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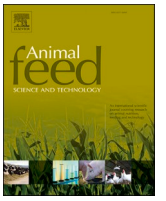

Effect of solid-state fermentation of Brewer's spent grain on digestibility and digestive function of european seabass (*Dicentrarchus labrax)* juveniles

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ABSTRACT

This study assessed the effects of dietary inclusion of solid-state fermented (SSF) brewer's spent grain (BSG) with *Aspergillus ibericus* on nutrient and energy digestibility, digestive enzyme activity, and intestinal histomorphology of juvenile European seabass (*Dicentrarchus labrax*). Five diets (18 % crude lipids and 45 % crude protein) were formulated, including a control diet (without BSG), two diets with 10 % and 20 % unfermented BSG (10BSG and 20BSG), and two other diets with 10 % and 20 % fermented BSG (10BSG-SSF and 20BSG-SSF). SSF affected the BSG's nutritional composition, including a 21 % increase in protein content and reductions in lipid (49 %), cellulose (30 %), hemicellulose (34 %), and lignin (7.3 %) content. Antioxidant activity and total phenolic compounds were minimal before SSF but significantly increased after SSF. Dietary incorporation of 20 % BSG-SSF increased the digestibility of dry matter ($p < 0.01$), protein ($p=0.03$), isoleucine ($p=0.03$), glutamate ($p=0.02$), lipids ($p=0.04$) and energy ($p=0.04$) compared to the 20BSG diet. Moreover, SSF also modulated digestive enzyme activity, reducing total protease ($p=0.03$) and trypsin ($p=0.01$) activities in fish fed the 10BSG-SSF diet compared to the control diet. Fish fed the 20BSG diet showed changes in intestinal histomorphology compared to those fed the control diet, and SSF of BSG appeared to mitigate these effects.

Overall, these results indicate that SSF is a promising technique for enhancing the nutritional quality of low-value agro-industrial by-products, such as BSG, increasing their potential use as feed ingredients and contributing to reducing the climate and environmental footprint of aquaculture production.

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1. Introduction

The world population is expected to surpass nine billion people by 2050, which will demand a 70 % increase in food production systems (FAO, 2022). To meet this demand, aquaculture production must also increase, aligning with the sustainable food production practices and food security objectives outlined in the United Nations' Sustainable Development Goals (FAO, 2022; Charles et al., 2010). For this purpose, aquaculture production needs to reduce the traditional ingredients in aquafeeds, such as fisheries and agricultural feedstuffs, which compete directly with human consumption, are expensive, and have a high carbon footprint (Colombo et al., 2022). In this context, low-cost agro-industrial by-products can be viable alternatives due to their abundant availability, minimal use in human consumption, and low carbon footprint (Berbel and Posadillo, 2018). Furthermore, these biomasses are often discarded, directly causing adverse environmental impacts. Therefore, the valorization of agro-industrial by-products as feedstuffs would simultaneously benefit the aquaculture industry and ecosystem integrity based on an eco-friendly and circular economic framework (Fernandes et al., 2021; Filipe et al., 2020; Diógenes et al., 2018; Obirikorang et al., 2015).

Brewer's spent grain (BSG) is the main by-product of the brewing industry, accounting for approximately 80 %–85 % of the total solid waste produced (Mussatto, 2014; Mussatto et al., 2006). In 2021, the EU produced approximately 32 billion liters of beer, generating over 6 billion tons of BSG (Eurostat, 2021). In addition to direct discarding, BSG is often used as feed for ruminants, but its further utilization is limited by its high moisture and sugar content, which hinders its transport and storage (Lao et al., 2020; Westendorf and Wohlt, 2002). Nonetheless, the valorization of BSG is particularly compelling, showing the potential to yield a diverse array of compounds, such as enzymes (Liguori et al., 2015), phenolic compounds (Leite et al., 2019), lactic acid (Mussatto, 2014), and ethanol (Parchami et al., 2022).

Despite its potential, the exploration of BSG nutritional improvement and its application in animal feeds, including aquatic feeds, has been limited. In aquaculture, studies using BSG as a substitute for fish meal or agricultural feedstuffs have only been conducted in omnivorous species, such as carp (*Cyprinus carpio* L.) (Morgan et al., 2013) and Nile tilapia (*Oreochromis niloticus*) (Tidwell et al., 2023). The high fiber (up to 50 % DM) and moderate protein content (between 20 % and 30 % DM) limit BSG inclusion in diets for carnivorous fish (Nazzaro et al., 2021; San Martin et al., 2020). Similar to other animals, fish lack intestinal enzymes capable of degrading non-starch polysaccharides (NSP), hindering the dietary inclusion of high levels of fiber-rich ingredients because they may negatively affect growth, health, nutrient digestion, and metabolism (Dawood and Koshio, 2020; Glencross et al., 2020; Kokou and Fountoulaki, 2018; Oliva-Teles et al., 2015; Sinha et al., 2011). Biotechnological approaches can be used to improve the nutritional value of BSG and increase its potential for use in aquafeeds. However, including bioprocessed BSG in aquafeeds has yielded mixed results. For example, dietary inclusion of enzymatically hydrolyzed BSG decreased lipids and protein weight gain and digestibility in rainbow trout (*Oncorhynchus mykiss*) (Estévez et al., 2022). Similarly, in sea bream (*Sparus aurata*), dietary replacement of fish meal with 20 % dry or hydrolyzed BSG reduced weight gain and feed efficiency (Estévez et al., 2021). In contrast, including up to 20–30 % enzymatically treated BSG in diets for rainbow trout (*Oncorhynchus mykiss*) did not affect protein digestibility, growth, and feed efficiency (Nazzaro et al., 2021).

Innovative microbial fermentation techniques are gaining attention as strategies for increasing the nutritional value of agro-industrial by-products with minimal environmental impact. Solid-state fermentation (SSF) involves the growth of selected microorganisms in a solid matrix that functions as a nutritional and physical support (Bhargav et al., 2008). SSF requires low energy and water volumes and usually uses low-cost and abundant by-products as fermentative substrates, decreasing operating costs and environmental impact and avoiding the formation of toxic compounds (Soccol et al., 2017). Fungi, in particular, are commonly used in SSF due to their ability to produce a wide range of enzymes that degrade the complex cell wall polysaccharides of agro-industrial by-products, such as BSG (Bhargav et al., 2008). The efficiency of SSF of BSG by different fungi, e.g. *Mortierella alpina; Rhizopus oryzae*, *Aspergillus niger* and *Aspergillus ibericus,* to enhance its nutritional value and produce fungal enzymes has been demonstrated through the degradation of the lignocellulosic matrix and the production of highly active lignocellulosic enzymes, particularly with the *Aspergillus ibericus* (Jacobs et al., 2009; Leite et al., 2019; Chin et al., 2022).

