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Nutritional and Growth Effect of Insect Meal Inclusion on Seabass (*Dicentrarchuss labrax*) Feeds

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Abstract: Abajo: se repite los tres en el resumen**.** This work studies the effect of high-level fish meal replacement with insect meal: YW meal (obtained from Tenebrio larvae fed a broiler diet), BSF meal (from hermetia larvae fed broilers diet), BSFm meal (obtained from hermetia larvae fed discard fish) on growth performance nutritive indices and in vitro digestibility of *Dicentrarchus labrax* juvenile. Three different insect meals were used: BSF meal from hermetia larvae fed broilers diet; BSF improve (BSFm) obtained from hermetia larvae fed discarded fish; YW meal obtained from the larvae of Tenebrio fed a broiler diet. Five diets were used, a control (C) diet and four experimental diets by replacing fishmeal with insect meal from BSF at 30% and 50% (BSF30 and BSF50) substitutions, BSFm at 50% substitution (BSF50 m) and YM at 50% substitution (YW50). Nutritional and growth indices worsened by including insect meal, especially for hermetia meal at 50% substitution, BSF50 and BSF50 m. The internal organs' weight reflected the growth of the fish fed each experimental diet. No differences were found in fillet composition. Nevertheless, under our experimental condition, YW replacement obtained better results than both BSF diets.

Keywords: sea bass; fishmeal; *Tenebrio molitor*; *Hermetia illucens*

1. Introduction

Nowadays the utilization of insect meal as an alternative protein source of fish meal arouses much interest. Insects are a good protein source with a high percentage of protein, similarly to the fish meal in *Boopedon flaviventris* (76.0%), *Melanoplus mexicanus* (77.1%) and *Sphenarium histrio* (74.8%) [1]. The amino acids (AA) profile depends on the taxon, and Diptera is the most similar one to fish meal in terms of essential and limiting AA [2]. From an environmental viewpoint, insect cultures are sustainable because there is no need to use large areas or much water, they contribute to waste recycling, and their culture gives quite low carbon footprint scores [3].

Several insects have been tested as aquafeed for some fish species [4–6], where both *Hermetia illucens* (Black Soldier Fly, BSF) and *Tenebrio molitor* (Yellow mealworm, YW) were the most promising insects given their ability to convert organic waste into protein, fat and energy [7] by incorporating the AA and fatty acids (FA) of manure and organic waste into their biomass. The obtained biomass is high in protein and fat [5,8].

The meal deriving from YM larvae has a high crude protein content within the 44%–69% range, fat content is 23%–47%, plus 6.6% fiber and 2.4% ash [9,10].

The BSF meal contains 35%–57% crude protein, 32.6% crude fat, 6.7% crude fiber and 8.6% ash content [9,10]. Nevertheless, the amount and quality of fat depend on diet type [5,11].

Currently, some companies rear these two insect species in mass and make meals available for animal feeding. However, prices are still uncompetitive as marketing is not yet operational.

Dicentrarchus labrax is a fish species that consumers greatly appreciate. European Union *D. labrax* production was 69.031 Tm, which is the fifth most produced species, and the fourth according to commercial demand [12]. The effect on *D. labrax* growth of including YW has been studied by [13], who concluded that YW meal can be used up to 25% inclusion. For BSF, Magalhães et al. [14] found that up to 19.5% BSF, corresponding to 22.5% total dietary protein, can replace FM in diets for juvenile *D. labrax* with no adverse effects on nutritional and growth indices. Nevertheless, high inclusion percentages can worsen growth performance and feed utilization because insect meal entails some nutritional inconveniences that condition it being included in aquafeeds, of which digestibility is the main one. The insect exoskeleton is composed of chitin and scleroprotein. Chitin is a polymer of N-acetyl-glucosamine with β-(1/4) linkages, a nondigestible crude fiber [15] for fish and interferes with protein use [16]. However, chitinolytic activities have been described in fish, and marked chitinase activities have been identified in the stomach of several fish species. Marked N-acetylhexosaminidase activity is distributed to the intestine and/or pyloric caeca [17]. An in vitro protein hydrolysis [18], like in vivo [19] digestibility, reduces with insect inclusion. Scleroprotein digestibility is low, and keratin is a scleroprotein with very stable S–S and S–H linkages that are not readily broken down by animals without processing [20].

