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Solid-State Hydrolysis (SSH) Improves the Nutritional Value of Plant Ingredients in the Diet of *Mugil cephalus*

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Abstract: The possibility of improving the nutritional quality of plant byproducts (brewers' spent grain and rice bran) through an enzyme treatment was tested in a formulated feed for grey mullet (*Mugil cephalus*). The enzyme treatment was carried out by Solid-State Hydrolysis (SSH) using a commercial preparation including carbohydrases and phytase. A feed prepared without the treatment and a commercial feed for carp were used as controls. In a preliminary short-term trial carried out at laboratory facilities, fish receiving the enzyme-treated feed showed significant improvement in both FCR and SGR when compared to those obtained with the untreated diet, although both experimental diets presented worse values than those obtained with the commercial feed. Different metabolic indicators including higher values of muscle glycogen and plasmatic triglycerides supported the positive effect of the enzyme treatment on the nutritional condition of the fish over those fed on the diet containing non-treated ingredients. Results of growth and feed efficiency that were obtained in a second long-term trial developed for 148 days under real production conditions evidenced the equivalence among the experimental and commercial diets and confirmed that enzyme pretreatment of plant ingredients by SSH may be a useful procedure to improve the nutritive value of high fiber plant byproducts when included in practical diets for this species and others with similar nutritional features.

Keywords: aquaculture feeds; plant byproducts; enzymatic pretreatment

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1. Introduction

Although in the last years important efforts have been carried out to reduce the levels of the conventional marine resources, i.e., fish meal and fish oil, in the diets of cultured fish, the sustainable development of marine aquaculture requires species that can be produced without the need to use high amounts of such ingredients, the availability of which is growing progressively more limited. Mugilidae (mullets) are a group of fish gaining increasing interest for aquaculture due to their rapid growth, resistance to a wide range of environmental conditions, and omnivorous profile. Over the past few years, the culture of these species, particularly of the grey mullet (*Mugil cephalus*), is considered a priority within the current strategies of aquaculture diversification in different parts of the world, with particular interest in some Mediterranean countries [1]. In addition to specific research aimed at completing its reproduction in captivity, the culture of grey mullet requires the development of suitable species-specific diets, the availability of which represents a bottleneck for their production under intensive systems. Recent studies show good results when testing highly nutritive diets based in the use of zooplankton species [2,3], and several others support the possibility of using high amounts of plant byproducts in feeds for these species, even during early stages of their development [4–8]. Nevertheless, in this latter

case the selected products presented a high nutritional quality (high protein, low fibre contents) and hence the potential of using other vegetable ingredients with a more limited nutritional value has not been properly tested.

As indicated previously, due to their low trophic level and opportunistic nature, mullets are ideal candidates to take advantage of feeds including high percentages of alternative products and byproducts, many of which have high interest for local use within the framework of the circular economy. Nevertheless, from a nutritional point of view, such plant byproducts may present important limitations linked to both their amino acid imbalances and reduced digestibility due to the presence of a wide variety of antinutritional compounds including alkaloids, lectins, digestive enzyme inhibitors, indigestible carbohydrates (mainly non-starch polysaccharides, NSPs) and phytate [9]. In this sense, the use of enzyme additives may be a powerful tool to counteract the potential negative effects derived from the presence of some of these compounds, such as phytate and NSP, thus increasing the whole nutritional value of the ingredients. A number of commercially available multienzyme complexes have been developed to improve the use of carbohydrates and phytate present in plant ingredients used in feeds for terrestrial animals. Nevertheless, they have been designed for optimal functioning under the body temperature and pH conditions existing in the digestive systems of pigs and poultry, which are notably different from those present in aquatic species. This may explain the limited effectiveness and somewhat contradictory results obtained when such products are tested in some fish species, such as Japanese sea bass, carp or rainbow trout [10–13]. The efficiency of hydrolysis produced by such enzymes is greatly conditioned by several aspects. As an example, there may be interactions between gastric and intestinal proteases produced by fish and the exogenous enzymes that can negatively affect their potential beneficial effects [14]. Furthermore, the effectiveness of the exogenous enzymes may be greatly reduced by the high temperatures reached during feed preparation or, in the case of being applied post-extrusion via oil top coating or spraying, by the time available for the enzymatic action inside the digestive system of the species, which is closely related to gut transit rates linked to water temperature.

