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Pelvetia canaliculata as an Aquafeed Supplement for Gilthead Seabream *Sparus aurata*: A Biorefinery Approach for Seaweed Biomass Valorisation

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Abstract: For sustainable and economically viable aquaculture, it is necessary to search for alternative sources of aquafeeds. Algae have been studied because of their bioactive compounds with several activities such as antioxidants. The direct incorporation of the macroalgae *Pelvetia canaliculata* in sunflower oil to increase oxidative stability and biological value results in waste with high nutritional value that may be used as an ingredient in aquaculture feed. This study aimed to evaluate the effect of incorporating algae powder (PEL 1%, PEL 10%) and algae waste obtained after sunflower oil supplementation (WO 1%, WO 10%) in aquafeeds for gilthead seabream. We studied the growth performance, haematological profile, oxidative stress and metabolic parameters, and intestine histomorphology. Experimental diets did not influence growth performance or somatic indexes, and barely affected the haematological profile. Catalase showed higher activity in seabream fed with PEL10 than with control diet. Total glutathione had a higher activity in fish fed with both WO diets. Plasmatic levels of cholesterol were higher in PEL1 and WO10. Triglyceride levels were higher in WO1 and total lipids were higher in both WO diets. The histomorphology of the intestine was slightly modulated by experimental diets but was not affected negatively. In general, supplementation with *Pelvetia* powder and algal waste oil may be used as an aquafeed for gilthead seabream according to the results obtained for growth, some haematological parameters, catalase and total glutathione, intestinal villi length, and the number of total and acid goblet cells.

Keywords: aquaculture; aquafeed; circular economy; seaweed

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1. Introduction

In 2019, the global production of gilthead seabream (*Sparus aurata*) reached 258,754 t, being one of the most cultivated and economically important marine fish species in the Mediterranean and southern Europe [1]. Currently, the development of the aquaculture industry is being driven by increased human consumption and market acceptance. Furthermore, it is expected that between 2020 and 2027, aquaculture growth will increase at a rate of over 7.1% [2]. The growth of the aquaculture industry must be economically viable and environmentally sustainable. Thus, there is a need to replace ingredients of marine origin, such as fishmeal, in aquafeed with other alternative sources of proteins and lipids, which should be eco-friendly but highly digestible and without compromising fish growth [3,4].

Seaweeds are one of the alternative ingredients that have drawn attention, for their great potential as low caloric source of proteins, soluble dietary fibres, minerals, vitamins, antioxidants, and polyunsaturated fatty acids. [5,6]. Several studies have demonstrated the benefits of incorporating seaweeds in the diets of fish farmed in aquaculture [4,7–12]. In addition, marine algae, especially those in the intertidal zone, proliferate in habitats that

are exposed to various stressful environmental conditions (e.g., variations of temperature and salinity, exposure to ultraviolet radiation, among others), having developed adaptive mechanisms that allow them to live in these conditions. These mechanisms are responsible for producing a wide range of secondary metabolites such as pigments, vitamins, phenolic compounds, sterols, and other bioactive compounds [13]. Currently, these compounds have commercial applications in several industries such as pharmacology/medical, nutraceutical, cosmeceuticals, agricultural, bioremediation, and biofuels [13–16]. The secondary metabolites produced by algae have several biological activities such as antioxidant, antibacterial, anti-inflammatory, antifungal, antiviral, anti-coagulation, antiproliferative, UV protection, among others [15,17]. Antioxidant compounds from seaweed have been of increasing interest as ingredients for functional feeds [18]. Several studies have been performed on the antioxidant activity of various compounds and extracts of brown macroalgae as well as their potential use in industry, namely, of the brown macroalgae *Pelvetia canaliculata* that is widely distributes in the North Atlantic [19–23]. This macroalgae species belonging to the Fucales family inhabits the upper limit of the intertidal zones. Due to its composition in carotenoids, it is one of the most stress-tolerant macroalgae, being able to resist desiccation and quickly adapt to high radiation [24]. Brown macroalgae (Phaeophyta) are characterised by organisms ranging from small filamentous forms to large/giant complex algae belonging to the class Pheophyceae. These macroalgae have several bioactive compounds such as carotenoids (fucoxanthin, carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin, and neoxanthin), pigments (chlorophylls a and c), phenolic compounds (phlorotannin, phytosterol, and polyphenol), polysaccharides (alginic acids, laminarans, and fucoidans) [15,22–26]. Furthermore, these specific metabolites with their several biological activities make algae potential functional ingredients.

The circular economy concept consists of using renewable resources and reusing waste as secondary raw materials, involving less energy consumption and non-renewable resources, in addition to having more benefits for the environment through the reuse of waste and lower emission of pollutants [24]. It is known that the food industry produces a large amount of waste, which raises serious management problems at an economic and environmental level, valuable materials with the potential to be reused in other production systems are wasted [27].

Sunflower oil with increased oxidative stability and biological value was produced by direct incorporation of the macroalgae *P. canaliculata* as a source of pigments and antioxidant compounds [28]. However, the algae residue, obtained after oil separation, still contains a high nutritional value. Following the concept of biorefinery, this algae waste may be applied as an ingredient in aquaculture feed formulations.

The present study aimed to evaluate the effect of incorporating powder algae *P. canaliculata* (1% and 10%) and algae waste obtained after sunflower oil supplementation (1% and 10%) in aquafeeds for gilthead seabream *(S. aurata*) by studying the growth performance, haematological profile, immunological, metabolic, oxidative stress parameters, and intestine histomorphology.

2. Materials and Methods

2.1. Ethics Statement

The current study was conducted according to the guidelines on the protection of animals used for scientific purposes from the European Directive 2010/63/EU and under a project authorisation 0421/000/000/2019.

2.2. Algae Collection and Processing

The brown seaweed *Pelvetia canaliculata* was harvested at the beach of Pedras do Corgo, Portugal (41°14′55.52″ N, 8°43′29.89″ W) in April 2021 (Figure 1). According to Martins et al., [29], this seaweed can be found at a depth of 0.5 to 0.75 m during the high tide period.

Figure 1. *P. canaliculata* growing on the beach of Pedras do Corgo, Portugal.

The algae material was cleaned from extraneous matter, washed with distilled water, freeze-dried, and powdered. *P. canaliculata* was previously characterised concerning proximate composition and pigments content [28], as shown in Table 1.

Table 1. Proximate composition and pigments content of *Pelvetia canaliculata* powder [28].

