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Original Article

Converting agricultural and fisheries waste into high-value products: Utilization of pea meal and shrimp protein hydrolysate as feed components for Nile Tilapia (*Oreochromis niloticus*)

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Abstract: This study aims to characterize pea meal and shrimp protein hydrolysate as alternative protein ingredients, replacing soybean meal (SBM) in tilapia feed. The presence of protease inhibitors and the *in vitro* digestion were evaluated for both raw and extruded pea meals, while the characteristics of shrimp protein hydrolysate were also studied. The nutritional quality of alternative ingredients was assessed to formulate a diet that meets tilapia juveniles' requirements, and its preliminary effects on growth were evaluated. Extrusion significantly reduced the inhibitory effect of pea meal on tilapia proteases (from 5.4 to 1.9%). *In vitro*, protein digestion of extruded pea meal (7.8±1.35%) did not differ from the control treatment with fish meal (11.8±1.94%). Additionally, shrimp protein hydrolysate presented 41.6±2.91% of DPPH radical scavenging activity. Tilapia fed a diet including 25% extruded pea meal and 10% shrimp protein hydrolysate, replacing soybean meal, presented comparable growth indicators to those exposed to the control diet. All the results in this study demonstrate that it is possible to include these ingredients derived from industrial waste in tilapia diets, improving the quality of the feed without affecting its performance.

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Introduction

The aquaculture sector is experiencing rapid growth and is poised to emerge as the primary source of fish for human consumption. The escalating demand for fish meal (FM), a fundamental protein source in aquafeeds, coupled with its surging prices, has necessitated the search for alternative protein sources (El-Sayed et al., 2000; Khieokhajonkhet et al., 2021). Historically, this demand was primarily met using soybean meal (SBM), which has now become the prevalent protein source for replacing FM in finfish diets (Khieokhajonkhet et al., 2021). However, the substantial consumption of SBM has led to a staggering price increase, soaring from $190 per metric ton in 2002 to over $601 per ton in 2022 (IndexMundi, 2022). Consequently, SBM has evolved into the costliest ingredient in fish feed, second only to FM (Pradhan et al., 2020). Furthermore, the cultivation of SBM is associated with an augmented carbon footprint due to its importation from producing countries to consuming nations (Tallentire et al., 2018). Adding to the challenges, SBM exhibits nutritional deficiencies attributable to amino acid imbalances and a high concentration of anti-nutritional factors, thereby limiting nutrient utilization (Qi et al., 2021). Therefore, exploring appropriate protein sources that promote SBM replacement is vital.

Numerous potential alternative ingredients derived from animal and plant sources have gained attention due to their application in aquafeeds. Pea (*Pisum sativum*), in particular, is a staple food source in many agricultural countries and is globally recognized as one of the most significant legumes (Magbanua and Ragaza, 2022). Pea meal (PM), a by-product typically of low commercial value and rarely featured in human

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diets, offers promise as an excellent protein source due to its low-fat content, gluten-free nature, and abundant minerals and vitamins (Ma et al., 2017).

Tilapia (*Oreochromis niloticus*), one of the world's most extensively farmed fish, exhibits a notable ability to tolerate elevated levels of plant-derived dietary protein owing to its omnivorous dietary preferences (Schulz et al., 2007). While PM holds potential as a protein source for tilapia feed, it presents certain challenges, including issues related to palatability, amino acid imbalances, and the presence of antinutritional compounds that could impede protein digestibility (Ribéreau et al., 2017). These concerns may be effectively addressed by subjecting PM to an extrusion treatment, which deactivates its antinutritional factors and enhances both palatability and functional properties (Ma et al., 2017; Ribéreau et al., 2017; Magbanua and Ragaza, 2022).

Shrimp protein hydrolysate (SH) emerges as a compelling dietary supplement in formulated diets featuring PM, given its capacity to supply essential amino acids and enhance palatability (Pereira et al., 2021). Dietary supplementation with SH in plant-based diets has demonstrated noteworthy benefits in terms of fish growth, feed utilization efficiency, digestibility, immunity, intestinal morphology, and disease resistance (Gisbert et al., 2018; Li et al., 2021). The processing of shrimp (*Pleoticus muelleri*) generates substantial head waste, which is typically discarded, contributing to adverse environmental consequences. These byproducts can be revalorized through the autolysis-based production of protein hydrolysates, which generally feature a favorable amino acid profile (Pereira et al., 2021).