An interesting alternative to overcome the aforementioned limitations is the pre-treatment of plant ingredients with the enzyme compound before the preparation of the feed pellets, using Solid-State Hydrolysis (SSH). SSH operates with a percentage of solid substrate greater than 15%, so there is little or no free water [15]. This process is routinely used to obtain specific products such as glucose or other sugars, or directly to increase the nutritional value of plant ingredients by reducing the content of NSP [16]. By using SSH, hydrolysis can be carried out under optimal conditions for the enzymes, and their activity is not affected either by the high temperatures reached during feed preparation or by the biochemical conditions present in the guts of fish.

Considering all the above, the aim of the present work was to assess whether pre-treatment of plant ingredients containing high proportions of NSP and phytate using a mixture of enzymes applied under an SSH protocol could improve nutritional value when used in the diet of *M. cephalus*. To achieve this objective two different trials were carried out: an initial short-term trial aimed at preliminary evaluation of the growth performance, feed efficiency and energy metabolism of fish fed on such diet, and a second long-term trial focused only on the evaluation of differences in growth and feed efficiency produced by the diet when evaluated under field production conditions.

2. Materials and Methods

2.1. Experiment 1. Short-Term Trial

2.1.1. Ingredients and Experimental Feeds

The experimental (EXP) diet was formulated taking the proximate composition (Table 1) of a commercial diet as a reference (AQUASOJA, SORGAL, Ovar, Portugal). This commercial diet (COM) was routinely used as maintenance feed in the culture of *M. cephalus* by the company providing the fish. It contains several animal ingredients (fishmeal, fish

hydrolysate, feather meal, meat and bone meal, poultry fat) and plant ingredients (soybean, wheat, bean and sunflower meals) to give a total amount of 35 g/100 g crude protein and 9 g/100 g crude fat. The EXP diet was formulated including more than 75% plant ingredients, of which 30% were high-fibre by-products such as rice bran and brewer's spent grain. To prepare the enzyme treated diet (EXP/enz), all plant ingredients were milled to a mesh size of 0.5 mm and mixed with citrate buffer (pH 5.0, 0.1 M) to obtain a moist mass (1:2 w/v), providing the optimal conditions for the action of the multienzyme complex under SSH. The product used was Rovabio®, a mixture of xylanases, glucanases, arabinofuranosidases and phytase produced by Adisseo (Auvergne, France). It was added to the mixture of plant meals by dissolving the dose recommended for terrestrial species by the manufacturer (0.2 mL/kg) in a certain amount of citrate buffer (0.1 M, pH 5.5) that was then carefully sprayed and mixed. The enzymes were allowed to act, keeping the mixture at 45 °C for 6 h with manual stirrming every hour to ensure the homogeneity of the reaction. After this time, the reaction was stopped by placing the mixture in a cold chamber at 4 °C until addition of the rest of the diet ingredients and preparation of feed pellets. The feeds were prepared using an extrusion machine with a mesh size of 2 mm, dried, and stored at 4 °C until use.

Table 1. Ingredients and proximate composition of the experimental feed used in the experiments.

Ingredient (in g/100 g d.w.)	EXP	COM
Fishmeal 67/10	10.00	
Soybean meal 47	18.83	
Defatted rice bran	10.00	
Soybean protein concentrate	8.00	
Corn gluten meal 60	8.00	
Guar meal (Korma)	11.16	
Brewer's spent grain	20.00	
Fish oil	3.25	
Sunflower oil	2.60	
Soy lecithin	0.65	
Vitamin/mineral premix	0.05	
Taurine	0.30	
Yeast	3.00	
Squid hydrolysate	1.50	
Proximate composition (in g/100 g)		
Crude protein	35.60	35.00
Crude fat	9.03	9.00
Digestible carbohydrates (starch + oligosaccharides)	10.56	4.00
NSP	27.73	
Ash	6.21	8.00
Phosphorus	0.85	1.30
Phytate P	0.35	
Gross Energy (MJ kg^{-1})	17.80	17.10