Proximate Composition	
Proteins (%)	7.72 ± 0.13
Lipids (%)	5.12 ± 0.41
Ash (%)	21.40 ± 0.04
Carbohydrates (%)	65.76 ± 0.43
Pigments	
Chlorophylls (pheophytin a /Kg)	602 ± 30
Carotenoids (mg β-carotene/Kg)	236 ± 12

Algae waste was obtained after sunflower oil supplementation with powdered *P. canaliculata*, added to the oil in a proportion of 12.5% (*m*/*v*), and subjected to ultrasound-assisted extraction for 20 min. Then, the mixture was filtered (paper filter, grammage 160 g m^{-2}, thickness 0.470 mm, pore 60–68 µm, and ashes < 0.15%) to separate the solid residue (algae waste) from the supplemented oil. The nutritional composition of *P. canaliculata* waste is described in Table 2.

Table 2. Proximate composition of *Pelvetia canaliculata* waste.

Proteins (%)	5.26 ± 0.09
Lipids (%)	35.27 ± 1.13
Ash (%)	14.60 ± 0.03
Carbohydrates (%)	44.86 ± 0.29

2.3. Experimental Diets

A specialised company (SPAROS, Portugal) formulated five isoproteic (48%), isolipidic (17%) diets (Table 3), considering two powder concentrations, 1% (PEL1) and 10% (PEL10), and two algae waste oil concentrations of 1 and 10% (WO1, WO10), and a control diet.

Table 3. Formulation and proximate composition of the experimental diets.

	CTRL	PEL1	PEL10	WO1	WO10
Ingredients (%)					
Fishmeal	20	20	20	20	20
Fish protein hydrolysate	5	5	5	5	5
Fish gelatine	2	2	2	2	2
Poultry meal	10	10	10	10	10
Pea protein concentrate	5	5	5	5	5
Wheat gluten	9.6	9.7	10.4	9.7	10.1
Corn gluten meal	6	6	6	6	6
Soybean meal	15	15	15	15	15
Wheat meal	15.5	14.4	5.1	14.7	8.4
Vitamin and mineral premix	1	1	1	1	1
Monocalcium phosphate	0.9	0.9	0.9	0.9	0.9
Fish oil	6	6	6	6	6
Soybean oil	6	6	5.6	5.7	2.6
Pelvetia powder		1	10		
Pelvetia waste				1	10
Proximate composition (%)					
Protein	48.9 ± 0.8	47.2 ± 0.7	48.6 ± 0.6	47.1 ± 2.0	47.9 ± 1.4
Fat	17.4 ± 0.2	17.0 ± 1.1	16.9 ± 0.6	17.6 ± 0.3	16.8 ± 0.4
Ash	6.33 ± 0.07	6.37 ± 0.08	8.22 ± 0.04	6.58 ± 0.05	7.73 ± 0.04
Moisture	7.08 ± 0.15	9.08 ± 0.11	7.97 ± 0.13	6.12 ± 0.05	6.49 ± 0.07
Carbohydrates	19.9 ± 0.6	19.9 ± 1.4	17.8 ± 0.3	22.1 ± 2.3	20.6 ± 1.1

2.4. Fish and Rearing Conditions

The gilthead seabream (*Sparus aurata*) juveniles (15.41 ± 3.69 g) (Figure 2) were purchased from EPPO—Aquaculture Research Station (Portuguese Institute for Sea and Atmosphere), and transported to the Aquaculture Laboratory of MARE—Polytechnic of Leiria (Peniche, Portugal). Then, they were kept in quarantine for a fortnight. After this period, the fish were weighed individually and randomly distributed (20 fish) through fifteen 60 L aquaria (triplicate for each treatment, Figure 3). The stocking density was 5.14 ± 0.26 kg m^{-3} and an acclimatisation period of one week elapsed before commencing the experiment. The trial was performed in closed water recirculation system under controlled conditions (water temperature, 20.49 ± 1.07 °C; salinity, 32.79 ± 0.35; pH, 8.07 ± 0.19 and dissolved oxygen, 91.87 ± 3.36%). Water quality parameters and mortality were monitored and recorded daily. The feeding period lasted 44 days, the fish were manually fed, ad libitum, three times a day, and a record was made of the feed consumed daily. The fish had a 24-h fasting period prior to sampling. For this, three fish from each tank were anaesthetised with 2-phenoxyethanol (0.5 mL L^{-1}), weighed, blood-drawn, and euthanised for liver collection for determination of antioxidant enzyme activity. Blood collection was done from the caudal vein using heparinised syringes and then placed in a microtube also heparinised with 20 µL of heparin (3000 U). Plasma was obtained after centrifugation of the blood at 10,000× *g* and 4 °C for 10 min. Moreover, three fish from each tank were also sampled to collect liver for metabolic parameters evaluation and the intestine was collected for histological analysis. Plasma and liver samples were kept at −80 °C until use.

Figure 2. Gilthead seabream (*Sparus aurata*) juveniles in aquarium during the feeding trial.

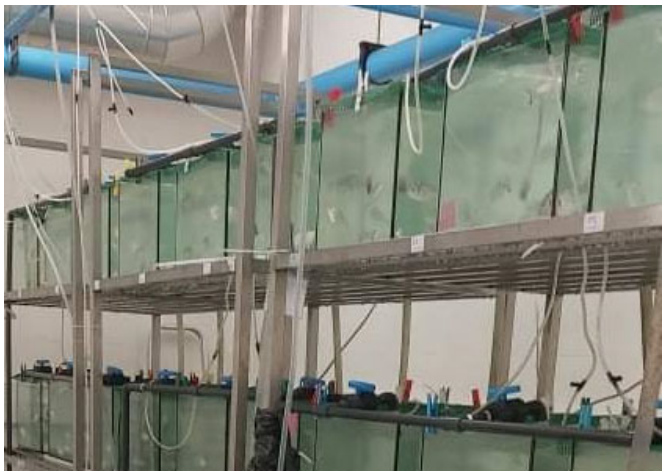


Figure 3. Experimental design with aquaria of feeding trial.

2.5. Growth Performance, Hepatosomatic and Viscerosomatic Index

Every fish was weighed in the beginning and end of the trial, and the average weight of each tank was used for the growth performance calculations:

Weight gain (%) = (Final weight − Initial weight) × 100/(Initial weight)

Average body weight = (Final weight + Initial weight) ÷ 2

Voluntary feed intake = (((Feed intake)/(Average body weight))/(44 days)) × 100

Daily growth index = ([Final weight] $^{(1/3)}$ − [Initial weight] $^{(1/3)}$)/(44 days) × 100

Feed conversion ratio = (Feed intake)/(Weight gain (g))

For the calculation of hepatosomatic and viscerosomatic indexes, three fish from each aquarium were sampled and weighted as well as their viscera and liver:

Hepatosomatic index (HSI %) = 100 × (liver weight (g)/fish weight (g))

Viscerosomatic index (VSI %) = 100 × (viscera weight (g)/fish weight (g))

2.6. Haematological Parameters

White and red blood cell counts (WBC and RBC, respectively), haematocrit (Ht), and haemoglobin (Hb, SPINREACT, Spain, ref. 1001230) formed the haematological profile of the fish. Additionally, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated [30].

