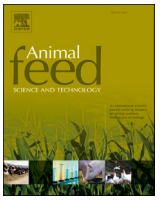
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Growth, oxidative stress, and intermediary metabolism of European seabass (*Dicentrarchus labrax*) juveniles fed diets including solid-state fermented distiller's dried grains with solubles

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ABSTRACT

Previous research demonstrated that solid-state fermentation (SSF) of distiller's dried grains with solubles (DDGS) by *Aspergillus ibericus* improved protein, reduced neutral and acid detergent fiber levels, and added phenolic compounds, xylanase, and cellulase. Fermentation of DDGS also increased protein, lipids, energy, and starch digestibility. This study evaluated the effects of unfermented and fermented DDGS on growth, oxidative stress, and intermediary metabolism of European seabass (*Dicentrarchus labrax*) juveniles. Five different diets were prepared: a control diet without DDGS and four with 10 % or 20 % unfermented or fermented DDGS (diets 10DDGS, 20DDGS, 10SSF-DDGS, and 20SSF-DDGS). Each diet was fed to triplicate seabass (30 ± 5 g) for 12 weeks. The 10SSF-DDGS diet increased the protein efficiency ratio, final body weight, and nitrogen retention (% N intake) compared to the 10DDGS diet, with no differences from the control diet. Both 20 % DDGS diets reduced growth performance, but the 20SSF-DDGS diet showed higher feed and protein utilization than the 20DDGS diet. Whole-body composition was unaffected except for 20SSF-DDGS, which had lower dry matter, lipids, protein, and gross energy content. The 10SSF-DDGS diet reduced hepatic glucose-6-phosphate dehydrogenase activity, while the 20SSF-DDGS diet decreased amino acid catabolism enzyme activity and increased glucokinase and pyruvate kinase activity. In intestinal oxidative status, 10DDGS reduced superoxide dismutase and increased glutathione reductase, glutathione peroxidase, and lipid peroxidation, whereas the 10SSF-DDGS diet mirrored the control diet. In conclusion, SSF of DDGS enhances feed utilization and maintains intestinal oxidative status, particularly at lower inclusion levels, highlighting its potential as an improved alternative to unfermented DDGS in aquafeeds. However, further research involving long-term trials, thermal stress challenges in the context of

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climate change, and disease resistance assessments is necessary to confirm its broader applicability in aquaculture.

1. Introduction

Ensuring food safety is a primary concern of today's humankind. According to FAO, food safety is achieved when "all individuals have consistent physical and economic access to sufficient, safe, and nutritious food that meets their dietary needs and preferences for an active and healthy life." (Food and Agricultural Organization of the United Nation, 2003). With the world population increasing by 1.6 % annually, the average consumption of aquatic products has more than doubled (FAO, 2022). This increased demand for aquatic foods requires higher production, making aquaculture a potential solution for the food production sector with the fastest growth rate. Throughout the previous few decades, aquaculture has registered a significant rise in production levels, with an average increase of 4 % per year from 2012 to 2020. In 2022, aquaculture accounted for 51 % of the world's aquatic animal production, making this industry a crucial contributor to this sector (FAO, 2024). Novel and sustainable feed ingredients are essential to support aquaculture growth, as reliance on fish meal and fish oil has been limited by production decline and increased costs (Mitra, 2021). Alternative dietary components such as insect meals, terrestrial animal byproducts, microbial products, and plant feedstuffs have been used as promising options for aquafeeds despite the inherent challenges hindering their use (Bandara and Tharindu Bandara, 2018; Tacon et al., 2022).

Plant feedstuffs are common protein sources in fish feeds due to their higher availability and lower overall market value than fish meal (Mugwanya et al., 2022). However, plant feedstuffs pose several challenges, such as low palatability and digestibility, nutritional imbalances, and anti-nutritional factors like fiber (Bandara and Tharindu Bandara, 2018; Dawood and Koshio, 2019). Mechanical, chemical, and biological pre-treatments were explored to improve the nutritional value of plant feedstuffs and reduce anti-nutritional factors (Batista Meneses et al., 2020). A promising approach is the addition of exogenous enzymes like phytase and carbohydrases to the diets or feedstuffs (Castillo and Gatlin, 2015). Monogastric animals do not produce carbohydrases that hydrolyze β-glucosidase bonds; these enzymes are usually present in low or negligible quantities in the digestive tract of non-ruminant animals such as fish (Sinha et al., 2011). Dietary carbohydrate supplementation may help to hydrolyze the complex and undigested NSPs, or non-starch polysaccharides, which interfere with nutrient digestion and absorption by shielding the diet from endogenous digestive enzymes (Castillo and Gatlin, 2015).

Corn distillers' dried grains with solubles (DDGS), a byproduct of corn ethanol plants, account for 44 million metric tons of production annually in the U.S. alone. DDGS has grown in exports from 5 million tons in 2009–10 million tons in 2022/2023, demonstrating an increasing commercial interest in this byproduct (U.S. GRAINS COUNCIL, 2024). During ethanol production, corn is milled and fermented, and after distillation, the non-fermentable products are centrifuged to separate the liquid fraction from the insoluble wet distiller's grains. The liquid fraction is condensed to obtain the condensed distiller's solubles (CDS), which, when mixed with the wet distiller's grains and solids and dried, produce DDGS (Kingsly et al., 2010).