Before introducing new ingredients as substitutes for soybean meal (SBM) in aquafeeds, it is essential to thoroughly assess their biochemical and nutritional properties. Accordingly, the primary objective of this study is to characterize two food industry waste-derived byproducts, namely PM and SH, for their potential utilization as alternative protein components in Nile tilapia feeds. Our approach commences with *in vitro* assays to assess the impact of extrusion treatment on PM protease inhibitors and digestibility. Subsequently, we delve into the characterization of SH, examining the nutritional quality of both ingredients. Finally, we investigate the preliminary effects of incorporating PM and SH into the diet as SBM substitutes on tilapia growth.

Materials and Methods

Tilapia enzyme extracts: A fish farm in Bolívar (Buenos Aires, Argentina) provided a viscera batch from *O. niloticus* juveniles. The stomachs and intestines of 24 fish weighing 21.1±3.02 g were removed from the visceral mass and placed on ice. For each organ, pools of 4 individuals were separately homogenized in ice-cold water (1:1 w/v) at pH 2 for stomach pools or pH 8 for intestine pools that were adjusted with 0.1 N HCl and 0.1 N NaOH, respectively. These preparations were centrifuged (10000 *g* for 30 min at 4°C), and the resulting supernatants were stored at -20°C as stomach enzyme extract (SE, n=6) and intestine enzyme extract (IE, n=6).

First, SE acid proteolytic activity was evaluated according to Anson (1938) using a substrate solution of 0.5% (w/v) bovine hemoglobin (Sigma H 2625) in a 200 mM glycine-HCl buffer. The reaction was incubated for 30 min at 25°C. The absorbance was measured at 280 nm using a microplate spectrophotometer with Gen5™ Software (Epoch BioTek). Total acid protease activity was expressed as activity unit per mL of enzyme extract (U/mL), where U = Abs 280 × mL total / 0.051 × min (0.051 represents the molar extinction coefficient of Tyrosine). Secondly, the methodology employed by García-Carreño (1992) and adapted by González-Zamorano et al. (2013) was used to determine the alkaline protease activity in IE. For this, 0.5% (w/v) azocasein (Sigma A 2765) in 50 mM Tris-HCl buffer at pH 8 was used as the substrate. The incubation was performed for 30 min at 25°C. The readings were performed using the microplate spectrophotometer, with the wavelength set at 366 nm. All assays were run in triplicate. Total protease activity was expressed as activity unit per mL of enzyme extract (U/mL). A unit of enzyme activity (U) was defined as a change in

absorbance per minute. Finally, to estimate specific activities, SE and IE soluble protein concentrations were determined according to Bradford (1976), using bovine serum albumin (Sigma A9647, St. Louis, MO) as the standard. Specific activity was expressed as the activity unit per mg of soluble protein (U/mg protein).

Protease inhibition assay: The inhibitory effect of PM on tilapia alkaline proteases was evaluated using the methodology of El-Sayed et al. (2000). For this, pea waste was provided by a regional producer (Buenos Aires, Argentina). The legume seeds were ground to obtain a meal (PM0). Next, a fraction of this PM was extruded at 150°C for 15 seconds using a hammer grinder model D5-50 (Nutriking, Tandil, Argentina) to inactivate the inhibition factors (PM150). Commercial FM of marine origin was obtained from Coomarpes Ltd. (Mar del Plata, Argentina) and used as a control treatment without inhibitors in the following protocol.

Prior to the inhibition assay, all meals were exposed to an *in vitro* simulation of tilapia stomach digestion. For this, 100 mg/mL of PM0, PM150, and FM were separately homogenized with 5 µL of SE and 955 µL of 200 mMGly-HCl buffer at pH 2. These mixtures were incubated for 60 min at 25°C. Then, they were centrifuged for 10 min at 2000 *g*. Subsequently, 5 µL of each supernatant was mixed with 250 µL of 50 mM Tris-HCl buffer at pH 8 for the alkaline protease inhibition assay. Immediately, 5 µL of IE was added to the reaction tubes, which were incubated for 60 min at 25°C under continuous agitation. Finally, alkaline protease activity was determined, as detailed in the previous section. To determine the inhibition rate, the alkaline proteolytic activity of IE in the absence of meals was set as 100%. All assays were run in triplicate.