2.7. Oxidative Stress Analysis

Liver extracts were obtained by homogenisation with 1:10 (m:v) ultrapure water, using a pellet mixer. For determination of lipid peroxidation (LPO) an aliquot of 200 µL was placed in a microtube containing 4 µL of 4% BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol. The remaining extract was mixed with the same volume of potassium phosphate buffer (0.2 M, pH 7.4), centrifuged at 10,000× *g* at 4 °C, for 20 min and stored at −80 °C. LPO [31], catalase activity (CAT) [32], superoxide dismutase activity (SOD) [33], glutathione-S-transferase activity (GST) [34,35], and total glutathione (tGSH) [36,37] were evaluated as hepatic oxidative stress biomarkers. Protein concentration was determined for sample normalisation (Pierce™ BCA Protein Assay Kit).

2.8. Metabolic and Immune Parameters

Thawed plasma samples were used to quantify glucose (Ref. 201001190), cholesterol (Ref. 201001090), triglycerides (Ref. 201001311), and total lipids (Ref. 201001270) with SPINREACT (Spain) kits.

2.9. Intestine Histology

For histological analysis, three fish were randomly selected per tank (9 per diet) and two parts of the intestine, namely anterior and posterior, were collected. After fixation in 10% buffered formalin for 24 h, samples were preserved in 70% ethanol until used. Both parts of the intestine were processed and embedded in paraffin. The sections (5 µm) were made in a microtome (Accu-Cut® SRM™ 200 Rotary, Sakura) and stained with Alcian blue/PAS (pH 2.5). An optical microscope (Leica DM2000 LED) with a digital camera (Leica MC 170 HD) was used to observe the intestinal sections. For assessing the morphology of the intestine, measurements of the following parameters were made in two intestinal sections per sample: muscularis externa (µm); outer longitudinal and inner circular layers of muscularis externa (µm); villus length and width (µm); and goblet cells (GC) count (GC no. per folds); divided into neutral and acid cells (Figure 4) as described in previous studies [9,38,39] using the Leica Application Suite version 4.4.0 software. To evaluate the morphological changes in both parts of the intestine, scores (1–5) were assigned according to the criteria from [40].

Figure 4. *Cont.*

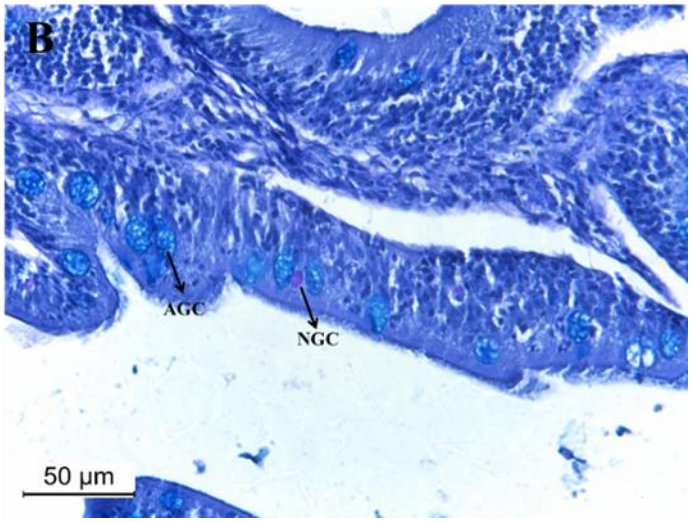


Figure 4. Histological sections of the intestine of gilthead seabream (*S. aurata*), Alcian blue/PAS (pH 2.5). (**A**) Parameters measured in intestinal sections (100×): 1—muscularis externa; 2—outer longitudinal layer; 3—inner circular layer; 4—villus length; 5—villus width. (**B**) Goblet cells in intestinal folds (400×): AGC—acid goblet cells (blue); NGC—neutral goblet cells (purple).

2.10. Statistical Analysis

All data were represented as mean ± SD and tested for significant differences using a one-way ANOVA, with each diet as factor. This was followed by multiple comparisons using Tukey's test, when the assumptions of normality and homogeneity of variance were verified. If not, a Kruskal–Wallis test was performed. The statistical significance used for all statistical tests was $p < 0.05$. SPSS software (v27, IBM, Armonk, New York, NY, USA) was used.

3. Results

3.1. Growth Performance, Somatic Indexes, and Mortality

The experimental diets did not induce a distinct response in growth performance, feed utilisation, or somatic indexes of seabream that underwent the trial for 44 days (Table 4).

Table 4. Growth performance and somatic indexes of seabream fed the experimental diets for 44 days.

	CTRL	PEL1	PEL10	WO1	WO10
Growth					
Initial body weight (g)	15.4 ± 3.5	15.6 ± 3.7	15.4 ± 3.4	15.5 ± 3.8	15.0 ± 3.3
Final body weight (g)	33 ± 8	34 ± 9	33 ±11	37 ±11	33 ± 9
Weight gain (%)	111 ± 3	119 ± 20	112 ± 5	144 ± 14	119 ± 14
Specific growth rate (% day^{-1})	0.69 ± 0.01	0.72 ± 0.08	0.70 ± 0.02	0.82 ± 0.05	0.72 ± 0.06
Voluntary feed intake (% BW day $^{-1}$)	2.26 ± 0.16	2.05 ± 0.19	2.17 ± 0.09	2.16 ± 0.13	2.18 ± 0.02
Daily growth index (BW day^{-1})	1.60 ± 0.05	1.70 ± 0.23	1.62 ± 0.06	1.96 ± 0.15	1.66 ± 0.15
Feed conversion rate	1.40 ± 0.11	1.22 ± 0.02	1.33 ± 0.09	1.14 ± 0.08	1.30 ± 0.12
Somatic indexes					
Hepatosomatic index	1.24 ± 0.07	1.30 ± 0.16	0.98 ± 0.26	1.15 ± 0.19	1.24 ± 0.04
Viscerosomatic index	8.67 ± 0.70	8.72 ± 1.62	8.28 ± 1.38	7.81 ± 0.54	8.74 ± 0.59

Values presented as mean ± standard deviation ($n = 60$ for initial and final body weight, $n = 9$ for the somatic indexes, and $n = 3$ for weight gain, specific growth rate, voluntary feed intake, daily growth index, and feed conversion ratio). No significant differences were registered, $p > 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil.