Declared ingredients in the commercial diet: Meat and bone meal, feather meal, fishmeal, fish hydrolysate, wheat meal, horse bean meal, sunflower meal, dehulled soybean meal, rice bran, fish oil, poultry fat, brewers' yeast. EXP: experimental; COM: commercial; NSP: Non-starch polysaccharides; P: phosphorus. Vitamins and mineral premix (IU or mg kg^{-1} diet);DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 16,650 IU; DL-cholecalciferol, 2000 IU; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; L-ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; copper sulphate heptahydrate, 25 mg; ferric sulphate monohydrate,100 mg; potassium iodide, 2 mg; manganese sulphate monohydrate,100 mg; sodium selenite, 0.05 mg; zinc sulphate monohydrate, 200 mg.

The effect of SSH on the feeds was assessed by chemical analysis of some specific compounds of which the relative concentrations were expected to be modified as a result of enzyme treatment: soluble protein, reducing sugars, pentoses, total phosphorus and phytate phosphorus. Soluble protein was analysed by the Bradford method [17] using a SIGMA

Total Protein Kit (TP0100). Reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS) following the method described by Miller [18]. Pentoses were measured by the phloroglucinol method described by Douglas [19]. Total phosphorus was determined by the molybdovanadate method after total digestion of the organic matter with concentrated nitric acid. Phytic acid was determined following the bipyridine method described by Haug and Lantzsch [20]. All the analyses were performed in triplicate on samples from each diet. The rest of compounds (total crude protein and lipids, moisture, ash) were analysed using AOAC protocols [21]. In brief, crude protein (N × 6.25) was evaluated using the Kjeldahl method, lipid content was determined by petroleum ether extraction (40–60 °C) using a Soxhlet System, moisture content was calculated by drying at 105 °C for 24 h, and ash content was determined using a muffle furnace at 550 °C for 5 h. In addition to these chemical analyses, change in the water retention capacity of feed pellets as a result of partial hydrolysis of the carbohydrate fraction was evaluated as described in Heywood et al. [22].

2.1.2. Feeding Trial, Samples Collection and Data Recording

Juvenile grey mullets (*Mugil cephalus*) were provided by PIMSL (Sevilla, Spain), transferred to the experimental facility (CTAQUA, Centro Tecnológico de la Acuicultura de Andalucía, El Puerto de Santa María, Cádiz, Spain) and acclimated to laboratory conditions for two weeks. Then, the fish (12.24 ± 1.05 g body weight) were randomly distributed in triplicate groups in nine 400 L tanks (n = 100 fish per tank, 300 fish per experimental diet) coupled to a recirculation aquaculture system (RAS) and equipped with physical and biological filters and programmable temperature and O_2 suppliers. Water flow of 5–6 L/tank/min ensured a daily renewal of ten times total volume, and was maintained at 20.3 ± 1.0 °C during the experiment. Experimental diets were offered to apparent satiation three times per day, with the orientative daily ration adapted according to weight controls carried out every 14 days. The experiment lasted six weeks. Total feed intake was recorded for each experimental unit to calculate growth performance parameters. At the end of the trial, overnight fasted fish (four fish per tank, twelve per experimental conditions) were randomly sampled and deeply anaesthetized with 2-fenoxiethanol in a lethal dose (1 mL/L SW) to obtain blood and tissue samples. Blood was drawn from caudal vessels with heparinized syringes and centrifuged at 3000× g for 15 min at 4 °C to separate plasma, which was then snap-frozen in liquid nitrogen and stored at −80 °C until used for biochemical analysis. Fish were cervically sectioned in order to obtain biopsies of different tissues; samples of liver were rapidly taken and weighed to calculate the hepatosomatic index (HSI) and, together with samples of white skeletal muscle, were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent biochemical analysis. Maintenance and sampling of the fish was carried out in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

