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Effect of Solid-Fermented Brewer's Spent Grain on Growth, Metabolism, and Oxidative Status of European Seabass (*Dicentrarchus labrax*)

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Abstract: Replacing traditional agricultural ingredients with biotechnologically improved agro-industry by-products in fish diets promotes sustainable aquaculture, reduces production costs and carbon footprint, and promotes a circular economy. Brewer's spent grain (BSG) is one such by-product. Solid-state fermentation (SSF) of BSG with *Aspergillus ibericus* enhances its nutritional value and digestibility for European seabass. The present study further evaluates the potential of dietary inclusion of BSG-SSF on growth performance, feed utilization, plasma metabolite profile, intermediary metabolism, and oxidative status of European seabass juveniles compared to the unfermented product. A practical diet (45% protein; 18% lipids) was tested against diets incorporating 10% or 20% of BSG or BSG-SSF, replacing plant-protein feedstuffs. Triplicate groups of European seabass juveniles (49 g initial weight) were fed for 10 weeks. Unfermented BSG (10% and 20%) reduced growth and feed efficiency. In comparison, the 20% BSG-SSF diet promoted growth and feed efficiency similar to the control group, while the 10% BSG-SSF diet surpassed the control diet. Whole-body protein content was unaffected, but lipid and energy content decreased with increasing BSG levels, regardless of fermentation. Plasma glucose and phospholipid levels and hepatic activities of glucokinase and malic enzymes decreased with increasing BSG, irrespective of fermentation. BSG-SSF incorporation increased plasma triglyceride levels and decreased hepatic transaminase activities but did not affect hepatic key enzyme activity of β-oxidation or lipogenesis. It also reduced antioxidant enzyme activity and lipid peroxidation. In conclusion, BSG negatively impacted growth performance, while BSG-SSF supported inclusion levels up to 20% without performance loss. Further, the 10% BSG -SSF diet outperformed the control diet.

Keywords: alternative ingredients; biocircularity; by-product; European seabass; fermentation; metabolism; oxidative stress

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Key Contribution: This study demonstrates that solid-state fermentation (SSF) of brewer's spent grain (BSG) mitigates growth performance constraints and improves oxidative status in European seabass. A 10% BSG-SSF inclusion surpasses the performance of the non-BSG control diet, highlighting its potential as a sustainable and functional feed ingredient.

1. Introduction

The growing global population is placing significant pressure on food production systems to meet the increasing demand for protein [1]. Addressing this challenge requires adopting eco-friendly strategies, prioritizing resource circularity, and alleviating the pressure on the global food supply chain [2]. In this context, the fed aquaculture industry must improve efficiency practices and implement sustainable measures to reduce reliance on traditional ingredients for aquafeed formulation [3].

Over the past decades, substantial efforts have been directed to decrease the dependency on fishmeal and fish oil. Plant proteins have become a significant source of protein and lipids in aquafeed, even for carnivorous fish [4]. However, including high-quality plant ingredients and their derivatives in aquaculture diets presents sustainability challenges, such as deforestation, increased carbon footprint, higher costs, and competition with human food [5]. To address these concerns and align with European Union recommendations [6], a circularity approach focused on recycling biomass that does not compete with food sources is essential [3].

Agro-industrial by-products present potential as alternative ingredients for fish feeds. Reintegrating these by-products into the food chain implies evaluating their feasibility and determining the most efficient processing treatment to improve their nutritional value [7]. A primary challenge in incorporating these by-products into aquafeeds is their low protein content and, more critically, their high levels of non-starch polysaccharides (NSPs), which can adversely impact fish growth and health [8].

NSPs are the main component of plant cell walls and include various undigestible polysaccharides and lignin [8,9]. The polysaccharides include cellulose (linear β-(1-4)-D-glucose chains, insoluble and resistant to hydrolysis), hemicellulose (branched, shorter chains of diverse sugars), and lignin (an amorphous, hydrolysis-resistant structure), and pectins [8]. Fish lack the ability to digest NSPs [10], and high dietary levels may impair digestion, absorption, and utilization of other nutrients [8,11]. However, appropriate processing treatments through physical, chemical, and biological methods can disrupt the complex structure of NSPs into simpler compounds [9].

Solid-state fermentation (SSF) is a biotechnological process capable of hydrolyzing agro-industrial substracts. In SSF, the substrate serves as a solid support and a nutritional source for microorganisms, which secrete hydrolytic enzymes that disrupt the substrate's structure [11]. Enzymes such as hemicellulases and cellulases, generated during SSF, break NSPs into monomers and low-molecular-weight oligosaccharides [12]. The type of enzymes produced depends on both the substrate and selected microorganism, with fungi being the preferred due to their adaptability to SSF conditions [13]. Further, thermophilic fungi produce thermostable enzymes, which offer several advantages for various applications [14]. Overall, SSF transforms plant by-products into nutritionally enriched biomasses with higher protein content, reduced anti-nutritional factors such as NSPs and phytate, and the addition of functional compounds, such as hydrolytic enzymes and antioxidant compounds [13].