While solid-state fermentation (SSF) has been employed to yield diverse bioactive compounds from agro-industrial by-products (Fernandes et al., 2021; Salgado et al., 2015; Filipe et al., 2020), there is currently a lack of studies assessing the effectiveness of SSF in enhancing the BSG nutritional value for fish nutrition. Therefore, this study aimed to evaluate 1) the nutritional changes in BSG after SSF with *Aspergillus ibericus* and 2) compare the effects of dietary inclusion of non-fermented and SSFed BSG on diet digestibility, intestinal function, and intestinal histomorphology of European seabass (*Dicentrarchus labrax*) juveniles.

2. Materials and methods

2.1. Solid-state fermentation

The brewer's spent grain (BSG) was supplied by Unicer-Bebidas de Portugal, SA (Matosinhos, Portugal). BSG was composed of barley, Pilsen malt, and corn Gritz and was obtained after the raw material filtration process. *Aspergillus ibericus* MUM 03.49 was used in the solid-state fermentation (SSF) of BSG and was supplied by Micoteca of the University of Minho (Braga, Portugal).

The details of the optimization of the SSF of BSG are described in Fernandes et al. (2022). Briefly, prior to SSF, BSG was sterilized (121 °C, 15 min), and the moisture content was adjusted to 75 % w/w (wet basis) with sterile distilled water. Then, 400 g of BSG (dry weight) was placed in each previously sterilized (121 °C, 15 min) fermentation tray and inoculated with 2 mL of spore solution at a concentration of $10^{\hat{}6}$ cells mL^{-1}. The trays were incubated at 25 °C for 7 days and manually stirred every day. At the end of the SSF process, the fermented BSG (BSG-SSF) was pooled and stored at 4 °C until the test diets were produced. For analytical purposes,

samples from 3 different trays were randomly collected to characterize the BSG-SSF (Table 1).

2.2. Experimental diets

A control diet (45 % crude protein, 18 % crude lipids) was formulated to contain 15 % fish meal and 65.8 % plant feedstuffs. Four other diets were formulated similar to the control diet but partially replacing the plant feedstuff mixture with 10 % or 20 % of unfermented (10BSG and 20BSG diets) or BSG-SSF (10BSG-SSF and 20BSG-SSF diets) products. Chromium oxide was used as a digestibility marker and added to the diets at 1 %. The ingredients were finely ground, thoroughly mixed, and pelleted using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 2 mm die. The diets were placed on trays and dried in an oven at 60 °C for 24 h. The ingredients and proximal composition of the experimental diets are presented in Table 2, and the amino acid composition is shown in Table 2.

2.3. Digestibility trial

The digestibility trial was approved by the Ethics Committee of CIIMAR and the National Competent Authority (Direção Geral de Alimentação e Veterinária - DGAV) (reference ORBEA_CIIMAR_27_2019). All procedures were performed by accredited scientists in compliance with the European Union (Directive 2010/63/EU) and Portuguese (DecretoLei n° 113/2013) guidelines on protecting animals for scientific purposes.

Juveniles of European seabass were purchased from commercial aquaculture (Maresa, Huelva, Spain). Fish were transported to the experimental facilities at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR, Matosinhos, Portugal) and quarantined for two weeks, during which they were fed a commercial diet (Neo Gold Blue seabream/seabass diet (48 % protein and 17 % lipids; Sorgal, S.A., Portugal). After this period, the fish were transferred to the experimental system and acclimatized for 2 weeks.

The experimental system consisted of a thermoregulated water recirculation system with 12 fiberglass tanks of 60 L capacity, equipped with a feces-settling column connected to the outlet of each tank. During the trial, the water flow in each tank was maintained at 5 L min^{-1}, the average water temperature was 22 ± 1°C, salinity was 35 ± 1 ‰, nitrogenous compounds were maintained below 0.02 mg/L, and dissolved oxygen was above 90 %. The photoperiod was adjusted to a 12/12 h light/dark cycle.

A completely randomized block design was used to test each diet in duplicate in two sequential periods (blocks) to obtain four replicates per treatment. The same group of fish (initial average body weight of 92 g) was used in each block and was randomized between blocks. The fish were adapted to the experimental diets for 7 days in each period before starting fecal collection. Fish were manually fed twice daily, at 9:00 a.m. and 4:00 p.m., until apparent visual satiety. Feces were collected for 22 consecutive days before the first daily feeding, centrifuged (3000 × g, 10 min), pooled for each tank, and stored at −20 °C until analysis. Forty-five minutes after the afternoon meal, the feces-settling columns and pipes were thoroughly cleaned to eliminate any residual feces or uneaten feed. The second block period was extended to 60 days of feeding for intestinal sampling purposes. Six hours after the morning meal, 4 fish per tank (8 per treatment) were randomly sampled and killed by an overdose of anesthesia (10 mL L^{-1}; ethylene glycol monophenyl ether), followed by cervical dislocation. The intestine was excised, freed of perivisceral fat and connective tissues, and divided into two regions: the anterior intestine (from the pyloric cecum to the distal intestine) and the distal intestine (visually distinguished by increased diameter, thicker mucosa, and darker mucosa). For histological analyses, tissue samples (approximately 0.5 cm) from each region were collected, washed in phosphate-buffered saline (PBS), fixed in phosphate-buffered formalin (4 % w/v; pH 7.4) for 24 h, and then transferred to 70 % v/v ethanol for storage until processing. The remaining tissue from both regions was pooled per fish and stored at −80 °C until the digestive enzyme activity was analyzed.

The apparent digestibility coefficients (ADC) of dry matter, protein, lipids, and energy in the experimental diets were calculated as follows:

$$ADC_{diet} = \left(1 - \left(\frac{\text{dietary } Cr_2O_3 \text{ level} \times \text{feces nutrient or energy level}}{(\text{feces } Cr_2O_3 \text{ level} \times \text{dietary nutrient or energy level})}\right)\right) \times 100$$

Table 1
Composition of brewer's spent grain before (non-fermented BSG) and after seven days of solid-state fermentation with Aspergillus ibericus MUM 03.49 (BSG-SSF).