Some cases of mortality also occurred during the feeding trial; however, no significant differences between treatments were registered (Figure 5).

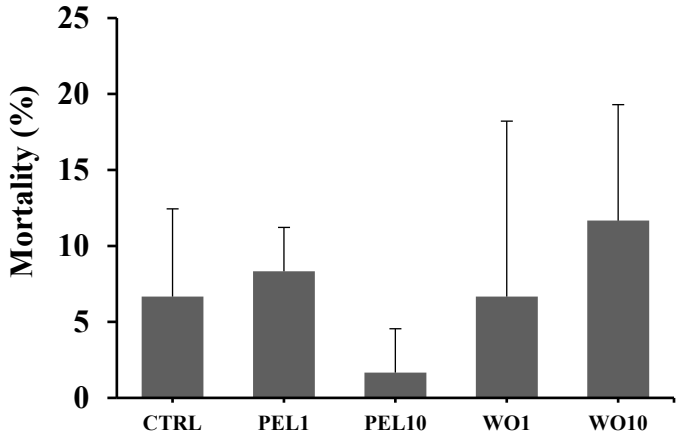


Figure 5. Mortality rates of seabream during the feeding trial (mean ± SD, n = 3). No significant differences were registered, $p > 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil.

3.2. Haematological Parameters

Few differences were recorded in the haematological profile of seabream fed the experimental diets (Table 5). Only white blood cells had a higher count in the control diet than PEL10 and WO1, while the haematocrit was increased in WO1 when compared to PEL1 and PEL10.

Table 5. Haematological profile of seabream fed the experimental diets for 44 days.

	CTRL	PEL1	PEL10	WO1	WO10
WBC ($\times 10^4$ μL^{-1})	9.5 ± 2.2 ^{a}	7.8 ± 1.5 ^{ab}	6.8 ± 1.3 ^{b}	6.9 ± 1.8 ^{b}	7.6 ± 1.9 ^{ab}
RBC ($\times 10^6$ μL^{-1})	2.58 ± 0.42	2.71 ± 0.70	2.23 ± 0.61	2.46 ± 0.48	2.35 ± 0.60
Ht (%)	29.6 ± 5.2 ^{ab}	26.0 ± 4.0 ^{a}	25.5 ± 6.1 ^{a}	33.2 ± 6.7 ^{b}	29.0 ± 1.5 ^{ab}
Hb (g dL^{-1})	1.39 ± 0.43	1.10 ± 0.11	1.04 ± 0.18	1.27 ± 0.31	1.04 ± 0.38
MCV (μm^3)	104 ± 47	101 ± 26	111 ± 18	129 ± 18	121 ± 25
MCH (pg $cell^{-1}$)	5.7 ± 2.4	4.2 ± 1.4	4.9 ± 1.2	4.9 ± 0.2	4.3 ± 1.8
MCHC (g 100 mL^{-1})	4.4 ± 0.3	4.1 ± 0.8	4.0 ± 0.4	3.9 ± 0.8	3.5 ± 1.0

Values presented as mean ± standard deviation (n = 9). Different letters in the same row stand for significant differences between dietary treatments, $p < 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil. WBC: white blood cells; RBC: red blood cells; Ht: haematocrit; Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration.

3.3. Oxidative Stress

Oxidative stress biomarkers are stated in Figure 6. The diets induced distinct responses in the antioxidant protection, seen by CAT, where the higher concentration of dried seaweed (PEL10) was responsible for higher activities than the control diet and lower seaweed inclusion (PEL1), and by tGSH, where the control diet had significantly lower activity than both waste oil diets.

3.4. Metabolic Parameters

Plasmatic metabolites showed differences in every parameter except for glucose (Figure 7). Cholesterol levels were elevated in PEL1 when compared to the CTRL diet and in WO10 against every diet excluding PEL1. WO1 displayed higher triglycerides concentration than CTRL, PEL1, and WO10. Regarding total lipids, WO1 was only superior to CTRL, WO10 was increased when compared to every diet but WO1.