Brewer's spent grain (BSG) is the primary by-product of the brewery industry, constituting 80% of total brewery waste. According to the Eurostat data from 2023, approximately

35 million liters of beer were produced in the EU in 2022, generating around 20 kg of BSG per 100 liters produced [15]. BSG contains moderate protein and high fiber, mineral, and vitamin levels [15]. However, its high fiber content limits its utilization in aquafeeds. BSG has been previously used as a substrate in SSF to produce modified biomass with increased protein content and reduced structural polysaccharides and to extract valuable compounds, such as enzymes and phenolic compounds with antioxidant capacity [16].

It was previously shown that extracts obtained from BSG after SSF have high potential as functional ingredients for European seabass [17]. Moreover, SSF improved the nutritional profile of BSG, increasing protein content and reducing cellulose and hemicellulose levels, and improved the digestibility of dry matter, energy, protein, and amino acids in European seabass juveniles [18].

The present study further investigated the potential impact of dietary inclusion of non-fermented and solid-fermented BSG (BSG-SSF) on growth performance, feed utilization, and oxidative status in European seabass juveniles.

2. Materials and Methods

2.1. Ethical Statement

This study was approved by the Animal Welfare Committee (ORBEA) of CIIMAR and the Portuguese National Authority for Animal Health (DGAV; reference ORBEA-CIIMAR-27-2019). The growth trial was performed by certified personnel in compliance with European Union Directive 2010/63/EU and Portuguese legislation (Decreto-Lei no. 113/2013).

2.2. Solid-State Fermentation (SSF)

Brewer's spent grain (BSG), comprising barley, Pilsen malt, and corn grits, was sourced from Unicer-Bebidas de Portugal, SA (Matosinhos, Portugal). *Aspergillus ibericus* MUM 03.49, provided by the University of Minho Micoteca (Braga, Portugal), was employed in the solid-state fermentation (SSF) of BSG.

SSF was performed in sterilized trays (121 °C, 15 min) containing 400 g of sterilized BSG (moisture adjusted to 75% *w*/*w*) inoculated with 2 mL of *A. ibericus* MUM 03.49 spore solution (10^6 cells mL^{-1}). The trays were incubated at 25 °C for 7 days with daily stirring. After fermentation, the BSG-SSF was pooled and stored at 4 °C until diet production.

2.3. Experimental Diets

Five approximately isoproteic and isolipidic diets (45% crude protein, 18% crude lipids) were formulated: a practical control diet including 15% fish meal and plant ingredients as protein sources, and 4 test diets including 10% or 20% of either BSG (diets 10BSG and 20BSG) or BSG-SSF (diets 10BSG-SSF and 20BSG-SSF). BSG and BSG-SSF replaced a mixture of soybean, rapeseed, sunflower, and wheat meal. Ingredients were ground, mixed thoroughly, and pelleted using a 2 mm die laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA). Diets were then dried in an oven at 55 °C for 24 h. Ingredients and proximate composition of experimental diets are presented in Table 1.

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

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF
	Ingredient (% dry matter)				
BSG [1]	—	10	—	20	—
BSG-SSF [2]	—	—	10	—	20
Fish meal	15	15	15	15	15

Table 1. *Cont.*

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF
Pea protein concentrate	10	10	10	10	10
Corn gluten	12.5	12.5	12.5	12.5	12.5
Soybean meal	17.6	15	15	10	10
Rapeseed meal	7.5	7	7	5	5
Sunflower meal	6.5	6.2	6.2	4.8	4.8
Wheat meal	11.7	4.6	4.6	—	—
Hemoglobin	—	—	—	2.2	2.2
Fish oil	14.0	14.4	14.4	14.9	14.9
Dicalcium phosphate	—	0.1	0.1	0.5	0.5
Constant componests [3]	5.1	5.1	5.1	5.1	1.2
Proximate composition (% dry matter)					
Dry matter, DM	88.5	87.3	87.7	88.2	87.3
Crude protein	45.3	45.6	46.9	45.9	46.3
Crude lipids	18.0	18.1	17.9	18.3	18.7
Gross energy (kJ g^{-1} DM)	23.1	23.2	23.7	23.2	23.7
Ash	6.3	6.8	6.4	6.0	6.30
Cellulose	4.2	6.2	4.7	7.0	5.35
Hemicellulose	2.5	2.4	1.8	2.1	1.79
Klason lignin	12.2	10.0	10.1	12.8	12.6
Essential amino acids (EAA, % protein)					
Lysine, Lys	4.8	4.2	4.6	4.9	4.6
Arginine, Arg	3.6	3.6	3.6	3.5	3.4
Histidine, His	1.5	1.6	1.6	1.9	1.7
Isoleucine, Ile	2.9	2.8	2.7	2.8	2.8
Leucine, Leu	11.7	12.0	12.6	12.3	12.1
Valine, Val	2.9	2.9	3.1	3.1	3.2
Methionine, Met	2.6	2.1	2.6	2.0	3.0
Cysteine, Cys	1.2	1.2	1.1	1.1	1.1
Phenylalanine, Phe	4.6	4.3	4.8	4.2	4.4
Tyrosine, Tyr	1.7	1.8	1.9	1.8	1.8
Threonine, Thr	2.5	2.5	2.4	2.9	3.0
Sum EAA	40.5	39.3	41.4	40.9	41.5
Non-essential amino acids (NEAA, % protein)					
Aspartic acid, Asp	6.20	6.03	5.40	5.98	5.81
Glutamic acid, Glu	8.73	8.76	8.92	8.62	8.01
Serine, Ser	9.87	11.1	9.84	10.4	10.0
Glycine, Gly	12.4	11.8	11.5	11.7	11.8
Alanine, Ala	11.0	11.4	12.1	10.7	11.5
Proline, Pro	11.3	11.6	10.8	11.7	11.4
Sum NEAA	59.5	60.6	58.6	59.1	58.4