Other factors that could limit insect meal inclusion percentages are fat levels, which are higher for insects than for fish, and the FA profile of insects. While fishmeal is rich in n-3 long-chain polyunsaturated FA (n-3 LCPUFAs), insects contain larger quantities of n-6 polyunsaturated FA (n-6 PUFAs) [21]. However, the FA profile of insects could be manipulated through feeding [22]. Barroso et al. [11,23] were able to increase n-3 LCPUFAS content in BSF larvae fed fish meal or fish offal.

This work studies the effect of high-level fish meal replacement with YM, BSF or fish fed BSF rich in long-chain FA n-3 on growth performance nutritive indices and in vitro digestibility.

2. Results

2.1. Nutritional and Growth Indices of Fish

Table 1 shows the obtained nutritional and growth indices results. The determined growth indices were SGR, DGC, weight gain and K. The lowest weight gain went to the fish fed BSF50 (60% less than the control diet) and BSF50m (48% less than the control diet). SGR and DGR showed the same trend; the highest values corresponded to the control diet (1.88 for SGR and 1.61 for DGC, respectively), followed by YW50, which was slightly higher, but not statically different, than BSF30. The lowest values corresponded to BSF50 (0.95 for SGR and 0.76 for DGC), which was slightly lower than BSF50m (1.16 for SGR and 0.94 for DGC). The K factor displayed the same trend, but no statistical differences were observed between YW50 and the control diet in this case.

Table 1. Fish performance indices (n = 3 mean ± SD) of *D. labrax* fed the five experimental diets.

Diets	SGR	FCE	FI %	DGC	FCR	K	PER	WG (g)
C	1.88 ± 0.02 ^{a}	0.56 ± 0.01 ^{b}	3.12 ± 0.07 ^{a}	1.61 ± 0.04 ^{a}	1.77 ± 0.02 ^{c}	162.26 ± 2.85 ^{a}	1.25 ± 0.02 ^{b}	16.10 ± 0.74 ^{a}
YW50	1.64 ± 0.02 ^{b}	0.56 ± 0.01 ^{b}	2.76 ± 0.08 ^{b}	1.38 ± 0.02 ^{b}	1.78 ± 0.03 ^{c}	153.00 ± 4.03 ^{ab}	1.25 ± 0.02 ^{b}	13.15 ± 0.48 ^{b}
BSF30	1.52 ± 0.05 ^{b}	0.49 ± 0.04 ^{b}	2.94 ± 0.15 ^{ab}	1.26 ± 0.07 ^{b}	2.03 ± 0.17 ^{c}	146.48 ± 8.74 ^{bc}	1.10 ± 0.09 ^{b}	11.78 ± 1.19 ^{b}
BSF50	0.95 ± 0.15 ^{c}	0.31 ± 0.04 ^{c}	3.06 ± 0.05 ^{a}	0.76 ± 0.12 ^{c}	3.31 ± 0.42 ^{a}	126.26 ± 3.41 ^{d}	0.68 ± 0.09 ^{c}	6.36 ± 1.15 ^{c}
BSF50 m	1.16 ± 0.13 ^{c}	0.38 ± 0.02 ^{c}	2.99 ± 0.16 ^{ab}	0.94 ± 0.13 ^{c}	2.67 ± 0.14 ^{b}	134.94 ± 7.58 ^{cd}	0.84 ± 0.05 ^{c}	8.32 ± 1.51 ^{c}

C: Control; YW50: Yellow mealworm 50% replacement; BSF30: Black Soldier Fly 30% replacement; BSF50: Black Soldier Fly 50% replacement; BSF50m: Enriched Black Soldier Fly 50% replacement; SGR: Specific growth ratio; FCE: Feed Conversion Efficiency; FI: Feed Intake; DGC: Daily Growth Coefficient; FCR: Feed Conversion Ratio; K: Condition Factor; PER: Protein Efficiency Ratio; WG: Weight Gain. Different letters in columns represent significant differences ($p < 0.05$) among treatments.

The nutritional indices showed statistical feed intake differences only between YW50, with lower feed intake (2.76%), and the control (3.12%). For FCR, a high BSF inclusion level (BSF50 and BSF50m) made FCR worse, which was especially low for BSF50. No statistical differences were found in FCR among the other treatments.

