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Research Article

Application of Fermented Brewer's Spent Grain Extract in Plant-Based Diets Improves *Pre-* and *Post-mortem* Oxidative Status of European Seabass (*Dicentrarchus labrax*)

H. Fernandes[1,2] **C. Castro**[1,2] **D. Filipe**[1,2] **P. Ferreira**[2] **J. M. Salgado**[3,4] **F. Moyano**[5] **A. Oliva-Teles**[1,2] **I. Belo**[3] **and H. Peres**[1,2]

[1]*Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre Ed. FC4, 4169-007 Porto, Portugal*
[2]*CIMAR/CIIMAR-Centro Interdisciplinar Marinha e Ambiental, Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, 4450-208 Matosinhos, Portugal*
[3]*CEB-Centre of Biological Engineering of University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[4]*Industrial Biotechnology and Environmental Engineering Group "BiotecnIA", Chemical Engineering Department, University of Vigo (Campus Ourense), As Lagoas s/n, Ourense 32004, Spain*
[5]*Dpto. Biología y Geología, CEIMAR, University of Almería, Almería, Spain*

Correspondence should be addressed to H. Fernandes; helenafernandes012@gmail.com

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In a previous study, it was shown that dietary supplementation with a carbohydrases and antioxidant-enriched extract (BSG-extract) produced by solid-state fermentation of brewer's spent grain (BSG) improved nutrients digestibility, feed, and protein utilization of European seabass juveniles. This work aims to investigate the effect of this BSG-extract on liver and intestine oxidative status and muscle lipid peroxidation (LPO) of European seabass. A plant-based diet (55.4% plant feedstuffs; PF) was used as a control. Four test diets were formulated by supplementing the control diet with the BSG-extract (0.4 or 0.8% diet), added directly to the diet (BSG4 and BSG8 diets), or used to pre-treat the PF mixture of the control diet (PreBSG4 and PreBSG8 diets). European seabass juveniles (IBW 22 ± 1 g) were fed the experimental diets for 66 days until apparent satiety. In fish fed BSG-extract diets, compared to the control diet, intestinal LPO was reduced up to 29-40% ($p < 0.05$); GPX, GR, and G6PD activities were increased compared to the control ($p < 0.05$). In the liver, LPO was similar among diets, but except for catalase and G6PD, fish fed BSG-extract diets had higher antioxidant enzyme activities than the control ($p < 0.05$). Except for the liver SOD activity, hepatic and intestinal antioxidant capacity was not affected by the dietary BSG-extract incorporation mode ($p > 0.05$). After 72 h of storage at 4°C, muscle LPO was higher than at time 0 h. Compared to the control, BSG-extract reduced muscle LPO levels up to 30% at time 0 h in fish fed diets BSG4, BSG8, and PreBSG8 ($p < 0.008$). Results indicate that dietary supplementation with BSG-extract increased the antioxidant level of diets, improved the intestine antioxidant capacity, and decreased LPO in European seabass juveniles' muscle.

1. Introduction

Aquaculture is an industrial sector in continuous expansion whose sustainable growth depends on searching for alternative, economically advantageous, and sustainable feedstuffs that overcome the present limitations of using fisheries-based ingredients [1]. Plant feedstuffs (PF) are readily available alternatives, but most have antinutritional factors, such as phytic acid, protein inhibitors, and indigestible carbohydrates, mainly non-starch polysaccharides (NSP) [2]. Dietary inclusion of NSP-rich PF is challenging, decreasing diet digestibility and its nutritional value for fish [3, 4].

Dietary supplementation with exogenous carbohydrases has been used to overcome NSP adverse effects and increase plant feedstuffs nutritional value. This is a common practice in poultry and pig production [5]. In fish, diet

supplementation with carbohydrases has provided promising results on growth [6–9], feed utilization [6, 10, 11], and health and welfare [4, 10, 12]. The global market of carbohydrases, mainly constituted by xylanases and glucanases, is rapidly increasing, but their high costs, primarily associated with high purity substrates, still limit their application [4]. Using waste biomass, such as lignocellulosic by-products, as a low-cost and renewable alternative to these traditional substrates may contribute to the sustainability of this industry [13].

Valorization and reuse of waste biomass to produce added-value compounds to improve the nutritional value of ingredients is an environmentally sound approach, following the circular economy concept. This is the case of brewer's spent grain (BSG), the most generated by-product from the brewery industry [14], totalizing 38.8 million tons in 2018 (wet BSG; [15]). The BSG's perishable nature hampers its transport and storage, being frequently used as cattle feed [15]. BSG is a lignocellulosic by-product with high moisture and sugar contents, mainly constituted of seeds, pericarp, and peel layers, being an excellent substrate for enzyme production by solid-state fermentation (SSF). SSF is a cost-effective and eco-friendly biotechnological process that may be used to produce a wide range of enzymes with high hydrolytic activity and thermostability [16]. Substrates used for SSF are mainly lignocellulosic agro-industrial by-products since they promote the growth of filamentous *Ascomycetes* fungi [17]. Besides enzymes production, phenolic compounds with antioxidant activity are also released from the lignocellulosic matrix of substrates during SSF [18]. In fish, phenolic compounds may prevent or delay lipid oxidation, increasing fish resistance under stressful conditions [19] and increasing shelf-life [20].

