota. *Aquaculture* **2022**, *552*, 738014. [CrossRef]

29. García-Meilán, I.; Valentín, J.M.; Fontanillas, R.; Gallardo, M.A. Different Protein to Energy Ratio Diets for Gilthead Sea Bream (*Sparus aurata*): Effects on Digestive and Absorptive Processes. *Aquaculture* **2013**, *412–413*, 1–7. [CrossRef]
30. García-Meilán, I.; Ordóñez-Grande, B.; Gallardo, M.A. Meal Timing Affects Protein-Sparing Effect by Carbohydrates in Sea Bream: Effects on Digestive and Absorptive Processes. *Aquaculture* **2014**, *434*, 121–128. [CrossRef]
31. García-Meilán, I.; Ordóñez-Grande, B.; Machahua, C.; Buenestado, S.; Fontanillas, R.; Gallardo, M.A. Effects of Dietary Protein-to-Lipid Ratio on Digestive and Absorptive Processes in Sea Bass Fingerlings. *Aquaculture* **2016**, *463*, 163–173. [CrossRef]
32. García-Meilán, I.; Ordóñez-Grande, B.; Valentín, J.M.; Hernández, M.D.; García, B.; Fontanillas, R.; Gallardo, M.A. Modulation of Digestive and Absorptive Processes with Age and/or after a Dietary Change in Gilthead Sea Bream. *Aquaculture* **2016**, *459*, 54–64. [CrossRef]
33. Santigosa, E.; García-Meilán, I.; Valentín, J.M.; Navarro, I.; Pérez-Sánchez, J.; Gallardo, M.Á. Plant Oils' Inclusion in High Fish Meal-Substituted Diets: Effect on Digestion and Nutrient Absorption in Gilthead Sea Bream (*Sparus aurata* L.). *Aquac. Res.* **2011**, *42*, 962–974. [CrossRef]
34. Santigosa, E.; García-Meilán, I.; Valentin, J.M.; Pérez-Sánchez, J.; Médale, F.; Kaushik, S.; Gallardo, M.A. Modifications of Intestinal Nutrient Absorption in Response to Dietary Fish Meal Replacement by Plant Protein Sources in Sea Bream (*Sparus aurata*) and Rainbow Trout (*Onchorynchus mykiss*). *Aquaculture* **2011**, *317*, 146–154. [CrossRef]
35. Kurokawa, T.; Suzuki, T. Structure of the Exocrine Pancreas of Flounder (*Paralichthys olivaceus*): Immunological Localization of Zymogen Granules in the Digestive Tract Using Anti-Trypsinogen Antibody. *J. Fish Biol.* **1995**, *46*, 292–301. [CrossRef]
36. Krogdahl, Å.; Sundby, A. Characteristics of Pancreatic Function in Fish. In *Biology of the Pancreas in Growing Animals*; Pierzynowski, S.G., Zabielski, R., Eds.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1999; pp. 437–458.
37. Morrison, C.M.; Pohajdak, B.; Tam, J.; Wright, J.R., Jr. Development of the Islets, Exocrine Pancreas, and Related Ducts in the Nile Tilapia, Oreochromis Niloticus (Pisces: *Cichlidae*). *J. Morphol.* **2004**, *261*, 377–389. [CrossRef] [PubMed]
38. Ballesteros, N.A.; Castro, R.; Abos, B.; Rodríguez Saint-Jean, S.S.; Pérez-Prieto, S.I.; Tafalla, C. The Pyloric Caeca Area Is a Major Site for IgM+ and IgT+ B Cell Recruitment in Response to Oral Vaccination in Rainbow Trout. *PLoS ONE* **2013**, *8*, e66118. [CrossRef] [PubMed]
39. Sun, H.; Jami, E.; Harpaz, S.; Mizrahi, I. Involvement of Dietary Salt in Shaping Bacterial Communities in European Sea Bass (*Dicentrarchus labrax*). *Sci. Rep.* **2013**, *3*, srep01558. [CrossRef] [PubMed]
40. Buddington, R.K.; Diamond, J.M. Pyloric Ceca of Fish: A "New" Absorptive Organ. *Am. J. Physiol. Gastrointest. Liver Physiol.* **1987**, *252*, G65–G76. [CrossRef] [PubMed]
41. Bauermeister, A.E.M.; Pirie, B.J.S.; Sargent, J.R. An Electron Microscopic Study of Lipid Absorption in the Pyloric Caeca of Rainbow Trout (*Salmo gairdnerii*) Fed Wax Ester—Rich Zooplankton. *Cell Tissue Res.* **1979**, *200*, 475–486. [CrossRef] [PubMed]
42. Sire, M.-F.; Lutton, C.; Vernier, J.-M. New Views on Intestinal Absorption of Lipids in Teleostean Fishes: An Ultrastructural and Biochemical Study in the Rainbow Trout. *J. Lipid Res.* **1981**, *22*, 81–94. [CrossRef] [PubMed]
43. Bakke, A.M.; Glover, C.; Krogdahl, A. Feeding, Digestion and Absorption of Nutrients. In *Multifunctional Gut of Fish*; Grosell, M., Farrell, A., Brauner, C., Eds.; Academic Press: Cambridge, MA, USA, 2011; pp. 57–75.