For protein utilization, no differences appeared among the control, YM50 and BSF30 diets. The worse PER values corresponded to the diet with BSF inclusion, with BSF50 being the lowest with 0.68, followed by BSF50m with 0.84, whereas the value for the control diet and YW50 was 1.25.

2.2. Proximal Composition and Morphology of Fish

Table 2 provides the data corresponding to the proximal composition of muscle. No significant differences were observed in any fillet component for crude protein, crude fat or ash. The crude protein percentage ranged from 72.4% in the fish fed BSF50m to 67.9% in those fed the control diet. The ash percentage was similar for all treatments (around 11%) with no statistical differences. The ether extract percentages ranged from 16.1% for the fish fed BSF50 to 21.3% for those fed the control diet, although differences were not significant.

Table 2. Proximal composition of fillet of *D. labrax* fed different experimental diets (n = 6).

	C	YW50	BSF30	BSF50	BSF50m
Dry matter (%)	98.3 ± 0.29	97.6 ± 1.25	96.7 ± 1.80	97.2 ± 1.21	97.5 ± 0.81
Ash (%)	11.4 ± 0.08	11.0 ± 0.76	12.3 ± 0.83	11.6 ± 0.82	11.3 ± 1.11
Organic matter (%)	88.6 ± 0.13	89.0 ± 0.76	87.7 ± 0.83	88.4 ± 0.82	88.7 ± 1.11
Crude fat (%)	21.3 ± 0.69	17.6 ± 3.21	20.4 ± 3.79	16.1 ± 1.53	17.3 ± 0.66
Crude protein (Nx6.25; %)	68.0 ± 4.01	71.7 ± 4.93	69.3 ± 3.71	69.6 ± 4.23	72.5 ± 0.36

C: Control; YW50: Yellow mealworm 50% replacement; BSF30: Black Soldier Fly 30% replacement; BSF50: Black Soldier Fly 50% replacement; BSF50m: Enriched Black Soldier Fly 50% replacement. Means (± SD) from six individual measurements. Different letters in columns represent significant differences ($p < 0.05$) among treatments.

Table 3 provides the fillet, digestive, liver and perivisceral fat weights. The control fish fillet weight was much higher than for other treatments, but no statistical differences were found for other treatments and digestive weights. Nevertheless, the experimental diets affected liver and perivisceral fat weight. Liver weight lowered with the high BSF inclusion level (diets BSF50 and BSF50m). Regarding perivisceral fat, the fish fed the control diet had the heaviest visceral fat weight, followed by YW50. The lightest perivisceral fat weight went to the fish fed diets BSF50 and BSF50m, while those fed BSF30 obtained intermediate values.

Table 3. Internal organs' weight (g) of *Dicentrarchus labrax* fed different experimental diets (n = 6)

Diets	Fillet	Digestive	Liver	Visceral Fat
C	25.16 ± 0.79 ^{a}	0.98 ± 0.06	0.55 ± 0.03 ^{a}	1.97 ± 0.39 ^{a}
YW50	19.66 ± 2.37 ^{b}	0.83 ± 0.12	0.38 ± 0.02 ^{a,b,c}	1.61 ± 0.28 ^{a,b}
BSF30	19.55 ± 2.55 ^{b}	0.90 ± 0.15	0.45 ± 0.11 ^{a,b}	1.20 ± 0.14 ^{b,c}
BSF50	17.72 ± 2.16 ^{b}	0.90 ± 0.09	0.31 ± 0.10 ^{b,c}	0.75 ± 0.32 ^{c}
BSF50m	15.25 ± 2.38 ^{b}	0.78 ± 0.05	0.30 ± 0.06 ^{c}	0.69 ± 0.13 ^{c}

C: Control; YW50: Yellow mealworm 50% replacement; BSF30: Black Soldier Fly 30% replacement; BSF50: Black Soldier Fly 50% replacement; BSF50m: Enriched Black Soldier Fly 50% replacement means (± SD) from nine individuals. Different letters in columns represent significant differences ($p < 0.05$) among treatments.

2.3. In Vitro Hydrolysis

The in vitro protein hydrolysis (Table 4) obtained the maximum hydrolysis for the control lots, with no statistical differences for BSF50m and YW50. The minimum hydrolysis was found for the BSF50 lot, while BSF30 showed intermediate hydrolysis capacity.