Organisms originate reactive oxygen species (ROS) during aerobic metabolic processes. The balance between ROS generation and elimination is ensured by conjugated endogenous and exogenous antioxidant defense mechanisms [21]. Endogenous mechanisms include non-enzymatic and enzymatic systems [22], and exogenous mechanisms include bioactive compounds, such as vitamins, amino acids, pigments, and phenolic compounds [23]. Diet, environmental, and stressful factors may compromise oxidative status, potentially affecting fish redox status and welfare [21, 24]. There is a growing interest in dietary supplementation with functional compounds as a strategy to improve fish oxidative status [23]. Phenolic compounds are safe and potent antioxidant active molecules, interacting with ROS and counteracting cellular damage caused by excessive ROS formation [25]. The use of natural phenolic compounds in aquafeeds is increasing, constituting promising and functional alternatives to synthetic antioxidants [20, 25].

Dietary carbohydrases supplementation may also contribute to modulating oxidative status by mitigating the adverse effects of dietary NSP on gut physiology, morphology, and microbiota composition [3]. However, although documented in terrestrial animals, the effect of exogenous dietary enzymes on fish oxidative status has been scarcely studied [26–28]. For instance, in common carp fry (*Cyprinus carpio*), dietary supplementation with a multi-enzymatic cocktail (Hostazyme X, Norel; containing xylanase, amylase, cellulase, α-galactosidase, β-glucanase, pectinase, lipase, and protease) increased hepatic antioxidant enzyme activity and resistance to oxidative damage [29].

Previously, it was shown that SSF of BSG with *Aspergillus ibericus* led to the production of an extract rich in carbohydrases, with high activity and thermostability, and high antioxidant activity [30]. Supplementation of a practical diet with this extract increased energy and cellulose digestibility [31] and improved feed utilization efficiency of European seabass (*Dicentrarchus labrax*) [32]. The present study aimed to evaluate the effect of dietary supplementation with this extract on the *pre-* and *post*mortem oxidative status of European seabass.

2. Materials and Methods

This study was approved by the ORBEA Animal Welfare Committee of CIIMAR (ORBEA; reference ORBEA_CIIMAR_27_2019) in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013) and conducted by certified scientists (functions a, b, c, and d defined in article 23 of European Union Directive 2010/63).

2.1. Solid-State Fermentation (SSF) of Brewer's Spent Grain. Brewer's spent grain (BSG) was supplied by Unicer-Bebidas de Portugal, S.A. (Matosinhos, Portugal). The fungus used in SSF was *Aspergillus ibericus* (MUM 03.49), provided by Micoteca of the University of Minho (Braga, Portugal).

SSF was performed in plastic trays measuring $43 \times 33 \times 7$ cm. Each tray was filled with 400 g of dry BSG, and moisture was adjusted to 75% with distilled water before sterilization at 121°C for 15 min. Then, 2×10^6 *A. ibericus* spores per gram of dry BSG were added to each tray and incubated for 7 days at 25 °C. After the incubation, an aqueous extraction of the fermented BSG was carried out (5 : 1 v/w; at room temperature, 30 min, constant stirring). The obtained extract was filtrated through a fine-mesh net, centrifuged (4000 *g*, 15 min, 4°C), and the supernatant was collected and lyophilized (-51 ± 1 °C; 30 to 50 mTorr). The lyophilized extract (named BSG-extract) was stored at 4 °C in darkness until use. The lyophilized BSG-extract contained 94.5 mg GAE g^{-1} phenolic compounds, 131.1 μmol TE g^{-1} antioxidant activity, 1178 U cellulase, and 8317 U xylanase g^{-1}.

2.2. Experimental Diets. Experimental diets were formulated and manufactured as described in Fernandes et al. [32]. A control diet was formulated with 50% crude protein, 18% crude lipids, and with high incorporation of PF (55.4%). Two test diets were formulated as the control diet but included 0.4% and 0.8% of the lyophilized BSG-extract, corresponding to 4000 U and 8000 U cellulase kg^{-1} diet, respectively (diets BSG4 and BSG8). Two other diets were formulated as the control diet, but the dietary PF mixture was previously treated with 0.4% and 0.8% of BSG-extract (diets PreBSG4 and PreBSG8). For that purpose, 0.4% or 0.8% lyophilized BSG-extract were added to the PF mixtures,

mixed with sodium citrate buffer (pH 4.8, 0.1 M) at a concentration of 1 : 0.4 (PF mixture: buffer with BSG-extract), and incubated for 4 h at 45 ˚C, with manual stirring each hour to ensure homogeneity of the reaction. Then, the hydrolysis was stopped by placing the mixture in a cold chamber at -20 ˚C, where it was kept until diets production.

All dietary ingredients were weighed, finely grounded, well-mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3-mm die. The diets were dried (24 h, 60 ˚C), frizzed in plastic bags, and stored at -20 ˚C until their use.

The ingredients and proximate composition of the diets are presented in Table 1. The proximate composition of diets was analyzed following the standard procedures of the Association of Official Analytical Chemists [33]. Dietary starch content was determined according to Beutler [34] and energy density by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

2.3. Growth Trial and Sample Collection. The growth trial was performed as described in Fernandes et al. [32]. Briefly, it was carried out in the experimental facilities of CIIMAR (Matosinhos, Portugal). European seabass juveniles were acquired from a certified hatchery (SONRÍONANSA; Cantabria, Spain). Before entering the CIIMAR bioterium, fish were held in quarantine for 15 days. During this time, fish were hand-fed with a commercial diet (NEO diet, Aquasoja; Sorgal, SA, Portugal). After this period, fish were transferred to the experimental system and acclimatized to the system for 30 days.

