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Solid-State Fermentation of Distiller's Dried Grains with Solubles Improves Digestibility for European Seabass (*Dicentrarchus labrax*) Juveniles

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Abstract: Aquaculture requires new, economical, and eco-friendly protein sources to replace traditional fisheries and plant ingredients. Using agriculture by-products as protein sources would reduce land-based feed production pressure and waste production, promoting a circular economy and sustainable aquaculture. Distiller's dried grains with solubles (DDGS) is the main by-product of bioethanol production. Corn DDGS has a high protein level, but its high fiber content limits its use as a feed ingredient, particularly for carnivorous fish. Solid-state fermentation (SSF) uses lignocellulosic-rich substrates, such as DDGS, for microbial growth in the near absence of water, promoting enzyme production that degrades the lignocellulosic matrix, increasing free reducing sugars, protein, and antioxidant levels of the substrate. In the present work, the SSF of corn DDGS with *Aspergillus carbonarius*, *A. ibericus*, and *A. uvarum* was tested. Then, the digestibility of the most promising fermented DDGS (in terms of upgraded nutritional composition) was tested by including it in a reference diet (70% of a reference diet; 48% crude protein; 15% crude lipids) for European seabass (*Dicentrarchus labrax)* juveniles (171 g averaged weight; trial duration of 52 days). Among the fungi tested, *Aspergillus ibericus* led a generally higher upgrading of the DDGS nutritional composition, leading to a high amount of protein (from 42.7 to 49.7 g N/kg DM), phenolic compounds (1.49 to 4.86 mg/g caffeic acid equivalents), free sugars (9.5 to 31.9 mg/g), and enzyme production (45 U/g and 68 U/g of cellulase and xylanase, respectively), and a high reduction in acid detergent fiber and neutral detergent fiber content (up to 29 and 43%, respectively). Compared to the unfermented DDGS, fermented DDGS presented increased protein, lipids, starch, and energy digestibility, while phosphorous digestibility was similar. Compared to the reference diet, dietary inclusion of unfermented or fermented DDGS increased trypsin and chymotrypsin activities. The activity of digestive enzymes was not affected by the inclusion of fermented DDGS, except for amylase activity, which was lower with the fermented DDGS than with the unfermented DDGS diet. In conclusion, SSF of DDGS enhanced its nutritional value, increasing DDGS digestibility when included in diets for European seabass juveniles.

Keywords: aquaculture; by-products; distiller's dried grains; digestibility; digestive enzymes; solid-state fermentation

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1. Introduction

Industrial production of bioethanol using cereal grains has increased significantly as a renewable fuel source [1,2]. Global ethanol production has registered a steady increase

in the past few years, reaching 103 million liters in 2021 [3]. This large-scale industrial operation generates a high amount of by-products, the main one being distiller's dried grains with solubles (DDGS) [4–7]. Corn is the most used cereal in ethanol production [8]. For each 3 kg of fermented corn, circa 1.4 L of ethanol and 1 kg of DDGS are produced [9].

Aquaculture has a crucial role in global food security, being responsible for providing 87.5 million tons of aquatic animals, representing 49.2% of the total fishery production in 2020 [10]. Until recently, aquaculture's sustainability was threatened by its reliance on fisheries feedstuffs, mainly fishmeal and fish oil, used in aquafeeds. However, research efforts have led to a significant reduction in fishmeal and fish oil use with the increased utilization of feedstuffs [11]. Many traditional agricultural feedstuffs used in aquafeeds, such as soybean, rapeseed, corn, and wheat, are internationally traded, used for direct human consumption, and therefore expensive, involving the use of arable land, water, and fertilizers. In order to foster the sustainability and cost-efficiency of aquafeeds, agro-industrial by-products should be explored as new and underutilized feedstuffs.

Corn DDGS is a granular by-product containing approximately 30–32% crude protein, 3–12% lipids, 8–11% fiber, and a higher energy value than corn, depending on the grain composition and the processing methods [12–15]. Corn DDGS color varies from light yellow to dark brown [16]. The dark brown color may be associated with the increased CDS levels added to WDG, increasing energy, crude lipid, and mineral content. However, the dark color may also be associated with overheating during the drying process of DDGS, reducing lysine digestibility [17]. Corn DDGS also holds spent yeast and yeast cell walls rich in beta-glucans and nucleotides, with potential functional properties in fish [18].

Corn DDGS is already considered an efficient protein source for poultry, swine, and beef [19,20]. Dietary inclusion of up to 20% DDGS did not affect swine growth performance [21] or milk production in dairy cattle. For beef cattle, DDGS may replace up to 40% of corn grain [19]. The use of DDGS in aquafeeds also showed promising results, particularly for omnivore species. Corn DDGS may be included up to 30% and 40% in diets for hybrid tilapia, *Oreochromis* spp. [22] and Nile tilapia, *Oreochromis niloticus*, respectively, without affecting growth or feed efficiency [23]. The replacement of up to 30% of soybean and corn meals by DDGS, without lysine supplementation, did not decrease the growth and feed utilization of hybrid channel catfish juveniles, *Ictalurus punctatus* × blue catfish *I. furcatus* [24], while the dietary supplementation with lysine increased the DDGS inclusion level up to 40% [25]. The use of DDGS in diets for carnivorous fish is more limited, averaging 15% for rainbow trout, *Oncorhynchus mykiss* [26], although the inclusion level may increase when combined with amino acid supplementation [23,26]. In gilthead seabream, *Sparus aurata*, voluntary feed intake, feed efficiency, or growth performance were not affected by the total dietary replacement of soybean meal with 35% of DDGS [27]. However, for turbot, *Scophthalmus maximus*, even low dietary inclusion of levels of DDGS, from 10 to 25% diet, reduced daily growth index and impaired nitrogen and energy metabolism [28].

New approaches and technologies have been developed to potentiate DDGS use in aquafeeds, including high-protein DDGS [29,30] or dietary supplementation with commercial exoenzymes [26,29,31]. DDGS is a suitable substrate for microbial fermentation [20], so solid-state fermentation (SSF) may also be applied to increase its nutritional profile. SSF is a bioprocess with various applications, including animal nutrition [32]. This type of fermentation is an ecological and economic bioprocess that uses low water volumes [33] and cellulosic materials as substrates for microbial growth, such as agro-industrial by-products such as DDGS [34]. The low free water and high moisture content during SSF make it more adequate for actinomyces and fungi growth [35] since filamentous fungi, with hyphal growth and excellent tolerance to low water activity, are specially adapted to this process. During SSF, microorganisms produce hydrolytic enzymes, including lignocellulolytic enzymes [36,37]. The SSF of DDGS may promote hydrolysis is non-starch polysaccharides (NSP), releasing sugars and reducing the DDGS fiber content [38], increasing its nutritional composition and potentiating DDGS utilization in aquafeeds [39].

The present study aimed to improve DDGS nutritional value by applying SSF with three *Aspergillus* sp. fungi, namely: *A. carbonarius*, one of the most widely used fungi in biotechnological processes [40]; *A. ibericus*, a recently discovered strain of the nigri section [41]; and *A. uvarum*. From these three fungi, the one resulting in a fermented DDGS with higher protein content and lignocellulolytic enzyme activity was selected. The fermentation process was then scaled up to include fermented DDGS in fish diets. The digestibility of diets containing both unfermented and fermented DDGS was determined in a digestibility trial with European seabass juveniles, *Dicentrarchus labrax*, also assessing digestive enzyme activities.

2. Material and Methods

2.1. Solid-State Fermentation (SSF)

Three fungi from the *Aspergillus* section Nigri were obtained from Micoteca of the University of Minho (Braga, Portugal) and utilized in SSF: *Aspergillus carbonarius* 04.43, *Aspergillus ibericus* MUM 03.49, *Aspergillus uvarum* MUM 08.01. Fungi (preserved at −80 °C in glycerol) were revived in malt extract agar (MEA) plates (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar), transferred to MEA slants, incubated for 7 days at 25 °C, and then stored at 4 °C until SSF.