Table 4. Protein hydrolysis (mg/mL of free amino acids) of intestines of the fish fed different treatments (n = 3).

Treatments	End Point (120′)
Control	159.2 ± 2.9 ^{a}
YW50	155.5 ± 1.4 ^{ab}
BSF30	122.8 ± 7.8 ^{bc}
BSF50	114.3 ± 14.5 ^{c}
BSF50M	150.2 ± 10.6 ^{ab}

C: Control; YW50: Yellow mealworm 50% replacement; BSF30: Black Soldier Fly 30% replacement; BSF50: Black Soldier Fly 50% replacement; BSF50m: Enriched Black Soldier Fly 50% replacement. Free amino acids ± standard deviation (mg/mL) corresponding to the hydrolysis of each enzyme extract with its corresponding diet. means (± SD) from nine individuals. Different letters in columns represent significant differences ($p < 0.05$) among treatments

3. Discussion

The idea of employing insects in fish feeding is widely accepted, but it is necessary to acquire further knowledge about the nutritional properties of the source for their real use in aquafeed.

The growth indices (Table 1) indicated better growth for the control fish, followed by BSF30 and YW50. For the BSF diets, lower weight values were obtained at high replacement levels (50%), which resulted in a slight improvement when BFSm were used, but not statistically different to BSF50. A similar trend was observed for the other treatment growth indices. This result contrasts with those reported by Magalhães et al. [14] in *D. labrax* also fed BSF at 45% fish meal replacement, although Magalhães et al. [14] employed defatted insect meal. The defatting of insect meal increased the crude protein percentage, which for crude protein was 55.8% vs. 43.27% on average, following the values described by Makkar et al. [5]. Compared to fish meal, insect meal is deficient in lysine and tryptophan, and is limited in threonine and sulfur AA [4,5]. At our high BSF replacement level, indispensable AA levels could be limiting, but the increased crude protein percentage of defatted meal entailed a bigger AA supply, which could avoid AA deficiency. Similar results have been obtained in rainbow trout (*Oncorhynchus mykiss*) or turbot (*Psetta maxima* L.), with decreasing growth performance and diet digestibility when BSF was used [24,25].

The defatting of insect meal is because its FA profile differs considerably from fishmeal. Nevertheless, the FA profile can be changed by feeding insect larvae [11,23]. The BSF50m insect meal was made by feeding BSF discarded fish to obtain a BSF meal rich in n-3 LCPUFAs. Nevertheless, the growth indices seemed better than for BSF50 and, albeit not statistically different, were clearly worse than those obtained for BSF30 and YW50 or the control. These results revealed that improving the FA of insect meal might prove insufficient. For the *D. labrax* juveniles, the minimum dietary n-3 LCPUFAs requirement for adequate growth has been set at 0.7% on a dry matter (DM) basis [26], and lower values worsen fish growth performance, without negatively affecting feed efficiency or PER. Nevertheless, the amount of n-3 LCPUFAs exceeded 0.7% in all our experimental diets, so reduced growth would not have been caused by this.

The YW meal replacement seemed more suitable for sea bass, with similar growth indices to BSF30, better than BSF50, but were lower than the control. A similar result was obtained by Gasco et al. [13] in sea bass, who reported lower dry matter digestibility and feeding rates compared to the control diet with a consequent 27% reduction in weight gain at a high YW inclusion level (50%), compared to the 19% reduction in weight gain herein obtained. Piccolo et al. [19] reported similar growth parameters when feeding sea bream (*Sparus aurata*) a diet with 50% YW meal replacement

compared to the fish fed a control diet. The growth indices improved when fish were fed a diet with 25% replacement.

The nutritive indices revealed a similar trend to the growth indices. FI was similar among treatments, except for YW50, while FCR and PER were worse for both treatments with a higher BSF replacement level (BSF50 and BSF50m), which explained the result obtained for both growth indices and weight gain. These results partially agree with those reported for *D. labrax* by Magalhães et al. [14], who observed worse PER with increased BSF inclusion, but no changes in FCR. Similar results have been found for *P. maxima* [25] or *O. mykiss* [27]. The opposite results have been reported for *S. aurata* [28], *Salmo salar* [29] and *O. mykiss* [30], with no differences in nutritional indices among diets.