The experimental system consisted of a thermoregulated recirculating water system (RAS) with 15 fiberglass tanks with 100 l water capacity and a continuous filtered seawater flow. At the beginning of the growth trial, groups of 18 fish with an initial body weight of 21.5 ± 1 g were established and randomly distributed to each tank. Diets were tested in triplicate and randomly assigned to triplicate tanks. Fish were hand-fed twice a day, 6 days per week, for 66 days. Throughout the trial, water quality was routinely checked. Water temperature was maintained at 23 ± 1 ˚C, salinity at 35 ± 1 g l^{-1}, nitrogenous compounds below 0.02 mg l^{-1}, dissolved oxygen above 7.5 mg l^{-1}, and photoperiod controlled to 12 h light and 12 h dark.

At the end of the trial, 3 fish per tank were randomly sampled and killed by a sharp blow to the head and immediately eviscerated in an ice-cooled tray. The intestine and liver were excised and frozen in liquid nitrogen and stored at -80 ˚C until measurement of antioxidant enzyme activity.

The right and left fillets were also removed, and the epaxial part of each fillet was sectioned (i.e., the lateral dorsal part above the lateral line) to assess lipid peroxidation during shelf-life. The right fillet was immediately frozen in liquid nitrogen. The left fillet was placed in individual trays and stored for 72 h in a fridge at 4 ˚C. At the end of this period, each fillet was frozen at -80 ˚C.

2.4. Carbohydrases Activity. Xylanase (endo-1,4-β-xylanase) and cellulase (endo-1,4-β-glucanase) activities in the lyophilized BSG-extract and experimental diets were determined as described by Fernandes et al. [35]. Briefly, for xylanase activity, the diluted samples were mixed with beechwood xylan (1% in sodium citrate buffer 0.05 N, pH 4.8) and incubated for 15 min. For cellulase activity, carboxymethyl cellulose (2% in sodium citrate buffer 0.05 N, pH 4.8) was used as a substrate and incubated for 30 min. Then, 3,5-dinitrosalicylic acid (DNS reagent) was added to each mixture and placed at 100 ˚C for 5 min. After cooling at room temperature, distilled water was added, and the absorbance was read at 540 nm. Standard curves using glucose or xylose in sodium citrate buffer (0.05 N, pH 4.8) were used, respectively, for cellulase and xylanase activities. An international unit (IU) of enzymatic activity was defined as the amount of enzyme needed to release 1 μmol of glucose or xylose per minute for cellulase or xylanase, respectively, under the standard assay conditions. Results are expressed in units per gram of dry BSG-extract (U g^{-1}).

2.5. Reducing Sugars. Free reducing sugars content (glucose and xylose) of experimental diets were determined using the DNS method [36] after an aqueous extraction (1 : 5; w/v).

2.6. Phenolic Compounds and Antioxidant Activity. Total phenolics content of BSG-extract and experimental diets was determined by the Folin-Ciocalteu, as described by Filipe et al. [37]. A calibration curve was performed using gallic acid as standard, and total phenolic compounds were expressed as mg gallic acid equivalents (GAE) per gram of sample. Orto-diphenols and flavonoids were assessed in the experimental diets. Orto-diphenols were determined as described by Gouvinhas et al. [38] using gallic acid as standard, and results were expressed as mg gallic acid equivalents (GAE) per gram of diet. Flavonoid content was assessed as described by Gouvinhas et al. [38], using quercetin as the standard. Results are expressed in mg of quercetin equivalents (QE) per gram of diet.

Antioxidant activity was measured in the BSG-extract and experimental diets using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay following the method described by Fernandes et al. [35]. Antioxidant activity was expressed in micromoles of Trolox equivalents (TE) per gram of sample.

2.7. Oxidative Enzyme Activity. Intestine, liver, and fillet samples were homogenized (1 : 4, w/v) in ice-cold 100 mM Tris-HCl buffer with 0.1% Triton X-100 and 0.1 mM EDTA, pH 7.8. The resultant homogenates were centrifuged at 30,000 g for 30 min at 4 ˚C, and supernatant aliquots were stored at -80 ˚C until analysis.

Antioxidant enzyme activity was assessed in the liver and the intestine as described by Castro et al. [39]. Briefly, superoxide dismutase (SOD; EC1.15.1.1) was measured by the ferricytochrome C method at 550 nm, and xanthine/xanthine oxidase was used as the source of superoxide radical. Catalase (EC 1.11.1.6) was determined by measuring the decrease of H_2O_2 concentration at 240 nm. Glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined by measuring NADPH consumption when GR reduces the oxidized

Table 1: Ingredients and proximal composition of the experimental diets.

