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Evaluation of Enzyme Additives on the Nutritional Use of Feeds with a High Content of Plant Ingredients for *Mugil cephalus*

Francisca P. Martínez [1,*], Laura Bermúdez [2], María J. Aznar [1] and Francisco J. Moyano [1]

[1] Department of Biology and Geology, University of Almeria, 04120 Almeria, Spain; maria_jesus_08@hotmail.com (M.J.A.); fjmoyano@ual.es (F.J.M.)

[2] CTAQUA, Commercial Dock S/N, El Puerto de Santa Maria, 11500 Cadiz, Spain; info@ctaqua.es

* Correspondence: fma996@inlumine.ual.es

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Abstract: The Mugilidae are a group of fish with a great interest for aquaculture due to their omnivorous profile, rapid growth, and resistance to environmental variations. The selection of feed ingredients for these species is currently focused on an extensive use of plant by-products, with this being limited by their content in anti-nutritive factors (mainly phytate and non-starch polysaccharides; NSPs). Nevertheless, specific enzymes can be used to counteract some of those negative effects. In the present study, the effect of pretreating two high-plant feeds with a mixture of enzymes (glucanases + phytase) on the digestive use of protein and phosphorus by juvenile mullets (*Mugil cephalus*) was assessed using both in vitro and in vivo assays. The enzymatic treatment significantly modified the potential bioavailability of some nutrients, such as a reduction of sugars, pentoses, and phytic phosphorus. Also, it increased the digestibility of protein in one of the feeds but reduced that of phosphorus in both of them. The potential usefulness of enzyme treatment and the information provided by the two types of assays are discussed.

Keywords: aquaculture; plant ingredients; enzymes

1. Introduction

Increasing concerns about the environmental and economic sustainability of aquaculture indicate that its future development cannot rely on intensive production systems of carnivorous species, due to the high impact linked to the need of using large quantities of fishmeal and oils in their feeds [1]. Therefore, multiple instances promote the development of semi-intensive and integrated aquaculture systems based on omnivorous or herbivorous species, as well as an extended use of plant ingredients and by-products [2]. This orientation is increasing in Asia and South America, but to date, it is not so extensive in Europe, where fish aquaculture is still primarily focused on the intensive production of carnivorous species with a high market value. Nevertheless, the need for diversification in Mediterranean marine aquaculture has promoted an increased interest in developing alternative models of aquaculture (aquaponics, recirculation systems, multitrophic aquaculture, etc.) as well as the cultivation of some omnivorous species. The common feature of these systems is the use of species placed at low trophic levels showing less demanding nutritional requirements, in terms of total amount or quality of feed ingredients, and whose feeding can be carried out largely using low-cost ingredients.

Within this context, it is worth noting the high potential presented by the species of the family Mugilidae (mullets), a group of fish living in temperate and subtropical coastal waters in both hemispheres. Mullets, which include species like *Mugil cephalus*, *Chelon labrosus*, *Liza aurata*, *Liza saliens*, and *Liza ramada*, present a great adaptability to different culture conditions, with their potential

for aquaculture being determined by their omnivorous profile, rapid growth, and resistance to environmental variations [3]. The culture of Mugilidae, particularly of the grey mullet (*Mugil cephalus*), is considered a priority within current strategies of European aquaculture. As an example, the EU Program DIVERSIFY considers the establishment of a basis for reproduction in captivity and the development of low-cost feeds adapted to its nutritional requirements as a main objective for this species. In contrast to the great amount of information available on the natural feeding habits of mullets, data on their nutritional requirements and use of artificial feeds are scarce [4]. This is due to the fact that the culture of mullets takes place under extensive or semi-intensive systems, implying that a great part of the food is naturally produced, and only supplemented with low-quality feeds [5,6]. However, the renovated interest to develop the culture of this species requires the development of suitable and more species-specific formulations. In this sense, the possibility of using high amounts of plant ingredients, even in feeds used during early stages of development, has been well demonstrated [5,7–10].