Many fish factors may lie behind these differences, such as age or species, or might be related to diet formulation or quality of ingredients. Indeed, insect meal quality is not standardized. Insect larvae feeding or the insect meal production process might affect the composition and, consequently, the nutritive value of meals [31,32]. Heat may cause certain proteins to interact with other proteins, oxidizing agents, sugars, polyphenols or tannins [33], and the drying method is able to affect the solubility of proteins [34]. In fact, our laboratory has obtained different results in sea bream fed similar diets made with distinct BSF lots (results not published). In *S. salar,* two BSF types have been tested, but only one allowed total FM replacement of diets without affecting fish performance [29].

The 50% YW replacement gave better nutritional indices than the 50% BSF replacement. FCR and PER were similar to the control, but these indices slightly worsened, which agrees with the results of Gasco et al. [13] in sea bass. The lower feed intake of the YW50 fish could justify the lower weight gain regarding the control fish, according to the results obtained for the *D. labrax* fed the diet with 50% YW meal replacement [13]. Based on our previous experience, YW might not be very palatable food because of some of its negative organoleptic characteristics.

Regarding fillets, the heaviest weight, higher liver weight and perivisceral fat corresponded to the control fish, which reflects the different growth rates and nutritional indices obtained among treatments. Finally, muscle composition remained unaltered after insect inclusion, which falls in line with previous reports about *D. labrax* fed YW [13] or *S. aurata* fed BSF [28].

In general, previous results have reflected that insect meal makes growth and nutritional indices worse by indicating lower feed efficiency, protein efficiency and growth. Decreased insect meal digestibility has been discussed because chitin is not degraded in the intestine [35] and can affect protein digestibility [36,37]. Marono et al. [38] found that the crude protein digestibility of BSF and YW correlates negatively with acid detergent fiber and chitin contents. BSF showed a positive correlation between crude protein and CP protein for in vitro digestibility. In this experiment, we determined the protein hydrolysis of each diet in vitro with the digestive enzymes of the fish fed each experimental diet. The results revealed lower protein hydrolysis for the diet that included BSF not enriched in long-chain n-3. Belghit et al. [39] did not find any differences in trypsin activities for insects or oil with insect inclusion, but reported apparent decreased protein digestibility in the *S. salar* fed BSF meal. The obtained data are consistent with those for *S. salar* as regards diets BSF30 and BSF50. Nevertheless, it is difficult to explain the high hydrolysis levels obtained herein for BSF50m. In the *S. salar* fed a diet with 85% insect meal and insect oil replacement, obtained from insect feed with seaweed to improve long-chain n-3 FA, no changes in trypsin activity were observed [39], while our results indicated increases in diets BSF30 and BSF50.

The YW meal did not seem to induce any changes in in vitro digestibility, which agrees with that observed in Tilapia fed a diet of 50% fish meal replacement with YW meal, and no differences in the in vitro protein hydrolysis were observed compared to the control, although alkaline protease increased [18]. In trout [40] and sea bream [19], apparent protein digestibility was significantly lower for the 50% YW inclusion.

Briefly, the inclusion of high insect meal levels made the nutritional and growth indices worse. Nevertheless, under our experimental conditions, YW replacement gave better results than both BSF diets. Although expectations for insects being used as a sustainable alternative to fishmeal are high, many unknown factors need to be clarified. It is hard to understand how several researchers have tested the same insects and fish species, but obtained different results. Therefore, more in-depth

studies about the effect on fish growth and physiology are needed, as is more information on the effect of larvae rearing, feeding and meal processing on the nutritive value of insect meal.

4. Materials and Methods

4.1. Diet Ingredients and Formulation

Three different insect meals were used for diet formulations: BSF meal from the hermetia larvae fed a broiler diet; BSFm obtained from the hermetia larvae fed discarded fish to improve the n-3 LCPUFAs profile of insect meal, as described by Barroso et al. [11] and YM meal obtained from the larvae of Tenebrio fed a broiler diet. Five diets were formulated, one control and four experimental, by replacing fishmeal with insect meal: BSF30 with BSF meal at 30% substitutions, BSF50 with BSF meal at 50% substitution; BSFm50 with BSF enriches in n-3 LCPUFAS at 50% substitution and YM50 (50% substitution; YW50; Tables 5 and 6). Insects were provided by Mealfood S.L. (Salamanca, Spain) and Entomotech S.L. (Almería, Spain). Diets were manufactured by the Experimental Diet Service of the University of Almería (Almería, Spain) and were extruded in 2-mm pellets.