The proximal composition of DDGS can differ with factors such as grain source and processing technology, typically containing 25–35 % crude protein (Lim et al., 2011). However, its high fiber content and reduced palatability have limited DDGS utilization in fish feeds (Naylor et al., 2009). To enhance the nutritional profile and bioavailability of DDGS, various pre-treatments have been used to reduce its fiber content. These include mechanical (e.g., grinding), physical (e.g., ultrasound), chemical (e.g., acid hydrolysis), and thermal (e.g., steam) processes (Iram et al., 2019). Biological methods, such as enzyme hydrolysis, have also been used to hydrolyze cellulosic material into simple sugars (Bals et al., 2006). Pre-enzymatic hydrolysis (Bals et al., 2006) or exogenous enzyme dietary inclusion (Silva et al., 2016) has been shown to improve improves the digestibility of DDGS. Recent technological advancements have led to the development of high-protein DDGS with lower fiber and fat contents. (Øverland et al., 2013a). Despite its potential, the high cost of this high-protein DDGS continues to limit its widespread use. Additionally, DDGS has shown promise as a fermentation feedstock for producing hydrolytic enzymes, organic acids, and biofuels (Iram et al., 2020).

Solid-state fermentation (SSF) is a fermentation process that requires little to no free water (Singhania et al., 2009) and uses microorganisms adapted to low water activity conditions, typically filamentous fungi such as *Aspergillus* fungi (Pandey, 2003). These fungi are known for their enzyme production, many of which are classified as GRAS (generally recognized as safe) by the FDA (Food and Drug Administration) (Schuster et al., 2002). Among the enzymes produced are carbohydrases, including cellulases and xylanases (Pandey et al., 1999). *Aspergillus* fungi are well suited to growth in fibrous substrates like DDGS (Yang et al., 2012), enabling its eco-friendly biodegradation through fermentation (Hölker and Lenz, 2005). This process typically results in a final product with increased protein content (Eliopoulos et al., 2022), reduced fiber (El-Bakry et al., 2015) and enriched with bioactive compounds like enzymes, antioxidants, organic acids, and antibiotics (Chilakamarry et al., 2022).

This study is a follow-up of our research study (Filipe et al., 2023), which assessed the effect of SSF of DDGS on its digestibility and nutritional value for the European seabass (*Dicentrarchus labrax*), a species highly produced in the Mediterranean aquaculture. The fermentation of DDGS increased the content of reducing sugars, total phenols, and soluble protein while reducing lignin, neutral and acid detergent fiber, and carbohydrate content. Among the fungi evaluated, *Aspergillus ibericus* demonstrated a significantly higher capacity to produce cellulase and reduce acid-detergent fiber and lignin than other fungi and was selected for further evaluation. In comparisons of fermented and nonfermented DDGS, the fermented DDGS exhibited higher digestibility of protein, lipids, starch, and energy. Based on these previous findings, the present study evaluates the potential of dietary incorporation of *A. ibericus*-fermented DDGS on feed utilization, growth performance, hepatic intermediary metabolism, and intestinal oxidative stress of the European

seabass juveniles.

2. Materials and methods

2.1. Solid-state fermentation (SSF)

SSF of corn DDGS was done as in our previous report (Filipe et al., 2023) using *A. ibericus* MUM 03.49 obtained from the Micoteca of the University of Minho (MUM, Braga, Portugal). Briefly, SSF was carried out in tray-type bioreactors (43 ×33 x 7 cm) for 7 days at 30 °C and with humidity adjusted to 75 % (*w/w* wet basis). In each tray, 400 g of dry autoclaved DDGS (15 min,121 °C) was inoculated with 80 mL of *A. ibericus* spore solution (0.1 % peptone and 0.001 % Tween-80), adjusted to 10^6 spores/mL. The different trays' fermented DDGS (SSF-DDGS) were pooled and dried (40 °C for 24 h) to reduce humidity to 10–15 %. The composition of DDGS before and after fermentation is presented in our previous report (Filipe et al., 2023).

2.2. Experimental diets

The control diet, formulated without DDGS, contained 44 % crude protein and 16 % crude lipids. Four additional feeds were formulated that differed from the control by including either 10 % or 20 % of unfermented or fermented DDGS at the expense of soybean and wheat meals (diets 10DDGS, 20DDGS, 10SSF-DDGS, and 20SSF-DDGS, respectively (Table 1). Dietary ingredients were ground, mixed, and pelleted with a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 4 mm die. Pellets were dried at 40 °C for 24 h and stored at −4 °C until the growth trial.

2.3. Growth trial

The growth trial was conducted following European Union guidelines (directive 2010/63/E.U.) and Portuguese law (Decreto Lei no. 113/2013, de 7 de Agosto) to protect animals used for scientific purposes. The trial was approved by CIIMAR's animal welfare committee (ORBEA-CIIMAR; reference ORBEA_CIIMAR_27_2019) and by the Direção Geral de Alimentação e Veterinária (DGAV) and followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

European Seabass (*Dicentrarchus labrax*) juveniles were purchased from a commercial hatchery (Maresa, Spain). Upon arrival, fish

Table 1
Diet formulation and proximate composition of the experimental diets used in the growth trial.