For many years, studies aimed at evaluating the incorporation of plant ingredients into feeds for aquaculture species has been one of the most active and productive research lines. Different reviews have identified the potentials and limitations of using these ingredients [2,11]. One is the deficiencies in essential amino acids in plant proteins, which has to be compensated for to obtain a protein profile for the feed that is suitable for the requirements of the species. Another key aspect is the presence of a wide variety of antinutritional factors in plant ingredients, such as alkaloids, protease inhibitors, saponins, lectins, as well as non-starch polysaccharides (NSPs) and phytate [12]. NSPs is the fraction of carbohydrates present in plant ingredients commonly named "undigestible fiber". It is formed by cellulose (glucose insoluble polymer) and a group of other complex sugar heteropolymers, such as mannose, xylose, arabinose, etc., which present important differences in their solubility, water retention capacity, and interaction with other feed ingredients or the intestinal microbiota. Significant effects on intestinal transit, nutrient absorption, or microbial diversity can be observed depending on the amount and type of NSP present in a plant ingredient [13]. On the other hand, phytate is an organic acid present in plant sources, particularly in some seeds and also in the fiber fraction. Phytate phosphorus is not bioavailable to monogastrics because they lack phytase, the enzyme able to hydrolyze such a compound; hence, it passes through the gastrointestinal tract and is finally eliminated in the feces. In addition, phytic acid combines with several nutritionally important minerals, such as calcium, magnesium, iron, and zinc, that become insoluble and are not absorbed in the intestine. Also, it is well known that phytate inhibits proteolytic enzymes [14,15].

The use of enzyme additives may be a powerful tool to counteract the potential negative effects derived from the presence of phytate and NSP, and thus increase the nutritional value of plant ingredients. Since, in many cases, NSPs form a matrix that hinders the access of digestive enzymes to the protein and starch present in cereal and leguminous seeds, the use of glucanases capable of totally or partially hydrolyzing them has shown positive effects on the nutritive use of feeds, including those feedstuffs [16]. In a similar way, the use of phytase has shown positive effects on different fish species in terms of their ability to improve the whole nutritional use of different ingredients and to reduce phosphorus discharge into the environment [17–19].

To date, all studies aimed at testing the potential effect of enzyme supplementation on fish feeds have been based on mixing with the rest of the ingredients, with their potential activity in the fish gut being affected by the processing conditions, mostly the high temperatures reached during pelleting. This could explain why in several cases, the results obtained were non-significant [20]. A possible alternative could be the external addition of the enzyme in the oil used to cover the pellets after extrusion; this has offered interesting results in the case of phytase added to trout feeds [21]. Another method, on which there are not published references, is the pre-treatment of the plant ingredients with specific enzymatic additives under controlled conditions prior to the preparation of the feed pellets. This solid-state hydrolysis (SSH) operates with a percentage of solid substrate greater than 15%, so little or no free water is present [22]. The procedure is routinely used in different

industrial processes aimed at obtaining specific products, such as glucose or other sugars, or directly to increase the nutritional value of plant ingredients for human or terrestrial animal consumption by reducing the content of NSP [23].

On the other hand, the preliminary selection of the suitability of a given ingredient and/or enzyme treatment for a given species can be performed using in vitro assays simulating the digestion process of such species. These assays, extensively used in the evaluation of the nutritional quality of foods and feeds for humans and terrestrial animals and more recently adapted to aquatic animals, may help to predict differences in the potential bioavailability of main nutrients (protein, carbohydrates, and fats) as well some minerals [24].

Considering the above-mentioned factors, the main objective of the present work was to evaluate the effect of an SSH enzymatic treatment of plant ingredients used in feeds for grey mullet on the potential bioavailability of nutrients. Changes in the bioavailability of sugars, amino acids, and phosphorus were evaluated using both in vitro and in vivo digestibility assays.