Dry matter basis	Non-Fermented BSG	BSG-SSF
Crude Protein (%)	26.7 ± 0.5	32.3 ± 0.2
Crude Lipids (%)	5.7 ± 0.04	2.8 ± 0.0
Klason lignin (%)	15.0 ± 0.4	13.9 ± 0.2
Cellulose (%)	21.1 ± 1.0	14.8 ± 0.1
Hemicellulose (%)	23.7 ± 1.4	15.7 ± 0.1
DPPH (μmol trolox equivalents g^{-1})	0.6±0.0	64.2 ± 5.2
Total phenolics (mg gallic acid equivalents g^{-1})	0.6 ± 0.1	11.8 ± 0.2
Cellulase (U g^{-1})	—	925.1 ± 46.3
Xylanase (U g^{-1})	—	67.7 ± 24.1

Values are presented as mean (n=3) ± standard deviation.

Table 2
Ingredients and proximal compositions of experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF
Ingredient (% dry matter)					
BSG[a]	—	10	—	20	—
BSG-SSF[b]	—	—	10	—	20
Fish meal[c]	15	15	15	15	15
Pea protein concentrate[d]	10	10	10	10	10
Corn gluten[e]	12.5	12.5	12.5	12.5	12.5
Soybean meal[f]	17.6	15	15	10	10
Wheat gluten[g]	11.7	4.6	4.6	—	—
Rapeseed meal[h]	7.5	7.0	7.0	5.0	5.0
Sunflower meal[i]	5.6	5.3	5.3	3.8	3.8
Hemoglobin[j]	—	—	—	2.2	2.2
Hydrolyzed shrimp[k]	1.2	1.2	1.2	1.2	1.2
Fish oil[l]	14	14.4	14.4	14.9	14.9
Vitamin premix[m]	1	1	1	1	1
Choline chloride (50 %)	0.5	0.5	0.5	0.5	0.5
Mineral premix[n]	1	1	1	1	1
Binder[o]	1	1	1	1	1
Dicalcium phosphate	—	0.1	0.1	0.5	0.5
Methionine[p]	0.1	0.1	0.1	0.1	0.1
Taurine[p]	0.3	0.3	0.3	0.3	0.3
Chromium oxide	1	1	1	1	1
Proximate composition (% dry matter)					
Dry matter (DM, %)	88.5	87.3	87.7	88.2	87.3
Crude protein (CP)	45.3	45.6	46.9	45.9	46.3
Crude lipid (CL)	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	6.33	6.84	6.49	6.00	6.30
Cellulose	4.21	6.20	4.71	7.07	5.35
Hemicellulose	2.54	2.43	1.85	2.11	1.79
Klason lignin	12.2	10.0	10.1	12.8	12.6
Essential amino acids (% dry matter)					
Lysine, Lys	2.17	1.94	2.26	2.15	2.14
Arginine, Arg	1.63	1.67	1.62	1.68	1.59
Histidine, His	0.71	0.74	0.89	0.78	0.82
Isoleucine,Ile	1.30	1.29	1.30	1.28	1.32
Leucine,Leu	5.24	5.53	5.66	5.91	5.63
Valine, Val	1.33	1.34	1.45	1.44	1.50
Methionine, Met	1.20	0.97	0.95	1.24	1.39
Phenylalanine, Phe	2.07	2.00	1.97	2.25	2.05
Threonine, Thr	1.14	1.16	1.34	1.14	1.43
Non-essential amino acids					
Tyrosine, Tyr	0.78	0.83	0.83	0.89	0.85
Aspartic acid, Asp	2.77	2.76	2.75	2.51	2.69
Glutamic acid, Glu	3.91	4.01	3.96	4.15	3.71
Serine, Ser	4.42	5.07	4.79	4.58	4.63
Glycine, Gli	5.53	5.39	5.37	5.37	5.45
Alanine, Ala	4.93	5.22	4.91	5.62	5.30
Proline, Pro	5.06	5.31	5.37	5.02	5.27

[a] Brewer's spent grain, Unicer-Bebidas de Portugal, SA. Matosinhos, Portugal; composition detailed in Table 1
[b] Fermented brewer's spent grain; composition detailed in Table 1.
[c] Pesquera Centinela, Steam Dried LT, Chile (CP: 66.8; CL: 9,6). Sorgal, S.A. Ovar, Portugal.
[d] Pea protein concentrate (CP:45.1 %, CL: 2.84 %)
[e] Corn gluten (CP:80.1 %; CL:4.07 %). Sorgal, S.A. Ovar, Portugal
[f] Soybean meal 48 (CP: 44.9 %; CL: 1.4 %), Sorgal, S.A. Ovar, Portugal.
[g] Wheat gluten (CP: 74.7 %; CL: 1.74 %), Sorgal, S.A. Ovar, Portugal.
[h] Rapeseed (CP: 34.6 %; CL: 2.6 %), Sorgal, S.A. Ovar, Portugal.
[i] Sunflower (CP: 28.5 %; CL: 1.48 %), Sorgal, S.A. Ovar, Portugal
[j] Hemoglobin powder AP310P; APC Europe S.A.
[k] Hydrolyzed shrimp (CP: 69.8 %; CL: 12.1 %). Sorgal, S.A. Ovar, Portugal.
[l] Fish oil Sorgal, S.A. Ovar, Portugal.
[m] 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.
[n] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 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).
[o] Aquacube. Agil, UK.

[p] Feed grade amino acids, Sorgal, S.A. Ovar, Portugal.

2.4. Chemical analysis

2.4.1. Proximal composition

The proximal composition of BSG, before and after SSF, dietary ingredients, diets, and feces were analyzed as follows: dry matter, by drying the samples at 105 °C until constant weight; ash, by incineration in a muffle furnace at 450 °C for 16 h; protein (N × 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipids were extracted with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046) for ingredients and diets, or following a gravimetric method (Folch et al., 1957) for feces because of the limited amount of sample available; gross energy, by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, II, USA; PARR model 1261). Cellulose, hemicellulose, and lignin contents were determined as described by Filipe et al. (2020). Dietary amino acid quantification was performed by high-performance liquid chromatography (HPLC), according to Cocuron et al. (2017).

2.4.2. Phenolic compounds and antioxidant activity

To quantify the phenolic compounds and antioxidant activity of BSG-SSF, an aqueous extraction was performed using distilled water at a solid/liquid ratio of 1:5 (w/v). The mixture was agitated at 150 rpm for 30 min at room temperature, filtered through a fine mesh, sieved, and centrifuged at (15,000 g for 10 min at 4°C). The resulting supernatant was filtered through a syringe filter (0.45 μm, ø25 mm), and the extract was stored at −20 °C until analysis.