	Control	10DDGS	10SSF-DDGS	20DDGS	20SSF-DDGS
Ingredients (% dry matter)					
DDGS[a]	–	10	–	20	–
SSF-DDGS[b]	–	–	10	–	20
Fish meal[c]	20	20	20	20	20
Corn gluten[d]	17.5	17.5	17.5	17.5	17.5
Wheat gluten[e]	5	5	5	5	5
Soybean meal[f]	20.1	16.3	15.3	12.6	10.5
Wheat meal[g]	15.3	10.1	11.1	4.8	7.0
Fish oil	14.6	14.0	14.0	13.4	13.4
Constant ingredients[h]	5	5	5	5	5
NaH_2PO_4	2.5	2.1	2.1	1.7	1.7
Proximate composition (% dry matter)					
Dry matter (DM, %)	92.8	94.3	94.8	90.0	89.0
Crude protein	44.2	44.8	44.1	44.2	45.9
Crude lipids	15.6	15.6	15.4	15.2	16.0
Ash	7.2	5.7	5.2	10.0	11.0
Energy (kJ/g DM)	21.3	21.6	21.4	21.5	21.5

[1] Vitamins (mg kg^{-1} diet): retinol, 18,000 (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; mascorbyl monophosphate, 50; inositol, 400.

[2] Minerals (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; dibasic calcium phosphate, 5.9 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).

[3] Binder (Aquacube. Agil, England).

[a] Corn Dried distillers' grains with solubles (CP: 22.6 %; CL: 3.4 %); Sorgal, S.A. Ovar, Portugal.

[b] Solid state fermented corn-dried distillers' grains with solubles (CP: 30.1 %; CL: 4.3 %)(26.)

[c] Fish meal (CP: 77.5 %; CL: 9,5 %); Pesquera Diamante, Peru.

[d] Corn gluten (CP: 72.1 %; CL:2.1 %); Sorgal, S.A. Ovar, Portugal.

[e] Wheat gluten (CP: 84.4 %; CL: 1.8 %), Sorgal, S.A. Ovar, Portugal.

[f] Non GOM Soybean meal (CP: 51.1; CL:2.6 %); Sorgal, S.A. Ovar, Portugal.

[g] Wheat meal (CP: 13.4 %; CL: 3.2 %); Sorgal, S.A. Ovar, Portugal.

[h] Constant compounds (% dry matter): 1 vitamin premix[1], 0.5 choline chloride (50 %), 1 mineral premix[2], 1 binder[3], 1 agar, 0.5 taurine.

were maintained for 15 days in quarantine, transferred to the experimental system, and acclimatized to the experimental conditions for 2 weeks before the growth trial. The experimental system consisted of a thermoregulated recirculating water system (RAS) equipped with 15 tanks of 100 L fiberglass and connected to a life support unit with mechanical and biological filtration and ozone disinfection. Water was continuously aerated using air stones, and a 4–5 L/min water flow was maintained in each tank. During the trial, water quality parameters were kept as follows: temperature, 22 ± 1 °C; salinity, 34 ± 1 ‰; dissolved oxygen, 7.3 ± 0.3 mg/L; pH, 8.3 ± 0.2; total ammonia-N, below 0.02 mg/L; and nitrite-N: below 0.2 mg/L. The photoperiod was set to 12 h light and 12 h dark.

At the start of the trial, fish were lightly anesthetized (0.3 mg/L ethylene glycol monophenyl ether), and 15 homogenous groups of 15 European seabass juveniles (initial body weight of 30 ± 5 g) were established. For 12 weeks, diets were randomly allocated to triplicate groups, and fish were fed twice daily to apparent satiation, six days per week, ensuring no feed was wasted.

2.4. Sampling

Ten fish from the initial stock, along with 3 fish per tank, were randomly sampled and euthanized using an anesthetic overdose at the start and end of the trial (1 mg/l ethylene glycol monophenyl ether) for whole-body composition. The remaining fish continued to be fed the same diets for three more days to recover from the stress caused by the final weighing manipulation. Then, 4 h after the morning meal, 3 fish per tank were euthanized by decapitation. The intestine and liver were sampled on an iced tray, separated from adjacent adipose tissue, frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.5. Chemical analysis

The proximate composition of ingredients, diets, and whole fish was determined following the standard procedures of the Association of Official Analytical Chemists (Association of Official Analytical Chemists et al., 1980). Dry matter was measured by drying at 105°C until constant weight. Ash content was determined by incineration using a muffle furnace at 450 °C for 16 h. Crude protein (Nx6.25) was measured using the Kjeldahl method through a 2-step process, acid digestion and distillation, with a Kjeltec digestion and distillation unit (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively). Crude lipids were determined using a petroleum ether extraction procedure (Tecator, Höganäs, Sweden). Gross energy was determined by combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

2.6. Enzymatic analysis

Intestine and liver samples were homogenized in an ice-chilled buffer, 0.1 mM– EDTA, 100 mM – Tris-HCl, and 0.1 % Triton X-100 (v/v), pH 7.8. Samples were centrifuged at 30 000 g for 30 min at 4 °C. Aliquots of the supernatant were stored at −80 °C until enzyme activity analysis. Enzymatic activity was measured at 37 °C using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). The protein content of the homogenates was determined using a Bio-Rad protein assay kit (ref. 5000,006).