2. Results

The effect of the enzymatic treatment on the potential availability of nutrients of the experimental feeds (composition detailed in Table 1) is shown in Table 2. The results show that enzyme pretreatment significantly increased the contents of the available reducing sugars and pentoses while it reduced that of phytate. The release of nutrients due to the action of the digestive enzymes of mullet under conditions simulating the digestion of the species is summarized in Table 3. A significantly higher amount of amino acids was released from feed 1 than 2. On the other hand, the enzymatic treatment determined a significant increase in the release of amino acids and pentoses from both feeds while, in contrast, the release of total P remained unaffected. The values of the apparent digestibility coefficients for protein, total phosphorus, and phytic phosphorus are detailed in Table 4. The digestibility values for both protein and P were within the normal ranges determined for these nutrients in other species; in fact, the values were high, considering the high content of vegetable ingredients used in the feeds. There were no significant differences in the digestive utilization of protein between both untreated feeds, but the enzyme addition resulted in a significant improvement in the protein digestibility of feed 1. In the absence of enzymatic treatment, the digestibility of total P was significantly higher for feed 2. Enzymatic treatment reduced the digestibility values in both feeds, with this reduction being significant in the case of feed 2. A similar result was obtained for phytate.

Table 1. Ingredients and proximate composition of the diets used in the experiment.

Ingredient (in g/100 g d.w.)	FEED 1	FEED 2
Fishmeal 67/10	15.0	10.0
Soybean meal 47	15.0	21.8
Rapeseed meal	15.0	-
Defatted rice bran	-	12.0
Soybean protein concentrate	8.0	10.0
Corn gluten meal 60	16.0	15.0
Guar meal (Korma)	15.0	20.0
Fish oil	4.9	4.9
Sunflower oil	3.9	3.9
Soy lecithin	1.0	1.0
Vitamin/mineral premix	0.1	0.1
Taurin	0.5	0.5
Yeast	0.7	0.8
Cr_2O_3	1.0	1.0
Starch	4.4	-

Table 1. *Cont.*

Proximate Composition (in g/100 g)		
Crude protein	45.00	45.01
Crude fat	13.00	13.00
Digestible carbohydrates (starch + oligosaccharides)	5.85	9.00
NSP	23.90	25.17
Ash	5.95	6.42
Phosphorus	0.89	0.87
Phytate P	0.28	0.33

Table 2. Differences in the nutrient content of the experimental feeds (g/100 d.m). Statistical comparisons between feeds (three samples per feed) prior to the enzyme treatment are detailed in capital letters while those made within each feed, with or without enzymatic treatment, are detailed in lowercase. Values not sharing the same letter differ significantly at $p < 0.05$.

Experimental Feed	Total Protein	Soluble Protein	Reducing Sugars	Pentoses	Phosphorus	Phytate
FEED 1	46.85 ± 1.18	6.19 ± 0.66 ^{Aa}	0.67 ± 0.07 ^{Aa}	0.24 ± 0.02 ^{Aa}	1.33 ± 0.03	0.44 ± 0.04 ^{Aa}
FEED 1 + enz	46.85 ± 1.18	4.12 ± 0.24 ^{b}	5.94 ± 0.34 ^{b}	0.83 ± 0.04 ^{b}	1.30 ± 0.01	0.21 ± 0.00 ^{b}
FEED 2	46.96 ± 0.84	2.94 ± 0.44 ^{Ba}	1.12 ± 0.04 ^{Ba}	0.28 ± 0.01 ^{Aa}	1.55 ± 0.20	0.96 ± 0.02 ^{Ba}
FEED 2 + enz	46.96 ± 0.84	4.82 ± 0.37 ^{b}	5.61 ± 0.29 ^{b}	0.82 ± 0.04 ^{b}	1.41 ± 0.10	0.56 ± 0.02 ^{b}

Table 3. Nutrients released after in vitro hydrolysis of the experimental diets. Data are expressed as a total amount or as a percentage of the nutrient initially present in the sample (protein, NSP (non-starch polysaccharide), or total P). Statistical comparisons between feeds (three samples per feed) prior to the enzyme treatment are detailed in capital letters while those made within each feed, with or without enzymatic treatment, are detailed in lowercase. Values not sharing the same letter differ significantly at $p < 0.05$.