Table 5. Ingredients, proximate composition and gross energy on a dry matter basis (DM) of the experimental diets.

	C	YW50	BSF30	BSF50	BSF50m
Ingredients (% DM)					
Fish meal	35.9	18.0	25.3	18.0	18.0
BSF	0.0	0.0	10.9	18.0	0.0
BSFm	0.0	0.0	0.0	0.0	18.0
YW	0.0	18.0	0.0	0.0	0.0
Wheat gluten	10.5	11.9	13.0	15.4	15.0
Soy cake	15.5	17.0	17.5	18.3	18.3
Fish oil	12.2	9.0	10.4	9.5	9.7
Soy lecithin	1.3	0.5	1.0	0.5	0.5
Wheat flour	16.6	17.6	13.9	12.4	12.6
Vitamins and minerals	2.0	2.0	2.0	2.0	2.0
Guar gum	2.0	2.0	2.0	2.0	2.0
Hemoglobin powder	4.0	4.0	4.0	4.0	4.0
Gross energy (MJ/kg)	16.8	17.0	16.7	16.5	16.6
Proximate composition (% DM)					
Crude protein	43.86	43.1	43.51	42.8	42.85
Crude fat	17.16	17.97	17.6	17.12	7.6
Ash	7.42	6.28	6.45	6.94	6.11

C: Control; YW50: Yellow mealworm 50% replacement; BSF30: Black Soldier Fly 30% replacement; BSF50: Black Soldier Fly 50% replacement; BSF50m: Enriched Black Soldier Fly 50% replacement.

Table 6. Fatty acids profile from yellow mealworm meal, Black Soldier Fly and Black Soldier Fly enriched with n-3 LCPUFAS (g/100 g total fatty acids).

	YW	***BSF***	***BSFm***
10:0	n.d.	1.10	1.11
12:0	0.67	48.5	46.1
14:0	3.67	9.22	9.55
16:0	16.2	13.0	14.7
16:1n7	1.70	1.79	3.13
18:0	2.95	2.17	2.17
18:1n9	36.7	9.58	8.50
18:1n7	n.d.	n.d.	0.67
18:2n6	35.4	11.0	7.58
18:3n3	1.41	1.10	1.06
18:4n3	n.d.	0.99	0.70
20:5n3 (EPA)	n.d.	n.d.	2.87
22:6n3 (DHA)	n.d.	n.d.	1.00

YM: Tenebrio molitor; BSF: Hermetia illucens; BSFm: Enriched Hermetia illucens (fed discarded fish).

4.2. The D. Labrax Feeding Trial

D. labrax juveniles were provided by Predomar (Carboneras, Spain). The experiment was carried out at the Aquaculture Research Centre of the University of Almería (Spain). All the procedures were performed following Council Directive 86/609/EEC Guidelines (European Communities, 1986) on the protection of animals used for experimental and other scientific purposes. *D. labrax* juveniles were weighed (10.7 g average weight), measured (10.6 cm average length) and placed inside 15 tanks (3 tanks/diet, 20 fish/tank) with a volume of 250 L at a replacement rate of 10.4 L/h. Fish were maintained at 20 °C under a natural photoperiod (12L:12D), and were fed ad libitum twice daily (09:30 h and 13:30 h) until their weight had tripled on day 49. Feed was weighed before and after feeding fish. After 15 min, uneaten pellets were removed by aspiration, dried and weighed to calculate the daily feed intake.

4.3. Fish Performance Indices

SGR (Specific Growth Ratio: g*day^{-1}) = [(LN W_f − LN W_i)/t] × 100, where W_f = final weight, W_i = initial weight and "t" time in experiment days.

FCE (Feed Conversion Efficiency: g) = wet weight gain/dry feed intake.

FI (Feed intake %) = (Daily feed intake/average body weight*) × 100. *Average between the final and initial weights.

DGC (Daily Growth Coefficient: g*day^{-1}) = [($W_f^{1/3}$ − $W_i^{1/3}$)/t] × 100.

FCR (Feed Conversion Ratio) = total feed intake/weight increase.