2.7. Intermediary metabolism enzyme activities

Enzymatic activity was measured using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). Intermediary metabolism enzyme activities were measured in the liver. Alanine aminotransferase (ALAT/GPT, EC 2.6.1.2) and aspartate (ASAT/GOT. EC 2.6.1.1) activities were measured with commercial kits (Spinreact, ASAT/GOT; 41,273; ALAT/GPT; 41,283). Glutamate dehydrogenase (GDH, EC 1.4.1.2), pyruvate kinase (PK, EC 2.7.1.40), and fructose 1,6-biphosphate (FBPase, EC 3.1.3.11) activities were measured according to a previous report. (Morales et al., 1990). Glucokinase (HK-IV, EC 2.7.1.2) and hexokinase (H.K., EC 2.7.1.1) were determined as described previously (Diógenes et al., 2018). Specific enzyme activities are expressed as nmol/mg of soluble protein.

2.8. Antioxidant enzyme activities

Enzymatic activity was measured using a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China). Antioxidant enzyme activities were measured in the intestine. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as described previously (McCord and Fridovich, 1969). A reaction cocktail mixture was done, including potassium phosphate buffer 50 mM pH 7.8, xanthine 1 mM, and cytochrome C 1 mM. This cocktail was used for either the control reaction with 200 µl of cocktail, 10 µl of water and 10 µl of oxidized xanthine or an experimental reaction that substituted the water for the sample. Absorbance was read for 3 minutes each 15 seconds at 550 nm.

Catalase (CAT, EC 1.11.1.6) activity was measured according to a previous report (Aebi, 1984), and sample dilutions were mixed with the substrate, hydrogen peroxide 10.6 mM, and read at 240 nm every 12 seconds for 3 minutes at 25 ºC.

Glutathione reductase (GR, EC 1.6.4.2) activities were measured according to Morales et al. (1990). For the reaction, 192 µl of NADPH (0.66 Mm), 8 µl of the sample, and 10 µl of oxidized glutathione (GSSG; 3.25 mM) were mixed, and absorbance was read for 3 minutes in 15-second intervals at 340 nm. The control was made with distilled water.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to (Flohé and Günzler 1984). A reaction cocktail of GR, NADPH, reduced glutathione, EDTA, and sodium azide was prepared. 180 µl of reaction cocktail, 10 µl of sample, and 10 µl of cumene hydroperoxide were mixed, and absorbance was read for 3 minutes in 15-second intervals at 340 nm. Control was made

without the sample.

Glucose-6-phosphate-dehydrogenase (EC 1.1. 1.49) was determined according to (Castro et al., 2015). A reaction mixture of 50 mm-imidazole–HCl buffer (pH 7·4), 5 mm-$MgCl_2$, 2 mm-NADP, and 1 mm-glucose-6-phosphate was prepared. Each sample was mixed in a ratio of 5 % sample to 95 % reaction mixture activity and screened for 10 minutes by measuring the reduction of $NADP^+$ at 340 nm in 15-second intervals.

Enzyme activity is measured in units (U), with one unit being the amount of enzyme that converts 1 μmol of substrate per minute under standard assay conditions. Activities are expressed as units (CAT) or milliunits (GR, G6PDH, and GPX) per milligram of soluble protein. SOD activity is defined as the quantity of enzyme needed to inhibit 50 % of ferricytochrome C and expressed as units of SOD per milligram of soluble protein.

2.9. Lipid peroxidation

Enzymatic activity was measured using a Multisksan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China). Lipid peroxidation was estimated by measuring the amount of thiobarbituric acid reacting substances, as described previously (Diógenes et al., 2018). Shortly, the sample was mixed with trichloroacetic acid 15 %, thiobarbituric acid 0.375 %, BHT 0.01 %, and HCL 0.25 N. The mixture was then incubated at 100 ºC for 15 minutes. Samples were then centrifuged, and the supernatant was read at 535 nm.

A calibration curve was done with several dilutions of MDA and expressed as nmol MDA/g tissue.

2.10. Statistical analysis

Results are expressed as the mean and standard error of the mean (SEM). Data were assessed for normal distribution and variance homogeneity. Non-orthogonal contrasts were used to verify significant differences between each diet relative to the control, unfermented versus fermented DDGS diets at the same inclusion level and between each inclusion level of either fermented or unfermented DDGS diets. All data were analyzed using the IBM SPSS Statistics 26 software (IBM, NY, USA). The level of significance used was $p < 0.05$ for all tests.