Experimental Feed	Amino Acids (mg)	Amino Acids (%)	Pentoses (mg)	Pentoses (%)	P (mg)	P (%)
FEED 1	110.65 ± 0.87 ^{Aa}	39.36 ± 0.31 ^{Aa}	1.32 ± 0.13 ^{Aa}	0.99 ± 0.09 ^{Aa}	4.63 ± 0.50 ^{A}	58.0 ± 6.21 ^{A}
FEED 1 + enz	115.76 ± 1.02 ^{b}	41.18 ± 0.36 ^{b}	3.32 ± 0.34 ^{b}	2.31 ± 0.27 ^{b}	4.62 ± 0.07	59.2 ± 0.85
FEED 2	72.73 ± 0.19 ^{Ba}	25.81 ± 0.07 ^{Ba}	1.14± 0.11 ^{Aa}	0.76 ± 0.08 ^{Aa}	3.07 ± 0.02 ^{B}	33.0 ± 0.26 ^{B}
FEED 2 + enz	85.91 ± 13.07 ^{b}	30.49 ± 4.63 ^{b}	3.48± 0.08 ^{b}	2.31 ± 0.05 ^{b}	2.51 ± 0.27	39.7 ± 3.15

Table 4. Apparent digestibility coefficients (ADCs) in g/100 g of protein total P and phytate for the experimental diets. Statistical comparisons between feeds (nine samples per feed) prior to the enzyme treatment are detailed in capital letters while those made within each feed, with or without enzymatic treatment, are detailed in lowercase. Values not sharing the same letter differ significantly at $p < 0.05$.

Experimental Feed	Protein	Phosphorus	Phytate
FEED 1	89.6 ± 1.0 ^{Aa}	47.9 ± 7.1 ^{Aa}	55.2 ± 2.5 ^{Aa}
FEED 1+ enz	92.3 ± 0.4 ^{b}	38.5 ± 3.9 ^{a}	54.6 ± 5.0 ^{a}
FEED 2	91.9 ± 0.1 ^{Aa}	70.1 ± 3.0 ^{Ba}	62.4 ± 1.5 ^{Ba}
FEED 2 + enz	91.9 ± 0.5 ^{a}	61.0 ± 6.0 ^{b}	52.9 ± 5.7 ^{b}

3. Discussion

Most studies testing the effect of enzyme addition to improve the nutritional use of plant ingredients have been carried out in freshwater fish species (tilapia, carp, catfish, sturgeon, rainbow trout) but few have investigated marine fish like the Japanese sea bass or mullet [16]. This could be explained considering the greater presence of herbivorous species in fresh water, which implies a preferential use of low-value plant ingredients in their feeds that would justify the use of enzymes. In contrast,

most marine species are carnivorous; although plant ingredients are also routinely used in their feeds, they are mostly high-quality protein concentrates with a reduced content of NSP. The present study was developed using grey mullet, an omnivorous marine fish, with the feeds designed to include a significant amount of plant ingredients (around 70 g/100 g diet) with a presumed high content in several antinutritional factors like NSP and phytic acid. This is justified considering that the profile of ingredients to be used in feeds for mullets should mostly be based on the use of low-cost meals (guar, rapeseed, etc.) and by-products (cereal brans, distiller's dried grains with soluble, etc.).