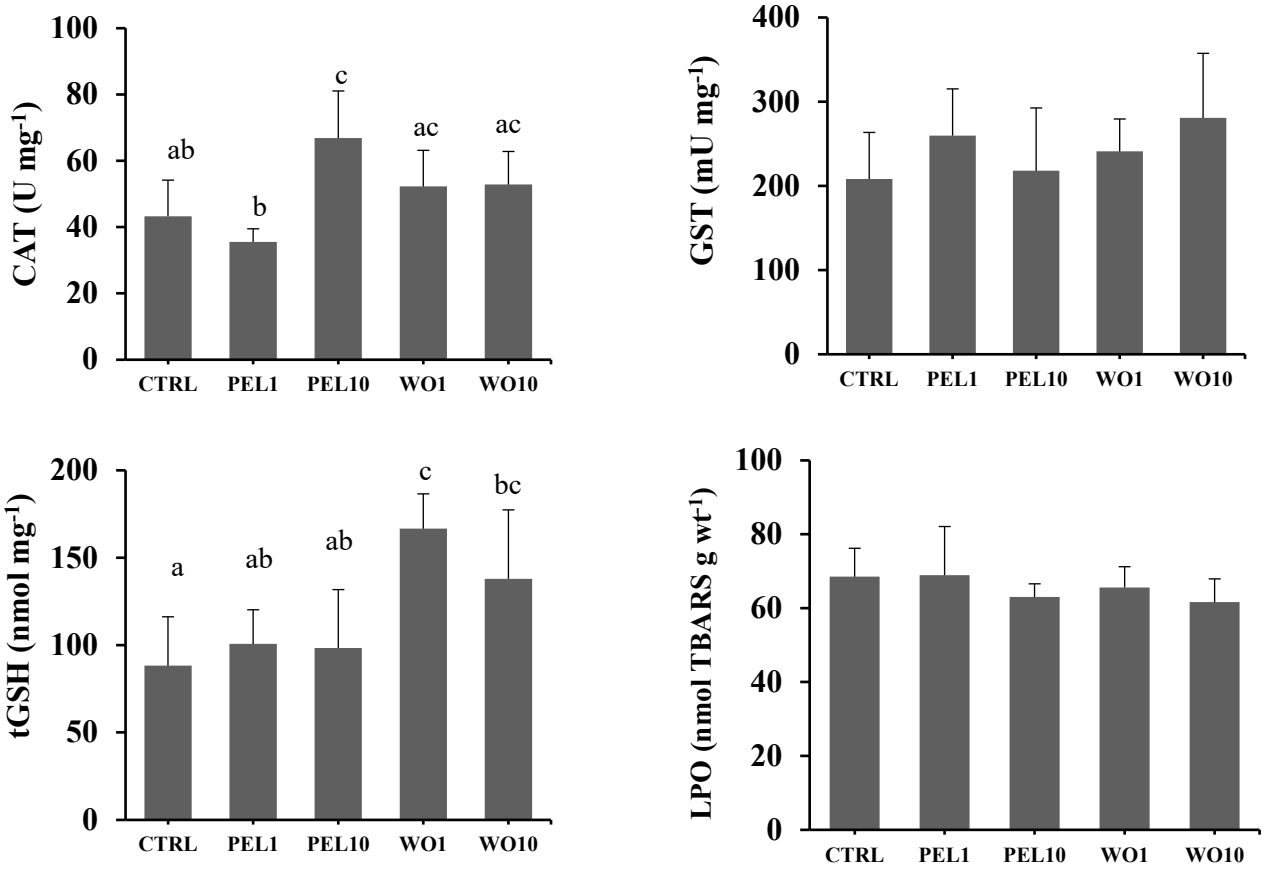


Figure 6. Oxidative stress biomarkers of seabream fed the experimental diets (mean ± SD, n = 9). Different letters stand for significant differences between dietary treatments, $p < 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil. CAT—catalase; GST—glutathione-S-transferase; tGSH—total glutathione; LPO—lipid peroxidation.

3.5. Intestine Histomorphology

The histomorphology analysis revealed differences in several parameters ($p < 0.05$) in both parts of the intestine of seabream when comparing the experimental diets (Table 6). In the anterior intestine, although there were no differences in the muscular externa of seabream fed with the experimental diets, when dividing the muscular layer into outer longitudinal and inner circular layers, there were significant differences. In the outer longitudinal layer, these differences were observed between fish that were fed CTRL, PEL1, and PEL10 diets when compared to WO10. On the other hand, in the inner circular layer, statistically significant differences were found between seabream fed the CTRL and PEL10 diets when compared to the remaining diets (PEL1, WO1, and WO10). The length of the intestinal villi in this part of the intestine increased significantly in fish fed the PEL1, PEL10, and WO1 diets compared to those fed the CTRL diet. Furthermore, in fish fed the PEL10 diet, there was a significant increase in villus length. In the WO10 diet, no statistically significant differences were observed in the villus length of the fish when compared to CTRL. Concerning WO1 diet, there were also no statistically significant differences when compared to PEL1 and PEL10. However, regarding villus width, a significant decrease was observed in seabream fed with diets PEL10, WO1, and WO10 when compared to diets CTRL and PEL1. On the WO10 diet, the intestinal villi of the fish had a significantly smaller width than the fish that were fed the PEL10 diet. The total number of GC increased significantly in seabream that was fed the PEL10 diet when compared to the other diets. When divided into neutral and acid GC, a significant increase in the number of these cells in the PEL10 diet was also observed. The number of neutral GC increased significantly in

fish fed the experimental diets (except the PEL1 diet) when compared to the CTRL diet. In addition, the number of acid GC increased in all experimental diets compared to the CTRL diet (Table 6). Histomorphological analysis of the posterior part of the intestine showed statistically significant differences in all parameters evaluated. The outer longitudinal layer was significantly thicker in seabream fed the PEL10 and WO10 diets when compared to fish fed with the other diets (CTRL, PEL1, and WO1). On the other hand, the thickness of this layer presented significantly higher values in the PEL10, WO1, and WO10 diets when compared to the CTRL and PEL1 diets. The thickness of the inner circular layer significantly decreased in fish fed the PEL1 diet, while in the PEL10 and WO10 diets fish showed a significant increase in the thickness of this muscle layer. In all seabream fed the experimental diets, an increase in the length of the intestinal villi was observed. However, in the PEL10 diet, the villus length was significantly greater than in the remaining diets, except for the PEL1 diet. Moreover, in fish fed the PEL10 diet, there was a significant increase in villus width. Nevertheless, it did not present significant differences when compared to the CTRL and WO10 diets. Concerning the total number of GC, a significant increase was observed in fish fed with the WO1 diet compared to fish fed with PEL1 and WO10. The number of neutral GC decreased significantly in fish fed experimental diets compared to fish fed the CTRL diet. The number of acid GC was significantly higher in fish fed with WO1 when compared to the other diets (Table 6).