[1] Brewer's spent grain, Unicer-Bebidas de Portugal, SA. Matosinhos, Portugal. Crude protein: 26.7%, crude lipids: 5.7%, cellulose: 21.1%, hemicellulose: 23.7%, lignin: 15.0% (% dry matter). [2] Fermented brewer's spent grain. Crude protein: 32.3%, crude lipids: 2.8%, cellulose: 14.8%, hemicellulose: 15.7%, lignin: 13.9% (% dry matter). [3] Constant components (% of the diet): vitamin premix, 1; mineral premix, 1; choline chloride, 0.5; shrimp hydrolysate, 1.2; binder, 1; methionine, 0.1; taurine, 0.3. Detailed composition of the ingredients, vitamins, and mineral premixes is presented in Estevão-Rodrigues et al. [18].

2.4. Growth Trial

European seabass (*Dicentrarchus labrax*) juveniles, sourced from commercial aquaculture (Maresa, Huelva, Spain), were transported by car in two closed containers completely filled with oxygenated seawater (using an oxygen bottle). Temperature, oxygen levels, and ammonia nitrogen were continuously monitored and controlled during transport. Upon arrival at the Bioterium of Aquatic Organisms of CIIMAR (Matosinhos, Portugal), the

fish were maintained in quarantine for 15 days in a 2000 L recirculating seawater system (RAS) and fed a commercial diet (NEOGOLD diet; 55% crude protein and 16% crude lipids; Aquasoja, Sorgal, S.A., Portugal). Then, fish were transferred to the experimental system consisting of a thermo-regulated RAS with 15 fiberglass tanks (300 L capacity) supplied by an aerated water flow. Throughout the trial, the water temperature was maintained at 22 ± 0.4 °C, dissolved oxygen averaged 7.2 mg mL^{-1}, salinity was 27 ± 3‰, and ammonia and nitrites levels were kept below 0.02 mg mL^{-1}. The photoperiod was set to 12 hours light and 12 hours dark (12L:12D).

At the beginning of the trial, 15 groups of 16 fish (initial body weight: 49 g) were randomly assigned to the tanks, and experimental diets were tested in triplicate. Fish were hand-fed to visual satiety twice daily, 6 days a week, for 10 weeks. At the end of the trial, fish were bulk-weighed under light anesthesia (0.3 mL L^{-1} ethylene glycol monophenyl ether) after one day of feed deprivation. Five fish from the initial stock and three fish per tank at the end of the trial were euthanized by anesthetic overdose (10 mL L^{-1} ethylene glycol monophenyl ether), pooled, and stored at −20 °C for determination of the proximate composition analysis.

2.5. Fish Sampling

Following the final weighing, the fish were fed for 3 additional days to mitigate stress resulting from handling. Subsequently, 4 hours after the morning meal, blood samples were collected from the caudal vein of 3 fish per tank using heparinized syringes. The collected blood was centrifugated (10,000× *g*, 10 min), and the resulting plasma was divided into aliquots and frozen at −80 °C until further analysis. The fish were then killed by decapitation, and the liver and whole intestine were dissected on an iced tray, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. The fish, liver, and viscera weights of these fish were recorded to determine the hepatosomatic (HSI) and visceral (VI) indices.

2.6. Proximate Composition Analysis

The proximate composition of dietary ingredients, diets, and whole-body fish was analyzed following standard AOAC methods. Dry matter was measured by drying samples at 105 °C until a constant weight was achieved. Ash content was determined through incineration in a muffle furnace at 450 °C for 16 hours. Protein content was analyzed using the Kjeldahl method (N × 6.25) with Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026). Total lipid content was quantified via petroleum ether extraction using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Cellulose, hemicellulose, and lignin content were analyzed following the method outlined by Vieira et al. [19].

2.7. Plasma Metabolites

Plasma glucose (REF. 1001191), cholesterol (REF. 1001090), triglyceride (REF. 1001312), and phospholipid (REF. 1001140) levels were determined using enzymatic colorimetric kits (Spinreact, Girona, Spain), following the manufacturer's protocols. The absorbances of all samples were read on a microplate reader (Multiskan GO Model5111 9200, Thermo Scientific, Nanjing, China).

2.8. Enzymatic Activity

Enzymatic analyses were performed at 37 °C, and absorbance changes were monitored using a microplate reader (Multiskan GO Model 5111 9200, Thermo Scientific, Nanjing,

China). Enzymatic activity was reported as milliunits (GPx, GR, and G6PDH) or units (CAT) per milligram of soluble protein, which was quantified using the Bradford method [20].