3. Results

Mortality was low (2.2–6.7 %) and similar between dietary treatments (Table 2). The diets were generally well accepted by the experimental fish, with no negative impact on voluntary feed intake between groups, except for a decrease in the 20SSF-DDGS group

Table 2
Growth performance and feed efficiency of European seabass fed the experimental diets for 12 weeks.

	Control	10DDGS	10SSF-DDGS	20DDGS	20SSF-DDGS	SEM
Initial body weight (IBW; g)	29.8	29.6	29.5	29.8	29.7	0.1
Final body weight (FBW; g)	80.5	79.8	86.3	67.6	66.3	2.2
Weight gain (WG; g/kg ABW/day)	10.3	10.3	11.0	8.7	8.6	0.3
Daily growth index (DGI)[1]	1.37	1.36	1.49	1.09	1.07	0.05
Feed intake (FI; g/kg ABW/day)	13.3	13.8	13.3	14.3	11.4	0.4
Feed efficiency (FE)[2]	0.79	0.77	0.87	0.66	0.79	0.0
Protein efficiency ratio (PER)[3]	1.80	1.71	1.97	1.49	1.74	0.08
Mortality (%)	2.2	2.2	4.4	6.7	4.4	1.4
N retention (NR; % N intake)[4]	32.4	28.9	34.9	27.7	29.5	0.9
Energy retention (ER; % E intake)[4]	37.4	33.1	37.3	30.6	35.6	0.9

#	Comparison	IBW	FBW	WG	DGI	FI	FE	PER	NR	ER
1	Control x 10DDGS									
2	Control x 10SSF-DDGS									
3	Control x 20DDGS		*	*	*		*	*	*	*
4	Control x 20SSF-DDGS		*	*	*					
5	10DDGS x 10SSF-DDGS		*					*	*	
6	20DDGS x 20SSF-DDGS					*	*	*		
7	DDGS levels (10 % vs 20 %)		*	*		*	*			
8	No-SSF x SSF					*	*	*	*	*
9	Interaction									

Samples are presented as means (n = 3) and standard error of the mean (SEM).
The orthogonal contrasts compare control versus each experimental diet (#1–4), unfermented versus fermented DDGS within each level (#5–6), DDGS levels (10 % vs. 20 %) (#7), fermentation effects (#8), and their interaction (#9). Significant differences ($P < 0.05$) are indicated by an asterisk (*).
ABW= (FBW+IBW)/2.
[1]DGI= $((FBW^{1/3} - IBW^{1/3})/$ time in days$) \times 100$.
[2]FE=wet weight gain / dry feed intake.
[3]PER=wet weight gain / crude protein intake. [4]Nitrogen and energy retention= $(FBW \times FBN - IBW \times IBN)/(NI)) \times 100$ or $(FBW \times FBE - IBW \times IBE)/(EI)) \times 100$, respectively.

compared to the 20DDGS group.

Compared to the control, dietary inclusion of 10 % of unfermented or fermented DDGS unaffected growth performance and feed utilization efficiency. Differently, the dietary inclusion of 20 % of unfermented or fermented DDGS reduced growth performance. Moreover, the dietary inclusion of unfermented DDGS also lowered feed utilization compared to the control. In contrast, the dietary inclusion of fermented DDGS led to an efficiency of feed utilization similar to that of the control diet.

At a 10 % DDGS dietary inclusion level, SSF increased final body weight, protein efficiency ratio (PER), and nitrogen (N) retention. At a 20 % inclusion level, SSF did not affect growth but reduced feed intake and improved feed efficiency (FE), PER, and N and energy retention. Regardless of fermentation, increasing DDGS inclusion from 10 % to 20 % decreased growth, feed intake, and FE. For both DDGS levels, SSF reduced feed intake but enhanced FE, PER, and N and energy retention.

Fish fed the 10DDGS and 20SSF-DDGS diets, compared to the control, showed lower whole-body protein content (Table 3). Fish fed the 20SSF-DDGS diet had reduced dry matter, lipids, and energy content compared to the control. Regardless of SSF treatment, the whole-body composition of fish fed the 10 % DDGS diets remained similar. However, among fish on the 20 % DDGS diets, those fed the 20 % SSF-DDGS diet exhibited lower dry matter and crude protein levels.

Glutamate dehydrogenase (GDH) activity was unaffected by the dietary treatments (Table 4). However, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) activities were higher in fish fed the control diet than in the 10DDGS and 20SSF-DDGS, respectively. HK activity was not modulated by the dietary treatments, but glucokinase (GK) and pyruvate kinase (PK) activities were increased in fish fed the 20SSF-DDGS diet compared to the control. Differently, FBPase activity was unaffected by the dietary composition. GDH and ASAT were significantly reduced by fermentation, while GK increased. Regardless of fermentation, the increase in DDGS increased ASAT activity. Irrespective of the DDGS level, fermentation decreased GDH and ASAT and increased GK.