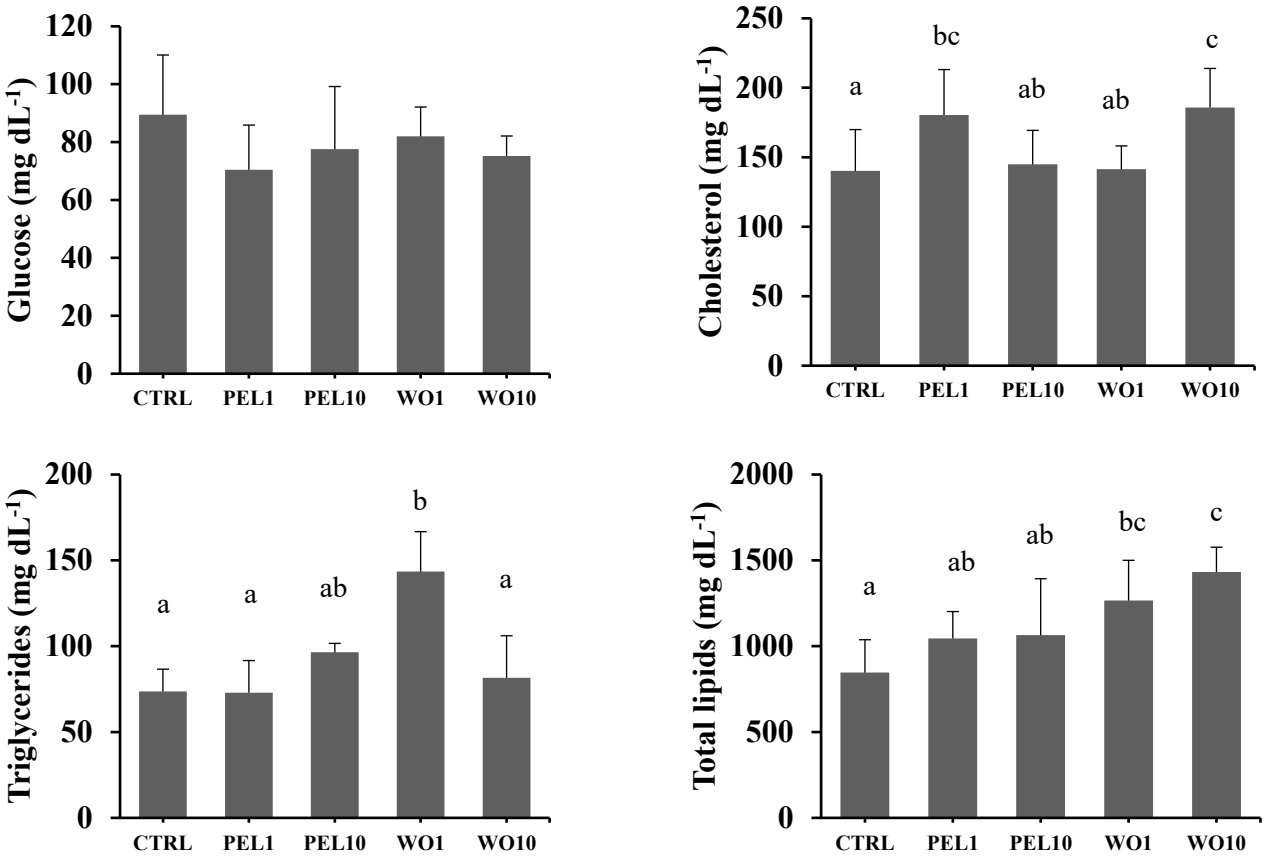


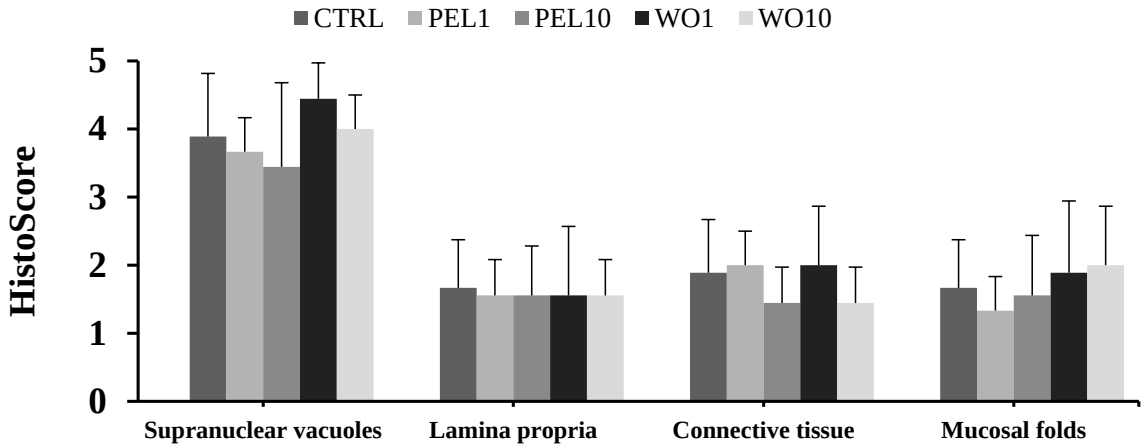
Figure 7. Plasma metabolites of seabream fed the experimental diets (mean ± SD, $n = 9$). Different letters stand for significant differences between dietary treatments, $p < 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil.

Table 6. Intestinal histomorphology of seabream (*S. aurata*) after feeding the experimental diets for 44 days.

	CTRL	PEL1	PEL10	WO1	WO10
Anterior intestine					
Muscularis externa (µm)	112 ± 16	117 ± 17	119 ± 29	116 ± 17	113 ± 17
Outer longitudinal layer (µm)	44 ± 13 ^{a}	44 ± 14 ^{a}	47 ± 18 ^{a}	42 ± 12 ^{ab}	41 ± 13 ^{b}
Inner circular layer (µm)	47 ± 12 ^{a}	43 ± 12 ^{b}	51 ± 21 ^{a}	44 ± 12 ^{b}	42 ± 12 ^{b}
Villus length (µm)	829 ± 242 ^{ab}	865 ± 203 ^{bd}	955 ± 283 ^{c}	878 ± 168 ^{cd}	827 ± 281 ^{a}
Villus width (µm)	183 ± 60 ^{a}	181 ± 64 ^{a}	153 ± 49 ^{b}	147 ± 46 ^{bc}	138 ± 41 ^{c}
Goblet cells (no. GC fold^{-1})	38 ± 18 ^{a}	44 ± 22 ^{a}	57 ± 29 ^{b}	42 ± 21 ^{a}	40 ± 22 ^{a}
Neutral GC (no. GC fold^{-1})	0.61 ± 0.88 ^{a}	0.14 ± 0.47 ^{b}	1.8 ± 1.9 ^{c}	1.4 ± 1.6 ^{c}	1.0 ± 1.3 ^{d}
Acid GC (no. GC fold^{-1})	36 ± 16 ^{a}	44 ± 22 ^{b}	56 ± 30 ^{c}	45 ± 15 ^{b}	39 ± 22 ^{a}
Posterior intestine					
Muscularis externa (µm)	133 ± 42 ^{ab}	119 ± 29 ^{a}	152 ± 37 ^{b}	120 ± 14 ^{a}	144 ± 28 ^{b}
Outer longitudinal layer (µm)	52 ± 21 ^{a}	53 ± 18 ^{a}	60 ± 20 ^{b}	60 ± 19 ^{b}	62 ± 24 ^{b}
Inner circular layer (µm)	44 ± 14 ^{a}	40 ± 12 ^{b}	48 ± 17 ^{c}	45 ± 13 ^{ac}	47 ± 14 ^{c}
Villus length (µm)	728 ± 192 ^{a}	760 ± 187 ^{ab}	810 ± 218 ^{b}	762 ± 290 ^{a}	697 ± 174 ^{a}
Villus width (µm)	168 ± 60 ^{ab}	158 ± 54 ^{a}	182 ± 65 ^{b}	165 ± 55 ^{a}	169 ± 48 ^{b}
Goblet cells (no. GC fold^{-1})	37 ± 18 ^{ab}	34 ± 17 ^{ac}	37 ± 18 ^{ab}	41 ± 22 ^{b}	30 ± 13 ^{c}
Neutral GC (no. GC fold^{-1})	0.63 ± 0.89 ^{a}	0.00 ± 0.00 ^{b}	0.11 ± 0.36 ^{c}	0.17 ± 0.48 ^{c}	0.20 ± 0.56 ^{c}
Acid GC (no. GC fold^{-1})	35 ± 17 ^{a}	34 ± 17 ^{a}	37 ± 18 ^{ab}	42 ± 22 ^{b}	31 ± 12 ^{a}

Values expressed as mean ±SD (n = 9), different letters in each line stand for statistically significant differences, $p < 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO1%—1% algae waste oil; WO 10%—10% algae waste oil.