Reduced-oil corn DDGS was used in the present trial. SSF of corn DDGS with each fungi species was performed in triplicate in 500 mL cotton-plugged Erlenmeyer flasks. SSF was carried out for 7 days at 30 °C using autoclaved corn DDGS (121 °C, 15 min), and humidity was adjusted to 75% (*w*/*w* wet basis). A sterile spores solution (0.1% peptone and 0.001% Tween-80) was added to the MEA slants. The spore concentration in each flask was adjusted to 10^6 spores/mL with a Neubauer counting chamber. The mixtures were incubated for 7 days at 25 °C. At the end of SSF, the fermented DDGS was frozen until the chemical composition and enzymatic and phenolic activity were analyzed. For enzymatic and phenolic activity analysis, an aqueous extraction was carried out in the fermented DDGS using a solution containing 1% NaCl and 0.5% Triton X-100 with a ratio of 1 g dry fermented solid to 5 mL of solution (1:5, *w*/*v*).

2.2. SSF Scale-Up

SSF of DDGS was scaled up to be included in the diets used in the digestibility trial with European seabass. The SSF scale-up was performed in tray-type bioreactors (trays measuring 43 × 33 × 7 cm) using the experimental conditions mentioned in Section 2.1. Afterward, the fermented DDGS (SSF-DDGS) was dried at 40 °C for 24 h to reduce the moisture content to 10–15% to be included in the experimental diets.

2.3. Experimental Diets

The chemical composition and enzymatic activity of reference ingredients are described in Table 1. A reference diet was formulated to include approximately 48% crude protein, 15% crude lipids, and an inert digestibility marker (chromium oxide). Two test diets were prepared by mixing 30% DDGS or SSF-DDGS with 70% of the reference diet (DDGS and SSF-DDGS diets, respectively). Dietary ingredients were grounded, mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 4 mm die. The resultant pellets were then dried at 40 °C for approximately 24 h and stored at −4 °C until further use. The formulation and composition of the reference and test diets are presented in Table 2.

Table 1. Chemical composition (% DM) and enzymatic activity (U/g DM) of test ingredients.

	Fishmeal [1]	**DDGS** [2]	**SSF-DDGS** [3]
Dry matter (%; DM)	85.8	85.3	95.5
Ash	10.6	3.8	5.1
Crude Protein	77.5	26.7	30.1
Crude Lipids	7.6	3.4	4.3
Cellulose	—	40.5	17.6
Hemicellulose	—	22.9	22.4
Lignin	—	17.6	17.6
Acid Detergent Fiber	—	28.2	35.3
Neutral Detergent Fiber	—	63.1	57.6
Starch	—	0.8	0.7
Phosphorus	2.4	0.6	0.6
Gross energy (kJ/g DM)	18.2	19.8	20.1
Cellulase	—	—	43.4
Xylanase	—	—	68

[1] Pesquera Diamante, Peru. [2] Distiller's dried grains with solubles, Sorgal, S.A. Ovar, Portugal. [3] Solid state fermented distiller's dried grains with solubles with *Aspergillus Ibericus*.

Table 2. Ingredient composition and proximate composition (% dry matter) of experimental diets.

Diets	**Reference**	**DDGS**	**SSF-DDGS**
Ingredients			
Fishmeal [1]	63.2	44.2	44.2
DDGS [2]	—	30	—
SSF-DDGS [3]	—	—	30
Pre-gelatinized corn starch [4]	22.1	15.4	15.4
Fish oil	10.2	7.2	7.2
Vitamins premix [5]	1	0.7	0.7
Choline chloride (50%)	0.5	0.4	0.4
Mineral premix [6]	1	0.7	0.7
Chromium oxide	1	0.7	0.7
Binder [7]	1	0.7	0.7
Proximate analysis			
Dry matter (%)	94.3	93.3	93.5
Crude protein	47.9	40.6	42.6
Crude lipids	14.6	12.6	12
Ash	14.6	13.3	14.2
Phosphorus	0.8	0.7	0.7
Cellulose	—	12.2	8.5
Starch	20.4	19.2	19.9
Gross energy (kJ/g)	20.2	20.6	20.8
Chromium oxide	0.76	0.56	0.55

[1] Pesquera Diamante, Peru. [2] Dried distillers' grains with solubles, Sorgal, S.A. Ovar, Portugal [3] Solid state fermented distiller's dried grains with solubles with *Aspergillus Ibericus* [4] Cerestar, France. [5] Vitamins (mg/kg diet): retinol, 18,000 (IU/kg diet); calciferol, 2000 (IU/kg diet); alpha-tocopherol, 35; menadione 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. [6] Minerals (mg/kg 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 diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.4 (g/kg diet). [7] Binder (Aquacube. Agil, UK).

2.4. Digestibility Trial

The digestibility trial was carried out at CIIMAR's Aquatic Organisms Bioterium (BOGA) under controlled conditions following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines for Reporting Animal Research. BOGA is an aquatic animal facility that is accredited by the Direcção Geral de Alimentação e Veterinária (DGAV), the Portuguese National Authority for Animal Health, as a breeder and user establishment, according to National Decree (Decreto Lei n.° 113/2013).

This trial was subjected to an ethical review process by the CIIMAR animal welfare body (ORBEA-CIIMAR; reference ORBEA_CIIMAR_27_2019) and further approved by DGAV. All procedures were performed by certified scientists in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto Lei no. 113/2013, de 7 de Agosto) for the protection of animals used for scientific purposes.

European seabass (*Dicentrarchus labrax*) juveniles were acquired at Maresa (Spain). After transport, fish were held in quarantine for 15 days. Fish were then transferred to the experimental system consisting of a thermo-regulated recirculation water system with nine 60 L fiberglass tanks, each equipped with fecal collectors (quadrangular tank; 62 × 42 cm; [42]). Seawater flow was maintained at 4–5 L/min, 22 ± 1 °C, 34 ± 1‰ of salinity, and 90% oxygen saturation. Nitrogenous compounds were kept below 0.02 mg/L. Fish were subjected to 12 h light and 12 h dark.

At the beginning of the trial, nine homogenous groups of five European seabass were established (initial average weight of 171 g). Experimental diets were randomly assigned to triplicate groups, and fish were fed twice a day until apparent satiation, seven days a week. After an adaptation period of 7 days, feces were collected daily for 45 days. The fecal collectors were emptied each day before the morning meal. Collected fecal samples were centrifuged (3000 g, 10 min), separated from supernatant, pooled for each tank, and stored at −20 °C until the end of the trial. Thirty minutes after the last daily meal, uneaten feed and feces were removed by draining one-third of the water in the tanks.

Apparent digestibility coefficients (ADCs) of dry and organic matter, protein, lipids, starch, energy, and phosphorus of the experimental diets were determined using the following formula:

$$ADC_{diet} = \left[1 - \left(\frac{dietary\ Cr_2O_3 level \times faeces\ nutrient\ or\ energy\ level}{(faeces\ Cr_2O_3 level \times dietary\ nutrient\ or\ energy\ level)}\right)\right] \times 100 \quad (1)$$

The apparent digestibility coefficients of the test ingredients, namely DDGS and SSF-DDGS, were determined according to Bureau et al. (1999) with the following formula:

$$ADC_{testingredient} = ADC_{testdiet} + \left[\left(ADC_{testdiet} - ADC_{refdiet}\right) \times \left(0.7 \times D_{ref.}/0.3 \times D_{ingr.}\right)\right] \quad (2)$$

where D_{ref} = % nutrient (or kJ/g) of reference diet (dry matter basis), and $D_{ingr.}$ = % nutrient (or kJ/g) of experimental ingredients (dry matter basis).

At the end of the trial, intestines were sampled to measure digestive enzyme activity. Sampling was conducted 4 h after feeding to ensure full intestines, using 3 fish per tank. Fish were killed by anesthetic overdose (10 mL/L of ethylene glycol monophenyl ether) followed by decapitation. The gastrointestinal tract was then dissected on chilled trays, cleaned of adipose and connective tissue, and separated from the stomach. The intestine with pyloric caeca was divided into two portions, the anterior and distal intestine, and stored at −80 °C until enzymatic activity analysis.