44. Buddington, R.K.; Krogdahl, A.; Bakke-Mckellep, A.M. The Intestines of Carnivorous Fish: Structure and Functions and the Relations with Diet. *Acta Physiol. Scand. Suppl.* **1997**, *638*, 67–80. [PubMed]
45. Mccords, J.M.; Fridovich, I. Superoxide Dismutase an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969**, *244*, 6049–6055.
46. Aebi, H. [13] Catalase In Vitro. *Methods Enzymol.* **1984**, *105*, 121–126. [CrossRef] [PubMed]
47. Bell, J.G.; Pirie, B.J.S.; Adron, J.W.; Cowey, C.B. Some Effects of Selenium Deficiency on Glutathione Peroxidase (EC 1.11.1.9) Activity and Tissue Pathology in Rainbow Trout (*Salmo gairdneri*). *Br. J. Nutr.* **1986**, *55*, 305–311. [CrossRef] [PubMed]
48. Habig, W.H.; Pabst, M.J.; Fleischner, G.; Gatmaitan, Z.; Arias, I.M.; Jakoby, W.B. The Identity of Glutathione S Transferase B with Ligandin, a Major Binding Protein of Liver. *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 3879–3882. [CrossRef] [PubMed]
49. Carlberg, I.; Mannervik, B. Purification and Characterization of the Flavoenzyme Glutathione Reductase from Rat Liver. *J. Biol. Chem.* **1975**, *260*, 5475–5480. [CrossRef]
50. Uchiyama, M.; Mihara, M. Determination of Malonaldehyde Precursor in Tissues by Thiobarbituric Acid Test. *Anal. Biochem.* **1978**, *86*, 271–278. [CrossRef] [PubMed]
51. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
52. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef] [PubMed]
53. Pfaffl, M.W. A New Mathematical Model for Relative Quantification in Real-Time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]
54. Wu, G.; Fang, Y.-Z.; Yang, S.; Lupton, J.R.; Turner, N.D. Glutathione Metabolism and Its Implications for Health. *J. Nutr.* **2004**, *134*, 489–492. [CrossRef] [PubMed]
55. Guerreiro, I.; Couto, A.; Pérez-Jiménez, A.; Oliva-Teles, A.; Enes, P. Gut Morphology and Hepatic Oxidative Status of European Sea Bass (*Dicentrarchus labrax*) Juveniles Fed Plant Feedstuffs or Fishmeal-Based Diets Supplemented with Short-Chain Fructo-Oligosaccharides and Xylo-Oligosaccharides. *Br. J. Nutr.* **2015**, *114*, 1975–1984. [CrossRef] [PubMed]

56. Castro, C.; Coutinho, F.; Iglesias, P.; Oliva-Teles, A.; Couto, A. *Chlorella* Sp. and *Nannochloropsis* Sp. Inclusion in Plant-Based Diets Modulate the Intestine and Liver Antioxidant Mechanisms of European Sea Bass Juveniles. *Front. Vet. Sci.* **2020**, *7*, 607575. [CrossRef] [PubMed]
57. Coutinho, F.; Castro, C.; Rufino-Palomares, E.; Ordóñez-Grande, B.; Gallardo, M.A.; Oliva-Teles, A.; Peres, H. Dietary Glutamine Supplementation Effects on Amino Acid Metabolism, Intestinal Nutrient Absorption Capacity and Antioxidant Response of Gilthead Sea Bream (*Sparus aurata*) Juveniles. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2016**, *191*, 9–17. [CrossRef] [PubMed]
58. Halliwell, B.; Zhao, K.; Whiteman, M. The Gastrointestinal Tract: A Major Site of Antioxidant Action? *Free Radic. Res.* **2000**, *33*, 819–830. [CrossRef] [PubMed]