2.8.1. Intermediary Metabolism

The liver samples were homogenized as described by Estevão-Rodrigues et al. [18]. Aspartate (ASAT/GOT, EC 2.6.1.1) and alanine aminotransferase (ALAT/GPT, EC 2.6.1.2) activities were assessed using commercial kits (Spinreact, ASAT/GOT: 41,273; ALAT/GPT: 41,283) adapted for fish by Diógenes et al. [21]. Malic enzyme (ME, EC 1.1.1.40), fatty acid synthase (FAS, EC 2. 3.1.38), 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35), hexokinase (HK, EC 2.7.1.1), glucokinase (GK, EC 2.7.1.2), and pyruvate kinase (PK; EC 2.7.1.40) activities were measured as described by Diógenes et al. [21].

2.8.2. Oxidative Stress

Liver and whole intestine samples were homogenized as described by Estevão-Rodrigues et al. [18]. Antioxidant enzyme activity analyses were performed as described by Diógenes et al. [21]. Catalase (CAT; EC 1.11.1.6) activity was determined by reducing the H_2O_2 concentration at 240 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR; EC 1.11.1.9) were analyzed by measuring NADPH oxidation at 340 nm, while glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was evaluated based on $NADP^+$ reduction at the same wavelength.

2.9. Lipid Peroxidation

Lipid peroxidation was measured by the malondialdehyde (MDA) concentration at 535 nm absorbance, with results expressed as nmol MDA g^{-1} tissue [21]. Homogenate supernatants were reacted with a solution containing trichloroacetic acid, thiobarbituric acid, and butylated hydroxytoluene, heated at 100 °C for 15 minutes, cooled to room temperature, and centrifuged. The absorbance of the supernatant was then measured at 535 nm. MDA levels were expressed as nmol MDA per g of wet tissue, calculated using a calibration curve.

2.10. Statistical Analysis

Data were assessed for normality and variance homogeneity using the Kolmogorov–Smirnov and Levene tests, respectively. Normalization procedures were applied when needed. Specific non-orthogonal contrast analyses were used to compare each test diet versus the control, BSG versus BSG-SSF diets, dietary BSG inclusion levels (10% versus 20%), and the interaction between BSG and SSF levels. All statistical analyses were performed using IBM SPSS Statistics software version 26 (IBM, Armonk, NY, USA).

3. Results

The fish readily accepted all of the diets, and the dietary treatments did not significantly affect voluntary feed intake (Table 2). Mortality was low and not significantly influenced by the dietary treatments. No fish died in the control and 10BSG groups. In the 10BSG-SSF, 20BSG, and 20BSG-SSF groups, one, three, and two fish died, respectively.

Irrespective of incorporation levels, the BSG diets led to a significant reduction of final body weight (FBW), weight gain (WG), daily growth index (DGI), feed efficiency (FE), and protein efficiency ratio (PER) compared to the control diet. In contrast, dietary BSG-SSF inclusion did not significantly reduce growth and feed utilization, with the 10% BSG-SSF diet promoting a better performance than the control diet. Daily nitrogen per kilogram of body weight was not significantly affected by the BSG level but increased with fermentation, while the opposite was observed for energy retention.

Table 2. Growth performance of European seabass fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Final body weight (g)	92.8	85.4	100.0	82.9	89.9	1.68
Weight gain (WG, g kg ABW^{-1} day^{-1})	9.14	8.03	10.1	7.42	8.71	0.26
Daily Growth Index (DGI) [1]	1.28	1.10	1.45	1.01	1.21	0.04
Feed intake (FI, g kg ABW^{-1} day^{-1}) [2]	12.8	13.1	12.6	12.0	11.5	0.24
Feed efficiency (FE) [3]	0.71	0.62	0.81	0.64	0.76	0.02
Protein efficiency ratio (PER) [4]	1.58	1.36	1.72	1.34	1.64	0.05
Nitrogen retention (NR, g kg ABW^{-1} day^{-1}) [5]	0.24	0.22	0.26	0.21	0.24	0.01
Energy retention (ER, kJ kg ABW^{-1} day^{-1}) [5]	26.8	28.9	34.1	27.4	26.9	1.16
Survival (%)	100	100	97.9	93.8	95.8	0.99

Non-orthogonal contrasts	**FBW**	**WG**	**DGI**	**FI**	**PER**	**FE**	**NR**	**ER**	**Survival**
Control vs. 10BSG	0.013 *	0.009 *	0.010 *	0.862	0.028 *	0.039 *	0.337	0.387	0.496
Control vs. 10BSG-SSF	0.016 *	0.018 *	0.015 *	0.851	0.124	0.042 *	0.229	0.038 *	0.060
Control vs. 20BSG	0.002 *	0.001 *	0.001 *	0.512	0.022 *	0.088	0.159	0.718	0.188
Control vs. 20BSG-SSF	0.267	0.241	0.245	0.053	0.453	0.261	0.766	0.495	0.423
BSG vs. BSG-SSF	0.000 *	0.000 *	0.000 *	0.212	0.000 *	0.000 *	0.008 *	0.439	0.998
10% vs. 20%	0.005 *	0.002 *	0.002 *	0.071	0.480	0.656	0.088	0.011 *	0.073
Interaction (BSG vs. SSF)	0.055	0.130	0.105	0.440	0.602	0.278	0.272	0.228	0.341

Values are presented as means (n = 3) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analyses; $p < 0.05$). IBW or FBW: initial or final body weight; ABW, average body weight: (IBW + FBW)/2. [1] DGI: ((final body weight$^{1/3}$ − initial body weight$^{1/3}$)/number of days) × 100. [2] Feed Intake: ((total intakex1000)/ABW) × number of days)). [3] FE: (wet weight gain/dry feed intake). [4] PER: (wet weight gain/crude protein intake) [5] NR and ER (g or kJ kg ABW^{-1} day^{-1}): ((FBW × carcass N or energy content) − (IBW × carcass N or energy content))/(ABW × number of days).