The Folin-Ciocalteu method was used to determine the total phenolic compounds, following Filipe et al. (2020). Briefly, 100 μL of the diluted sample (1:2) (or 100 μL of distilled water as a blank), 2 mL of 15 % Na_2CO_3, 500 μL of Folin–Ciocalteu reagent, and 7.4 mL of distilled water were mixed in a glass tube and incubated at 50 °C for 5 min. After cooling, the mixtures were mixed, and the absorbance was read at 740 nm using a calibration curve with caffeic acid (CA) at concentrations ranging from 0 to 2 g L^{-1}. The total phenolic compounds were expressed in milligrams of gallic acid equivalents per gram of dry solids.

Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH) as described by Blois (1958) and adapted by Filipe et al. (2020). Briefly, 200 μL of several dilutions of each sample were added to a microplate and mixed with 100 μL of DPPH. Methanol was used as a blank. The reaction was performed in the dark for 30 min, and the absorbance was read at 517 nm. A calibration curve was prepared using the standard 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox). The results are expressed in μmol of Trolox equivalents (TE) per gram of dry solid substrate (μmol TE g^{-1}).

2.4.3. Cellulase or xylanase activity

To measure xylanase (endo-1,4-β-xylanase) and cellulase (endo-1,4-β-glucanase) activities in BSG-SSF, carboxymethylcellulose 2 % w/v (CMC) and xylan 1 % w/v in 0.05 N sodium citrate buffer (pH 4.8) were used as substrates, respectively. The enzyme activity was measured as described by Filipe et al. (2020). One unit of cellulase or xylanase activity was defined as the amount of enzyme required to release 1 μmol of glucose or xylose per minute under assay conditions.

2.4.4. Digestive enzyme activity

The whole intestine was homogenized (1:4 w/v) using an ice-cold buffer solution (100 mM Tris-HCl, 0.1 mM EDTA, and 0.1 % (v/v) Triton X-100, pH 7.8) and centrifuged (30,000 × *g* for 30 min at 4 °C). The resulting supernatants were collected, divided into aliquots, and stored at −80 °C until analysis.

The activities of alpha-amylase (EC 3.2.1.1), trypsin (EC 3.4.21.4), and lipase (EC 3.1.1.3) were determined as described by Magalhães et al. (2015). Total alkaline protease (TAP) activity was measured according to Walter (1984) and adapted by Hidalgo et al. (1999), using casein as a substrate (1 % w/v) at pH 8, using 0.1 M tris HCl as a buffer. The reaction was stopped with 8 % trichloroacetic acid (w/v), and the absorbance was read at 280 nm against the blank.

All enzyme activities were determined at 37 °C using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.4.5. Histological processing

The anterior and posterior intestine samples were processed following routine procedures and stained with hematoxylin and eosin. The histomorphology of the two intestinal sections was evaluated using a double-blind semiquantitative scoring system ranging from 1 to 5, according to the criteria suggested by Krogdahl et al. (2003): 1) widening and shortening of intestinal folds, (2) number of goblet cells, (3) widening of the lamina propria, (4) size and regularity of supranuclear vacuoles, (5) changes in enterocytes nucleus position. Images were acquired using the Zen software (Blue edition; Zeiss, Jena, Germany).

2.5. Statistical analysis

Data are presented as mean and pooled standard error of the mean (SEM). Data were checked for normality and homogeneity of variance and normalized when necessary. Non-orthogonal contrasts were performed to compare each test diet with the control diet, non-fermented BSG versus BSG-SSF diets, dietary incorporation level (10 versus 20), and interactions of incorporation level versus BSG

processing. Non-parametric Kruskal-Wallis tests followed by pairwise comparisons with Bonferroni adjusted p-values were used for histological data because they did not follow normality and homogeneity of variance principles. To test the effect of dietary inclusion level (10–20 %) and fermentation process (fermented and non-fermented) on histological data, the Kruskal-Wallis test was used, excluding the control diet. All statistical analyses were performed using IBM SPSS Statistics software version 26 (IBM, NY, USA).

3. Results

The composition and enzymatic activity of BSG before and after 7 days of SSF with *Aspergillus ibericus* are presented in Table 1. SSF increased the crude protein content of BSG by 21 %, from 26.7 % to 32.3 %, but decreased the lipid content by 49 %, from 5.7 % to 2.8 %. Furthermore, SSF reduced lignin, cellulose, and hemicellulose contents by 7.3 %, 30 %, and 34 %, respectively, compared to unfermented BSG. Antioxidant activity and total phenolic compounds were nearly zero in BSG, and after SSF, increased significantly to 64 μmol Trolox equivalents g^{-1} and 12 mg gallic acid equivalents g^{-1}, respectively. Xylanase (68 U g^{-1} DM) and cellulase (925 U g^{-1} DM) activities were also detected after SSF, while no activity was observed in the unfermented product.

The ADC of proteins, lipids, energy, and amino acids are shown in Table 3. Dietary inclusion of 10 % BSG reduced the ADC of energy (p=0.04), whereas dietary inclusion of 20 % BSG decreased the ADC of dry matter ($p < 0.01$), protein (p=0.01), lipids (p=0.04), and energy ($p < 0.01$). However, irrespective of the dietary inclusion level, BSG-SSF restored ADC to values similar to those observed in the control group. Regardless of the dietary inclusion level, the ADC of dry matter, lipids, and energy of the BSG-SSF-based diets were higher than those of the BSG-based diets. Regardless of the treatment, the ADC of dry matter, protein, and energy of the diets with 20 % incorporation of BSG products were lower than those of diets with 10 % BSG products. Compared to the unfermented product, the ADC of dry matter ($p < 0.01$), protein (p=0.03), lipids (p=0.04), and energy (p=0.04) were higher in the diets containing 20 % BSG-SSF, but no differences between groups were observed at the 10 % incorporation level. Amino acid digestibility was not significantly affected by the ingredient source or dietary incorporation level. Nonetheless, the ADC for isoleucine (p=0.03) and glutamic acid (p=0.02) were higher in the 20BSG-SSF diet than in the 20BSG diet. The ADC of aspartic acid was lower in the BSG-SSF diets than in the BSG diets (p=0.04). The ADC of proline was higher in the 20BSG diet than in control (p=0.04), and the ADC of glutamate (p=0.02) and isoleucine