Condition Factor (K:g/cm) = 100 × body weight/total length^3.

PER (Protein Efficiency Ratio g) = wet weight gain/crude protein intake.

WI (Weight Increase: g) = W_f − W_i.

4.4. Sampling

Before sampling, fish were fasted for 24 h. All the fish in each tank were lightly anesthetized with clove oil to be weighed and measured. Three fish per tank (9 fish per diet) were slaughtered by an overdose of anesthesia (clove oil 100 mg/L solution) and were immediately eviscerated. Muscle, digestive (stomach and intestine), liver and fat were weighed. Intestines and muscles were frozen separately at −80 °C for further analyses.

4.5. Analytical Methods

4.5.1. Chemical Composition of Diets and Fish

All the proximal composition analyses were performed according to the Association of Official Analytical Chemists [41]. Dry matter (DM) and ash were determined gravimetrically after drying at 105 ± 0.5 °C and combustion at 500 °C in a muffle furnace, respectively. Crude protein content (Nx6,25) and total fat content were determined by the Kjeldahl method and diethyl ether extraction (Soxhlet technique), respectively. All the analyses were performed in triplicate.

4.5.2. Enzymatic Extract.

Five pools (1 per diet) were made with fish intestines (10 per pool) at 4 °C. Tissues were ground and homogenized by ultrasound in distilled water at a ratio of 0.5 g/L. Samples were centrifuged (12,000 rpm, 20 min, 4 °C) and supernatants were stored at −20 °C to be used in the alkaline protease and enzymatic hydrolysis analyses.

4.5.3. Alkaline Protease Activity

The enzyme extract supernatant was utilized to determine alkaline protease with Tris/HCl pH 9 buffer solution and casein as a substrate according to Vizcaíno et al. [42]. After a 30-min incubation at 37 °C, the reaction was stopped with 20% trichloroacetic acid solution. Samples were centrifuged (12,000 rpm, 15 min, 4 °C). The absorbance of the supernatant was measured at 280 nm by spectrophotometry in a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). All these analyses were performed in duplicate. One unit of protease activity was defined as 1 μg of tyrosine released per minute.

4.5.4. In Vitro Protein Hydrolysis

In vitro hydrolysis was carried out in jacketed glasses connected to a constant water flow at 37 °C with continuous stirring for 120 min. Six hydrolyzes were done with each enzyme extract and its corresponding diet. Fishmeal was used as a control. According to Vizcaíno et al. [42], the enzyme extract and substrate (diet) were added to Tris/HCl pH 9 buffer solution to obtain 200 U and 80 mg of protein, respectively. Samples (0.1 mL for soluble protein, 0.05 mL for free AA) were taken at reaction times 0, 15, 30, 60, 90 and 120 min. The soluble protein samples were heated for 5 min in boiling water to stop the enzymatic reaction. To stop the reaction of the free AA samples, 0.05 mL of 6% trichloroacetic acid solution was used. All these analyses were performed in duplicate. The hydrolysis samples were centrifuged (12,000 rpm, 20 min, 4 °C) and supernatants were stored at −20 °C.

4.5.5. Soluble Protein

A commercial kit (Pierce BCA™ Protein Assay Kit, Thermo Scientific™, Rockford, IL, USA) was used to calculate soluble protein. Samples were incubated with the reagent for 30 min at 37 °C and measured at 561 nm by a spectrophotometer. The absorbance results were extrapolated using albumin as a standard. The results were expressed as mg of soluble protein/mL of enzymatic extract. All these analyses were performed in duplicate.

4.5.6. Free Amino Acids (OPA)

In order to know the protein degradation of diets, the free AA concentration after protein hydrolysis was measured according to Church et al. [43]. The sample was incubated at room temperature for 2 min with the reagent before measuring absorbance at 340 nm. The obtained data were extrapolated with a standard line made by taking L-leucine as a standard. The results were expressed as mg of free AA/mL of enzymatic extract. All these analyses were performed in duplicate.

4.6. Statistical Analysis

The experimental results were expressed as means ± SD for three different determinations. The Kruskal–Wallis test and comparisons among pairs using Tukey–Kramer HSD tests (JMP 9.0.0 for Macintosh) were performed to compare the results of the fish growth performance indices, proximal composition, organ weights and the in vitro protein hydrolysis.

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