2.1.3. Growth Performance and Biometric Parameters

The following growth parameters were evaluated:

$$\text{specific growth rate (SGR)} = (100 \times (\ln \text{final body weight} - \ln \text{initial body weight})/\text{days} \quad (1)$$

$$\text{weight gain percent (WG)} = (100 \times (\text{body weigh increase})/\text{initial body weight} \quad (2)$$

$$\text{feed conversion ratio (FCR)} = \text{total feed intake}/\text{weight gain} \quad (3)$$

$$\text{condition factor} = (100 \times \text{body weight})/\text{fork length}^3 \quad (4)$$

$$\text{hepatosomatic index (HSI)} = (100 \times \text{liver weight})/\text{fish weight} \quad (5)$$

2.1.4. Biochemical Parameters

Glucose (Ref. 1001200), lactate (Ref. 1001330) and triglycerides (Ref. 1001311) in plasma and tissues were measured using commercial kits (Spinreact, St. Esteve d'en Bas, Girona, Spain) adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA Protein Assay Kit (Ref. 23225, Thermo Fisher Scientific Pierce, Waltham, MA, USA,) using BSA as the standard. Glycogen concentration was quantified using the method described by Decker and Keppler [23], while glucose obtained after glycogen breakdown with amyloglucosidase (Ref. A7420; Sigma-Aldrich, St. Louis, MO, USA) was determined using the same commercial kit described above. To analyse biochemical parameters in liver and muscle, frozen tissues were homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralized using 1 M KCO_3, and centrifuged (30 min, 3220× *g* and 4 °C); the supernatants were then isolated to determine tissue metabolites. All assays were performed using a PowerWaveTM 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using the KCjunior™ data analysis software (Bio-Tek Instruments, Winooski, VT, USA) for Microsoft®

2.2. Experiment 2. Field Trial

Experimental Feeds, Feeding Trial, Samples Collection and Data Recording

The same three experimental feeds described and evaluated in Experiment 1 were used in this trial. In this case, the experiment was carried out in the facilities of PIMSL (Sevilla, Spain); 1200 juvenile fish with a 39.63 ± 1.14 g initial mean body weight were randomly distributed in six 8 m^3 concrete outdoor tanks in duplicate groups (n = 200 fish per tank) coupled to a recirculation aquaculture system (RAS) equipped with physical and biological filters. Water temperature varied within a wide range during the 148 days of the experimental period according to season, from June to December 2020 (19.5 ± 3.14 °C). Fish were fed twice a day at an initial ration of 2.0%bw that was adjusted after one weight control carried out at an intermediate point during the experiment. Total feed intake and weight increase were recorded for each experimental group in order to calculate FCR and SGR at the end of the experiment, as indicated in Section 2.1.4.

2.3. Statistical Analysis

After a preliminary evaluation to determine the normality of the data using the Shapiro–Wilk test, homoscedasticity analysis was conducted using the Brown–Forsythe test. Due to the different composition of the two diets used as a reference (commercial and experimental without enzyme), separate comparisons were carried out among the diets, on one hand comparing the commercial diet with each of the two experimental diets, and on the other comparing only these latter between themselves. The objective was to assess whether any of the two experimental feeds were equivalent to the commercial diet, while in the second case the objective was to check whether the enzyme treatment could improve on the performance obtained with the experimental diet. The first was carried out by one-way ANOVA followed by the Bonferroni test, and the second was performed using Student *T*-test. The significance level was established at $p < 0.05$. Data expressed in percentage were previously arc-sin transformed. All analyses were carried out using Statgraphics Centurion software (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Experiment 1

The analysis of the diets showed significantly higher values of some compounds (soluble protein, reducing sugars and phytate) and lower values of total phosphorus in EXP feed when compared to COM feed, reflecting differences in the ingredients used in their elaboration. The Solid-State enzymatic Hydrolysis significantly increased the amount of potentially available reducing sugars and pentoses, and reduced the amount of phytate (Table 2). Furthermore, physical transformation resulting from the enzyme

treatment determined a significant reduction in water retention capacity in the EXP/enz diet (Table 2).

Table 2. Differences in the nutrient content and water retention of experimental feeds (g/100 d.m.).