2.5. Chemical Analyses

Chemical analyses of ingredients, including fishmeal, DDGS and SSF-DDGS, diets, and feces were performed following standard procedures [43]: dry matter by drying samples at 105 °C until constant weight; ash by incineration in a muffle furnace at 450 °C for 16 h; crude protein (N × 6.25) using the Kjeldahl method with acid digestion, in a Kjeltec digestor and distillation units (Tecator Systems, Hõganäs, Sweden; model 1015 and 1026, respectively); lipids by petroleum ether extraction using a Soxtec system (Tecator Systems, Hõganäs, Sweden; extraction unit model 1043 and service unit model 1046); and gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Total phosphorus content was determined using the colorimetric method described by Fiske [44], and starch content following Beutler [45]. Chromium oxide in diets and feces was determined by acidic digestion [46]. Cellulose, hemicellulose, and Klason lignin content were determined with a two-stage acid treatment described

by [47], preceded by the removal of starch. Reducing sugars were measured using the 3,5-dinitrosalicylic acid method (DNS; [48]), soluble protein by the Bradford method [49], and total phenols using the Folin–Ciocalteau method (Commission Regulation (EEC) No. 2676/90), using caffeic acid for calibration.

2.6. Enzymes Activity Analyses

2.6.1. Lignocellulolytic Enzymes

The activity of lignocellulolytic enzymes in SSF-DDGS and experimental diets was determined by mixing SSF-DDGS or diet (1:5 w/v) with a solution containing 1% NaCl and 0.5% Triton X-100, stirred for 60 min at room temperature, filtered through a fine-mesh net (300 µm pore size), and centrifuged (7000 g, 10 min). The enzymatic activities were measured in the collected supernatants. Cellulase (endo-1,4-β-glucanase) activity was measured using an enzymatic kit "Azo-CM-Cellulose S-ACMC 04/07" (Megazyme International, Ireland). One unit of cellulase activity (U) was defined as the amount of enzyme required to release 1 µmol of glucose-reducing sugar equivalents from carboxymethylcellulose under the assay conditions (40 °C; pH 4.5). Xylanase (endo-1, 4-β-xylanase) activity was measured with an enzymatic kit "Azo Wheat arabinoxylan AWX 10/2002" (Megazyme International, Ireland). One unit of xylanase activity (U) was defined as the amount of enzyme that released 1 µmol of xylose-reducing sugar equivalents from the substrate under the assay conditions (40 °C; pH 4.5).

2.6.2. Digestive Enzymes Activity

Each portion of the intestines was homogenized in ice with ultrapure water buffer (pH 8.0), centrifuged (23,000× g; 30 min; 4 °C), and the supernatants were stored at −80 °C until analysis. Alpha-amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), and total proteases were measured according to Magalhaes et al. (2015). Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were determined following the methods described by [50]. Soluble protein concentration was determined using the Bradford method [49]. All digestive enzyme activity was expressed as mU/mg of soluble protein. One unit (U) of enzymatic activity was represented as µmol of product generated per minute under the specific assay conditions.

2.7. Statistical Analyses

All data were checked for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) and normalized if necessary. Data regarding DDGS composition after SSF and ADC of experimental diets were analyzed by one-way analysis of variance (ANOVA). ADC of DDGS and SSF-DDGS diets were analyzed using a *t*-test. Digestive enzyme activity was analyzed by a two-way ANOVA with the intestine section and diets as fixed factors; when interaction was significant, a one-way ANOVA was performed for each intestine section. Significant differences among groups ($p < 0.05$) were determined with Tukey's multiple-range test. All data were analyzed in SPSS software package version 24.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Solid-State Fermentation

The chemical composition and enzymatic activity of DDGS before and after SSF with *A. carbonarius*, *A. ibericus*, and *A. uvarum* is presented in Table 3. Despite not being statistically significant, the total nitrogen content of DDGS decreased by 20% after SSF with *A. uvarum*, while it increased by 16% and 5% with *A. ibericus* and *A. carbonarius*, respectively. DDGS soluble protein content increased after SSF irrespective of fungi species. Total phenolic compounds and reducing sugar levels increased after SSF with each fungi species, attaining a maximum with *A. carbonarius* (9.6 mg/g and 38 mg/g SSF-DDGS, respectively). DDGS cellulose content decreased by circa 48 to 59% after SSF with all the fungi species, while hemicellulose content decreased by circa 35 and 9% after SSF with *A. uvarum* and

A. carbonarius, respectively. Lignin content decreased by up to 27% after SSF with *A. ibericus*. NDF and ADF content decreased by circa 32 and 43% after SSF with *A. carbonarius* and *A. ibericus*, respectively. Ash content was unaffected after SSF.

Table 3. Chemical composition (g/kg DM) and enzymatic activity (U/g) of distiller's dried grains with solubles (DDGS) before (unfermented) and after solid-state fermentation (SSF) with *Aspergillus ibericus*, *Aspergillus uvarum*, and *Aspergillus carbonarius*.

		After SSF		
	Unfermented	***A. ibericus***	***A. uvarum***	***A. carbonarius***
Total nitrogen	42.7 ± 1.1	49.7 ± 0.7	34.0 ± 2.2	44.9 ± 0.5
Soluble Protein	0.28 ^{a} ± 0.00	5.27 ^{b} ± 0.24	5.31 ^{b} ± 0.69	4.71 ^{b} ± 0.15
Total phenols (mg caffeic acid/kg DM)	1.49 ^{a} ± 0.03	4.86 ^{b} ± 0.03	5.06 ^{b} ± 0.49	9.57 ^{c} ± 0.02
Reducing sugars (mg/kg DM)	9.47 ^{a} ± 0.45	31.9 ^{b} ± 0.74	28.4 ^{b} ± 1.8	38.1 ^{c} ± 0.60
Hemicellulose	229.3 ^{a} ± 2.1	242.9 ^{a} ± 15.1	149.0 ^{b} ± 17.1	209.1 ^{ab} ± 20.9
Cellulose	405.2 ^{a} ± 19.6	204.4 ^{b} ± 6.4	165.4 ^{b} ± 11.0	212 ^{b} ± 20.2
Lignin	176.4 ^{a} ± 11.0	129.0 ^{b} ± 0.0	240.7 ^{c} ± 5.9	171.4 ^{a} ± 10.2
NDF	810.9 ^{a} ± 16.8	576.3 ^{ab} ± 15.2	592.5 ^{ab} ± 25.2	555.1 ^{b} ± 26.1
ADF	581.6 ^{a} ± 0.2	333.4 ^{b} ± 4.6	383.4 ^{ab} ± 7.1	406.1 ^{ab} ± 14.0
Ash	38.1 ± 2.5	54.3 ± 3.1	43.5 ± 8.3	54.9 ± 1.1
Enzymatic activity				
Xylanase	—	68.0 ^{ab} ± 2.05	152.1 ^{b} ± 42.31	31.2 ^{a} ± 1.0
Cellulase	—	44.6 ^{a} ± 1.58	27.4 ^{b} ± 7.17	3.75 ^{c} ± 1.46

DM: dry matter. Values are presented as means (n = 3) ± standard deviation. Means in the same row with different superscripts are significantly different (Tukey's test, $p < 0.05$).

Lignocellulolytic enzyme activity was dependent on the fungi species used in SSF. SSF with *A. uvarum* and *A. ibericus* yielded the highest xylanase activity (152.1 U/g and 68.0 U/g SSF-DDGS, respectively), while *A. ibericus* led to the highest cellulase activity (44.6 U/g SSF-DDGS).

Based on the protein content and enzymatic activity of fermented DDGS obtained after SSF, *A. ibericus* was selected. SSF of DDGS with *A. ibericus* was then scaled up to produce enough fermented DDGS to be included in the test diet and used in the digestibility trial.

3.2. Diets and Ingredient Digestibility

The ADCs of the experimental diets are presented in Table 4. The reference diet had the highest ADCs of dry matter, organic matter, lipids, and starch. The energy digestibility of the reference diet was similar to the SSF-DDGS diet and higher than the DDGS diet. Comparing DDGS diets, lipid and energy ADCs were higher with the SSF-DDGS diet than with the DDGS diet, while ADCs of dry matter, organic matter, protein, starch, and phosphorus were similar among DDGS-based diets.

Table 4. Apparent digestibility coefficients (%) of the experimental diets.

Diets	**Reference**	**DDGS**	**SSF-DDGS**	**SEM**
Dry matter	83.7 ^{b}	74.9 ^{a}	75.1 ^{a}	0.6
Organic matter	85.1 ^{b}	75.2 ^{a}	75.1 ^{a}	0.7
Protein	91.9	91.9	93.6	0.4
Lipids	98.9 ^{c}	93.4 ^{a}	96.0 ^{b}	0.7
Starch	88.1 ^{b}	79.3 ^{a}	81.8 ^{a}	0.5
Energy	93.9 ^{b}	86.8 ^{a}	92.7 ^{b}	1.2
Phosphorus	92	87.2	88.4	1.1

Values are presented as means (n = 3) and pooled standard error of the mean (SEM). Mean values in the same row with different superscripts are significantly different (Tukey's test, $p < 0.05$).