	Control	*BSG4*	*BSG8*	*PreBSG4*	*PreBSG8*
Ingredients (%)					
Fish meal[a]	20	20	20	20	20
Soluble fish-protein concentrate[b]	5	5	5	5	5
Plant feedstuffs mixture[c]	55.4	55.4	55.4	—	—
Pretreated plant feedstuffs mixture	—	—	—	55.4	55.4
Hemoglobin[d]	3	3	3	3	3
Hydrolyzed shrimp[e]	1.2	1.2	1.2	1.2	1.2
Fish oil[f]	11.2	11.2	11.2	11.2	11.2
Vitamin premix[g]	1	1	1	1	1
Mineral premix[h]	1	1	1	1	1
Choline chloride (50%)[i]	0.5	0.5	0.5	0.5	0.5
Betaine[j]	0.2	0.2	0.2	0.2	0.2
Binder[k]	1	1	1	1	1
Taurine[l]	0.5	0.5	0.5	0.5	0.5
BSG-extract directly added to the diets	—	0.34	0.68	—	—
BSG-extract used for pretreatment∗	—	—	—	0.19	0.38
Proximate composition (% dry matter)					
Dry matter (%)	94.5	95.2	96.1	95	94.5
Crude protein	49.2	49.2	48.8	47.6	47.1
Crude lipid	17.2	17.0	17.9	19.7	18.5
Ash	7.5	7.6	7.6	7.8	7.8
NFE + crude fiber[m]	24.9	26.3	25.7	26.6	27.7
Starch	9.4	10.2	9.3	9.5	7.0
Energy (kJ g^{-1})	25.0	26.3	26.3	26.3	26.5

Per g of lyophilized BSG-extract: cellulase: 1178 U; xylanase: 8317 U; phenolic compounds: 94.5 mg GAE; antioxidant activity: 131.1 μmol TE. ∗Quantity of BSG-extract used for plant feedstuffs pretreatment = % plant feedstuffs (55.4% of the diet) × % BSG-extract level of BSG4 and BSG8 diets[a]CP: 79.7; CL: 10.5; Pesquera Centinela, steam dried LT, Chile.[b]CP: 80.2%; CL: 15.4%; Sopropeche, France. [c]Plant feedstuffs mixture (Sorgal, S. A, Portugal): including 21.8% wheat gluten (CP: 80%; CL: 1.74); 20.6% soy protein concentrate (CP: 48.57%; CL: 2.52%); 15.3% sunflower (CP: 40.4%; CL: 1.0%), 18.1% rice bran (CP: 14.20%; CL: 13.20%), 14.4% repressed (CP: 41.14%; CL: 5.80%), and 9.7% whole-wheat (CP: 12.23%; CL: 3.19%).[d] APC Europe S. A. (AP310P). [e]CP: 69.8%; CL: 12.1%; Sorgal, S.A. Ovar, Portugal. [f] Fish oil Sorgal, S.A. Ovar, Portugal. [g]Vitamin premix (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.[h]Minerals premix (mg kg^{-1} diet): cobalt sulfate, 1.91; copper sulfate, 19.6; iron sulfate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet). [i]Choline chloride (50%) Sorgal, S.A. Ovar, Portugal. [j]Sorgal, S.A. Ovar, Portugal. [k]AQUACUBE. Agil, UK. [l]Sorgal, S.A. Ovar, Portugal. [m]Nitrogen-free extract (NFE) + crude fiber = 100 − (crude protein + crude lipid + ash).

glutathione (GSSG) generated by GPX to reduce glutathione (GSH). Glutathione reductase (GR; EC 1.6.4.2) activity was assayed by monitoring the oxidation of NADPH-dependent reduction of GSSG to reduced GS. Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) was determined following the reduction of $NADP^+$, using a reaction mixture with glucose-6-phosphate. GR, GPX, and G6PD were assayed at 340 nm.

For SOD, one unit of activity corresponds to the amount of enzyme necessary to produce 50% of inhibition of ferricytochrome C reduction rate. For the other enzymes, one unit of enzymatic activity was defined as the quantity of enzyme required to transform 1 μmol of substrate min^{-1} under the assay condition. Enzymatic activities were expressed as units (catalase) or milliunits (G6PD, GR, and GPX) per mg of soluble protein. Soluble protein was determined according to Bradford [40] using bovine serum albumin as standard.

2.8. Lipid Peroxidation. Lipid peroxidation (LPO) of the muscle, liver, and intestine was assessed using malondialdehyde (MDA) as a marker. Supernatants were mixed with a solution containing 15% (w/v) TCA (Sigma), 0.375% (w/v) thiobarbituric acid (Sigma), 80% (v/v) HCl 0.25 N, and 0.01% (w/v) butylated hydroxytoluene (Sigma). The mixture was heated for 15 min, cooled at room temperature, and centrifuged at 1500 *g* for 10 min, and the absorbance was read at 535 nm. MDA concentration was expressed as mg MDA kg^{-1} wet tissue in muscle and nmols MDA g^{-1} in the intestine and liver, calculated from a calibration curve.

2.9. Statistical Analysis. All data were checked for normality and homogeneity of variances and were transformed when needed. The effect of the experimental diets on the intestine and hepatic oxidative stress was assessed by predefined orthogonal contrasts, namely, control diet versus all test

diets; control diet versus BSG4 diet; control diet versus BSG8 diet; control diet versus PreBSG4 diet; control diet versus PreBSG8 diet; BSG diets versus PreBSG diets; level (4) diets versus level (8) diets; and interaction of BSG diets versus PreBSG diets. The effect of the experimental diets on muscle LPO was assessed by repeated measures ANOVA with time (0 h and 72 h) as a within-subject factor and diets as a between-subject factor. One-way ANOVA was also carried out for muscle LPO within each time. Statistical significance was considered at $P \leq 0.05$. All statistical analyses were carried out using IBM SPSS *Statistics* software version 26 (IBM, NY, USA).