	Soluble Protein	Reducing Sugars	Pentoses	Phosphorus	Phytate	Water Retention
COM	3.13 ± 0.05^{A}	0.45 ± 0.00^{A}	0.29 ± 0.01^{A}	1.31 ± 0.02^{A}	0.22 ± 0.00^{A}	305.77 ± 6.89^{A}
EXP	6.83 ± 1.13^{Ba}	2.08 ± 0.02^{Ba}	0.28 ± 0.00^{Aa}	0.83 ± 0.10^{Ba}	0.34 ± 0.01^{Ba}	305.45 ± 3.87^{Aa}
EXP/enz	5.58 ± 0.24^{Ba}	2.83 ± 0.03^{Bb}	0.36 ± 0.05^{Bb}	0.76 ± 0.03^{Ba}	0.21 ± 0.02^{Bb}	280.94 ± 9.29^{Bb}

Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase. Each assay was performed in triplicate. Values not sharing the same letter differ significantly with $p < 0.05$. COM: commercial; EXP: experimental; EXP/enz: experimental enzyme treated.

3.1.1. Growth Performance

Results of growth performance and feed utilisation are presented in Table 3. No mortality occurred during the experiment, and all groups presented an increase in body mass, accounting for 11% to 33% of their initial mean body weight. Fish presented a normal condition factor for the species (K = 1.07–1.12). The results presented in Table 4 show that the experimental diet, irrespective of being enzymatically treated or not, clearly offered worse results in growth and feed efficiency than the control diet. Nevertheless, it was also clear that the Solid-State enzymatic Hydrolysis significantly improved the same parameters when comparing fish fed on EXP/enz to those obtained with EXP.

Table 3. Growth and feed efficiency measured in fish fed on the experimental diets.

Parameter	COM	EXP	EXP/enz
Initial body mass (g/fish)	12.02 ± 0.33^{A}	11.89 ± 0.55^{Aa}	12.38 ± 0.16^{Aa}
Final body mass (g/fish)	16.60 ± 0.60^{A}	13.21 ± 0.49^{Ba}	15.36 ± 0.04^{Ab}
Feed consumption (g/fish)	10.02 ± 0.17^{A}	8.65 ± 0.45^{Ba}	9.23 ± 0.17^{Ab}
FCR (g feed/g fish)	2.19 ± 0.11^{A}	5.89 ± 0.60^{Ba}	3.11 ± 0.19^{Bb}
SGR (%/day)	0.75 ± 0.02^{A}	0.25 ± 0.05^{Ba}	0.50 ± 0.03^{Bb}
HIS (%)	1.27 ± 0.07^{A}	0.93 ± 0.18^{Ba}	0.83 ± 0.20^{Bb}
Condition factor (K)	1.11 ± 0.12^{A}	1.07 ± 0.12^{Ba}	1.07 ± 0.17^{Ba}

Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase. COM: commercial; EXP: experimental; EXP/enz: experimental enzyme treated; FCR: feed conversion ratio; SGR: specific growth rate; HIS: hepatosomatic index.

Table 4. Metabolites measured in plasma and tissues of fish fed on the different experimental diets.

Parameter	COM	EXP	EXP/enz
In plasma (mg/dL)			
Glucose	80.54 ± 26.21^{A}	92.12 ± 29.61^{Aa}	76.76 ± 14.81^{Aa}
Lactate	67.28 ± 32.89^{A}	56.71 ± 11.07^{Ba}	47.13 ± 22.73^{Bb}
Protein	32.60 ± 5.46^{A}	34.62 ± 10.08^{Aa}	38.23 ± 4.35^{Aa}
TAG	38.09 ± 7.60^{A}	34.13 ± 4.76^{Aa}	42.53 ± 6.48^{Bb}
In liver (mg/g *w*/*w*)			
Glucose	1.41 ± 0.48^{A}	1.37 ± 0.56^{Aa}	1.34 ± 0.29^{Aa}
Glycogen	7.26 ± 1.26^{A}	2.61 ± 1.52^{Ba}	3.31 ± 1.32^{Ba}
In muscle (mg/g *w*/*w*)			
Glucose	0.77 ± 0.30^{A}	0.84 ± 0.28^{Aa}	0.72 ± 0.30^{Aa}
Glycogen	0.78 ± 0.45^{A}	0.38 ± 0.14^{Ba}	0.79 ± 0.31^{Ab}
Lactate	24.57 ± 6.63^{A}	26.35 ± 7.62^{Ba}	24.96 ± 3.90^{Aa}
TAG	10.48 ± 5.14^{A}	10.79 ± 3.83^{Aa}	11.17 ± 7.15^{Aa}

Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase.