It is noteworthy that most studies aimed at evaluating enzymes as additives do not reproduce the real conditions used during manufacturing, since experimental feeds are usually prepared by cold granulation [25–27]. Nevertheless, most feeds currently used in aquaculture are extruded at high temperatures, so the enzymes to be used should be highly resistant to this process. As previously indicated, one possible alternative is to add the enzymatic mixtures in the final coating of the grain [21]. Another one, used in the present work, is to carry out enzymatic pretreatment of the ingredients before preparation of the pellets. As shown in Table 2, the enzyme pretreatment demonstrated a clear modification of the nutritional profile of the diets, which showed an increased amount of reducing sugars and pentoses, as well as a decrease in the amount of phytate phosphorus. To date, only one published study developed a similar approach [28] and presents results on the biochemical transformation of the ingredients used in trout feeds after enzymatic action. In that study, plant ingredients made up 45% of the feed (34% crude protein); this resulted in NSPs accounting for 8% of the proximate composition. These figures are considerably different to those of the feeds used in the present study, where the content of plant ingredients was much higher (60%–70%) and the estimated NSP contents (including cellulose) exceeded 20%. The enzymatic action in the work of Denstadli et al. (2011) [28] determined a reduction in the NSP content between 10% and 13% when using soy flour as the main ingredient and only 4% to 6% when using rapeseed flour. Additionally, the authors did not obtain significant changes in the contents of pentoses and reducing sugars. In the present study, changes in the amount of NSP were not measured directly, but, as previously indicated, the products of hydrolysis multiplied their concentrations by three to four times, suggesting that the enzymatic hydrolysis was remarkably higher.

The in vitro model of gut hydrolysis by the digestive enzymes of mullet, which was used to evaluate differences in the potential bioavailability of some nutrients, showed some interesting results when compared to those obtained in the analysis of the feeds. As an example, a significantly lower amount of available amino acids was measured for feed 2 when compared to feed 1 despite both feeds presenting the same crude protein contents. This could be explained considering the higher proportion of plant ingredients included in the former (78 vs. 68 g/100 g diet). Interestingly, the enzyme treatment significantly increased the amount of available amino acids in both diets, but the increase was higher for feed 2 than for feed 1 (4% and 18%, respectively). As identified by Castillo and Gatlin (2015) [16], this increased accessibility of protein to the action of digestive enzymes could be explained considering that NSP are present as part of the cell wall, thus shielding substrates from contact with the digestive enzymes, or as part of cell content, where their presence may interfere with digestion and absorption due to their chemical nature. This is in line with the observed significant increase in the amount of pentoses released from the enzyme-treated feeds. On the other hand, a low potential bioavailability of total P was determined for the experimental feeds, irrespective of the enzyme treatment. This could be explained considering that phytate accounted for more than 60% of total P present in feed 2 but represented only one third of that present in feed 1. This was also reflected by the lower bioavailability of this element observed in the digestive simulation despite the observed reduction in phytate produced by the enzyme treatment.

The results obtained in the in vivo evaluation of digestibility were somewhat surprising. No significant differences were measured between ADC values of protein in untreated diets, and the enzyme treatment only significantly improved the value obtained for feed 1. The higher bioaccessibility of the protein fraction found in the in vitro assay for such feed could explain this result to a certain

extent. An opposite result was obtained for ADC of total P or phytate, since a much higher digestive utilization of this element was measured for feed 2 in untreated feeds and the enzyme treatment resulted in a decreased digestibility in both feeds. The observed reduction in the efficiency of the digestive utilization of P after enzyme treatment of phytate could be explained considering that the increase in available P resulting from phytate hydrolysis was not in parallel with an equivalent ability for its absorption at the intestinal level. It must be considered that the experiment was performed using very young fish, on which the functionality of the intestine was still under development, and this could limit the absorption of the extra amount of P produced by the action of phytase. Intestinal transport of P is complex and proceeds via two distinct mechanisms: One component is developed by a sodium-phosphorus co-transporter (Na-Pi-II) and appears to saturate at low P concentrations, whereas the second mechanism relies on the luminal P concentration and does not show saturation, resembling passive diffusion [29]. The presence of divalent cations in sea water, which is continuously ingested by young marine fish, could lead to the formation of insoluble phosphate compounds that may limit gastrointestinal phosphorus bioavailability [30]. Also, it has been reported that an increase in the availability of dietary P typically decreases the efficiency of P utilization, thereby increasing the amount of P excreted [31].