3. Results

Results of the growth trial were not the aim of this study and were published elsewhere [32]. Briefly, compared to the control, dietary BSG-extract used either as pre-treatment of PF or mixed directly into the diet decreased feed intake by about 13% and increased feed and protein utilization by about 18% and 22%, respectively, without affecting growth performance or whole-body composition. Pre-treatment of PF with BSG-extract was more effective in improving protein utilization than when directly mixed into the diet.

The phenolic compounds composition and antioxidant and enzymatic activities of the experimental diets are present in Table 2. Total phenolic compounds content was similar (4.3-4.9 mg GAE g^{-1}) in the control, BSG4, and BSG8 diets but lower than that of the PreBSG4 (5.9 mg GAE g^{-1}) and PreBSG8 (7.6 mg GAE g^{-1}) diets. Antioxidant activity of the test diets was higher (101-131 μmol TE g^{-1}) than in the control diet (48 μmol TE g^{-1}). Maximum enzymatic activity was measured in diet BSG8. Cellulase activity in the BSG8 diet was 7.2 U g^{-1}, while, in the other diets, it ranged between 2.3 and 4.7 U g^{-1}. Xylanase activity was higher in the diets in which BSG-extract was directly added than when used to pre-treat the dietary PF.

The specific activity of oxidative stress-related enzymes and LPO in the intestine is presented in Table 3. Except for SOD, all enzymatic activities were higher in fish fed with diet PreBSG8 than in control. Catalase and GPX activity were higher in diets with higher BSG-extract levels (BSG8 and PreBSG8 diets) than with lower BSG-extract levels (BSG4 and PreBSG4 diets). GPX was higher in fish fed BSG8, PreBSG4, and PreBSG8 diets than in the control diet. GPX, GR, and G6PD activities were lower, and LPO was higher in fish fed the control diet than in the test diets. GR activity was higher in fish fed BSG4, PreBSG4, and PreBSG8 diets, while G6PD activity was higher in fish fed the PreBSG8 diet than with the control diet. The BSG-extract application method (pre-treatment of PF or direct incorporation into the diet) and the level (4 and 8, corresponding to 4000 U and 8000 U cellulase kg^{-1} diet) had no significant effect on the intestine oxidative stress enzyme activity, except for SOD that was higher in BSG diets than in PreBSG diets. LPO was lower in fish fed the experimental diets than in control, and no differences in LPO levels were noticed among test diets.

The specific activity of oxidative stress-related enzymes and LPO in the liver is presented in Table 4. Except for catalase and G6PD, fish fed the test diets generally had higher antioxidant enzyme activities than the control. SOD activity was higher in fish fed the highest BSG-extract levels (BSG8 and PreBSG8 diets) than in the control diet. GPX activity was also higher in fish fed the BSG8 diet than in the control. GR activity was higher in fish fed with the BSG8, PreBSG4, and PreBSG8 diets than in the control diet. The BSG-extract application method (pre-treatment of PF or direct incorporation into the diet) and the level (4 and 8) had no significant effect on the hepatic oxidative stress enzyme activity. No differences between groups were observed in LPO levels.

Irrespective of the diet, muscle LPO was higher after 72 h of storage at 4 °C than at 0 h (Table 5). Repeated measures ANOVA did not extract differences between diets, but one-way ANOVA for time 0 h showed ($p < 0.008$) that muscle LPO of the control diet was higher than that of BSG4, BSG8, and PreBSG8 diets.

4. Discussion

Modern aquaculture relies on alternative ingredients, mainly plant origin, to replace traditional fisheries products. Despite the environmental, economic, and social advantages of their use, PF present different nutritional constraints that may impair optimal growth and welfare [41]. Several strategies have been explored to enhance the potential of PF in aquafeeds, including the use of exogenous enzymes, such as carbohydrases, to promote feed utilization efficiency (revised by [4, 42, 43]). Recently, health and antioxidant benefits have also been attributed to carbohydrases supplementation beyond their original purpose [27].

Besides having a high carbohydrases activity, the BSG-extract used in this study also had high phenolic compounds content (95 mg g^{-1} lyophilized extract) and antioxidant activity (131 μmol TE g^{-1} lyophilized extract). Overall, BSG-extract utilization doubled the antioxidant activity of the test diets compared to the control diet. Moreover, diets including pre-treated PF (PreBSG4 and PreBSG8 diets) had higher antioxidant activity and phenolic content than diets including BSG-extract directly added. During PF pre-treatment, BSG-extract enzymes hydrolyzed the lignocellulosic structure of PF, which may concomitantly lead to the release of non-soluble phenolic compounds present in the PF cell wall, increasing their availability. Dietary phenolic compounds have antioxidant properties with protective effects against animal oxidative stress, acting through different mechanisms, including preventing and neutralizing ROS production, repairing biological molecules, modulating enzyme activity, inhibiting receptors activity, and inducing and/or inhibiting gene expression [25, 44, 45]. Thus, BSG-extract carbohydrases and phenolic compounds may exert a synergetic effect on the oxidative status of fish.