In vitro digestibility of protein sources: The *in vitro* protein digestion of PM0, PM150, and FM was determined using a pH-stat method based on Yasumaru and Lemos (2014). Tilapia digestion includes an acid phase in the stomach, followed by an alkaline one in the intestine; thus, determining the hydrolysis degree in both conditions becomes essential. The assessments were conducted using a pH-meter (Orion Star A211) and an automatic titrator (TIM 856). SE (1:3 w/v) and IE (1:1 w/v) extracts were utilized for these assays.

A proper volume of distilled water, adjusted to pH 2 with 0.1 N HCl, was added to each protein substrate (PM0, PM150, or FM), resulting in a final solution of 80 mg of protein per mL. The proper amount of protein was estimated based on the proximal composition of each ingredient. Then, 130 µL SE was added to each solution and incubated at pH 2 and 25±0.2°C in a magnetic stirrer. After the acid digestion, the resulting solutions were adjusted to pH 8 by adding 0.1 N NaOH to simulate the alkaline protein digestion. Once the desired pH was reached, each solution was incubated with the IE (10 µL of enzyme per mL of solution). The solutions were constantly mixed in a magnetic stirrer and maintained at pH 8 and 25±0.2°C. The pH was stabilized by adding the titrant (0.1 N HCl or 0.1 N NaOH). These assays were carried out in triplicate.

The hydrolysis degree of protein (AcHD) exposed to stomach acid conditions was calculated based on the formula of Diermayr and Dehne (1990): $AcHD\ (\%) = [(V \times N)/E] \times (1/P) \times F_{pH} \times 100$. Where V is the volume of acid consumed in the hydrolysis reaction (mL); N represents the normality of such acid; E is the mass of substrate protein (g); P denotes the number of peptide bonds cleaved (mol/g protein), for proteins which amino acid composition is not determined, P is generally suggested as 8.0; F_{pH} is 1.08 (correction factor for pH 2.0 at 25°C). The alkaline hydrolysis (AkHD) estimated at intestinal conditions was calculated according to Adler Nissen (1986): $AkHD(\%) = B \times Nb \times 1/a \times 1/MP \times 1/H_{tot} \times 100$. In which B is the volume of alkali consumed (mL); Nb represents the normality of the alkali; α is the average degree of dissociation of the α-NH groups (1/α=1.50 for pH 8.0 at 25 °C); MP denotes the mass of substrate protein (g); and H_{tot} is a total number of peptide bonds in the protein substrate [7.6-9.2 meqv/g protein, according to the source of protein]. Finally, a total protein digestibility (TPD) was estimated for each meal (FM, PM0, and PM150) by the sum of the acid (AcHD) and alkaline (AkHD)

hydrolysis degree.

Preparation of shrimp hydrolysate: SH was elaborated through enzyme autolysis using *Pleoticus muelleri* processing waste, composed of cephalothoraxes, based on the method described by Leal-Goncaves et al. (2010). The raw material was ground in distilled water (1:1 v/v) and then submitted to digestion in a jacketed stirred reactor connected to a thermostatic bath at 45°C for 10 min. A sample was taken to determine alkaline protease activity as previously described, and afterward, enzyme deactivation was performed by raising the temperature (100°C, 10 min). The solid and liquid fractions were separated by centrifugation at 10000 *g* for 15 min. The obtained supernatant, defined as SH, was employed to estimate the hydrolysis degree (HD) and its antioxidant properties in the following section.

Shrimp hydrolysate characterization: The HD of SH was estimated according to the methodology of Baek and Cadwallader (1995). For this, 500 µL of HD was mixed with 1000 µL of 0.3M TCA, incubated at room temperature for 20 min, and then filtered. Twenty-five microliters of the resultant filtrate were mixed with 225 µL of distilled water, 1250 µL of 0.5N NaOH, and 250 µL of 1.0 N Folin and Ciocalteu's phenol reagent (Sigma F9252). The resulting solution was incubated at 30°C for 15 min and then centrifuged at 2000 *g* for 10 min. Supernatant absorbance was measured at 578 nm. A sample of SH was taken from the reactor prior to the hydrolysis reaction (initial time) and used as a blank for HD. The assay was run in triplicate. HD was defined as follows: $HD(\%) = (Abs_t - Abs_{t0}) / Abs_{max} \times 100$. Where: Abs_t represents the absorbance after ten minutes of hydrolysis, Abs_{t0} is the absorbance at zero time, and Abs_{max} is the maximum amount of 0.3 M TCA soluble peptides as tyrosine determined after the hydrolysis of 0.1 g shrimp substrate with 4 ml 6 N HCl at 110°C for 24 hr.