In any case, the correlation between the results obtained with in vitro and in vivo assays was not directly clear. This can be explained considering that both types of assays measure different things. In vitro tests allow an estimation of the potential bioavailability of nutrients; that is, that fraction that would be available for biological functions once absorbed by the intestine (something that may not occur in practice, as indicated in the case of P). In contrast, in vivo digestibility assays estimate the net result of such intestinal absorption [32]. The results obtained in the present work support such differences in the approaches; the significantly lower values of potential P bioavailability measured in vitro for feed 2 should reflect a much slower release from the feed matrix. As a result, a better digestive efficiency and decreased fecal loss was observed in vivo, probably due to the decreased saturation of the intestinal transporters. Hence, the assessment of in vivo digestibility could be considered as a post-absorption method to estimate bioavailability while in vitro assays can provide a pre-absorption estimation of this parameter [24]. Moreover, in vitro models can simulate the physical or chemical transformations of food components but not other aspects, such as the effect of antinutritional factors or the influence of carbohydrates, transformed or not, on the intestinal microbiota. In the case of mullet, this last aspect is of great importance because it is an omnivorous fish with a well-developed intestine in which microbiota are presumed to play a fundamental role in the transformation of nutrients. This could explain why the clear differences in amino acid release measured between feeds in vitro were not equally reflected in the ADC of their protein fraction. It follows that in vitro bioavailability assays can correlate better with other indicators of biological efficiency like the conversion index or the specific growth rate, as suggested by the results obtained by Dimes et al. (1994) [33] or Rungruangsak-Torrissen et al. (2002) [34].

4. Materials and Methods

4.1. Ingredients and Experimental Feeds

The different ingredients used in the fabrication of the experimental feeds were supplied by DIBAQ S.A. (Segovia, Spain). Two different diets, detailed in Table 1, were designed considering the following points: (a) The inclusion of a low amount of fish meal (10–15 g/100 g); (b) use of some different ingredients in both diets (diet 1 contained rapeseed meal while diet 2 included rice bran); (c) adaption of the diets to the nutritional requirements of juvenile mullets (thus they contained 45% crude protein and 13% total fat); and (d) contain a similar amount of NSP (24–5 g/100) and phytate (0.3%).

Each of the two diets was prepared with or without enzyme treatment, resulting in a total of four experimental diets. The enzymes used were Viscozyme™ (Sigma-Aldrich RN V2010), a mixture of xylanase, cellulase, and hemicellulose containing 100 fungal beta glucanase units/g at a dose of 10 g/kg

feed and Quantum™ phytase (AB Enzymes, Germany), containing 2500 phytase units/g at a dose of 0.4 g/kg feed. To carry out the treatment, the vegetable meals in each diet were milled to a mesh size of 0.5 mm and mixed carefully with citrate buffer (pH 5.0, 0.1 M; 1:3 w/v) to obtain a moist mass with the optimal conditions for the action of the enzymes, which were solubilized in water and added to the feed mixtures by spraying. The enzymes were allowed to act by keeping the mixture at 45 °C for 4 h, with manual stirring 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 the addition of the rest of diet ingredients and preparation of feed pellets. Cr_2O_3 was included in all diets as an inert marker to evaluate digestibility. The feeds were prepared using a lab-scale extrusion machine provided with a mesh size of 2 mm, and dried and stored at 4 °C until used.

4.2. Analytical Techniques Used

Samples of each feed were used for the analysis of total protein, soluble protein, reducing sugars, free pentoses, total phosphorus, and phytic phosphorus according to the following methodologies:

Total nitrogen was analyzed by CNHS elementary analysis, with the contents in N (g/100 g sample) transformed into protein using a conversion factor of 6.25. Soluble protein was analyzed by the Bradford method (1976) [35] using the SIGMA Total Protein Kit (Sigma-Aldrich TP0100). Reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS) following the method described by Miller (1959) [36]. Free pentoses were measured by the phloroglucinol method described by Douglas (1981) [37]. Total phosphorus in feeds and feces was determined by the molybdovanadate method after total digestion of the organic matter with concentrated nitric acid [38]. Phytic acid was determined following the bipyridine method described by Haug and Lantzsch (1983) [39]. All the analyses were performed in triplicate samples from each diet.