3.1. Intestinal oxidative stress-related enzymatic activity and lipid peroxidation values

Fish fed the 10DDGS diet showed lower superoxide dismutase (SOD) and G6PDH activities

and higher glutathione reductase (GR), glutathione peroxidase (GPX) activities, and lipid peroxidation (LPO) than the control (Table 5). Similarly, fish fed the 20DDGS also presented higher GPX activity and LPO than the control. No differences relative to the control were observed in all parameters measured in fish fed the 10SSF-DDGS and 20SSF-DDGS diets, further supported by the significant differences in the treatments. Regardless of fermentation, superoxide dismutase (SOD) activity increased at the 20 % DDGS inclusion level. At both inclusion levels, fermentation reduced glutathione reductase (GR), glutathione peroxidase (GPX), and lipid peroxidation (LPO) while enhancing glucose-6-phosphate dehydrogenase (G6PDH) activity.

4. Discussion

Omnivorous fish generally tolerate higher levels of DDGS in their diets than carnivorous fish (Sándor et al., 2021; Zhou et al., 2010). Replacing fish meal with DDGS in carnivorous fish diets remains challenging [33] due to the high fiber content of DDGS, which can impair diet digestibility by increasing digesta viscosity and reducing nutrient digestion and absorption rate (Bonvini et al., 2018).

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

	Initial	Control	10DDGS	10SSF-DDGS	20DDGS	20SSF-DDGS	SEM
Dry matter, DM	25.2	32.5	31.4	32.2	31.5	29.5	0.29
Crude Protein	15.6	17.1	16.4	17.0	17.2	16.1	0.12
Crude Lipids	4.90	11.8	11.0	11.6	10.4	9.45	0.31
Ash	5.41	4.05	4.20	4.10	4.19	4.35	0.05
Gross energy (GE, kJ/g FW)	5.70	8.44	7.98	8.01	8.11	7.76	0.08

#	Comparison	DM	Protein	Lipids	Ash	GE
1	Control x 10DDGS		*			
2	Control x 10SSF-DDGS					
3	Control x 20DDGS					
4	Control x 20SSF-DDGS	*	*	*		*
5	10DDGS x 10SSF-DDGS					
6	20DDGS x 20SSF-DDGS	*	*			
7	DDGS levels (10 % vs 20 %)		*			
8	No-SSF x SSF					
9	Interaction	*	*			

Samples are presented as means (n = 3) and standard error of the mean (SEM).
The orthogonal contrasts compare control versus each experimental diet (#1–4), unfermented versus fermented DDGS within each level (#5–6), DDGS levels (10 % vs. 20 %) (#7), fermentation effects (#8), and their interaction (#9). Significant differences ($P < 0.05$) are indicated by an asterisk (*).

Table 4
Specific hepatic activities (nmol/mg soluble protein) of glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glucokinase (GK), hepatic hexokinase (HK), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphate dehydrogenase (G6PDH) of European seabass fed the experimental diets.

	Control	10DDGS	10SSF-DDGS	20DDGS	20SSF-DDGS	SEM
Amino acid catabolism						
GDH	83.9	95.9	67.6	87.2	61.3	4.57
ASAT	945.0	776.5	763.5	1055.3	617.2	39.1
ALAT	367.7	210.7	340.4	309.6	272.6	20.5
Glycolysis						
GK	6.73	6.07	7.04	6.84	8.38	0.21
HK	1.82	1.41	1.86	1.64	1.92	0.14
PK	76.8	73.2	89.2	93.6	110.6	4.13
Gluconeogenesis						
FBPase	40.4	36.4	41.7	41.5	36.4	1.16

#	Comparison	GDH	ASAT	ALAT	GK	HK	PK	FBPase
1	Control x 10DDGS			*				
2	Control x 10SSF-DDGS							
3	Control x 20DDGS							
4	Control x 20SSF-DDGS		*		*		*	
5	10DDGS x 10SSF-DDGS							
6	20DDGS x 20SSF-DDGS		*		*			
7	DDGS levels (10 % vs 20 %)		*					
8	No-SSF x SSF	*	*		*			
9	Interaction		*					

Samples are presented as means (n = 9) and standard error of the mean (SEM). The orthogonal contrasts compare control versus each experimental diet (#1–4), unfermented versus fermented DDGS within each level (#5–6), DDGS levels (10 % vs. 20 %) (#7), fermentation effects (#8), and their interaction (#9). Significant differences ($P < 0.05$) are indicated by an asterisk (*).

Table 5
Specific enzyme activity of superoxide dismutase (SOD; U/mg protein), glucose-6phosphate dehydrogenase (G6PDH; mU/mg protein), catalase (CAT; U/mg protein), glutathione reductase (GR; mU/mg protein), glutathione peroxidase (GPX; mU/mg protein), lipid peroxidation (LPO; nmol MDA/g tissue) of European seabass intestine fed the experimental diets.