In this study, BSG-extract either directly included in feeds or used to pre-treat PF modulated the intestinal oxidative status of European seabass. Besides its direct ROS scavenging activity, dietary BSG-extract also activated the

Table 2: Reducing sugars, phenolic compounds composition, and antioxidant and enzymatic activities of the experimental diets (dry matter basis).

	Control	BSG4	BSG8	PreBSG4	PreBSG8
Glucose (%)	0.27	0.38	0.38	0.71	0.90
Xylose (%)	0.22	0.30	0.31	0.56	0.71
Phenolic compounds (mg GAE g^{-1})	4.60	4.29	4.92	5.93	7.63
Flavonoids (mg QE g^{-1})	1.18	1.57	1.48	1.76	1.73
Ortho-diphenols (mg GAE g^{-1})	0.90	1.35	1.18	1.31	1.10
Antioxidant activity (μmol TE g^{-1})	48.1	101.0	126.7	114.9	130.9
Enzymatic activity (U g^{-1})					
Cellulase	—	4.7	7.2	2.3	4.4
Xylanase	—	16.2	45.6	2.0	3.5

Table 3: Specific activity of antioxidant enzymes (U mg^{-1} protein) and lipid peroxidation (nmols MDA g^{-1} tissue) in the intestine of European seabass fed the experimental diets.

	Control	BSG4	BSG8	PreBSG4	PreBSG8	SEM
SOD	145.7	168.1	160.7	130.6	144.2	5.69
Catalase	557.7	603.6	724.5	548.8	810.8	29.7
GPX	2.65	4.32	5.26	5.00	6.98	0.34
GR	16.63	21.8	18.1	22.3	21.2	0.65
G6PD	12.1	15.7	17.3	17.7	19.1	1.04
LPO	79.7	47.0	51.1	56.0	47.8	3.81
Orthogonal contrast for the effects of the experimental diets	SOD	Catalase	GPX	GR	G6PD	LPO
Control diet versus all test diets			*	*	*	*
Control diet versus BSG4 diet				*		*
Control diet versus BSG8 diet			*			*
Control diet versus PreBSG4 diet			*	*		*
Control diet versus PreBSG8 diet		*	*	*	*	*
BSG diets versus PreBSG diets	*					
Level (4) diets versus level (8) diets		*	*			
Interaction of BSG diets versus PreBSG diets						

Values are presented as means ± standard error of the mean (SEM) (n=9).SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase; LPO, lipid peroxidation Orthogonal contrast, * denotes a significant effect ($P<0.05$).

antioxidant enzyme system, leading to a lower intestinal LPO than the control diet. Within the antioxidant enzymatic response, SOD is the first-line antioxidant enzyme responsible for the dismutation of superoxide radicals by producing hydrogen peroxides that are consequently scavenged into water and oxygen by catalase and GPX. In this study, even though SOD activity was not affected by diet composition, catalase and GPX activity were increased in fish fed the experimental diets with higher BSG-extract content. Catalase scavenges hydrogen peroxide directly into water and oxygen, while GPX scavenges hydrogen peroxide, using reduced glutathione as a donor of electrons. The resulting oxidized glutathione is then converted to its reduced form by the action of GR. However, GR activity did not increase in the present study, suggesting that, although both glutathione-dependent and independent systems of hydrogen peroxide detoxification were activated, the glutathione-independent pathway was the most active.

Present results align with other studies where phenolic compounds are also shown to activate the antioxidant enzymatic system. For instance, the addition of ferulic acid to Nile tilapia diets led to an increase in SOD, catalase, and GPX activities in the serum and the intestine, while decreased MDA levels [46]. Also, in Japanese seabass, *Lateolabrax maculatus*, the inclusion of condensed tannins in diets with oxidized fish oil improved serum GPX and catalase activities, decreased LPO, and exerted a protective effect against oxidative damage in intestinal epithelial cells exposed to hydrogen peroxide [47]. In red carp, *Cyprinus carpio* var. *xingguonensis*, the dietary inclusion of *Angelica sinensis* extracts increased the intestine and hepatopancreas antioxidant capacity by increasing SOD, catalase, GR, and GPX activities, while LPO was decreased [48]. In rainbow trout, *Oncorhynchus mykiss*, dietary supplementation with different phytobiotics (rosemary; *R. officinalis*; sea buckthorn, *Hippophae rhamnoides*; and ginger, *Zinziber*

Table 4: Specific activity of antioxidant enzymes (U mg^{-1} protein) and lipid peroxidation (nmols MDA g^{-1} tissue) in the liver of European seabass fed the experimental diets.

	Control	BSG4	BSG8	PreBSG4	PreBSG8	SEM
SOD	59.30	71.40	64.22	64.50	78.95	1.71
Catalase	412.6	310.4	374.5	399.7	368.4	20.3
GPX	26.37	40.67	44.09	27.77	41.13	2.40
GR	6.39	7.47	7.85	8.14	9.42	0.24
G6PD	311.3	379.7	308.2	337.0	403.4	18.3
LPO	13.74	14.14	12.58	14.46	13.80	0.36
Orthogonal contrast for the effects of the experimental diets				SOD	GPX	GR
Control diet versus all test diets				*	*	*
Control diet versus BSG4 diet				*		
Control diet versus BSG8 diet					*	*
Control diet versus PreBSG4 diet						*
Control diet versus PreBSG8 diet				*		*
BSG diets versus PreBSG diets						*
Level (4) diets versus level (8) diets						
Interaction of BSG diets versus PreBSG diets				*		

Values are presented as means ± standard error of the mean (SEM) (n =9). SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase; LPO, lipid peroxidation. Orthogonal contrast, * denotes a significant effect ($p < 0.05$).