Table 3
Apparent digestibility coefficients (%) of European seabass juveniles fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Dry Matter	62.8	60.7	60.6	51.8	63.7	1.1
Organic matter	68.9	65.7	66.1	60.9	68.3	1.1
Protein	88.4	88.4	88.4	85.8	88.0	0.4
Lipids	94.2	93.6	93.7	91.0	94.5	0.4
Energy	71.4	67.7	69.1	64.2	68.3	0.7
Essential amino acids						
Lysine, Lys	90.0	89.9	90.8	90.0	90.9	0.4
Arginine, Arg	86.8	89.4	89.8	87.0	86.3	0.6
Histidine, His	89.2	88.2	90.2	88.7	88.6	0.4
Isoleucine, ILe	87.6	85.1	88.3	88.7	84.2	0.7
Leucine, Leu	90.5	92.8	87.1	91.5	90.8	0.7
Valine, Val	83.3	85.4	85.7	82.1	85.0	0.7
Methionine, Met	86.9	88.4	88.2	91.9	94.0	0.7
Phenylalanine, Phe	86.2	85.7	84.9	88.5	87.7	05
Threonine, Thr	87.3	86.7	88.5	87.0	88.1	06
Non-essential amino acids						
Tyrosine, Tyr	89.3	90.8	86.5	86.7	86.8	0.7
Aspartic acid, Asp	86.6	85.4	84.1	92.9	86.6	1.1
Glutamic acid, Glu	84.4	84.9	82.8	82.1	87.8	0.7
Serine, Ser	87.7	88.7	83.7	87.7	88.8	0.6
Glycine, Gly	87.6	87.9	85.0	88.9	88.4	0.6
Alanine, Ala	86.7	86.7	84.2	90.2	87.5	0.9
Proline, Pro	90.3	87.6	85.8	87.8	88.6	1.0

Non-orthogonal contrast

	Dry Matter	**Protein**	**Lipids**	**Energy**	**Lys**	**Ile**	**Leu**	**Asp**	**Glu**	**Pro**
Control vs 10BSG	0.24	0.97	0.59	0.04*	0.95	0.17	0.19	0.67	0.78	0.19
Control vs 10BSG-SSF	0.26	0.99	0.63	0.18	0.57	0.07	0.87	0.98	0.09	0.39
Control vs 20BSG	<0.01*	0.01*	0.04*	<0.01*	0.60	0.68	0.06	0.40	0.37	0.04*
Control vs 20BSG-SSF	0.63	0.65	0.81	0.08	0.97	0.50	0.54	0.04*	0.21	0.23
BSG vs BSG-SSF	0.03*	0.07	0.04*	0.04*	0.92	0.83	0.29	0.03*	0.42	0.29
Level (10 vs 20)	<0.01*	0.03*	0.15	0.03*	0.43	0.55	0.02*	0.09	0.15	0.72
10BSG vs 10BSG-SSF	0.96	0.96	0.95	0.42	0.92	0.06	0.45	0.02*	0.13	0.91
20BSG vs 20BSG-SSF	<0.01*	0.03*	0.04*	0.04*	0.95	0.0.3*	0.04	0.39	0.02*	0.18
Interaction (Level, SSF)	<0.01*	0.13	0.13	0.25	0.98	<0.01*	0.05	0.23	0.01*	0.38

Values are presented as mean (n = 4) and pooled standard error of the mean (SEM).

* Denotes significant differences (non-orthogonal contrast analysis) of control group versus each test group (10BSG, 10BSG-SSF, 20BSG, 20BSG-SSF); BSG versus BSG-SSF diets; BSF level 10 % versus SF level 20 %; 10BSG versus 10BSG-SSF; 20BSG versus 20BSG-SSF; and interaction (level and SSF).

(p=0.03) were higher in the 20BSG diet than in the 20BSG-SSF diet.

The intestinal enzyme activities of European seabass fed the experimental diets are presented in Table 4. Compared to the unfermented product, enzyme activity was not affected by SSF. However, fish fed the 10BSG-SSF diet had lower total protease (p=0.03) and trypsin activities (p=0.01) than those fed the control diet. Irrespective of the treatment, amylase (p=0.02) activity was higher in fish fed the 20 % diets than in those fed the 10 % diets.

Histological assessment of the anterior and distal intestines of European seabass fed the experimental diets is presented in Table 5. In the anterior intestine, fold height and lamina propria width were not affected by dietary treatments. The number of goblet cells was lower in fish fed the experimental diets than in the control. Compared to the control diet, the position of enterocytes was the same in fish fed the 10BSG, 10BSG-SSF, and 20BSG-SSF diets than the control diet. Fish fed the 20BSG (p=0.05) diet had higher scores in the position of the enterocyte nuclei than fish fed the control diet. In the distal intestine, only supranuclear vacuolation (p=0.02) was affected by diet composition, and it was higher in fish fed the 20BSG diet than in those fed the control diet (Fig. 1). In the anterior intestine, no differences between the experimental groups were observed in the mean score. However, the mean score was higher in fish fed the 20 % diets than in the control, irrespective of treatment. In the posterior intestine, no differences in the mean scores were observed between the groups.

4. Discussion

4.1. SSF of BSG with Aspergillus ibericus

Plant feedstuff cell walls are composed of highly complex carbohydrates that are not digested by monogastric animals, including fish, hindering plant feedstuff digestibility and nutrient absorption and negatively affecting fish growth, feed utilization, intestinal function, and overall health status (Glencross et al., 2020; Kokou and Fountoulaki, 2018; Oliva-Teles et al., 2015). This is particularly relevant for carnivorous fish, for which only low-fiber and relatively high-protein plant feedstuffs, such as soybean, rapeseed, wheat gluten, and corn gluten, are used in the formulation of plant-based diets (Gatlin et al., 2007). Nonetheless, the sustainability and cost-effectiveness of aquafeeds may be compromised by using only traditional feedstuffs in the diet formulation. Therefore, technological approaches that reduce the complexity of plant carbohydrates and increase the nutrient bioavailability of underutilized feedstuffs using the circular economy concept may be of high practical value. This is the case for SSF, a relatively simple and low-technological process that hydrolyzes plant feedstuff fiber content and increases protein and other nutrient contents while also releasing enzymes, organic acids, vitamins, and other compounds that may increase the nutritional value of fermented feedstuffs (Leite et al., 2021).