	Control	10DDGS	10SSF-DDGS	20DDGS	20SSF-DDGS	SEM
SOD	221.2	188.2	208.7	226.0	220.2	4.82
G6PDH	10.85	8.15	11.87	9.43	11.73	0.43
CAT	152.8	162.3	153.2	168.0	161.5	4.95
GR	21.64	27.07	20.80	23.66	21.98	0.73
GPX	91.56	128.1	105.8	145.1	98.04	5.62
LPO	33.81	49.03	24.79	54.00	38.45	2.35

#	Comparison	SOD	G6PDH	CAT	GR	GPX	LPO
1	Control x 10DDGS	*	*		*	*	*
2	Control x 10SSF-DDGS						
3	Control x 20DDGS					*	*
4	Control x 20SSF-DDGS						
5	10DDGS x 10SSF-DDGS		*		*		*
6	20DDGS x 20SSF-DDGS					*	*
7	DDGS levels (10 % vs 20 %)	*					
8	No-SSF x SSF		*		*	*	*
9	Interaction						

Samples are presented as means (n = 9) and standard error of the mean (SEM).
The orthogonal contrasts compare control versus each experimental diet (#1–4), unfermented versus fermented DDGS within each level (#5–6), DDGS levels (10 % vs. 20 %) (#7), fermentation effects (#8), and their interaction (#9). Significant differences ($P < 0.05$) are indicated by an asterisk (*).

Nonetheless, replacing plant ingredients with DDGS has shown some potential in carnivorous fish. (Diógenes et al., 2019; Øverland et al., 2013b). Accordingly, in the present study, incorporating 10 % DDGS in the diets to replace soybean and wheat meals did not negatively affect European seabass juveniles' growth performance or feed utilization efficiency. However, increasing the dietary DDGS to 20 % adversely affected growth performance.

Previously, we have shown that biotechnological pre-treatment of DDGS through SSF using *A. ibericus* reduced neutral detergent

fiber, increased protein content, and improved the nutrient digestibility of DDGS in European seabass (Filipe et al., 2023). In this study, we observed that fish fed with the 10 % fermented DDGS (10SSF-DDGS) diet presented a higher final body weight, PER, and N retention than those fed with the 10 % nonfermented product (10DDGS diet). Nonetheless, when the inclusion level of SSF-DDGS in the diets increased to 20 %, voluntary feed intake decreased, reducing growth performance but increasing FE compared to the 20DDGS diet. Similarly, European seabass fed a diet including a 20 % plant protein mixture fermented by *A. niger,* growth performance was also not affected, and FE and PER were also increased (Diógenes et al., 2019). However, when the fermented plant protein mixture was increased to 40 %, feed intake was also reduced, and this was not due to dietary mycotoxins. Indeed, both *A. niger* and *A. ibericus* are classified as GRAS by the FDA, reducing concerns about mycotoxins production during fermentation (Meyer and Schmidhalter, 2012). In Atlantic Salmon (*Salmo salar*), the dietary replacement of fish meal with 15 % lupin fermented with *Lactobacillus plantarum* led to a higher growth rate and PER than the 15 % unfermented lupin diet (Rodríguez-Estrada et al., 2020). The reduced voluntary feed intake observed with the 20DDGS-SSF diet may be due to changes in diet palatability, a concern noted in studies involving fermented ingredients with *Aspergillus* strains in aquaculture species (Rombenso et al., 2013). However, in other species, such as turbot and trout-fed *Bacillus* spp. fermented ingredients, no effect on feed intake was observed (Dan et al., 2022; Yamamoto et al., 2010). Despite the reduced feed intake, nitrogen and energy retention (% intake) increased by about 6 and 16 %, indicating higher protein and energy efficiency utilization. Several authors have also demonstrated an inverse relationship between protein intake and protein retention efficiency (Council, 2011), including in the European seabass (Boujard et al., 2004).

In the present study, DDGS inclusion in the experimental diets only slightly impacted whole-body composition. Previous studies have shown varying effects of DDGS inclusion on whole-body composition. As in this study, no impact in whole-body composition was observed in grass carp (Abouel Azm et al., 2021), but a higher lipidic content was observed in turbot (Diógenes et al., 2018), and a lower protein content was observed in channel catfish (Li et al., 2010).

Fermented DDGS had a higher effect on whole-body composition, particularly at a 20 % inclusion level, where dry matter, protein, lipids, and energy levels were reduced compared to the control, and dry matter and protein were reduced compared to the 20 % DDGS group. This is likely related to decreased feed intake and final weight in the SSF-DDGS group. Previously, a reduction in whole-body lipid content was observed in European seabass under restricted feeding, while protein content remained unchanged due to enhanced utilization of digestible protein when the fish were fed above maintenance requirements (Boujard et al., 2004). The influence of fermented ingredients on whole-body composition differs according to the inclusion level, feedstuffs, and species. In the European seabass, the whole-body composition was impacted by the inclusion level of the fermented plant mixture; a 20 % inclusion increased whole-body protein and energy content, whereas a 40 % inclusion reduced them (Vieira et al., 2023). Intermediary metabolism is affected by dietary nutrient content, availability, and balance between nutrients (Pérez-Jiménez et al., 2009). In the present work, dietary inclusion of DDGS, at 10 or 20 % of the diet, did not affect amino acids and glucose metabolism, except for a decreased transaminase activity (ALAT), consistent with gilthead seabream (*Sparus aurata*) fed DDGS-based diets (Diógenes et al., 2019). Contrarily, the 20SSF-DDGS diet reduced ALAT activity and increased GK and PK activity compared to that observed in fish fed the 20DDGS and control diets. This was likely due to the reduced feed intake in that group, which led to a protein-sparing and increased use of carbohydrates for energy. This agrees with the higher PER and nitrogen retention (% intake) observed with the 20SSF-DDGS diet compared to the 20DDGS diet. Similarly, previous research found that feeding a restricted protein-level diet did not affect GDH activity but reduced transaminase activity in the European seabass (Pérez-Jiménez et al., 2007) and other species (Gómez-Requeni et al., 2004). GK, a key enzyme of glucose metabolism, phosphorylates glucose to glucose-6-phosphate with lower affinity to glucose, contrarily to HK, acting as a sensor of high glucose levels (Polakof et al., 2009). Previously, it was observed that GK activity responds positively to dietary carbohydrate levels (Li et al., 2019) and feeding status (Panserat et al., 2014). The increased GK and PK activity also reflects higher carbohydrate availability with the 20SSF-DDGS diet, as indicated by its higher energy digestibility compared to DDGS (Filipe et al., 2023).