The ADCs of the tested ingredients (DDGS and SSF-DDGS) are presented in Table 5. ADCs of protein, lipids, starch, and energy were higher with SSF-DDGS than with DDGS, while ADCs of dry and organic matter and phosphorus were similar.

Table 5. Apparent digestibility coefficients (%) of distiller's dried grains with solubles (DDGS) and solid-state fermented distiller's dried grains with solubles (SSF-DDGS) with *Aspergillus ibericus*.

Ingredients	DDGS	SSF-DDGS	SEM
Dry matter	54.6	54	2.1
Organic matter	54.3	55.2	2.1
Protein	88.5	96.6 *	2
Lipids	87.9	98.6 *	2.4
Starch	58.8	65.3 *	2.7
Energy	70	89.9 *	1.3
Phosphorus	73.4	77.5	1.7

Values are presented as means (n = 3) and pooled standard error of the mean (SEM). A significant difference between DDGS and SSF-DDGS is indicated by an asterisk (*t*-test, $p < 0.05$).

The digestive enzymes specific activities in the anterior and distal intestine sections are presented in Table 6. Irrespective of the diet, digestive enzyme activity was higher in the distal intestine than in the anterior intestine, except for chymotrypsin and amylase. Total protease activity was similar among diets, while trypsin, chymotrypsin, and lipase activity were higher in fish fed the DDGS or SSF-DDGS diets than in those fed with the reference diet. Amylase activity was higher with the DDGS diet than with the other diets.

Table 6. Specific activity of protease, trypsin, chymotrypsin, lipase, and amylase (mU/mg protein) in anterior and distal intestine sections of European seabass fed the experimental diets.

Diets	Reference	DDGS	SSF-DDGS	SEM
Total proteases				
Anterior intestine	467.4	487.4	422.2	13.7
Distal intestine	342.6	302.9	403.9	21.1
Trypsin				
Anterior intestine	5.4	10.5	12.8	0.9
Distal intestine	10.9	17	16.4	1.2
Chymotrypsin				
Anterior intestine	694.3	1192.8	1283.4	70.7
Distal intestine	976.2	1469.8	1336.3	71.2
Lipase				
Anterior intestine	365.9 ^{a}	497.0 ^{ab}	541.2 ^{b}	0.5
Distal intestine	478.4 ^{a}	864.6 ^{b}	694.6 ^{b}	0.5
Amylase				
Anterior intestine	6.2	7.1	6.5	30.3
Distal intestine	6.7	9.8	6.5	44.3
Two-Way ANOVA ^{1}				

Table 6. *Cont.*

Diets	Reference		DDGS	SSF-DDGS		SEM
	Diet	Intestine section	Interaction	Diets		
				Reference	DDGS	SSF-DDGS
Proteases	ns	***	*	—	—	—
Trypsin	***	**	ns	a	b	b
Chymotrypsin	***	ns	ns	a	b	b
Lipases	***	***	*	—	—	—
Amylase	*	ns	ns	ab	c	b

Values are presented as means (n = 9) and pooled standard error of the mean (SEM). [1] Two-way ANOVA: ns: non-significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. If the two-way ANOVA interaction was significant, a one-way ANOVA was performed for each intestine section ($p < 0.05$). Mean values in the same row with different superscripts are significantly different.

4. Discussion

4.1. Solid-State Fermentation

Solid-state fermentation has many applications of interest for animal nutrition, for example, the production of functional compounds, such as enzymes and phenolic compounds, and nutritionally enriched feedstuffs. During SSF, fungi grow on solid substrates, such as agriculture by-products, and produce lignocellulolytic hydrolyzing enzymes that decompose cellulose and hemicellulose into simpler sugars, reducing the content of indigestible fiber and increasing nutrient bioavailability [51,52]. *Aspergillus* sp. are efficient producers of lignocellulolytic enzymes [53] and have been used to hydrolyze different lignocellulosic agro-industrial by-products [47,54–56]. In the present study, the enzyme production depended on the fungus, but irrespective of the species, xylanase production was higher than cellulase production. This suggests that DDGS hemicellulose is more readily available for enzymatic hydrolysis than cellulose, probably due to the crystalline nature of cellulose and its connection with lignin and the higher exposure of the hemicellulose fraction [57]. Previously, it was confirmed that DDGS fermentation with fungal stains produced higher cellulase and xylanase activity than with bacterial strains [58]. Among the fungal strains tested, *A. carbonarius* led to the highest xylanase and cellulase production, followed by *Trichoderma reesei* [58]. Moreover, higher xylanase and cellulase activities may also be obtained when supplementing DDGS with a nitrogen source. Supplementation of DDGS with urea, before SSF with *Trichoderma reesei*, increased cellulase yield from 1.5 to 2.4 U/g and xylanase yield from 16.3 to 51.7 U/g [34]. SSF of DDGS with *Bacillus subtilis* and *Lactobacillus plantarum* resulted in 70 U/g of xylanase and 27 U/g of cellulase [38].

Solid-state fermentation has been used to reduce substrate lignocellulosic compounds, as the enzymes produced during this process act upon these substrates. In the present study, the ADF content of DDGS decreased more than the NDF content, which may indicate a higher degree of hydrolysis in hemicellulose than cellulose. This result aligns with the higher activity levels of xylanase than cellulase obtained after SSF of DDGS. Hemicellulose is a branched heteropolysaccharide with a low polymerization degree and is more easily prone to hydrolysis than cellulose [59]. In previous studies, SSF of different lignocellulosic substrates led to higher production of xylanase than cellulase [47,60]. However, using DDGS as a substrate, SSF with *Bacillus subtilis* and *Lactobacillus plantarum* reduced 1.1, 5, and 20.4% of lignin, ADF, and NDF content, respectively [38]. SSF of corn straw with *Trichoderma afroharzianum* reduced the crude fiber content by about 23.5% after 21 days of fermentation [61], while SSF of corn meal with *Lactobacillus plantarum* reduced the crude fiber content by about 77% [62,63].

The increase in protein content of low-cost feedstuffs as by-products is of utmost importance as access to affordable and quality feedstuffs is one of the most significant constraints of animal nutrition. SSF may increase protein content through the lignocellulosic matrix degradation or fungal biomass retaining in the substrate [64], as previously observed for DDGS submitted to SSF with probiotics [65]. In the present study, SSF of DDGS with

A. ibericus increased nitrogen content by about 16%, and soluble protein content was 18 times higher than with the unfermented DDGS. Similarly, SSF of DDGS with *A. niger* increased protein content circa 22%, from 9.5% to 11% wet weight [66]. SSF with *Pleurotus sapidus* also increased rice bran protein content from 7.4% to 12.8% [67].

Degradation of lignocellulosic components of the plant's cell wall opens phenol rings entrapped in the lignin fraction and may release phenolic compounds with antioxidant activity [63,68]. In the present study, SSF led to a more than three-fold increase in the total phenolic content of DDGS. Similarly, the effect of SSF on increasing substrate phenolic content has been described. For example, the SSF of corn kernels by a co-culture of *Monascus anka*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* increased the total phenols by about 18-fold [69]. SSF of *Ulva rigida* increased by 6-fold the phenolic content [70], and SSF of olive and winery by-products doubled the antioxidant content after the third day of fermentation, before it decreased [56]. The increase in phenolic content and antioxidant availability through the SSF is particularly interesting for aquafeed, particularly under the actual restriction on the European Union of using ethoxyquin and butylated hydroxyanisole (synthetic compounds) to inhibit the oxidation of the highly unsaturated fatty acids present in aquafeeds.