Table 5: Lipid peroxidation (LPO; mg MDA kg^{-1} tissue) of fish muscles at 0 h and 72 h.

	Time (h)	Control	BSG4	BSG8	PreBSG4	PreBSG8	SEM
LPO	0	2.16^{b}	1.49^{a}	1.52^{a}	1.60^{ab}	1.39^{a}	0.08
	72	5.97	5.55	5.20	5.0	5.52	0.30
Repeated-measures ANOVA	*p*-value						
Time	<0.001						
Diet	n.s.						
Time∗Diet	n.s.						

Values are presented as means ± standard error (n=9).For each time, means without a common superscript letter differ (one-way ANOVA, Tukey's test $p<0.05$).

officinale) reduced intestine LPO [49]. However, other studies failed to observe a protective effect of dietary antioxidants compounds on intestine oxidative status. For example, in gilthead seabream, *Sparus aurata*, dietary supplementation with olive oil bioactive compounds did not affect intestine antioxidant capacity since no differences were observed in catalase and GPX activities and LPO level [50].

BSG-extract is not a purified extract but is composed of several water-soluble molecules, including polyphenols, exoenzymes, soluble proteins, reducing sugars, and oligosaccharides. Thus, the mechanisms for antioxidant action of the BSG-extract on fish intestinal oxidative status cannot be exclusively attributed to its phenolic compounds composition since the potential action of other bioactive molecules with a single or complementary mode of action, such as enzymes and oligosaccharides, cannot be discarded. Nevertheless, the increased antioxidant enzyme activities induced by the higher BSG-extract level in the experimental diets align with the higher antioxidant activity and total phenolic compounds determined in these diets.

Previously, it was observed that the BSG-extract increased cellulose and hemicellulose digestibility [31], which may also have contributed to the reduction of the oxidative intestine damage of European seabass since the dietary complex carbohydrates negatively impact fish intestine oxidative status. Moreover, by disrupting cell walls through enzymes action, embedded phenolic compounds may also become available, thus enhancing their direct antioxidant action. The effect of dietary carbohydrate complexity on the intestine oxidative status has been poorly studied. However, dietary inclusion of moderate levels of soluble NSP improved the intestine antioxidant status of white seabream, *Diplodus sargus*. In contrast, higher levels induced the opposite effect [51]. Further, the enhanced NSP digestibility, promoted by the BSG-extract, may have increased the substrate availability for intestinal microbiota growth, and this may have beneficially modulated microbiota diversity and intestinal oxidative status, as revised by Hoseinifar et al. [52]. Indeed, previous studies have confirmed the role of exogenous enzymes on microbiota modulation, increasing

microbiota richness and diversity in turbot, *Scophthalmus maximus* [53], and in grass carp, *Ctenopharyngodon idella* [54], or contrarily, reducing the microbiota diversity in African catfish, *Clarias gariepinus* [55].

The antioxidant capacity of phenolic compounds depends not only on its level but also on the conjugation with other dietary nutrients that may modulate phenolic compounds' bioaccessibility [56]. In the present study, even though diets including pre-treated PF with BSG-extract had higher phenolic content and antioxidant activity, as was also the case of diets with higher BSG-extract levels, the intestine LPO levels were similar among test diets. Nonetheless, intestine LPO was significantly lower in fish fed the BSG-extract including diets than in the control. Contrarily, hepatic LPO was similar among control and test diets being generally much lower than the intestine. These results suggest that the overall effects of dietary BSG-extract related to the phenolic compounds and exoenzyme activity were more important at the local extra-organism level. However, it cannot be discarded that the hepatic lower LPO level in the liver than in the intestine precludes, evidencing the potential effects of BSG-extract at the hepatic level.

Compared to the liver, the intestine has a higher cellular turnover and is exposed to dietary components that may induce higher ROS production [39]. In the present study, the hepatic LPO level was 17-fold lower than that of the intestine in the control group. Compared to the liver, higher oxidative stress susceptibility of the intestine has been reported in other studies in this species [39] and other species [50, 57, 58]. Accordingly, in the present study, antioxidant enzyme activity in the liver was lower than in the intestine, except for GPX and G6PD. Besides providing NADPH required for glutathione reduction, G6PD also provides NADPH for fatty acid biosynthesis. So, the much higher G6PD activity observed in the liver was probably related to the high reduction power required for fatty acid biosynthesis processes. Similarly, higher G6PD activity in the liver than in the intestine has been previously reported in this species [39, 59] and also in gilthead seabream [60].