3.1.2. Biochemical Parameters

Data on blood and tissue biochemistry are detailed in Table 4. The parameters measured in plasma show significantly lower values of lactate in fish fed any of the experimental diets when compared to those obtained in fish fed the COM diet. Additionally, the amount of TAG measured in fish fed the EXP/enz diet was significantly higher than in fish fed the EXP diet. Liver glycogen measured in fish fed any of the experimental diets was also significantly lower than in fish fed the COM diet. Indeed, muscle glycogen measured in fish fed on the EXP diet was significantly reduced compared to the other two groups.

3.2. Experiment 2. Field Trial

The results on growth performance and feed efficiency in the field trial are presented in Table 5. Average fish growth during the whole experimental period ranged from 0.32 to 0.43%, although it was low or even absent during a great part of the period due to low winter temperatures. Nevertheless, all of the experimental groups doubled their initial weight by the end of the experiment. Moreover, all groups presented reasonably good FCR values, ranging from 2.33 to 2.67, and in contrast to those obtained in experiment 1, the values of FCR were significantly enhanced when fish were fed on the EXP/enz diet.

Table 5. Growth and feed efficiency in fish fed on the experimental diets in the field trial.

Parameter	COM	EXP	EXP/enz
Initial body mass (g/fish)	40.13 ± 0.18^{A}	38.25 ± 0.35^{Ba}	40.50 ± 0.71^{Ab}
Final body mass (g/fish)	88.05 ± 11.53^{A}	94.65 ± 13.08^{Aa}	104.70 ± 13.86^{Aa}
Feed consumption (g/fish)	18.58 ± 0.96^{A}	17.83 ± 1.68^{Aa}	17.76 ± 0.01^{Aa}
FCR (g feed/g fish)	2.65 ± 0.12^{A}	2.67 ± 0.29^{Aa}	2.33 ± 0.58^{Bb}
SGR (%/day)	0.32 ± 0.08^{A}	0.38 ± 0.09^{Aa}	0.43 ± 0.09^{Aa}

Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase.

4. Discussion

The "extreme" experimental feed (EXP) used in the present work was designed to assess the limits of including a high amount of plant ingredients of limited nutritional value in practical diets for *M. cephalus*, as well as the potential benefits derived from the enzymatic treatment of such plant ingredients. For this reason, it included a low proportion of fishmeal (10%), a high amount of fibrous byproducts containing significant levels of phytate and NSP (10% rice bran, 20% brewer's spent grain) and no supplementation with lysine or methionine. Although experiment 1, developed with small juveniles, was not maintained for enough time to allow the fish to at least double their weight, it offered preliminary insight on the potential performance of the diets and two clear results: on the one hand, it demonstrated that the EXP diet did not fulfill the nutritional requirements of the species at this early stage of development; on the other, it showed that enzyme pretreatment of the plant ingredients had a significant positive effect on the nutritional value of such a diet.

Related to the first point, despite the values of FCR and SGR obtained with diets EXP being not directly comparable to those obtained with diet COM (as both types of diets were differently formulated and contained quite different ingredients in terms of acceptance, digestibility and nutritional value), it was clear that the use of the EXP diet impaired the growth of the fish. Similarly, El-Gendy et al. [24] reported a clear reduction in FCR and SGR when feeding juveniles of *M. cephalus* with diets prepared with increasing amounts of plant ingredients (from 20 to 100%), in that case including a significant proportion of cereal bran. Certainly, a different selection of plant ingredients with a higher nutritional value should have produced much better results; in this way, Gisbert et al. [8] reported good results in terms of SGR, digestive physiology and fish condition when feeding small (0.2 g) juveniles of *M. cephalus* on feeds including a blend of corn and wheat gluten and soy protein concentrate, supplemented with crystalline L-lysine and DL-methionine. Nevertheless,

as mentioned previously, the objective of the present study was to assess to what extent feeds for *M. cephalus* could include high amounts of plant byproducts and no specific supplementation to minimize the final cost of the diet without compromising growth.