Organisms have an endogenous antioxidant defense mechanism to protect against reactive oxygen species (ROS) that includes both enzymatic (SOD, CAT, GR, and GPX) and non-enzymatic mechanisms (glutathione, vitamins) (El-Aal, 2012). Exogenous antioxidants, such as the phenolic compounds produced during the SSF of DDGS (Filipe et al., 2023), may also contribute to counterattack ROS (Taştan and Salem, 2021). Intracellular metabolism produces ROS as byproducts of aerobic energy production in the mitochondria, and a disproportional ROS production may induce oxidative stress that can damage tissues and cells by oxidizing DNA, proteins, and lipids. Lipid peroxidation is widely recognized as a significant form of oxidative damage in biological systems (Lushchak, 2011), as it disrupts the lipid bilayer structure of cellular membranes, compromising both their structural integrity and functional capacity (Ighodaro and Akinloye, 2018). Fish are particularly vulnerable to lipid peroxidation due to the high amount of highly unsaturated fatty acids in their phospholipids, making them more prone to oxidative damage (Fontagné et al., 2008). In this particular study, fish fed the 10 % DDGS diet presented higher intestinal GR and GPX activities and lower SOD and G6PDH activities compared to the control, and at 20 % DDGS inclusion, GPX activity further increased. Moreover, higher LPO in fish-fed diets containing DDGS than in the control diet suggests increased ROS levels. Similarly, substituting soybean meal with DDGS in gilthead seabream reduced oxidative stress enzymes and increased liver LPO (Diógenes et al., 2019). The impact of DDGS on intestinal oxidative status and LPO damage appears to be species and level-dependent (Barbacariu et al., 2022; Diógenes et al., 2019). In juvenile hybrid grouper and common carp diets, including DDGS, increased intestinal LPO (Barbacariu et al., 2022; Zhu et al., 2022), while in turbot, no adverse effects on hepatic and posterior intestine oxidative stress were observed (Diógenes et al., 2018). In contrast, in pacu (*Piaractus mesopotamicus*), replacing soybean meal with DDGS increased intestinal CAT activity and reduced LPO (Oliveira et al., 2021).

SSF DDGS-based diets, however, showed no differences in intestinal oxidative stress-related enzyme activities and LPO levels compared to the control diet, suggesting that phenolic compounds released during SSF may have mitigated intestine oxidative damage. Moreover, SSF DDGS-based diets increased G6PDH activity, likely increasing their oxidative-reducing power relative to nondemented

DDGS-based diets. G6PDH activity generates NADPH, which is necessary for glutathione regeneration (Wu et al., 2004).

5. Conclusions

This study demonstrates that up to 10 % DDGS or SSF-DDGS can replace soybean and wheat meals in the diets of European seabass juveniles without compromising growth or feed efficiency compared to the control diet. At this inclusion level, SSF-DDGS enhanced growth and feed utilization compared to nonfermented DDGS. Furthermore, while the inclusion of DDGS increased intestinal oxidative stress in European seabass, the incorporation of SSF-DDGS restored the oxidative balance status of the fish. These findings highlight the potential of SSF to enhance the nutritional value of DDGS while mitigating oxidative stress, making agroindustrial by-products such as DDGS more appealing to aquaculture through a sustainable biotechnological method. However, further research is necessary to validate these results under long-term feeding trials, particularly under thermal stress conditions reflecting the challenges of climate change. Additionally, studies evaluating disease resistance and overall fish health are recommended to assess the applicability of SSF-DDGS in aquaculture.

Author contributions

Formal analysis was performed by Diogo Filipe, Helena Fernandes, José Manuel Salgado, and Rui Magalhães. The investigation was accomplished by Diogo Filipe and Rui Magalhães. Resources were facilitated by José Manuel Salgado, Isabel Belo, Aires Oliva-Teles, and Helena Peres. Validation was done by Aires Oliva-Teles and Helena Peres. Both funding acquisition and project administration were executed by Helena Peres. The first draft of the manuscript was written by Diogo Filipe, and all authors commented and reviewed subsequent versions of the manuscript. All authors read and approved the final manuscript.

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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.

Data Availability

The data that support the findings of this study are not openly available for privacy reasons and are available from the corresponding author upon reasonable request.

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