The DPPH radical scavenging activity by DH was evaluated using the method of Shimada et al. (1992). An SH sample of 1500 µL was added to 1500 µL of DPPH in 95% ethanol. The mixture was homogenized and incubated in the absence of light for 30 min. After standing, its absorbance was measured at 517 nm. The sequestration capacity of the DPPH free radical, defined as the scavenging effect (SCE), was calculated as follows: $SCE\% = (1 - (Abs_{sample} / Abs_{control})) \times 100$, where $Abs_{control}$ is the absorbance without sample and Abs_{sample} is the absorbance with sample.

Proximate and amino acid analyses: Feed ingredients and experimental diets (elaborated on 2.7 seccion) proximate compositions were determined at the "Laboratorio de análisis industriales" of the "Universidad Tecnológica Nacional" (UTN-FRMdP, Mar del Plata, Argentina) and at the "Instituto Nacional de Tecnología Industrial" (INTI, Mar del Plata, Argentina), as recommended by the Association of Official Analytical Chemists (AOAC, 2013). Amino acid profiles were performed at the "Laboratorio Fares Taie" (Mar del Plata, Argentina) using the DAD/FLD HPLC method, according to AOAC (1997).

Experimental diets: Two isonitrogenous (33% crude protein) and isocaloric (378 kcal/100 g) diets, referred to as the control diet and PM150+SH diet, were formulated to feed *O. niloticus* juveniles (Table 1). The ingredients were mixed in a laboratory blender, and the resulting dough was pelleted using a manual pelleting machine with a 2mm die. Then, the pelleted feed was oven-dried at 55°C. Feed formulae were designed according to the nutritional requirements of this species (NRC, 2011; FAO, 2022). The control-fed ingredients were selected based on a formulation routinely used by the "Laboratorio de Acuicultura" (UTN-FRMdP) for Nile tilapia production. The PM150+SH diet was formulated using linear programming in Excel Solver from Windows 2010, in which SBM was replaced with 10% SH and 25% PM150. According to Leal-Goncaves et al. (2010), liquid SH was mixed with PM150, and the dough was dried at 65°C for 24 h. Table 1 shows the percentual formulation and proximate composition of the formulated feeds.

Fish and experimental conditions: All experimental work involving fish was revised and approved by the Institutional Animal Welfare and Ethical Review Committee at Universidad Nacional de Mar del Plata

Table 1. Diet formulation and proximate composition of the formulated feeds.

	Formulated feeds	
Ingredients[1] (%)	Control	PM150+SH
Pea meal	-	25
Shrimp hydrolysate	-	10
Soybean meal	16.05	-
Fish meal	20	20
Wheat gluten	13.07	13.07
Cornmeal	6.93	6.93
Vegetable oil	3	3
Cornstarch	7	7
Vitamins and minerals premix[2]	1	1
Wheat bran	32.95	14
Proximal composition (% of dry matter basis)		
Moisture	6	8.7
Ash	7.1	6.5
Lipids	8.5	5.6
Protein	32.8	34.5
Carbohydrates	45.6	44.7
Gross energy (Kcal/100g)	390.1	367.2

[1]The nutritional composition of the experimental diets was calculated by linear programming using Excel Solver from Windows 2010. [2]Each kilogram of premix contains the following vitamins and minerals: molybdenum 240 mg; thiamine 163 mg; riboflavin 156 mg; pyridoxine 213 mg; calcium pantothenate 250 mg; biotin 250 mg; niacin 500 mg; folic acid 25 mg; B12Hcl 20 mg; Rovimix STAY C 781 ascorbic acid mg; menadione 240 mg; inositol 300 mg; chlorinated choline 200 mg; α tocopherol acetate 1500 mg wheat semolina csp; calcium 1000mg; magnesium 500 mg; potassium 99 mg; zinc 30 mg; iron 10 mg; copper 2mg; iodine 150 µg; selenium 200 µg; molybdenum 500 µg.