The results obtained from the scores to assess the inflammatory changes in the intestinal tissue in both the anterior and posterior parts of the intestine indicated a normal morphology in all diets. However, in the supranuclear vacuoles, a high score was obtained in both parts of the intestine. Furthermore, in the posterior intestine, statistically significant differences were observed in the connective tissue between the CTRL and PEL1 diets when compared to the PEL10 diet (Figure 8).



(A)

Figure 8. *Cont.*

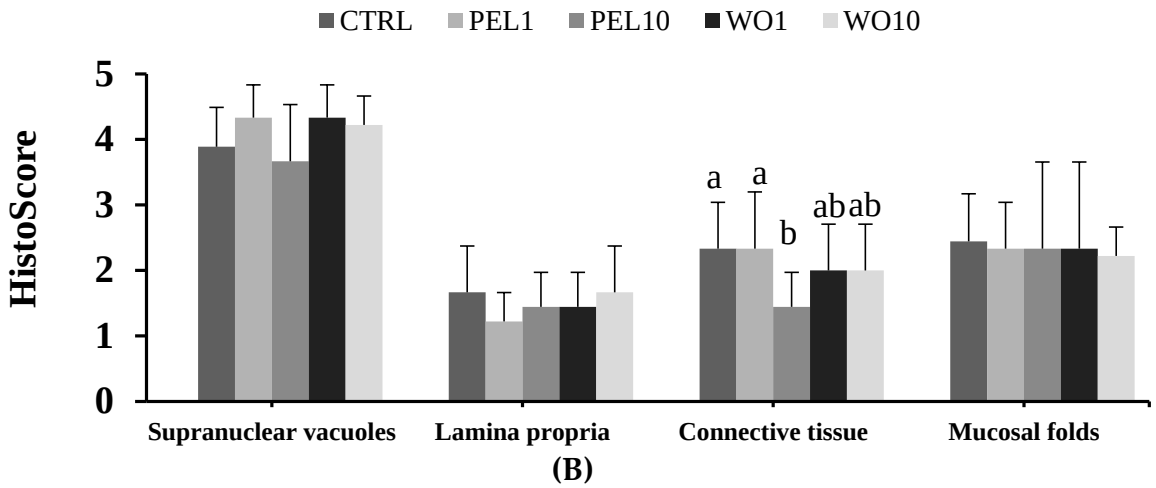


Figure 8. Score of intestinal morphology of seabream (*S. aurata*) feed with experimental diets for 44 days. (**A**) Anterior intestine; (**B**) posterior intestine. Intestinal sections from nine fish per diet were scored according to criteria: supranuclear vacuoles; lamina propria; connective tissue; mucosal folds. Normal morphology is associated with a score of "1–2" while intestinal tissue damage is represented by the score "5". Different letters represent statistically significant differences, $p < 0.05$. CTRL—control feed; PEL 1%—1% algae powder; PEL 10%—10% algae powder; WO 1%—1% algae waste oil; WO 10%—10% algae waste oil.

4. Discussion

Algae have been studied as alternative ingredients to fish meals in aquaculture. Moreover, the concept of the circular economy comes with the opportunity to reuse waste from the food industry, which provides a new source of economically viable and environmentally sustainable ingredients. In the present study, the dried powder macroalgae *P. canaliculata* and algae waste obtained after sunflower oil supplementation were incorporated into aquafeeds for gilthead seabream. However, it was found that the effect of supplementation with PEL and WO seems to be different, which can be explained by the formulation of the diets. The PEL diet uses powder-dried seaweed and the WO diet results from the incorporation of waste from sunflower oil with seaweed extract. The algae components present in diets are different. In a study carried out by Valente et al. [4], after physical processing of *Gracilaria gracilis*, it was found that the ability of fish to digest diets rich in seaweed was vastly improved without significantly affecting fish growth. In this study, experimental diets did not affect the growth performance, feed utilisation, and somatic indexes. Nevertheless, the WO1 diet demonstrated a tendency towards a better growth performance of the fish compared to other diets, which can be explained by the fact that it has a higher carbohydrate content.

In aquaculture, the utilisation of haematological parameters is an important tool for disease diagnosis and evaluation of the nutritional status of the fish [41]. The white blood cells (WBC) are an important parameter to evaluate the health status in fish because they are involved in immune responses and reflect the ability of the organism to fight infection [42]. In fish fed with CTRL, the WBC count was higher than PEL10 and WO1 [42]. Fish that have a higher level of WBC will be able to fight infection more effectively [42]. Moreover, the value of the haematocrit was superior in fish feed with WO1 than with PEL 1 and PEL10. There is a relationship between the increase of fish size and increased red blood cell (RBC), haematocrit (Ht), and haemoglobin (Hb) values [43], which could justify the superior value of the Ht for diet WO1. However, in this study, there is no correlation between the fish size and RBC and Hb. Furthermore, Passos et al. [8] verified this and suggested that there is a pattern of haematological influence associated with algae inclusion.

Antioxidant enzymes, such as CAT, are a defence mechanism against the imbalance of the oxidative state and are considered nonspecific immune biomarkers in fish [8]. The

higher activity of CAT showed their importance in protection against ROS formation. In stressful conditions, such as hypoxia, gilthead seabream fed with *Gracilaria* diets exhibited lower CAT activity [44]. In the present study, the antioxidant protection exhibited different responses in fish fed with experimental diets. CAT showed higher activity in seabream fed with PEL10 than CTRL, indicating that these diets could protect fish against ROS formation. Glutathione is involved in the reduction of oxidative damage and their lower levels occur in stressful situations [45]. In fish fed with both WO diets, tGSH had significantly higher activity than diet CTRL. Moreover, the inexistence of differences in lipid peroxidation between treatments suggests that the different diets did not cause oxidative imbalances. However, the dietary inclusions seemed to induce a higher protection level against hypothetical stress factors. This protective effect may be related to the high carotenoid constitution of *P. canaliculata* [29], which may be passed on through the supplemented aquafeed. The two different experimental diets may explain the higher activity of CAT in the PEL10 diet due to the carotenoid content and, in both WO diets, tGSH there is a significantly higher activity, possibly due to the seaweed extract present in the residue.