The dietary treatments had no statistically significant effect on the whole-body dry matter and protein content of the European seabass (Table 3). Lipid and energy content were significantly higher in fish fed the 10% rather than the 20% BSG diets, regardless of fermentation. Whole-body ash content was significantly higher in fish fed the 20% BSG diets, irrespective of fermentation, and, regardless of inclusion level, in the BSG-SSF diets than in the BSG diets. The energy content was significantly higher in fish fed the 20% rather than the 10% BSG diets, irrespective of fermentation. The HSI was significantly higher in fish fed the 10% rather than the 20% BSG diets, regardless of fermentation, and it was also significantly higher in fish fed the 10BSG-SSF diet rather than the control and 10BSG diets. VI was not significantly affected by BSG incorporation level or fermentation but was higher in fish fed the 10BSG diet compared to the control diet.

Table 3. Whole-body composition (% wet weight) of European seabass fed the experimental diets.

	Initial	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Dry matter, DM	28.5	30.0	32.7	31.5	31.4	29.7	0.45
Protein	15.3	15.9	16.1	16.2	16.2	16.1	0.12
Lipids	6.82	9.85	12.4	11.3	10.9	8.76	0.46
Energy (kJ g^{-1})	6.47	7.51	8.30	8.28	7.90	7.32	0.15
Ash	3.77	3.87	3.71	4.06	4.16	4.42	0.07
HIS [1]	ND	0.96	0.99	1.23	0.96	1.09	0.02
VI [2]	ND	8.88	10.49	10.28	9.20	9.13	0.20

Non-orthogonal contrast	**DM**	**Protein**	**Lipids**	**Energy**	**Ash**	**HSI**	**VI**
Control vs. 10BSG	0.326	0.707	0.071	0.075	0.295	0.831	0.039 *
Control vs. 10BSG-SSF	0.091	0.565	0.296	0.105	0.356	0.007 *	0.123
Control vs. 20BSG	0.282	0.641	0.411	0.382	0.124	0789	0.412
Control vs. 20BSG-SSF	0.368	0.686	0.413	0.682	0.008 *	0.788	0.683
BSG vs. BSG-SSF	0.187	0.921	0.087	0.301	0.027 *	0.068	0.495
10% vs. 20%	0.154	0.953	0.044 *	0.039 *	0.007 *	0.014 *	0.088
Interaction (BSG vs. SSF)	0.819	0.851	0.614	0.443	0.744	0.038 *	0.923
10BSG-SSF vs. 10BSG						0.001 *	
20BSG-SSF vs. 20BSG						0.998	

Values are presented as mean (n = 3) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analyses; $p < 0.05$). [1] Hepatosomatic index: (Fish weight/Liver weight) × 100. [2] Visceral index: (Fish weight/Visceral weight) × A100.

Plasma cholesterol levels were not significantly affected by diet composition (Table 4). Further, compared to the control, plasma metabolites were unaffected at the 10% BSG inclusion level, whether unfermented or fermented, except for a significant reduction in phospholipid levels in the BSG-SSF group. At the 20% inclusion level, independently

of fermentation, plasma glucose, phospholipid, and triglyceride (only in the BSG diet) levels decreased.

Table 4. Plasmatic metabolites levels (mg dL^{-1}) of European seabass fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Glucose	135.5	120.8	131.1	105.5	109.4	3.08
Cholesterol	104.7	90.2	97.0	91.9	93.3	2.45
Triglycerides	799.3	690.0	874.3	562.0	686.8	34.35
Phospholipids	1146.7	904.9	1118.6	699.6	709.1	41.55

Non-orthogonal contrast	Glucose	Cholesterol	Triglycerides	Phospholipids
Control vs. 10BSG	0.087	0.068	0.299	0.024 *
Control vs. 10BSG-SSF	0.599	0.448	0.474	0.786
Control vs. 20BSG	0.001 *	0.149	0.028 *	0.000 *
Control vs. 20BSG-SSF	0.004 *	0.107	0.285	0.000 *
BSG vs. BSG-SSF	0.245	0.538	0.036 *	0.155
10% vs. 20%	0.004 *	0.753	0.032 *	0.000 *
Interaction (BSG vs. SSF)	0.688	0.477	0.038 *	0.121
10BSG vs. 10BSG-SSF			0.075	
20BSG vs. 20BSG-SSF			0.223	

Values are presented as mean (n = 9) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analyses; $p < 0.05$).