(UNMdP, CICUAL 6-2041/19RD 378). Sex-reversed Nile tilapia (n = 56, mean weight = 7.28±0.186 g) were obtained from the "Laboratorio de Acuicultura" (UTN-FRMdP). Groups of seven fish were stocked in each of eight 70-L plastic tanks (60 × 35 × 25 cm, width × length × height). These tanks were connected to mechanical and biological filtration through a recirculating system, and the water flow in each tank was 1 L min^{-1} $tank^{-1}$. After 7 days of acclimatization, the PM150+SH and control diet were randomly assigned to four tanks.

Fish were fed the experimental diets for 40 days at a feeding rate of 5% of the aquaria's biomass in three equal rations. The daily feed was adjusted weekly by batch weighing in a water container after a 24-hour deprivation period. Although no significant feed scrap was observed, the aquaria were siphoned once daily and submitted to a 50% water replacement due to feces accumulation and to maintain water quality. Throughout the experiment, a 10:14 light: dark cycle was used; the temperature was maintained at 28±1°C, while the pH was 7.5±0.15. The water temperature and pH were measured daily, while ammonia-nitrogen, nitrite-nitrogen, and nitrate-nitrogen were measured once per week. All these water parameters were within the acceptable range for Nile tilapia culturing (Boyd and Tucker, 2012). To monitor growth, fish were weighed and measured before and after the onset of the feeding trial. In the end, fish were sacrificed, and their livers and gastrointestinal tracts (both counted as viscera) were excised and weighed.

Growth performance: Growth performance and organosomatic indexes were assessed by weight gain (WG), specific growth rate (SGR), specific total large rate (SLTR), condition factor (K), hepatosomatic index (HSI), and viscero-somatic index (VSI). Calculations were carried out using the following formulae: WG = 100 × [Final body weight – Initial body weight] / Initial body weight; SGR = 100 × [ln(Final body weight) – ln(Initial body weight)] / Days of feeding trial; STLR = 100 × [ln(Final total large) – ln(Initial total large)] / Days of feeding trial;

Table 2. Proximate composition and amino acid profile of extruded pea meal and shrimp protein hydrolysate (g/100g sample).

Sample	PM150		SH	
Moisture	7.5		91.7	
	Wb	Db	Wb	Db
Ash	6.4	6.9	0.9	10.8
Lipids	5	5.4	0.4	4.8
Protein	33.4	36.1	7.3	88
Carbohydrates	47.7	51.6	19.5	234.9
Energetic value[1]	369.4		N/D	
Essential amino acids (%)				
Arginine	1.5		1.8	
Histidine	5.4		5.7	
Isoleucine	0.2		0.3	
Leucine	0.3		0.3	
Lysine	2.7		3.2	
Methionine	0.3		0.3	
Phenylalanine	3.6		2.6	
Threonine	4.1		8.6	
Tryptophan	0.3		0.0	
Valine	0.3		0.5	

Wb, wet basis; Db, dry basis; [1]Kcal/100g; N/D Data not determined

$K = 100 \times [\text{Final body weight (g)} / \text{Total length (cm)}^3]$; HSI = 100 × [Liver weight (g) / Body weight (g)]; VSI = 100 × [Visceral weight (g) / Body weight (g)].

Statistical analysis: Data sets were presented as mean ± standard error (SE). Statistical analysis was carried out using NCSS10 software for Windows. Data were checked for normality and variance homogeneity using Shapiro-Wilks and Levene tests. When the assumptions were met, the data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine significant differences. If data violated these conditions, a Kruskal-Wallis test was used. Differences between treatments were then determined using a Mann-Whitney U test. The protease inhibition assay data were analyzed differently using a student's t-test. Differences are reported as statistically significant when $P<0.05$.