59. Trenzado, C.; Hidalgo, M.C.; García-Gallego, M.; Morales, A.E.; Furné, M.; Domezain, A.; Domezain, J.; Sanz, A. Antioxidant Enzymes and Lipid Peroxidation in Sturgeon *Acipenser naccarii* and Trout *Oncorhynchus mykiss*. A Comparative Study. *Aquaculture* **2006**, *254*, 758–767. [CrossRef]
60. Li, J.; Liu, J.; Sun, X.; Liu, Y. The Mathematical Prediction Model for the Oxidative Stability of Vegetable Oils by the Main Fatty Acids Composition and Thermogravimetric Analysis. *LWT* **2018**, *96*, 51–57. [CrossRef]
61. Tan, P.; Dong, X.; Xu, H.; Mai, K.; Ai, Q. Dietary Vegetable Oil Suppressed Non-Specific Immunity and Liver Antioxidant Capacity but Induced Inflammatory Response in Japanese Sea Bass (*Lateolabrax japonicus*). *Fish Shellfish Immunol.* **2017**, *63*, 139–146. [CrossRef] [PubMed]
62. Box, A.; Sureda, A.; Galgani, F.; Pons, A.; Deudero, S. Assessment of Environmental Pollution at Balearic Islands Applying Oxidative Stress Biomarkers in the Mussel *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2007**, *146*, 531–539. [CrossRef] [PubMed]
63. Saera-Vila, A.; Benedito-Palos, L.; Sitjà-Bobadilla, A.; Nácher-Mestre, J.; Serrano, R.; Kaushik, S.; Pérez-Sánchez, J. Assessment of the Health and Antioxidant Trade-off in Gilthead Sea Bream (*Sparus aurata* L.) Fed Alternative Diets with Low Levels of Contaminants. *Aquaculture* **2009**, *296*, 87–95. [CrossRef]
64. Rønnestad, I.; Rojas-Garcia, C.R.; Skadal, J. Retrograde Peristalsis; a Possible Mechanism for Filling the Pyloric Caeca? *J. Fish Biol.* **2000**, *56*, 216–218. [CrossRef]
65. Jones, B.S.; Keightley, L.J.; Harris, J.O.; Wiklendt, L.; Spencer, N.J.; Dinning, P.G. Identification of Neurogenic Intestinal Motility Patterns in Silver Perch (*Bidyanus bidyanus*) That Persist over Wide Temperature Ranges. *Neurogastroenterol. Motil.* **2021**, *33*, e14037. [CrossRef] [PubMed]
66. Kikuchi, K.; Noh, H.; Numayama-Tsuruta, K.; Ishikawa, T. Mechanical Roles of Anterograde and Retrograde Intestinal Peristalses after Feeding in a Larval Fish (*Danio rerio*). *Am. J. Physiol. Gastrointest. Liver Physiol.* **2020**, *318*, 1013–1021. [CrossRef]
67. Verdile, N.; Pasquariello, R.; Scolari, M.; Scirè, G.; Brevini, T.A.L.; Gandolfi, F. A Detailed Study of Rainbow Trout (*Onchorhynchus mykiss*) Intestine Revealed That Digestive and Absorptive Functions Are Not Linearly Distributed along Its Length. *Animals* **2020**, *10*, 745. [CrossRef] [PubMed]
68. Benson, A.A.; Lee, R.F. The Role of Wax in Oceanic Food Chains. *Sci. Am.* **1975**, *232*, 76–86. [CrossRef] [PubMed]
69. Grenne, C.W. The Fat Absorving Function of the Alimentary Tract of the King Salmon. *Bull. Bur. Fish.* **1914**, *33*, 149–175.
70. Jansson, B.-O.; Olsson, R. The Cytology of the Caecal Epithelial Cells of Perca. *Acta Zool.* **1960**, *41*, 267–276. [CrossRef]