Dietary BSG-extract also activated the hepatic antioxidant enzyme system as observed in the intestine. However, contrary to what was detected in the intestine, SOD activity was enhanced in fish fed the test diets, while catalase activity was not affected by the BSG-extract inclusion. As in the intestine, GPX and GR activities were higher in fish fed the experimental diets suggesting that contrarily to the intestine, the glutathione-dependent system of hydrogen peroxide detoxification was more active in the liver. Although SOD, catalase, and GPX activities are intertwined in the antioxidative response, a clear relationship between the activity of these enzymes is not always observed [22]. As in the present study, turbot juveniles fed a diet supplemented with resveratrol had higher hepatic SOD activity, while catalase activity was unaffected [61]. In gilthead seabream, green tea-supplemented diets increased catalase activity without affecting LPO levels [62], while in Wuchang bream, *Megalobrama amblycephala*, dietary green tea polyphenols increased hepatic catalase, GR, and SOD activities [63]. Further, the dietary supplementation with *Moringa oleifera* leaf extract increased hepatic SOD and catalase activities in Nile tilapia [64], while ferulic acid supplementation increased SOD, catalase, and GPX activities in the serum and the liver [65].

The observed role of BSG-extract in increasing NSP digestibility (see [31]) may also have modulated the activity of hepatic antioxidant enzymes. The effect of dietary supplementation with exogenous enzymes on liver oxidative stress has been evaluated with variable results. For example, in yellow perch, *Perca flavescens*, dietary incorporation of a carbohydrases complex (Viscozyme® L) did not affect liver antioxidant enzyme activities (SOD, catalase, and GPX; [7]). Similarly, in large yellow croaker, *Larimichthys crocea*, dietary supplementation with xylanase did not affect liver SOD activity and LPO [66]. Contrarily, in common carp fry, diet supplementation with a commercial xylanase complex (Hostazyme X) increased hepatic SOD, catalase, and GPX activities [29]. In gilthead seabream, supplementation of corn distillers dried grains with soluble (DDGS)-based diet with a carbohydrases complex counteracted the increased susceptibility to hepatic oxidative stress induced by the DDGS by decreasing G6PD and SOD activity, while LPO level was unaffected [10]. Further, the modulatory action of dietary carbohydrates complexity on liver antioxidant status is also documented. For example, in Nile tilapia, glucose or maltose-based diets lead to higher hepatic SOD, catalase, GPX, and glutathione S-transferase activities than in dextrin or corn starch-based diets [67]. In common dentex, *Dentex dentex*, a maltodextrin-based diet, reduced hepatic GR and G6PD activities compared to dextrin or pre-gelatinized starch-based diets [68]. Brook trout juveniles, *Salvelinus fontinalis*, fed glucose- and sucrose-supplemented diets exhibited lower hepatic SOD and catalase activities than fish fed dextrin, raw, or pregelatinized corn starch [69].

The polyunsaturated fatty acid content of fish fillets accelerates the oxidative process, increasing fish fillet susceptibility to lipid peroxidation [20]. In this study, the muscle LPO level significantly decreased at time 0 h by the dietary application of BSG-extract, indicating that the BSG-extract effectively reduced fillet peroxidation. As expected, after 72 h stored at 4 °C, overall muscle LPO increased, but differences between groups were not significant compared to the control. Given their ability to retard lipid peroxidation, the utilization of phenolic-rich extracts obtained from natural sources in seafood preservation is receiving much attention [20]. For example, Motamedi-Tehrani et al. [70] observed that in common carp, the dietary inclusion of pistachio (*Pistacia vera*) hull extract increased total dietary phenolic compounds and decreased fish fillet LPO levels. Also, the dietary inclusion of antioxidants from thyme essential oils (*Thymbra capitata* and *Thymus zygis*, subspecies *gracilis*) and rosemary (*Rosmarinus officinalis*) extract in diets for gilthead seabream lowered lipid oxidation in muscle stored for 21 days at 4 °C [71, 72]. Monteiro et al. [73] observed that LPO levels decreased in the muscle of meagre, *Argyrosomus regius*, when fed a macroalga extract (*Fucus vesiculosus*) rich in phenolic compounds, even when it was exposed to acute stress. Also, in Nile tilapia fingerlings (*Oreochromis niloticus*), muscle LPO was reduced when fish were fed with

garlic- and ginger powder-supplemented diets [74]. However, the effect of dietary inclusion of phenolic extracts on fillet peroxidation is not always observed. For instance, in unstressed Nile tilapia, dietary supplementation with lycopene did not affect fillet LPO levels, while under stress conditions, lycopene reduced fillet LPO [75]. In rainbow trout, dietary supplementation with ribwort plantain (*Plantago lanceolata*) extract decreased muscle LPO after 60 days of feeding but not after 90 days [76]. Similarly, in the present study, a significant reduction of fillet lipid peroxidation was observed immediately *post*-mortem, but the effect was much reduced after 72 h of storage.

5. Conclusions

Agro-industrial by-products represent a biomass resource with enormous potential to produce eco-friendly, alternative, and functional feeds. The application of SSF to BSG simultaneously contributes to the circular economy and to the development of functional diets for aquaculture. The present study showed that the reutilization of BSG through SSF allowed the production of a functional extract with promising effects on European seabass oxidative status.

Overall, results showed that dietary application of BSG-extract significantly improved the intestine antioxidant status, increasing its ability to cope with ROS formation. BSG-extract also significantly improved the lipid oxidation status of fish fillets at time 0 h.

Data Availability

The datasets obtained and analyzed in the present study are not available for public consultation. Please contact the authors for data requests.

Conflicts of Interest

The authors declare that they do not have competing interests.

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