Results

Protease inhibition assay: Prior to the *in vitro* assays, total and specific acid protease activities were 36.5±7.66 U/mL and 6.4±1.33 U/mg protein for SE, while the alkaline ones that were determined in the IE showed values of 9.0±0.73 U/mL and 0.4±0.03 U/mg protein. A low protease inhibition was observed when IE was incubated with either PM0 or PM150. However, PM0 showed a significantly higher inhibition percentage (5.4±2.31%) compared to PM150 (1.9±0.95%), and as expected, FM did not affect the activities of the enzyme extracts.

In vitro protein digestion assay: The results obtained from the *in vitro* digestion assay of the different ingredients (PM0, PM150, and FM) are displayed in Figure 1. The results revealed that FM (control treatment) presented a significantly higher TPD than PM0, while no significant difference was found between FM and PM150. Considering the results obtained through the *in vitro* assays, PM150 was selected for its supplementation with SH to evaluate the effects of their inclusion in tilapia-formulated feeds.

Shrimp protein hydrolysate characteristics: Autolysis of shrimp heads presented a proteolytic activity of 0.2±0.01 U/mL and an HD of 9.7±2.25%. In addition, the SH showed 41.6±8.74% of DPPH radical scavenging activity.

Proximate composition and amino acid profile: The proximate composition of PM150 and SH are illustrated in Table 2. The crude protein content of PM150 was found to be 33.4 g/100 g (36.1 g/100 g on a dry weight basis), while SH contained 7.3 g/100 g (88.0 g/100 g on a dry weight basis). The amino acid profiles of these feed ingredients are shown in Table

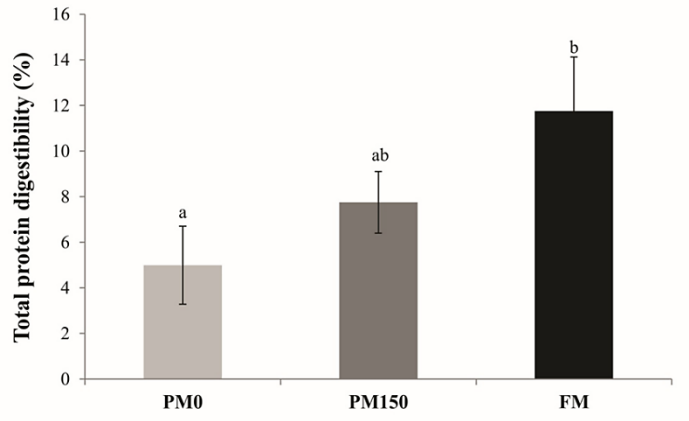

Figure 1. Total protein digestibility of FM, PM0, and PM150. Means with different letters (a-b) indicate significant differences between the treatments (P˂0.05). Error bars represent the standard error values. PM0, raw pea meal; PM 150, extruded pea meal; FM, fishmeal.

2. In addition to the data presented in Table 2, a high content of non-essential amino acids flavor enhancers was observed in SH, such as Glutamic acid (37.8%), Aspartic acid (26.1%), Serine (10.5%), and Threonine (8.6%).

Formulated feed characteristics: A balanced diet including 25% PM150 and 10% SH was obtained, with SBM completely replaced (Table 1). The feed formulated displayed that both ingredients provided good content of essential amino acids (1.7% Arginine, 2.5% Histidine, 1.1% Isoleucine, 2.7% Leucine, 2.3% Lysine, 0.7% Methionine, 2.3% Phenylalanine, 2.8% Threonine, 0.3% Tryptophan, 1.3% Valine) managing to meet tilapia nutritional requirements. However, the dietary content of Valine was slightly below the value established by NRC (2011).

Growth performance: The fish fed actively on both experimental diets, and mortality during the trial was very low and not affected by dietary treatments. At the end of the trial, the two groups had no differences in growth performance. The replacement of SBM by PM150+SH in the tilapia-formulated feed did not significantly affect weight gain, specific growth rate, specific total large rate, condition factor, or organosomatic indexes (Table 3).

Table 3. Growth performance of juvenile tilapia after 40 days of feeding a control diet and a diet containing recycled ingredients (PM150+SH) in total replacement of soybean meal.