The diet composition did not significantly affect the activity of GDH, HK, HOAD, and FAS (Table 5). Fermentation significantly decreased the activities of ASAT, ALAT, and PK, regardless of the dietary BSG incorporation level. In contrast, independently of fermentation, GK and ME activities were significantly reduced at the higher BSG incorporation level. Compared to the control group, GK and ME activities were reduced in the 20% group, independently of fermentation, while ASAT and ALAT also decreased but only in the 20% BSG-SSF group.

Table 5. Enzymatic activity (mU mg^{-1} protein) of European sea bass fed experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Amino acid catabolism						
Glutamate dehydrogenase (GDH)	48.6	55.1	52.1	45.7	49.3	2.06
Aspartate aminotransferase (ASAT)	482.1	431.9	329.1	460.5	362.0	19.8
Alanine aminotransferase (ALAT)	319.9	247.2	228.0	335.4	199.3	15.6
Glycolysis						
Hexokinase (HK)	1.65	1.30	1.67	1.26	1.42	0.10
Glucokinase (GK)	5.41	4.69	5.17	4.04	3.90	0.20
Pyruvate kinase (PK)	9.72	9.71	8.85	9.47	7.91	0.25
β-Oxidation						
Hydroxyacyl Co-A dehydrogenase (HOAD)	14.76	14.18	13.55	14.78	13.74	0.45
Lipogenesis						
Malic enzyme (ME)	4.86	4.94	3.83	3.42	3.33	0.19
Fatty acid synthase (FAS)	0.81	0.83	1.84	0.92	1.30	0.17

Non-orthogonal contrast	Amino acid catabolism			Glycolysis			β-Oxidation	Lipogenesis	
	GDH	ASAT	ALAT	HK	GK	PK	HOAD	ME	FAS
Control vs. 10BSG	0.339	0.406	0.126	0.301	0.231	0.984	0.697	0.876	0.975
Control vs. 10BSG-SSF	0.602	0.014 *	0.049 *	0.947	0.692	0.147	0.417	0.052	0.066
Control vs. 20BSG	0.676	0.720	0.733	0.241	0.015 *	0.744	0.991	0.008 *	0.852
Control vs. 20BSG-SSF	0.911	0.051	0.011 *	0.479	0.026 *	0.020 *	0.494	0.005 *	0.371
BSG vs. BSG-SSF	0.950	0.022 *	0.022 *	0.272	0.685	0.016 *	0.455	0.106	0.076
10% vs. 20%	0.211	0.471	0.365	0.543	0.027 *	0.380	0.716	0.009 *	0.560
Interaction (BSG vs. SSF)	0.493	0.959	0.079	0.647	0.459	0.000	0.852	0.170	0.422
10BSG vs. 10BSG-SSF						0.153			
20BSG vs. 20BSG-SSF						0.044			

Values are presented as means (n = 9) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analyses; $p < 0.05$).

In the liver, diet composition did not significantly affect CAT activity, except for a decreased activity in the 20% BSG-SSF group compared to the control group (Table 6). G6PDH activity decreased with increasing BSG levels, regardless of fermentation, while fermentation increased G6PDH activity, irrespective of BSG level. SSF significantly decreased GR activity independently of dietary BSG inclusion levels, and GR activity was

lower in the 20% BSG-SSF group than in the control group. SSF also significantly decreased LPO levels independently of dietary BSG inclusion level, and LPO was lower in the 20% BSG-SSF group than in the control group.

Table 6. Enzymatic activity of European sea bass fed experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Liver						
Catalase (CAT, U mg^{-1} protein)	153.9	141.4	134.7	138.1	120.5	3.41
Glutathione reductase (GR, mU mg^{-1} protein)	2.20	2.14	1.86	2.61	1.14	0.16
Glucose 6-phosphate dehydrogenase (G6PDH, mU mg^{-1} protein)	171.0	128.9	153.5	90.4	111.7	6.14
Lipid peroxidation (LPO, nmol MDA g^{-1} tissue)	20.14	28.12	23.82	24.69	20.02	0.92
Intestine						
Catalase (CAT, U mg^{-1} protein)	232.6	200.6	144.2	170.0	140.2	10.90
Glutathione reductase (GR, mU mg^{-1} protein)	24.24	23.14	16.67	22.01	17.45	0.96
Glutathione peroxidase (GPx, mU mg^{-1} protein)	36.09	36.62	20.29	48.24	31.67	3.15
Glucose 6-phosphate dehydrogenase (G6PDH, mU mg^{-1} protein)	12.18	10.54	15.18	9.57	14.02	0.85
Lipid peroxidation (LPO, nmol MDA g^{-1} tissue)	14.18	10.89	9.71	19.07	14.17	0.66

Non-orthogonal contrast	Liver				Intestine				
	CAT	GR	G6PDH	LPO	CAT	GR	GPx	G6PDH	LPO
Control vs. 10BSG	0.221	0.903	0.006 *	0.005 *	0.318	0.695	0.956	0.536	0.032 *
Control vs. 10BSG-SSF	0.064	0.488	0.233	0.175	0.008 *	0.010 *	0.114	0.259	0.004 *
Control vs. 20BSG	0.124	0.403	0.000 *	0.096	0.055	0.428	0.207	0.326	0.002 *
Control vs. 20BSG-SSF	0.002 *	0.034 *	0.000 *	0.965	0.006 *	0.019 *	0.643	0.487	0.995
BSGF vs. BSG-SSF	0.096	0.014 *	0.030 *	0.022 *	0.061	0.008 *	0.022 *	0.019 *	0.006 *
10% vs. 20%	0.226	0.709	0.000 *	0.062	0.444	0.927	0.104	0.569	0.000 *
Interaction (BSG vs. SSF)	0.448	0.089	0.868	0.923	0.556	0.633	0.986	0.958	0.084

Values are presented as means (n = 9) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analyses; $p < 0.05$).