4.3. In Vitro Digestive Hydrolysis Assay

Changes in the potential bioavailability of the nitrogen fraction, pentoses, and P present in feeds, enzymatically treated or not, were evaluated in vitro under conditions simulating the digestive tract of a mullet. The assays were performed using membrane bioreactors modified from that described in Morales and Moyano (2010) [40]. The device consists of two chambers separated by a semi-permeable membrane of 3500 kDa MWCO (Molecular Weight Cut- Off). Fish enzyme extracts and feed samples were placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. Hydrolysis products passing across the membrane into the lower chamber were recovered at different time intervals during the reaction time. The whole system was maintained at 25 °C within a temperature-controlled chamber.

The enzyme extracts used in the assays were obtained from adult individuals of mullet (*M. cephalus)* (n = 4, 2.2 kg average weight) on which intestinal pH and total protease activity were measured according to the method of Kunitz as modified by Walter (1984) [41]. The operation of the in vitro simulation required the use of an enzyme:substrate ratio close to that existing in the gut of live fish. This was estimated considering, on the one hand, the average total protease production measured in the intestine of the fish and on the other hand, the mean protein intake of such fish in a single meal. As a result of the aforementioned estimation, an enzyme/substrate ratio of 20 U/mg protein and a pH of 8.5 were used in the assays. All the assays were carried out in triplicate. The release of products from feeds in the absence of enzyme hydrolysis was assessed by running assays on which the enzyme extracts were heat inactivated (placed in a water bath at 100 °C for 5 min). Hydrolysis products were analyzed using the same methodologies described for the feeds while the release of amino acids was quantified by the orthophthaldehyde (OPA) method described by Church et al. (1983) [42].

4.4. In Vivo Digestibility Test

A total of 1200 fish (9.3 ± 1.1 g) were distributed into 12 tanks (120 L; 100 fish per tank) provided with a settling column for stool removal (Guelph method). Each of the four experimental feeds were

evaluated in triplicate. The fish were fed manually every day in two meals. Feces were removed daily for 3 weeks, dried, and processed to determine their nitrogen, total phosphorus, and phytate contents as previously detailed. Fecal samples obtained on three different days were pooled to form one sample and three different samples were obtained from each tank (a total of $3 \times 3 = 9$ samples per diet). The determination of the total chromium of feeds and feces was carried out using the diphenylcarbazide method [43]. Apparent digestibility coefficients (ADC) were calculated as follows:

$$ADC\ nutrient = 100 - [\left[\!\left[\frac{(\%\ \text{of indicator in food})}{(\%\ \text{of indicator in feces})} \right]\!\right] \left[\!\left[\frac{(\%\ \text{of nutrient in feces})}{(\%\ \text{of nutrient in food})} \right]\!\right] * 100].$$

4.5. Statistical Analysis

Data were analyzed by ANOVA followed by the Bonferroni test using the software Statgraphics Centurion (Statgraphics Technologies, The Plains, VI. EE.UU.). The significance level was established at $p < 0.05$. When required, data expressed in percentage were previously arc-sin transformed.

5. Conclusions

In conclusion, the results obtained in the present study suggest that:

Pretreatment of plant ingredients prior to feed elaboration with enzyme mixtures is a suitable way to modify their nutritional profile, increasing the potential bioavailability of different nutrients.

The characteristics of enzyme treatment should be carefully adapted to the physiological features of the target species, mostly in the case of non-adult fish that present a still underdeveloped digestive tract. Excessive hydrolysis of some substrates (i.e., phosphorus) causes an increase in their concentration at the gut level that may impair absorption, resulting in decreased digestibility due to fecal loss.

In vitro assays oriented to assess potential differences in the bioavailability of nutrients derived from enzyme treatments may help to explain and predict results obtained in vivo to a certain extent. Refinement of the conditions for developing such assays could represent a powerful tool to gain a better understanding of variations in gut nutrient availability.

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