4.2. Diets and Ingredient Digestibility

Dietary incorporation of DDGS into monogastric animal feeds, including for aquaculture fish species, has been limited by its high indigestible crude fiber content [71]. In the present study, the dietary inclusion of unfermented DDGS reduced dry matter, organic matter, lipids, starch, and energy digestibility. This is undoubtedly due to the higher DDGS crude fiber. Fish, mainly carnivorous fish, have a limited capacity to digest carbohydrates [72] and, if present in high quantities, impair intestinal function and health and, ultimately, reduce fish growth and feed efficiency [73–75]. For European seabass, the digestibility of DDGS was determined to be relatively high for protein, amino acids, and lipids but low for energy, reflecting the undigestible lignocellulosic nature of DDGS [76]. In grass carp, *Ctenopharyngodon idellus*, dietary replacement of rapeseed meal by increasing levels of DDGS linearly decreased dry matter and energy digestibility, while protein digestibility was little affected [77]. Similarly, in turbot juveniles, the dietary inclusion of 17.5% of DDGS decreased energy digestibility, although protein and amino acid digestibility were not affected [28].

Dietary incorporation of the fermented DDGS (SSF-DDGS diet) increased protein, lipid, starch, and energy digestibility, compared to the unfermented DDGS diet (DDGS diet). These results correlated well with the modification of DDGS composition after SSF, namely in the reduction in NDF and ADF content, increase in soluble protein content, and lignocellulolytic enzyme yield. The NDF and ADF levels of the SSF-DDGS decreased by about 29 and 43%, with a high impact on the overall diet digestibility. Indeed, high dietary NSP levels reduce the accessibility of endogenous fish enzymes to substrates, reducing the gastric emptying rate, increasing digesta viscosity, and impairing nutrient absorption [78]. Moreover, the lignocellulosic activity in the SSF-DDGS diet may contribute to the increase in diet digestibility. Indeed, it has been observed that dietary supplementation with fungi enzymes increases diet digestibility, depending on diet formulation, type and incorporation levels of the enzymes, fish species and size, and water temperature (revised by Castillo and Zheng [75,79]).

The effect of dietary incorporation of SSF of agro-industrial by-products on the digestibility of fish diets has been little studied. However, there is increased evidence of the positive impact of dietary supplementation with SSF products. For example, dietary supplementation with a commercial SSF product (Synergen^{TM}, Altech) increased lupin diet digestibility in rainbow trout [80] and DDGS-based diet in turbot [31]. Moreover, supplementing a plant feedstuff-based diet with an extract produced by SSF of brewer's spent grain increased the digestibility of dry matter, starch, cellulose, glucans, and energy

in European seabass [37]. To the best of the authors' knowledge, this is the first study evaluating the digestibility of SSF-DDGS in fish.

In the present study, dietary incorporation of unfermented or SSF-DDGS modulates the activity of the digestive enzymes, increasing the trypsin, chymotrypsin, and amylase activity. The reference diet was formulated to be devoid of indigestible carbohydrates, while DDGS and SSF-DDGS diets are rich in insoluble NSP. Therefore, the positive impact of dietary inclusion of DDGS and SSF-DDGS on digestive enzyme activity may be due to the presence of more accessible NSP and the presence of lignocellulolytic enzymes. In line with the present results, in European seabass and meagre, replacing 30% of the diet with DDGS increased amylase and lipase activity, while protease activity was unaffected [76]. However, for turbot, the dietary replacement of fishmeal with DDGS reduced amylase and lipase activities but did not affect protease activity [28]. Moreover, previous studies reported a negative impact of dietary soluble NSP but not insoluble NSP on digestive enzyme activities in fish [81,82].

Studies on the effects of dietary supplementation with enzymes on fish digestive endogenous enzyme production are still scarce, and the results are divergent [79]. For example, carbohydrase complex dietary supplementation did not affect protease, lipase, and amylase activity in seabass [83], but in white seabream increased the amylase activity [84]. Diet supplementation with a β-glucanase and xylanase complex increased amylase and protease activities in Nile tilapia [85], while a commercial carbohydrase complex increased amylase but not protease and lipase activity in hybrid tilapia [86]. In Jian carp, *Cyprinus carpio* var. Jian, xylanase dietary supplementation increased trypsin, chymotrypsin, amylase, and lipase activity [87], while in rohu, *Labeo rohita*, and grass carp, *Ctenopharyngodon idellus*, cellulase dietary supplementation increased protease, amylase, and lipase activity [88,89]. Dietary supplementation with a commercial protease increased endogenous protease activity in the anterior intestine of crucian carp, *Carassius auratus* [90]. For DDGS-based diets, carbohydrate supplementation increased lipase activity in turbot juveniles [31].

5. Conclusions

SSF is a cost-effective and biological treatment that effectively improves DDGS nutritional value, reduces NSP content, and increases protein and phenolics content, along with enriching lignocellulolytic enzymes. In this work, *Aspergillus ibericus* resulted in a higher protein increase, NSP reduction, and considerable lignocellulolytic enzyme production. Dietary incorporation of SSF-DDGS significantly increased protein, lipid, starch, and energy digestibility compared to in fish fed the unfermented DDGS-based diet. Overall, it was observed that nutrient digestibility increased with SSF-DDGS compared to with the unfermented DDGS, which may allow higher dietary inclusion levels in the diets of carnivorous fish such as European seabass.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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References