In the current study, SSF of BSG by *A. ibericus* increased the protein content and decreased the cellulose, hemicellulose, lignin, and lipid contents of the fermented product. Previously, we have also observed that SSF with *A. ibericus* increased the protein content and decreased the lipid content of DDGS (Filipe et al., 2023) or of a mixture of plant feedstuffs (soybean, rapeseed, sunflower, and rice bran) (Amaral et al., 2023, Vieira et al., 2022). In other studies where BSG was fermented by *Rhizopus oligosporus* or *Rhizopus sp.*, there was a decrease in lipid, fiber, sugars, and neutral detergent fiber, and an increase in the protein content of the fermented products (Cooray and Chen, 2018; Ibarruri et al., 2019). Moreover, SSF with BSG *Aspergillus oryzae* increased the protein content and decreased

Table 4
Intestine enzymes activity (mU mg^{-1} protein) of European seabass juveniles fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Total proteases	5.0	3.7	3.5	3.8	4.3	0.2
Trypsin	135.9	108.3	77.01	124.6	113.6	7.4
Amylase	122.7	106.3	87.6	115.4	159.4	8.4
Lipase	7.0	6.3	5.4	6.0	7.0	0.4

Non-orthogonal contrasts

	Total proteases	**Trypsin**	**Amylase**	**Lipase**
Control vs 10BSG	0.26	0.23	0.47	0.55
Control vs 10BSG-SSF	0.03*	0.01*	0.13	0.18
Control vs 20BSG	0.06	0.61	0.75	0.37
Control vs 20BSG-SSF	0.63	0.31	0.12	0.97
BSG vs BSG-SSF	0.84	0.18	0.44	0.94
Level (10 vs 20)	0.46	0.09	0.02*	0.46
10BSG vs 10BSG-SSF	0.26	0.16	0.42	0.46
20BSG vs 20BSG-SSF	0.16	0.62	0.06	0.40
Interaction (Level, SSF)	0.07	0.52	0.06	0.26

Values are presented as mean (n = 8) and pooled standard error of the mean (SEM).

* Denotes significant differences (non-orthogonal contrast analysis) of control group versus each test group (10BSG, 10BSG-SSF, 20BSG, 20BSG-SSF); BSG versus BSG-SSF diets; BSF level 10 % versus SF level 20 %; 10BSG versus 10BSG-SSF; 20BSG versus 20BSG-SSF; and interaction (level and SSF).

Table 5
Score-based evaluation of the anterior and distal intestine histology of European seabass fed the experimental diets[a].

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Anterior intestine						
Fold height	1.0	2.1	2.0	2.6	1.8	0.2
Goblet cells	1.2[a]	2.3[a,b]	2.8[b]	2.6[b]	2.5[b]	0.2
Lamina propria[b]	2.2	2.1	2.3	3.0	2.1	0.1
Supranuclear vacuolization	1.2[a]	1.6[a,b]	1.2[a]	2.2[b]	2.0[a,b]	0.1
Enterocyte nucleus position	1.2[a]	1.6[a,b]◆	1.2[a]◆	2.2[b]	2.0[a,b]	0.1
Mean	1.5[a]	2.0[a,b]	1.9[a,b]	2.5[b]	2.1[b]	0.2
Distal intestine						
Fold height	3.2	2.3	2.2	1.9	2.1	0.1
Goblet cells	2.0	3.0	3.5	2.2	2.7	0.3
Lamina propria[b]	2.0	2.6	2.2	2.3	3.1	0.2
Supranuclear vacuolization	2.0[a]	2.8[a,b]	2.7[a,b]	4.7[b]	3.1[ab]	0.3
Enterocyte nucleus position	1.0	1.0	1.0	1.0	1.0	0.0
Mean	2.9	2.6	2.4	2.6	2.8	0.2

Values are presented as mean scores (n = 8) and pooled standard error of the mean (SEM). Different lower-case letters represent statistical differences between dietary groups determined by comparing all Kruskal-Wallis pairs. Significance values were adjusted by Bonferroni correction for multiple tests. ◆ Denotes significant differences ($p < 0.05$) between the BSG levels (10 % versus 20 %).

[a] Scores from 1 to 5, with 5 indicating major alterations.

[b] Width and cellularity of the lamina propria

the lipid content of the fermented product (Ogunjobi et al., 2011). Differences in the nutrient composition of the BSG and SSF conditions, such as microorganism species, time, temperature, and bed height, led to differences in the final composition of the SSF products (Yang et al., 2021). The BSG nitrogen and carbon contents affect fungal growth, as nitrogen is used for fungal conidiation, and carbon is used as an energy source. Thus, the final composition of the SSF product depended on the total fungal biomass produced.

Filamentous fungi, particularly *Aspergillus* spp., are known to produce a wide range of enzymes, including carbohydrases, with higher enzymatic yields than those produced by yeast or bacteria (Ghorai et al., 2009). These enzymes are responsible for the degradation of the complex lignocellulosic matrix of the substrate. In the present study, high NSP content of BSG induced the production of cellulase and xylanase, with higher cellulase than xylanase activity. Similarly, SSF of different oilseed cakes by *A. ibericus* and *A. niger* led to higher cellulase and xylanase production than SSF of *Rhyzopus oryzae*, which in turn led to higher production of β-glucosidase (Sousa et al., 2018). Enzymatic production differs among species of the genus *Aspergillus*. For example, in the SSF of DDGS with *A. ibericus*, *A. uvarum*, and *A. carbonarius*, higher cellulase production was obtained with *A. ibericus*, whereas higher xylanase production was obtained with *A. uvarum* (Filipe et al., 2023). Maximum enzymatic production also depends on the duration of fermentation. For instance, in the SSF of rice bran with *A. terreus*, xylanase production reached its maximum after 8 days, aminoglycosidase after 6 days, and β-glucosidase after 10 days, and decreased afterward (Liang et al., 2011).

Bioactive compounds, such as alkaloids and phenolic compounds entrapped in the substrate lignocellulosic matrix, are released during the SSF process (Martins et al., 2011; Devasagayam et al., 2004) during the SSF process. The release of phenolic compounds is particularly interesting owing to their potent antioxidant properties (Bhanja-Dey et al., 2016). In the present study, SSF of BSG increased the total phenolic content by more than 50 times. Similar results were obtained by the SFF of a mixture of ago-industrial by-products (exhausted grape marc, vine trimming shoots, and exhausted olive pomace) with *Aspergillus ibericus* (Filipe et al., 2020). An increase in total phenolic content was also observed in the SSF of dried distiller grains with solubles of *Aspergillus ibericus*, Aspergillus *uvarum,* and *Aspergillus carbonarius* (Filipe et al., 2023). However, a reduction in phenolic compounds after SSF can also occur because of enzymes produced by fungi, such as laccases and peroxidases (Rodrıguez et al., 2004). For example, a reduction in phenolic compounds was observed in the SSF of olive pomace, exhausted grape marc, and vine shoot trimmings by *Aspergillus uvarum* (Salgado et al., 2015) or in the SSF of exhausted olive pomace by *Aspergillus Ibericus*, *Aspergillus uvarum,* and *Aspergillus niger* (Sousa et al., 2018).