Regarding the second point, it is clear that Solid-State enzymatic Hydrolysis of the EXP diet with a commercial mixture of xylanases, β-glucanases and pectinases resulted in a significant improvement in both FCR and SGR compared to the values obtained with the untreated diet, suggesting a clear positive effect of the enzyme complex. Similar positive effects have been reported in terrestrial species such as pigs [25] and poultry [26], being associated to a great extent with the modification of the structure of carbohydrates present in plant ingredients, which increases the bioaccessibility of nutrients to the action of digestive enzymes. In the present study, such modification was indirectly evidenced by the significant reduction in the water retention capacity, as well as by the significant increase in bioavailable monosaccharides and the reduction in phytate measured in the EXP/enz feed (Table 2). The improvement observed in the FCR and SGR over those obtained in fish fed the EXP diet was higher than 50%, suggesting that partial hydrolysis of the antinutritive factors (NSP and phytate) exerted a positive impact on the nutritional value of the feed, and hence on the performance indicators.

In the present study, the measurement of different metabolites was intended to evaluate the potential impact of the EXP diet on fish energy orchestration, as well as whether enzyme treatment yielded significant effects on metabolic homeostasis. As presented herein, no significant differences were observed in glucose levels measured in plasma, muscle or liver irrespective of the diet or enzyme treatment, even when this latter treatment significantly increased the amount of available dietary sugars measured in the fish receiving the EXP/enz diet. In contrast to what has been described for carnivorous fish [27], this suggests a good ability of the grey mullet to use carbohydrates as a source of energy, demonstrating a homeostatic load of this metabolite that might be considered sufficient for the potential growth of this species. The unfavourable nutritional status of fish fed on the EXP diet was reflected by several indicators. First, a significantly lower accumulation of liver glycogen was correlated with the lower weight of this organ, which determined decreased values of HSI as well as lower plasma levels of lactate and could reflect an impairment between the total energy incorporated through the feed and the demand for physiological processes such as growth. Furthermore, a significantly lower concentration of muscle glycogen was measured in fish receiving the EXP diet. Under normal conditions, if the feed is able to cover nutritional needs excess glucose may be stored as glycogen (glycogenesis) or converted into lipids (lipogenesis) instead of being oxidized for energy; however, under conditions of food deprivation or nutritionally unbalanced feeding, glucose requirements are satisfied either by glycogen depletion to glucose (glycogenolysis) or by de novo glucose synthesis through gluconeogenesis from lactate, glycerol or certain amino acids [28]. In contrast, the comparatively improved nutritional status associated with consumption of the EXP/enz feed was supported by significantly higher values of muscle glycogen and plasma TAG. Moreover, the results also suggest that de novo gluconeogenesis in the muscle may contribute to higher glycogen content, although to elucidate whether this fact is a cause or a consequence of better feed utilisation would require further investigation related to the key role of several metabolic enzymes in this and other tissues.

The results obtained in experiment 2 were noticeably different, for two main reasons: the initial size of the juvenile fish was much higher (nearly 40 g average weight) and the experimental period was long enough that the fish were able to double their initial weight (Table 5). In addition, the environmental conditions were different, as the experiment was carried on outdoors in standard facilities used for rearing the intermediate stages of growing fish. Under such conditions, both the EXP and EXP/enz diets appeared to be equivalent to the COM diet, and Solid-State enzymatic Hydrolysis confirmed its positive effect on the nutritional value of the ingredients. Although the growth rates of *M. cephalus* were relatively low at the end of the 28-week feeding period, they were comparable to those reported in previous studies carried out with this and other similar species that evidence

the slow growth rate of mugilids [29–31]. Legarda et al. [32] reported an SGR of 0.56%/day for *Mugil cephalus* juveniles fed 1%bw daily in a biofloc system maintained at 28 ± 1 °C. Nengas et al. [33] also recorded low SGR values (around 0.45%/day) in juveniles of *Liza aurata* fed once per day at 2% of their biomass at temperatures ranging from 12 to 26 °C.