1. European Renewable Ethanol Renewable Ethanol Production by End-Use. Available online: https://epure.org/media/1929/renewable-ethanol-production-by-end-use-2018.jpg (accessed on 24 March 2020).
2. Renewable Fuels Association. Ethanol & Co-Product Trade Exports and Imports. Available online: https://ethanolrfa.org/statistics/ethanol-co-product-trade/ (accessed on 24 March 2020).
3. Renewable Fuels Association. Annual Ethanol Production U.S. and World Ethanol Production. Available online: https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production (accessed on 24 March 2020).
4. Rausch, K.D.; Belyea, R.L.; Ellersieck, M.R.; Singh, V.; Johnston, D.B.; Tumbleson, M.E. Particle Size Distributions of Ground Corn and DDGS from Dry Grind Processing. *Trans. Am. Soc. Agric. Eng.* **2005**, *48*, 273–277. [CrossRef]
5. Bothast, R.J.; Schlicher, M.A. Biotechnological Processes for Conversion of Corn into Ethanol. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 19–25. [CrossRef] [PubMed]
6. Kwiatkowski, J.R.; McAloon, A.J.; Taylor, F.; Johnston, D.B. Modeling the Process and Costs of Fuel Ethanol Production by the Corn Dry-Grind Process. *Ind. Crops Prod.* **2006**, *23*, 288–296. [CrossRef]
7. Ganesan, V.; Rosentrater, K.A.; Muthukumarappan, K. Methodology to Determine Soluble Content in Dry Grind Ethanol Coproduct Streams. *Appl. Eng. Agric.* **2006**, *22*, 899–903. [CrossRef]
8. Renewable Fuels Association Feedstock Use and Co-Product Output. Available online: https://ethanolrfa.org/statistics/feedstock-use-co-product-output/ (accessed on 24 March 2020).
9. Mohammadi Shad, Z.; Venkitasamy, C.; Wen, Z. Corn Distillers Dried Grains with Solubles: Production, Properties, and Potential Uses. *Cereal Chem.* **2021**, *98*, 999–1019. [CrossRef]
10. FAO. *The State of World Fisheries and Aquaculture 2022*; FAO: Rome, Italy, 2022; ISBN 978-92-5-136364-5.
11. Tacon, A.G.J.; Metian, M.; McNevin, A.A. Future Feeds: Suggested Guidelines for Sustainable Development. *Rev. Fish. Sci. Aquac.* **2022**, *30*, 135–142. [CrossRef]
12. Belyea, R.L.; Rausch, K.D.; Tumbleson, M.E. Composition of Corn and Distillers Dried Grains with Solubles from Dry Grind Ethanol Processing. *Bioresour. Technol.* **2004**, *94*, 293–298. [CrossRef]
13. Shurson, G.; Spiehs, M.; Whitney, M. The Use of Maize Distiller's Dried Grains with Solubles in Pig Diets. *Pig News Inf.* **2004**, *25*, 75N–83N.
14. Spiehs, M.J.; Whitney, M.H.; Shurson, G.C. Nutrient Database for Distiller's Dried Grains with Solubles Produced from New Ethanol Plants in Minnesota and South Dakota. *J. Anim. Sci.* **2002**, *80*, 2639–2645. [CrossRef]
15. Nuez Ortín, W.G.; Yu, P. Nutrient Variation and Availability of Wheat DDGS, Corn DDGS and Blend DDGS from Bioethanol Plants. *J. Sci. Food Agric.* **2009**, *89*, 1754–1761. [CrossRef]
16. Lim, C.; Li, E.; Klesius, P.H. Distiller's Dried Grains with Solubles as an Alternative Protein Source in Diets of Tilapia. *Rev. Aquac.* **2011**, *3*, 172–178. [CrossRef]
17. Cozannet, P.; Primot, Y.; Gady, C.; Métayer, J.P.; Lessire, M.; Skiba, F.; Noblet, J. Energy Value of Wheat Distillers Grains with Solubles for Growing Pigs and Adult Sows. *J. Anim. Sci.* **2010**, *88*, 2382–2392. [CrossRef]
18. Shelby, R.A.; Lim, C.; Yildrim-Aksoy, M.; Klesius, P.H. Effect of Distillers Dried Grains with Solubles-Incorporated Diets on Growth, Immune Function and Disease Resistance in Nile Tilapia (*Oreochromis niloticus* L.). *Aquac. Res.* **2008**, *39*, 1351–1353. [CrossRef]
19. Shurson, J.; Noll, S.L. Feed and Alternative Uses for DDGs. In Proceedings of the Energy from Agriculture: New Technologies, Innovative Programs & Success Stories, St. Louis, MO, USA, 14–15 December 2005; pp. 1–11.
20. Iram, A.; Cekmecelioglu, D.; Demirci, A. Distillers' Dried Grains with Solubles (DDGS) and Its Potential as Fermentation Feedstock. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 6115–6128. [CrossRef]
21. Stein, H.H.; Shurson, G.C. Board-Invited Review: The Use and Application of Distillers Dried Grains with Solubles in Swine Diets. *J. Anim. Sci.* **2009**, *87*, 1292–1303. [CrossRef]
22. Coyle, S.D.; Mengel, G.J.; Tidwell, J.H.; Webster, C.D. Evaluation of Growth, Feed Utilization, and Economics of Hybrid Tilapia, *Oreochromis niloticus* x *Oreochromis aureus*, Fed Diets Containing Different Protein Sources in Combination with Distillers Dried Grains with Solubles. *Aquac. Res.* **2004**, *35*, 365–370. [CrossRef]
23. Schaeffer, T.W.; Brown, M.L.; Rosentrate, K.A. Performance Characteristics of Nile Tilapia (*Oreochromis niloticus*) Fed Diets Containing Graded Levels of Fuel-Based Distillers Dried Grains with Solubles. *J. Aquac. Feed. Sci. Nutr.* **2009**, *1*, 78–83. [CrossRef]
24. Zhou, P.; Zhang, W.; Davis, D.A.; Lim, C. Growth Response and Feed Utilization of Juvenile Hybrid Catfish Fed Diets Containing Distiller's Dried Grains with Solubles to Replace a Combination of Soybean Meal and Corn Meal. *N. Am. J. Aquac.* **2010**, *72*, 298–303. [CrossRef]
25. Lim, C.; Yildirim-Aksoy, M.; Klesius, P.H. Growth Response and Resistance to *Edwardsiella Ictaluri* of Channel Catfish, *Ictalurus Punctatus*, Fed Diets Containing Distiller's Dried Grains with Solubles. *J. World Aquac. Soc.* **2009**, *40*, 182–193. [CrossRef]
26. Cheng, Z.J.; Hardy, R.W. Nutritional Value of Diets Containing Distiller's Dried Grain with Solubles for Rainbow Trout, *Oncorhynchus mykiss*. *J. Appl. Aquac.* **2004**, *15*, 101–113. [CrossRef]
27. Diógenes, A.F.; Basto, A.; Estevão-Rodrigues, T.T.; Moutinho, S.; Aires, T.; Oliva-Teles, A.; Peres, H. Soybean Meal Replacement by Corn Distillers Dried Grains with Solubles (DDGS) and Exogenous Non-Starch Polysaccharidases Supplementation in Diets for Gilthead Seabream (*Sparus aurata*) Juveniles. *Aquaculture* **2019**, *500*, 435–442. [CrossRef]

28. Diógenes, A.F.; Castro, C.; Miranda, A.C.; Oliva-Teles, A.; Peres, H. Dietary Replacement of Fishmeal by Corn Distillers Dried Grains with Solubles (DDGS) in Diets for Turbot (*Scophthalmus maximus*, Linneaus, 1758) Juveniles. *Aquaculture* **2018**, *492*, 113–122. [CrossRef]
29. Goda, A.M.A.S.; Ahmed, S.R.; Nazmi, H.M.; Aboseif, A.M.; Taha, M.K.S.; Fadda, S.H.; Baromh, M.Z.; El-Haroun, E.; Davies, S.J. Assessment of a High Protein Distillers Dried Grain (HP-DDG) Augmented with Phytase in Diets for European Sea Bass, *Dicentrarchus Labrax* Fingerlings on Growth Performance, Haematological Status, Immune Response and Related Gut and Liver Histology. *Aquaculture* **2020**, *529*, 735617. [CrossRef]
30. Overland, M.; Krogdahl, Å.; Shurson, G.; Skrede, A.; Denstadli, V. Evaluation of Distiller's Dried Grains with Solubles (DDGS) and High Protein Distiller's Dried Grains (HPDDG) in Diets for Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture* **2013**, *416–417*, 201–208. [CrossRef]
31. Diógenes, A.F.; Castro, C.; Carvalho, M.; Magalhães, R.; Estevão-Rodrigues, T.T.; Serra, C.R.; Oliva-Teles, A.; Peres, H. Exogenous Enzymes Supplementation Enhances Diet Digestibility and Digestive Function and Affects Intestinal Microbiota of Turbot (*Scophthalmus maximus*) Juveniles Fed Distillers' Dried Grains with Solubles (DDGS) Based Diets. *Aquaculture* **2018**, *486*, 42–50. [CrossRef]
32. Parmar, A.; Patel, V.; Usadadia, S.; Rathwa, S.; Prajapati, D. A Solid State Fermentation, Its Role in Animal Nutrition: A Review. *Int. J. Chem. Stud.* **2019**, *7*, 4626–4633.
33. Graminha, E.B.N.; Gonçalves, A.Z.L.; Pirota, R.D.P.B.; Balsalobre, M.A.A.; da Silva, R.; Gomes, E. Enzyme Production by Solid-State Fermentation: Application to Animal Nutrition. *Anim. Feed Sci. Technol.* **2008**, *144*, 1–22. [CrossRef]
34. Yang, S.; Lio, J.Y.; Wang, T. Evaluation of Enzyme Activity and Fiber Content of Soybean Cotyledon Fiber and Distiller's Dried Grains with Solubles by Solid State Fermentation. *Appl. Biochem. Biotechnol.* **2012**, *167*, 109–121. [CrossRef]
35. Fleuri, L.F.; Kawaguti, H.Y.; Pedrosa, V.A.; Vianello, F.; Lima, G.P.P.; Novelli, P.K.; Okino-Delgado, C.H. Exploration of Microorganisms Producing Bioactive Molecules of Industrial Interest by Solid State Fermentation. In *Food Quality, Safety and Technology*; Springer: Vienna, Austria, 2013; pp. 147–161.
36. Sharma, R.K.; Arora, D.S. Fungal Degradation of Lignocellulosic Residues: An Aspect of Improved Nutritive Quality. *Crit. Rev. Microbiol.* **2015**, *41*, 52–60. [CrossRef]
37. Fernandes, H.; Moyano, F.; Castro, C.; Salgado, J.; Martínez, F.; Aznar, M.; Fernandes, N.; Ferreira, P.; Gonçalves, M.; Belo, I.; et al. Solid-State Fermented Brewer's Spent Grain Enzymatic Extract Increases in Vitro and in Vivo Feed Digestibility in European Seabass. *Sci. Rep.* **2021**, *11*, 22946. [CrossRef]
38. Wang, C.; Su, W.; Zhang, Y.; Hao, L.; Wang, F.; Lu, Z.; Zhao, J.; Liu, X.; Wang, Y. Solid-State Fermentation of Distilled Dried Grain with Solubles with Probiotics for Degrading Lignocellulose and Upgrading Nutrient Utilization. *AMB Express* **2018**, *8*, 1–13. [CrossRef]
39. Naylor, R.L.; Hardy, R.W.; Bureau, D.P.; Chiu, A.; Elliott, M.; Farrell, A.P.; Forster, I.; Gatlin, D.M.; Goldburg, R.J.; Hua, K.; et al. Feeding Aquaculture in an Era of Finite Resources. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15103–15110. [CrossRef] [PubMed]
40. Schuster, E.; Dunn-Coleman, N.; Frisvad, J.; van Dijck, P. On the Safety of *Aspergillus Niger*—A Review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 426–435.
41. Serra, R.; Cabañes, F.J.; Perrone, G.; Castellá, G.; Venâncio, A.; Mulè, G.; Kozakiewicz, Z. *Aspergillus Ibericus*: A New Species of Section *Nigri* Isolated from Grapes. *Mycologia* **2006**, *98*, 295–306. [CrossRef]
42. Cho, C.Y.; Slinger, S.J.; Bayley, H.S. Bioenergetics of Salmonid Fishes: Energy Intake, Expenditure and Productivity. *Comp. Biochem. Physiol. Part B Comp. Biochem.* **1982**, *73*, 25–41. [CrossRef]
43. Association of Official Analytical Chemists; Horwitz, W.; Association of Official Agricultural Chemists (U.S.). *Official Methods of Analysis of the Association of Official Analytical Chemists*; The Association: New York, NY, USA, 1980; ISBN 0935584145.
44. Fiske, C.; Chem, Y.S. The Colorimetric Determination of Phosphorus. *J. Biol. Chem.* **1925**, *66*, 375–400. [CrossRef]
45. Beutler, H. Methods of Enzymatic Analysis. In *Methods of Enzymatic Analysis*; Elsevier: Amsterdam, The Netherlands, 1984; pp. 2–10.
46. Furukawa, A.; Tsukahara, H. On the Acid Digestion Method for the Determination of Chromic Oxide as an Index Substance in the Study of Digestibility of Fish Feed. *Bull. Jpn. Soc. Sci. Fish.* **1966**, *32*, 502–508. [CrossRef]
47. Leite, P.; Salgado, J.M.; Venâncio, A.; Domínguez, J.M.; Belo, I. Ultrasounds Pretreatment of Olive Pomace to Improve Xylanase and Cellulase Production by Solid-State Fermentation. *Bioresour. Technol.* **2016**, *214*, 737–746. [CrossRef]
48. Miller, G.L. Modified DNS Method for Reducing Sugars. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
49. Bradford, M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
50. Moutinho, S.; Peres, H.; Serra, C.; Martínez-Llorens, S.; Tomás-Vidal, A.; Jover-Cerdá, M.; Oliva-Teles, A. Meat and Bone Meal as Partial Replacement of Fishmeal in Diets for Gilthead Sea Bream (*Sparus aurata*) Juveniles: Diets Digestibility, Digestive Function, and Microbiota Modulation. *Aquaculture* **2017**, *479*, 721–731. [CrossRef]
51. El-Bakry, M.; Abraham, J.; Cerda, A.; Barrena, R.; Ponsá, S.; Gea, T.; Sánchez, A. From Wastes to High Value Added Products: Novel Aspects of SSF in the Production of Enzymes. *Crit. Rev. Environ. Sci. Technol.* **2015**, *45*, 1999–2042. [CrossRef]
52. Leite, P.; Sousa, D.; Fernandes, H.; Ferreira, M.; Costa, A.R.; Filipe, D.; Gonçalves, M.; Peres, H.; Belo, I.; Salgado, J.M. Recent Advances in Production of Lignocellulolytic Enzymes by Solid-State Fermentation of Agro-Industrial Wastes. *Curr. Opin. Green Sustain. Chem.* **2021**, *27*, 100407. [CrossRef]