4.2. Digestibility of unfermented and SSFed BSG

BSG presents a potential and valuable source of nutrients for animal nutrition. However, its high fiber content (up to 50 %) may compromise nutrient availability (Mitri et al., 2022), limiting its utilization in aquafeeds, particularly for carnivorous fish species. In the present study, the replacement of a plant feedstuff mixture (soybean, rapeseed, sunflower, and wheat gluten meal) with BSG reduced the digestibility of dry matter, protein, lipids, and energy, and these effects linearly increased with the dietary replacement level. This is mainly related to the high fiber content of BSG. High dietary fiber levels reduce the digestibility of energy and may compromise the digestion of dietary nutrients by affecting the physical characteristics of the digesta and access of endogenous enzymes to the substrate (Adeola and Bedford, 2004). In European seabass, the detrimental effects of excessive dietary incorporation of complex polysaccharides on nutrient digestibility have been well documented (Fountoulaki et al., 2022; Magalhães et al., 2015; Enes et al., 2011; Tibaldi et al., 2006). In contrast, in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), dietary incorporation of 20 % BSG replacing fishmeal did not affect protein digestibility but decreased lipid digestibility (Nazzaro et al., 2021). However, in another study, the replacement of plant ingredients (soybean meal, wheat gluten, and wheat starch) with up to 15 % BSG

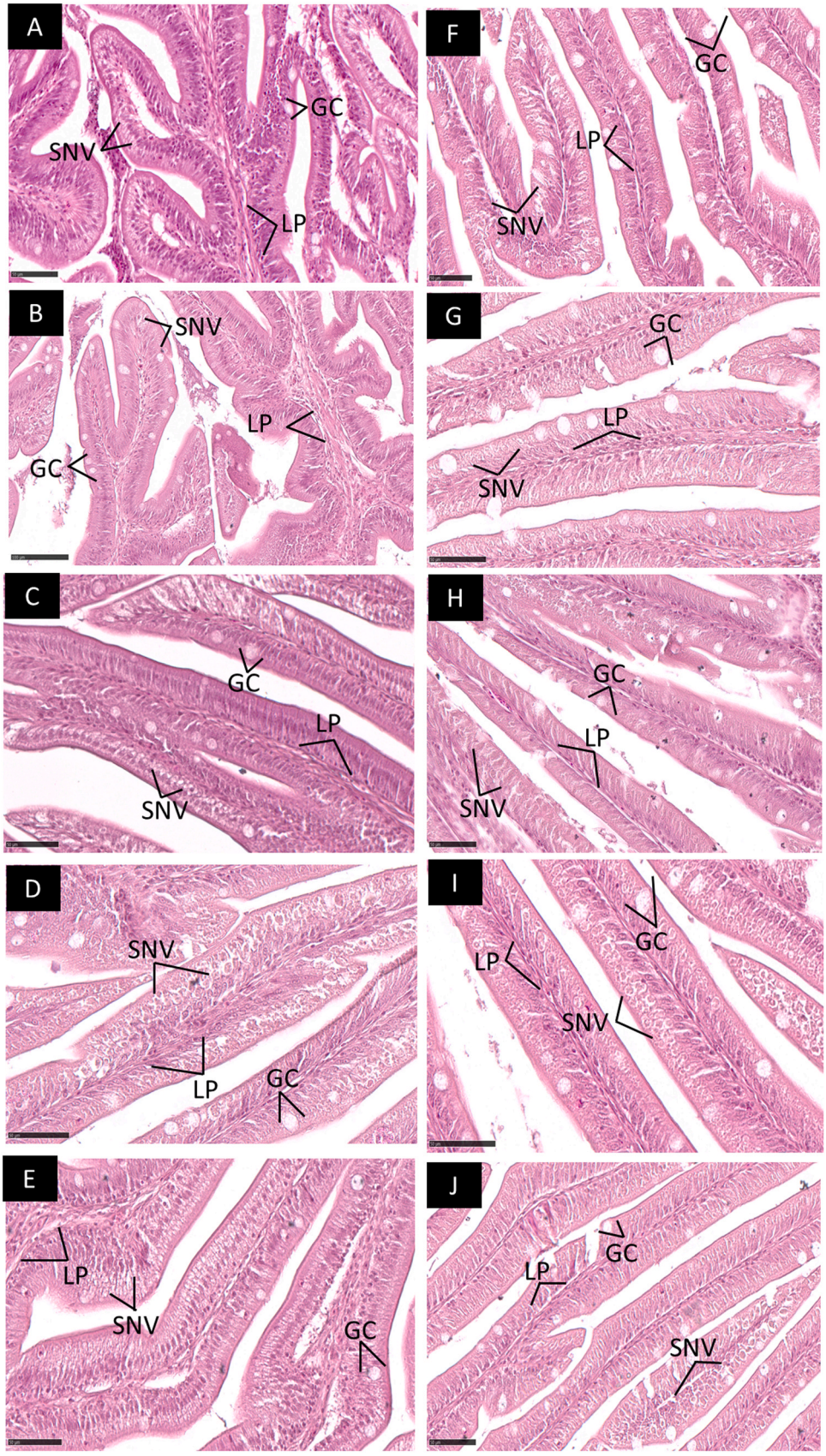

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Fig. 1. Detailed of anterior (Figs. A to E) and distal (Figs. F to J) intestine of European seabass fed the control (A and F), 10BSG (B and G), 10BSG-SSF (C and H), 20BSG (D and I), and 20BSG-SSF (E and J) for 68 days. Lamina propria: LP, goblets cells (GC); lamina propria: (LP) and supranuclear vacuoles (SNV). Scale bar: 25 mm; HeE staining.

did not affect protein and lipid digestibility in rainbow trout and gilthead seabream (Estevéz et al., 2022). This indicates that the adverse effects of dietary incorporation of BSG are related to species and dietary incorporation levels.