In the intestine, CAT and GR activities were lower in fish fed the BSG-SSF diets than in the control (Table 6). Independently of dietary inclusion level, GR and GPX activities were also lower in fish fed the BSG-SSF rather than the BSG diets. LPO levels were also lower in fish fed the BSG-SSF rather than the BSG diets, but it increased with the dietary inclusion level.

4. Discussion

Low-protein, plant-derived ingredients are nutritionally problematic for use in feed for carnivorous fish, such as European seabass (trophic level 3.5 ± 0.5, FishBase), due to their high fiber content [22] (REF). The results of this study showed that dietary incorporation of BSG reduced the growth performance of European seabass juveniles independently of the incorporation level. Differently, in gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), dietary incorporation levels of BSG up to 10–15% did not impair growth performance [23,24]. On the other hand, for omnivorous fish species, such as striped catfish (*Pangasianodon hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*), dietary BSG levels as high as 50% have been successfully used without adversely affecting growth performance [25,26].

The lower palatability of the diets, and consequently a reduced feed intake, cannot explain the decreased growth performance of European seabass juveniles, as diet composition did not affect feed intake. The higher cellulose content of the BSG diets contributes to explaining the lower energy available for growth, as feed intake was not adjusted to the dietary energy intake, as is generally observed in fish [27], including European seabass. Indeed, it was previously shown that European seabass fed a 20% cellulose-supplemented diet increased voluntary feed intake, matching the digestible energy intake to the non-supplemented diet [28].

Solid-state fermentation has emerged as a promising strategy to address the challenges of utilizing low-valued agro-industrial by-products as feedstuffs [29–31]. In the present

study, BSG-SSF significantly enhanced growth performance and feed utilization of European seabass compared to the unfermented product and promoted a growth performance similar to the control diet with dietary incorporation of 20%. Moreover, dietary incorporation of 10% BSG-SSF promoted higher growth performance and feed utilization than in the control group. These results may be attributed to the improved nutritional profile and nutrient digestibility of BSG-SSF compared to BSG. Indeed, a previous study showed that SSF of BSG reduced cellulose and hemicellulose content and increased crude protein content and the apparent digestibility of dry matter, lipids, and energy [18]. Additionally, the dietary methionine content of BSG-SSF was higher than that of BSG, which was reflected in a dietary methionine content similar to that of the control diet. Previously, in European seabass, it was also shown that dietary incorporation of a 20% SSF plant feedstuff mixture (rapeseed, soybean sunflower, rice bran, 25% each) with *A. niger* increased feed utilization efficiency and energy digestibility without compromising growth [19]. Also, in rohu (*Labeo rohita*) fingerlings, the dietary inclusion of 40% solid-fermented groundnut oil cake with yeast (*Pichia kudriavzevii*) increased the growth and PER [29]. Similarly, in gibel carp (*Carassius auratus gibelio*), a low inclusion level (3%) of plant ingredients (sprayed corn husk, rapeseed meal, soybean meal, palm meal, and rice bran) fermented with a *Lactobacillus* spp. and *Bacillus* spp. enhanced growth performance [30]. For Nile tilapia, up to 37% of fishmeal in the diet can be replaced with fermented soybean meal; however, higher replacement levels reduced growth and feed utilization efficiency [31].

Body composition is a valuable indicator of animal nutritional status, reflecting the balance between nutrients and energy intake, expenditure, and storage [32]. In the current study, the body composition of fish fed the experimental diets was similar to that of fish fed the control diet. Similarly, the inclusion of a 20% SSF plant-based ingredient mixture in the diet had no impact on the body composition of European seabass [19]. However, in the test diets, whole-body lipid and energy content and HSI decreased with the increased dietary inclusion of BSG and BSG-SSF. HSI was also higher in fish fed the 10% SSF-BSG diet compared to the 10% BSG diet. This seems related to the increased cellulose content in the diets, leading to lower available energy to be deposited as lipids in viscera and muscle. Similar increases in HSI have been reported in largemouth bass (*Micropterus salmoides*) and African catfish (*Clarias gariepinus*) fed diets containing non-fermented and fermented ingredients [33,34], while other studies reported the opposite effects, such as in Furong crucian carp [35], or found no effects, as observed in rainbow trout [36].