53. Mansour, A.A.; Arnaud, T.; Lu-Chau, T.A.; Fdz-Polanco, M.; Moreira, M.T.; Rivero, J.A.C. Review of Solid State Fermentation for Lignocellulolytic Enzyme Production: Challenges for Environmental Applications. *Rev. Environ. Sci. Biotechnol.* **2016**, *15*, 31–46. [CrossRef]
54. Salgado, J.M.; Abrunhosa, L.; Venâncio, A.; Domínguez, J.M.; Belo, I. Screening of Winery and Olive Mill Wastes for Lignocellulolytic Enzyme Production from *Aspergillus* Species by Solid-State Fermentation. *Biomass Convers. Biorefin.* **2014**, *4*, 201–209. [CrossRef]
55. Fernandes, H.; Salgado, J.M.; Ferreira, M.; Vršanská, M.; Fernandes, N.; Castro, C.; Oliva-Teles, A.; Peres, H.; Belo, I. Valorization of Brewer's Spent Grain Using Biological Treatments and Its Application in Feeds for European Seabass (*Dicentrarchus labrax*). *Front. Bioeng. Biotechnol.* **2022**, *10*, 732948. [CrossRef]
56. Filipe, D.; Fernandes, H.; Castro, C.; Peres, H.; Oliva-Teles, A.; Belo, I.; Salgado, J.M. Improved Lignocellulolytic Enzyme Production and Antioxidant Extraction Using Solid-State Fermentation of Olive Pomace Mixed with Winery Waste. *Biofuels Bioprod. Biorefining* **2020**, *14*, 78–91. [CrossRef]
57. Dashtban, M.; Schraft, H.; Qin, W. Fungal Bioconversion of Lignocellulosic Residues; Opportunities & Perspectives. *Int. J. Biol. Sci.* **2009**, *5*, 578.
58. Iram, A.; Cekmecelioglu, D.; Demirci, A. Screening of Bacterial and Fungal Strains for Cellulase and Xylanase Production Using Distillers' Dried Grains with Solubles (DDGS) as the Main Feedstock. *Biomass Convers. Biorefin.* **2020**, *11*, 1955–1964. [CrossRef]
59. Jönsson, L.J.; Martín, C. Pretreatment of Lignocellulose: Formation of Inhibitory by-Products and Strategies for Minimizing Their Effects. *Bioresour. Technol.* **2016**, *199*, 103–112. [CrossRef]
60. Fernandes, H.; Salgado, J.M.; Martins, N.; Peres, H.; Oliva-Teles, A.; Belo, I. Sequential Bioprocessing of Ulva Rigida to Produce Lignocellulolytic Enzymes and to Improve Its Nutritional Value as Aquaculture Feed. *Bioresour. Technol.* **2019**, *281*, 277–285. [CrossRef]
61. Li, W.; Zhao, L.; He, X. Degradation Potential of Different Lignocellulosic Residues by *Trichoderma longibrachiatum* and *Trichoderma afroharzianum* under Solid State Fermentation. *Process Biochem.* **2022**, *112*, 6–17. [CrossRef]
62. Terefe, Z.K.; Omwamba, M.N.; Nduko, J.M. Effect of Solid State Fermentation on Proximate Composition, Antinutritional Factors and in Vitro Protein Digestibility of Maize Flour. *Food Sci. Nutr.* **2021**, *9*, 6343–6352. [CrossRef] [PubMed]
63. Adebo, O.A.; Medina-Meza, I.G. Impact of Fermentation on the Phenolic Compounds and Antioxidant Activity of Whole Cereal Grains: A Mini Review. *Molecules* **2020**, *25*, 927. [CrossRef] [PubMed]
64. Ajila, C.M.; Gassara, F.; Brar, S.K.; Verma, M.; Tyagi, R.D.; Valéro, J.R. Polyphenolic Antioxidant Mobilization in Apple Pomace by Different Methods of Solid-State Fermentation and Evaluation of Its Antioxidant Activity. *Food Bioprocess Technol.* **2012**, *5*, 2697–2707. [CrossRef]
65. Sánchez, C. Lignocellulosic Residues: Biodegradation and Bioconversion by Fungi. *Biotechnol. Adv.* **2009**, *27*, 185–194. [CrossRef]
66. Fan, W.; Huang, X.; Liu, K.; Xu, Y.; Hu, B.; Chi, Z. Nutrition Component Adjustment of Distilled Dried Grain with Solubles via *Aspergillus Niger* and Its Change about Dynamic Physiological Metabolism. *Fermentation* **2022**, *8*, 264. [CrossRef]
67. Omarini, A.B.; Labuckas, D.; Zunino, M.P.; Pizzolitto, R.; Fernández-Lahore, M.; Barrionuevo, D.; Zygadlo, J.A. Upgrading the Nutritional Value of Rice Bran by Solid-State Fermentation with *Pleurotus sapidus*. *Fermentation* **2019**, *5*, 44. [CrossRef]
68. Martins, S.; Mussatto, S.I.; Martínez-Avila, G.; Montañez-Saenz, J.; Aguilar, C.N.; Teixeira, J.A. Bioactive Phenolic Compounds: Production and Extraction by Solid-State Fermentation. A Review. *Biotechnol. Adv.* **2011**, *29*, 365–373. [CrossRef]
69. Chen, G.; Chen, B.; Song, D. Co-Microbiological Regulation of Phenolic Release through Solid-State Fermentation of Corn Kernels (*Zea Mays* L.) to Improve Their Antioxidant Activity. *LWT* **2021**, *142*, 111003. [CrossRef]
70. Fernandes, H.; Martins, N.; Vieira, L.; Salgado, J.M.; Castro, C.; Oliva-Teles, A.; Belo, I.; Peres, H. Pre-Treatment of Ulva Rigida Improves Its Nutritional Value for European Seabass (*Dicentrarchus labrax*) Juveniles. *Algal Res.* **2022**, *66*, 102803. [CrossRef]
71. Buenavista, R.M.E.; Siliveru, K.; Zheng, Y. Utilization of Distiller's Dried Grains with Solubles: A Review. *J. Agric. Food Res.* **2021**, *5*, 100195. [CrossRef]
72. Enes, P.; Panserat, S.; Kaushik, S.; Oliva-Teles, A. Dietary Carbohydrate Utilization by European Sea Bass (*Dicentrarchus labrax* L.) and Gilthead Sea Bream (*Sparus aurata* L.) Juveniles. *Rev. Fish. Sci.* **2011**, *19*, 201–215. [CrossRef]
73. Sinha, A.K.; Kumar, V.; Makkar, H.P.S.; de Boeck, G.; Becker, K. Non-Starch Polysaccharides and Their Role in Fish Nutrition—A Review. *Food Chem.* **2011**, *127*, 1409–1426. [CrossRef]
74. Castro, C.; Peréz-Jiménez, A.; Coutinho, F.; Díaz-Rosales, P.; Alexandra, C.; Serra, R.; Panserat, S.; Corraze, G.; Peres, H.; Oliva-Teles, A. Dietary Carbohydrate and Lipid Sources Affect Differently the Oxidative Status of European Sea Bass (*Dicentrarchus labrax*) Juveniles. *Br. J. Nutr.* **2015**, *114*, 1584–1593. [CrossRef]
75. Castillo, S.; Gatlin, D.M. Dietary Supplementation of Exogenous Carbohydrase Enzymes in Fish Nutrition: A Review. *Aquaculture* **2015**, *435*, 286–292. [CrossRef]
76. Magalhães, R.; Coutinho, F.; Pousão-Ferreira, P.; Aires, T.; Oliva-Teles, A.; Peres, H. Corn Distiller's Dried Grains with Solubles: Apparent Digestibility and Digestive Enzymes Activities in European Seabass (*Dicentrarchus labrax*) and Meagre (*Argyrosomus regius*). *Aquaculture* **2015**, *443*, 90–97. [CrossRef]
77. Azm, F.R.A.; Kong, F.; Tan, Q.; Zhu, Y.; Yu, H.; Yao, J.; Luo, Z. Effects of Replacement of Dietary Rapeseed Meal by Distiller's Dried Grains with Solubles (DDGS) on Growth Performance, Muscle Texture, Health and Expression of Muscle-Related Genes in Grass Carp (*Ctenopharyngodon idellus*). *Aquaculture* **2021**, *533*, 736169.