BSG is a highly heterogeneous lignocellulosic biomass, and differences in lignocellulosic composition may also have contributed to the observed results (Lynch et al., 2016). In the present study, the BSG included in the diets contained approximately 45 % NSP (24 % hemicellulose and 21 % cellulose) and 15 % lignin. Studies carried out by Nazzaro et al. (2021) and Estevéz et al. (2022) indicated that the enzymatic hydrolysis of BSG reduced crude fiber content by 11.7 % compared to untreated BSG. Different carbohydrate fractions affect digestibility in divergent ways (Irvin et al., 2016). For example, starch is well tolerated (Oliva-Teles et al., 2015), while cellulose has little effect on protein digestibility but may decrease dry matter and energy digestibility (Glencross et al., 2020; Hansen and Storebakken, 2007; Kraugerud et al., 2007). In seabass, the dietary inclusion of up to 20 % insoluble dietary fiber (cellulose) had little impact on protein and dry matter digestibility (Dias et al., 1998), digesta characteristics, gut histology, and evacuation (Bonvini et al., 2018). However, 30 % sunflower meal (41 % NSP) decreased the dry matter but did not affect protein digestibility (Fountoulaki et al., 2022).

Previously, it was demonstrated that SSF of BSG with *A. ibericus* MUM 03.49 allowed the production of a highly active carbohydrases extract that can contribute to increasing dry matter and energy digestibility (Fernandes et al., 2021). In the present study, SSF of BSG increased the digestibility of dry matter and nutrients to a level similar to that of the control diet. These positive results can be attributed to the reduction in fiber content and the presence of fungal carbohydrases in the SSF product.

4.3. Digestive function and intestine histomorphology

Irrespective of the treatment, increased dietary inclusion of BSG reduced trypsin and amylase activity. These results align with the observed reduction in the ADC of protein and energy in diets.

The total proteases and trypsin activities were significantly lower in fish fed the 10BSG-SSF diet than in those fed the control diet. In absolute values, the activity of these enzymes was also lower in fish fed 20BSG-SSF than in the control, but the differences were not statistically significant. However, the reduction in proteolytic activity in the BSG-SSF groups was not reflected in differences in the ADC of the protein. During the SSF process, proteins may have been partially hydrolyzed by fungal proteases, and fungal proteases may have remained active in BSG-SSF. This may contribute to explaining the reduction in endogenous total proteases and trypsin activities. Previous studies have also observed that the dietary inclusion of SSF ingredients reduces endogenous digestive activity without compromising diet digestibility. For example, also in European seabass, the dietary inclusion of SSF *Ulva rigida* by *A. ibericus* (Fernandes et al., 2022) or SSF plant feedstuff mixture by *A. niger* (Vieira et al., 2022) decreased total intestinal proteases activity compared to the unfermented feedstuff. In Nile tilapia, digestive protease, and amylase activities also decreased when SSF guar and copra meal were included at the expense of fishmeal (Dileep et al., 2021).

The increase in dietary fiber can negatively affect the digestive process of fish (Raskovic et al., 2011); however, this was not observed in the present study. High dietary fiber levels may also negatively affect fish intestinal histomorphology, although European sea bass has been reported to have a relatively high fiber tolerance, and dietary fiber inclusion of up to 16 % does not affect gut morphology (Bonvini et al., 2018). In the present study, histomorphological alterations due to diet were primarily observed in the anterior intestine, and except for increased supranuclear vacuolization, no major differences were observed in the posterior intestine. The increase in goblet cells observed in the anterior intestine of fish fed diets containing fermented and non-fermented BSG may be associated with an increase in the fiber level of the diet, as the goblet cells protect the intestinal lining and help increase mucus production, facilitating fecal expulsion (Machado et al., 2013).

Goblet cells secrete mucin, which is crucial for the intestinal immune barrier in fish (Machado et al., 2013). While more goblet cells typically denote intestinal health, excessive numbers can thicken the mucus layer, hindering nutrient absorption (Kim and Khan, 2013). Studies show that high dietary fiber levels lead to mucus efflux from the intestine, possibly due to increased chyme volume physically scraping the mucus layer (Christelle et al., 2005). This suggests a link between high fiber and intestinal mucus efflux, requiring more goblet cells to maintain the mucus layer. Considering the harmful effects of dietary fiber on the fish intestinal epithelium, the increased number of goblet cells observed in the present study may indicate an attempt to diminish or repair the mucosal damage imparted by the high dietary fiber content of BSG contacting diets, as previously observed in other fish species (Liu et al., 2022).

Even though nutrient absorption in the distal portion of the intestine is limited, the number of absorptive cells in this region indicates that the distal intestine of some fish plays a vital role in the uptake of intact proteins and other large-size molecules (Bakke-Mckellep et al., 2000). Additionally, the fiber level in the diet may have impaired selective permeability of the enterocytes' membrane in the distal part of the intestine. Thus, the increase in the size of supranuclear vacuolation of enterocytes may result from the indiscriminate absorption of dietary components that have no routes of entry into the body and, therefore, accumulate in vacuoles. Converging with current research, Couto et al. (2016) reported that meagre juveniles (*Argyrosomus regius*) fed diets containing high levels of carob germ seed meal also showed increased supranuclear vacuolation of intestinal enterocytes with concomitant accumulation of an amorphous hyaline substance of unknown nature, associated with a potential deleterious effect of tannins on the selectivity of cell membranes.

5. Conclusions

The present study showed that SSF of BSG by *A. ibericus* increased the protein content and decreased the cellulose and hemicellulose fractions of the fermented product. Including BSG in the diet reduced digestibility, whereas SSF-BSG did not. Moreover, SSF also decreased the deleterious effects of the dietary incorporation of BSG. Overall, present results indicate that SSF increases the nutritional value of BSG, a lignocellulosic-rich agro-industrial by-product, increasing the potential of its use in fish feeds and contributing to reducing the climate and environmental footprint of aquaculture production. Further studies, including a growth trial, are needed to evaluate the impact of these ingredients on growth performance and feed utilization.

CRediT authorship contribution statement

Tássia Estevão-Rodrigues: Investigation, Formal analysis, Writing - Original Draft. **Helena Fernandes:** Investigation, Writing - Review & Editing. **Sara Moutinho:** Investigation, Methodology. **Diogo Filipe:** Investigation. **Filipa Fontinha:** Investigation, Formal analysis. **Rui Magalhães:** Investigation. **Ana Couto:** Writing - Review & Editing, Resources. **Marta Ferreira:** Resources, Writing - Review & Editing. **Margarida Gamboa:** Investigation, Resources. **Carolina Castro:** Writing - Review & Editing. **Isabel Belo:** Writing - Review & Editing, Resources. **José Salgado:** Supervision, Writing - Review & Editing – Resources. **Aires Oliva-Teles:** Conceptualization, Writing - Review & Editing – Resources. **Helena Peres:** Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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