Plasma metabolites are also important biomarkers of fish nutritional status. In the present study, plasma glucose levels were reduced in fish fed the 20% BSG and BSG-SSF diets, likely due to the lack of wheat meal, the main source of starch (glucose) in the experimental diets. However, fermentation of ingredients has been shown to enhance plasma glucose levels, as observed in European seabass [19] and rainbow trout [37], suggesting that fermentation increases carbohydrate bioavailability. In the present study, fermentation of BSG did not affect plasma glucose levels despite reductions in dietary cellulose and hemicellulose content. While plasma cholesterol was unaffected by diet composition, plasma triglycerides and phospholipids were lower in the BSG diets and decreased with dietary levels, which may be related to dietary fiber content. The impact of dietary fiber on plasma lipid parameters in European seabass remains inconsistent. While some studies reported no effect on plasma cholesterol and triglycerides [38], others observed reductions in both parameters [21,39]. Fermentation of BSG increased the plasma triglycerides, probably due to the increased lipid digestibility observed in BSG-SSF diets [18].

The dietary inclusion of BSG decreased the PER and N retention, which indicates increased amino acid catabolism. However, no changes in hepatic GDH activity, which is the primary amino acid deaminase, were observed. However, ALAT and ASAT activities

were higher in fish fed the BSG diets rather than the BSG-SSF diets, suggesting an increased need for amino acid interconversion to compensate for eventual amino acid imbalances in the BSG diets. In contrast, some studies have reported increased [40] or unchanged [19,41] hepatic transaminase activity in fish fed diets containing fermented ingredients.

While hepatic β-oxidation (HOAD) and lipogenesis (FAS) enzyme activities were unaffected by the diet composition, GK and PK activity decreased with the dietary BSG and BSG-SSF levels, which aligns with the reduced plasma glucose levels observed in fish fed these diets and, therefore, the lower available glucose for glycolysis [42]. ME activity, a NADPH-generating enzyme, was not affected by fermentation but was reduced with increasing BSG levels, which may be attributed to the reduced energy availability in diets with higher BSG inclusion. In seabass, a positive relationship between dietary digestible energy levels and ME and FAS activity has been reported [43]. In seabass and *Labeo rohita*, a positive relationship was also observed between dietary incorporation of fermented ingredients and ME and FAS activity, suggesting higher energy availability [19,29]. However, in this study, FAS activity remained unaffected.

The antioxidant system of animals is composed of enzymatic and non-enzymatic mechanisms that interact to regulate the balance between the generation and elimination of reactive oxygen species [44]. Several endogenous and exogenous factors can disrupt this balance, increasing susceptibility to oxidative damage of lipids, proteins, and DNA [45]. Among these, exogenous factors such as abiotic parameters and diet composition are highly relevant. In the present study, dietary incorporation of BSG did not affect hepatic LPO levels but increased intestinal LPO levels. However, in both tissues, regardless of the inclusion level, fermentation of BSG restored LPO levels, decreased GR and GPX activities (GPX was only measured in the intestine), and increased G6PDH activity. Compared to the control group, hepatic LPO levels were unaffected by diet composition (except for fish fed the 10BSG diet), while LPO levels were lower in fish fed the BSG-SSF diets rather than the BSG diets. On the other hand, in the intestine, LPO levels were lower in fish fed the 10% BSG and BSG-SSF diets than in the control group, while LPO levels were also lower in fish fed the BSG-SSF diets rather than the BSG diets.

Fermentation of BSG has been reported to release polyphenolic and other antioxidant compounds, including those present in BSG [17] and other by-products [46], enhancing the antioxidant potential of fermented ingredients and mitigating oxidative damage in species such as European seabass [47], coho salmon (*Oncorhynchus kisutch*) [48], and Nile tilapia [49]. As observed in this study, another study of European seabass also reported decreased hepatic LPO levels in diets including SSF ingredients without affecting antioxidant enzyme activity [50], suggesting that the antioxidant benefits of SSF of BSG may not be attributable to enzymatic antioxidant pathways but rather to the improved nutritional profile of BSG, with a reduction in cellulose and hemicellulose content and an increase in antioxidant levels.

The increased intestinal LPO levels observed with BSG diets may be due to their higher fiber content, which affects the intestinal oxidative status without triggering a full antioxidant response, as seen in sea bream and rainbow trout [21,51]. The impact of dietary fiber on intestinal oxidative status appears to depend on its type and inclusion level [52]. Oxidative damage associated with excessive dietary fiber may be mitigated by hydrolyzing the fiber, as observed with the addition of exogenous enzymes to the diet in carp [53] and sea bream [54], or through pre-treatment processes like fermentation, as observed in the present study.

5. Conclusions

Dietary inclusion of BSG led to reduced growth, feed utilization efficiency, plasma glucose, and triglycerides, and a decrease in hepatic glucokinase (GK) and malic enzyme (ME) activity. Solid-state fermentation (SSF) of BSG alleviated these limitations, improving growth and overall feed and protein utilization. While BSG impaired liver and intestine oxidative status, SSF-BSG countered this effect in both tissues.

Overall, the results of this study indicate that BSG-SSF can be included at levels of up to 20% in the diets of European seabass juveniles without affecting growth, feed utilization, and hepatic and intestine oxidative status, thus alleviating the negative results observed with the dietary inclusion of BSG. These results suggest that BSG-SSF could be a valuable alternative to more traditional feed ingredients, offering both environmental and economic benefits.

Further studies are needed to fine-tune the incorporation levels of BSG-SSF for different fish species and to explore its long-term effects on fish well-being and health, ultimately contributing to enhanced sustainability in the aquaculture industry.

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