78. Bedford, M.R.; Cowieson, A.J. Exogenous Enzymes and Their Effects on Intestinal Microbiology. *Anim. Feed Sci. Technol.* **2012**, *173*, 76–85. [CrossRef]
79. Zheng, C.C.; Wu, J.W.; Jin, Z.H.; Ye, Z.F.; Yang, S.; Sun, Y.Q.; Fei, H. Exogenous Enzymes as Functional Additives in Finfish Aquaculture. *Aquac. Nutr.* **2020**, *26*, 213–224. [CrossRef]
80. Davies, S.J.; El-Haroun, E.R.; Hassaan, M.S.; Bowyer, P.H. A Solid-State Fermentation (SSF) Supplement Improved Performance, Digestive Function and Gut Ultrastructure of Rainbow Trout (*Oncorhynchus mykiss*) Fed Plant Protein Diets Containing Yellow Lupin Meal. *Aquaculture* **2021**, *545*, 737177. [CrossRef]
81. Deng, J.; Zhang, X.; Sun, Y.; Mi, H.; Zhang, L. Effects of Different Types of Non-Starch Polysaccharides on Growth, Digestive Enzyme Activity, Intestinal Barrier Function and Antioxidant Activity of Rainbow Trout (*Oncorhynchus mykiss*). *Aquac. Rep.* **2021**, *21*, 100864. [CrossRef]
82. Glencross, B.; Rutherford, N.; Bourne, N. The Influence of Various Starch and Non-Starch Polysaccharides on the Digestibility of Diets Fed to Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture* **2012**, *356*, 141–146. [CrossRef]
83. Magalhães, R.; Díaz-Rosales, P.; Diógenes, A.F.; Enes, P.; Oliva-Teles, A.; Peres, H. Improved Digestibility of Plant Ingredient-Based Diets for European Seabass (*Dicentrarchus Labrax*) with Exogenous Enzyme Supplementation. *Aquac. Nutr.* **2018**, *24*, 1287–1295. [CrossRef]
84. Magalhães, R.; Lopes, T.; Martins, N.; Díaz-Rosales, P.; Couto, A.; Pousão-Ferreira, P.; Oliva-Teles, A.; Peres, H. Carbohydrases Supplementation Increased Nutrient Utilization in White Seabream (*Diplodus sargus*) Juveniles Fed High Soybean Meal Diets. *Aquaculture* **2016**, *463*, 43–50. [CrossRef]
85. Lin, S.; Mai, K.; Tan, B. Effects of Exogenous Enzyme Supplementation in Diets on Growth and Feed Utilization in Tilapia, *Oreochromis niloticus* x *O. Aureus*. *Aquac Res* **2007**, *38*, 1645–1653. [CrossRef]
86. Liang, C.H.; Syu, J.L.; Mau, J.L. Antioxidant Properties of Solid-State Fermented Adlay and Rice by *Phellinus linteus*. *Food Chem.* **2009**, *116*, 841–845. [CrossRef]
87. Jiang, T.; Feng, L.; Liu, Y.; Jiang, W.; Jiang, J.; Li, S.; Tang, L.; Kuang, S.; Zhou, X. Effects of Exogenous Xylanase Supplementation in Plant Protein-enriched Diets on Growth Performance, Intestinal Enzyme Activities and Microflora of Juvenile J Ian Carp (*Cyprinus carpio* Var. J Ian). *Aquac. Nutr.* **2014**, *20*, 632–645. [CrossRef]
88. Xavier, B.; Sahu, N.P.; Pal, A.K.; Jain, K.K.; Misra, S.; Dalvi, R.S.; Baruah, K. Water Soaking and Exogenous Enzyme Treatment of Plant-Based Diets: Effect on Growth Performance, Whole-Body Composition, and Digestive Enzyme Activities of Rohu, *Labeo rohita* (Hamilton), Fingerlings. *Fish. Physiol. Biochem.* **2012**, *38*, 341–353. [CrossRef] [PubMed]
89. Zhou, Y.; Yuan, X.; Liang, X.-F.; Fang, L.; Li, J.; Guo, X.; Bai, X.; He, S. Enhancement of Growth and Intestinal Flora in Grass Carp: The Effect of Exogenous Cellulase. *Aquaculture* **2013**, *416*, 1–7. [CrossRef]
90. Shi, X.; Luo, Z.; Chen, F.; Wei, C.; Wu, K.; Zhu, X.; Liu, X. Effect of Fish Meal Replacement by Chlorella Meal with Dietary Cellulase Addition on Growth Performance, Digestive Enzymatic Activities, Histology and Myogenic Genes' Expression for Crucian Carp *Carassius auratus*. *Aquac. Res.* **2017**, *48*, 3244–3256. [CrossRef]