In our study, the glucose levels in the blood of seabream, which are a stress indicator, were similar among the experimental groups indicating that the diets did not cause stress. Triglyceride and cholesterol levels should be monitored because they are important markers for health. In this study, cholesterol, triglycerides, and total lipids in the seabream blood were affected by the different diets, being higher in diets algae waste. These results are not in agreement with Vizcaíno et al. [46] who showed a decrease in plasma lipid content and glucose levels in seabream fed diets supplemented with *Ulva* and *Gracilaria* sp. at 25%. According to the study by Guerreiro et al. [47], levels of glucose and cholesterol were lower when gilthead seabream was fed experimental diets with *Ulva*, *Chondrus* sp., and both algae. However, plasmatic triglycerides levels were higher in fish fed with *Ulva* and *Chondrus* sp. separately. In a study by Basto-Silva et al. [48], the plasma levels of cholesterol and total lipids were higher in gilthead seabream fed with fishmeal than plant feedstuffs. On the other hand, plasma triglycerides showed lower levels in fish fed diets with less levels of protein and higher carbohydrate content. The results present in a study by Valente et al. [4] evidence the metabolic capacity induced by the dietary inclusion of *Gracilaria* on European seabass (*Dicentrarchus labrax*), through a significant reduction of plasma cholesterol in fish fed diet free nucleotides and a reduction in triglycerides in fish fed with *Gracilaria* processed, phythogenic compounds, and alginate oligosaccharide diets. On the other hand, in gilthead seabream, dietary supplementation of *Gracilaria* by-products did not show changes in plasma levels of glucose, triglycerides, and cholesterol 24 h after feeding, suggesting that there are no variations in the digestibility of carbohydrates and lipids [49].

The inclusion of macroalgae *P. canaliculata* and the algae waste in the experimental diets for seabream affected the histomorphology of the anterior and posterior parts of the intestine. The muscle layers (muscularis externa, outer longitudinal, and inner circular layers) in both parts of the intestine showed greater thickness in seabream fed with PEL10 when compared to the other diets. Moreover, in the studies by Passos et al. [9], with the inclusion of *Gracilaria gracilis* in the diet of European seabass (*D. labrax*), and Batista et al. [50], with the inclusion of two probiotics in the diet of Senegalese sole (*Solea senegalensis*), statistically significant differences were found in the thickness of the muscle layers. In both intestinal sections, significantly longer villi length was observed in fish fed with diets PEL10, WO1, and PEL1 while in fish fed with diet WO10, the villi length was significantly shorter. Regarding villi width, values were variable in both parts of the intestine. In the anterior intestine, there was a significant decrease in all experimental diets (except the PEL1 diet) when compared to the CTRL diet. In the posterior intestine, villi width was significantly bigger in seabream fed with PEL10 and WO10 diets. Several studies have been evaluated the effects of macroalgae incorporation in the feeding of several fish species produced in aquaculture. Histomorphological analysis of the intestine has revealed significant alterations in the length and width of the intestinal villi. Some studies have observed a decrease

in villi length and width which is related to a decrease in the villi absorption area [9,51,52]. Therefore, the digestive and absorptive processes are negatively affected which could be a factor in the reduction of nutrient intake by fish [52]. However, some studies demonstrate an increase in the length and width of the intestinal villi of fish after the feeding period with experimental diets containing various levels of macroalgae inclusion [4,7,12]. Several factors are associated with the effects that seaweed has on intestinal histomorphology, such as fish and seaweed species, diet processing, and the presence of other ingredients [47]. In the present study, it is possible to observe differences in the histomorphology of the intestine between the experimental diets that could be explained by the constituents and inclusion levels of dry macroalgae and waste oil of the experimental diets. In the intestine of many fish, there are goblet cells (GCs) that synthesise neutral and sulphated mucins and sialomucins (with sialic acid) [53]. GCs are important active components in the host's defence being involved in the immune response, in addition to their primary function consisting of maintaining the integrity of the intestinal barrier through the production of mucus [17]. In the present study, after feeding seabream with the experimental diets, in both parts of the intestine, the distribution of GC was found variable. In the anterior intestine of seabream fed the PEL10 diet, there was a significant increase in the total number of GC as in the number of AGC and NGC. On the other hand, in the posterior intestine, fish-fed diet WO1 had a significantly higher total number of GC and AGC when compared to the other diets. Furthermore, the number of NGC in the posterior intestine was significantly higher in the CTRL diet when compared to the remaining experimental diets. In addition, in the posterior intestine of fish fed with WO10 diet, there was a significant decrease in the total number of GC as in the number of AGC. Passos et al. [9] verified a significant increase in the number of GC in fish fed the experimental diets; on the other hand, Valente et al. [4] observed differences in the number of AGC in the anterior intestine of fish after feeding with the different diets. According to Batista et al. [39], when there is an increase in the total number of GC from the anterior to the posterior intestine, it indicates an increase in mucus production. In this study, the total number of GC from the anterior to the posterior intestine did not increase. On the contrary, there was a decrease in diets PEL1, PEL10, and WO10, while in the CTRL and WO1 diets, the GC values were similar in both parts of the intestine. These differences can be explained by the density of GC that could be influenced by several factors, such as nutritional, physiological, immunological, and microbiological [54]. In this study, another methodology was used to assess inflammatory changes in the intestinal tissue, and it was found that in both parts of the intestine the tissue had a normal morphology in all experimental diets. Although in the posterior intestine, the connective tissue presented a greater thickness in the CTRL and PEL1 diets when compared to the PEL10 diet. The study by Guerreiro et al. [47] showed that the experimental diets with macroalgae (*Chondrus crispus* and *Ulva lactuca*) did not affect the integrity of the intestinal tissue of seabream, and no signs of hypertrophy or hyperplasia of GC were observed, the number of intraepithelial leukocytes was similar, the thickness of the lamina propria was thin, and width of the submucosa were similar in all treatments. In this study, in the criteria, "supranuclear vacuoles" scores were higher than 1–2, which corresponds to the absence or presence of small supranuclear vacuoles in the intestinal villi. Therefore, in the intestine of seabream that was fed the CTRL diet, the absence or presence of small supranuclear vacuoles was observed, as in the experimental diets. One of the inflammatory reactions associated with several morphological changes is the loss of supranuclear vacuoles in the intestinal enterocytes [40]. Castro et al. [55] studied the effects of vegetable oils and carbohydrates on the histomorphology of the intestine of seabream and found changes in the normal structure of the enterocytes, with loss of the typical supranuclear vacuolisation in the anterior intestine, when the diet contained vegetable oils and carbohydrates.

5. Conclusions

In conclusion, under the experimental conditions of this study, no detrimental effects of including *Pelvetia* powder or algae waste oil in the diets for gilthead juveniles were

observed, although oxidative stress parameters suggest that the diets did not cause oxidative imbalances and seemed to induce higher protection against stress factors. In addition, the diets induced slight changes in intestine histomorphology but were not significantly negative. Thus, it is possible to use *Pelvetia* seaweed and waste oil as supplementation in diets for gilthead seabream. In particular, the WO1 diet showed potential to be an aquafeed according to growth performance, some haematological parameters, total glutathione, intestinal villi length, and the number of total and acid goblet cells. Nevertheless, further studies will be needed to verify that there are no changes in fish metabolism.

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Data Availability Statement: Data will be available on request to the authors.

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