Values of FCR obtained with any of the EXP diets were clearly improved in relation to those obtained in experiment 1, being in this case equivalent or even better than obtained with the COM diet. This could be thanks to for two main reasons: on the one hand, as indicated previously, the experiment was carried out using older fish, and presumably their ability to digest fibrous materials improved greatly with age, this being associated with the maturation and development of the digestive function. These changes in digestive capability with development have been previously reported in other mullet species including the thick-lipped grey mullet, *Chelon labrosus* [34,35]. This suggests that the possibility of using a high amount of fibrous plant byproducts in diets for this species is strongly conditioned by the age of the fish, and probably linked to the ability, acquired with development, to properly digest such ingredients. In addition, it must be considered that the conditions in which the fish were maintained in this latter experiment, mainly a lower stocking density, could result in lower stress and a positive impact on feeding behaviour, resulting in better food utilization. Although there are few studies on the nutrition of mullets under field conditions, the study carried out by [33] on *Liza aurata* showed that growth and feed utilization of the fish was not significantly affected by variations in the dietary protein level. Such absence of a clear effect could be due to the possible complementary effect of natural food present in the water mass. In the present study, the only source of nutrients was the artificial feeds, and no other food source was available; hence, the observed response was representative of the nutritional value of the feeds.

The results further confirmed the positive effect of the enzyme treatment already pointed out in experiment 1, which is in line with other previous studies. A 10% improvement in FCR was reported by Maas et al. [36] when including enzymes in diets for tilapia formulated solely using vegetable ingredients, and a 14% improvement in FCR was obtained after addition of multi-enzyme complexes (Natuzyme® or Hemicell®) in diets for Caspian salmon [37]. As previously indicated, a number of beneficial effects have been reported resulting from the hydrolysis of NSP [38,39]. In addition, the significant reduction in phytate derived from Solid-State enzymatic Hydrolysis (Table 2) could result in not only an increased availability of phosphorus, but also in a decrease in some other negative effects associated with the presence of phytate on the digestive bioavailability of proteins and some minerals, as described by several authors [40,41]. Furthermore, the citrate buffer used to develop the process of SSH could enhance the solubilisation of certain minerals, such as Fe or Mn [42].

The results support the suitability of SSH as a method for proper application of exogenous enzymes, as the hydrolysis is performed under optimal conditions and the enzyme activity is not affected by either the high temperatures reached during feed preparation or by the biochemical conditions present in the gut of the fish. This overcomes most of the physiological and technical limitations described when enzymes are included in the feed. To date, only one study, carried out by Denstadli et al. [43] on trout feeds, has tested this way of performing enzyme treatment. In that study, the feeds included 450 g/kg feed of plant ingredients and an estimated amount of NSPs accounted for 80 g/kg feed, values much lower than those used in the present study where the feed contained nearly 700 g plant ingredients per kg of feed and the estimated amount of NSP exceeded 200 g/kg. While in that work the enzyme treatment determined a 10–13% reduction of NSP content when using soybean meal as the main ingredient and of 4–6% when using rapeseed meal, in the present study the hydrolysis of NSP was not measured directly; however, the observed changes in the bioavailability of pentoses and reducing sugars suggested a modification of the nutritional value of the feed that impacted the results obtained on growth and nutritive utilization. From a practical point of view, performing such treatment by SSH allows adaptation of the more suitable operative conditions (dose, reaction time, etc.) to the

specific features of different plant ingredients. In addition, because the enzyme mixture is used prior to pelleting, inactivation due to thermal processing should eliminate any further undesired effects. Although the potential application of such procedures requires further research, it offers interesting possibilities for a wider utilization of different inexpensive byproducts in feeds for herbivorous/omnivorous fish species such as *M. cephalus*, which has highly positive features from an environmental perspective.

5. Conclusions

Considering the aforementioned, the present study strongly suggests that enzyme pretreatment of highly fibrous plant ingredients by SSH using a commercial mixture of different carbohydrases and phytase may be a useful procedure to improve the nutritive value of high fiber plant byproducts for inclusion in practical diets for *M. cephalus* and other fish with similar nutritional features.

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