Parameter	Diet	
	Control	SH+PM150
IW[1] (g)	7.57±0.237	7.00±0.225
FW[2] (g)	11.52±0.388	10.99±0.616
ITL[3](cm)	7.71±0.102	7.48±0.106
FTL[4](cm)	9.23±0.102	8.95±0.163
WG[5](%)	3.61±0.340	3.99±0.460
SGR[6](% day^{-1})	0.98±0.103	1.12±0.091
STLR[7]	0.45±0.039	0.45±0.016
K[8]	1.49±0.040	1.54±0.082
HSI[9] (%)	0.69±0.231	1.06±0.282
VSI[10] (%)	6.23±0.589	6.6±0.518

Values are presented as mean ± SE. [1] IW, Initial weight; [2] FW, Final weight (g); [3] ITL, Initial total length (cm); [4] FTL, Final total length (cm); [5] WG, Weight gain; [6] SGR, Specific growth rate; [7] STLR, Specific total large rate; [8] K, Condition factor; [9] HSI, Hepatosomatic index; [10] VSI, viserosomatic index.

Discussions

The present study aims to identify sustainable ingredients to replace SBM, which has been widely used in the aquafeed industry but has seen a recent increase in global consumption and price in the international market. We examined two protein sources — PM and SH — due to their potential to provide a valuable source of nutrients for fish, in addition to improving waste disposal. Interestingly, the protease inhibition assay revealed that incubation of tilapia enzymes with PM0 and PM150 resulted in a low reduction of protease activity compared to the control extracts. However, PM extrusion significantly reduced its inhibition effects on tilapia proteases (from 5.4 to 1.9%). These results are in agreement with Ma et al. (2017) and Wang et al. (2003) findings which showed a reduction of up to 84% in PM trypsin inhibitory activity after heat treatment exposure. It is encouraging to compare our results with those of El-Sayed et al. (2000), who found that tilapia proteases were strongly inhibited (between 60-80%) when exposed to raw and heat-treated SBM. With respect to the *in vitro* digestion assay, the most notable finding was that PM0 had a negative effect on digestibility, while PM150 managed to achieve the digestibility obtained with FM. These results were in accordance with those reported previously by other authors (Diermayr and Dehne, 1990; Ma et al., 2017; Qi et al., 2021), who found that heat treatments – including the extrusion process - positively affected the *in vitro* digestion of PM. A possible explanation is that the

reduction of antinutritional compounds and partial denaturation of pea proteins caused by extrusion conditions make them more bioavailable (Qi et al., 2021).

Considering the results obtained through the *in vitro* assays, PM150 was selected and supplemented with SH with the aim of evaluating the effects of its inclusion in diets for tilapia. We proposed the SH elaboration using a simple and low-cost method (autolysis for just 10 min) without using commercial enzymes, which are very expensive. At the end of the hydrolysis reaction, this protein hydrolysate reached an HD of 9.7±2.25%. Previous studies have shown that limited hydrolysis (low HD) could be associated with improvements in the functional properties of the protein hydrolysates (Gbogouri et al., 2004). Also, the SH showed 41.6±8.74% of DPPH radical scavenging activity. The high antioxidant power of SH may be explained by the intrinsic free radical scavenging capacity of the substrate used - shrimp tissues and enzymes - as well as the release of peptides with an antioxidant effect (Pereira et al., 2021). This result agrees with the findings of Pereira et al. (2021), who reported a DPPH radical scavenging activity of 63.06% in *P. muelleri* hydrolysate by autolysis. Other previous works that determined the *in vitro* antioxidant effect of marine protein hydrolysates have also shown that their inclusion in the diet could improve the activity of the SOD enzyme and the antioxidant defense system of fish (Javaherdoust et al., 2020).

Regarding chemical analysis, the PM150 protein content was 33.44%, whereas protein values reported in previous works ranged from 19% to 35% (Ma et al., 2017; Magbanua and Ragaza, 2022). The lipid value was low and comparable to the findings of other authors (Ma et al., 2017; Magbanua and Ragaza, 2022). The protein content of SH was found to be 87.95% on a dry weight basis, while the dry basis protein values reported by other authors were between 55 and 91% (Ruttanapornvareesakul et al., 2005; Cao et al., 2009). The amino acid profile of PM150 revealed that it had high values of some essential amino acids, such as histidine, Lysine, Phenylalanine, and Threonine, but low content of others, such as Isoleucine, Leucine, Methionine, and Valine. This amino acid imbalance is consistent with other studies (Schulz et al., 2007), which reported limited amounts of Lysine and Methionine in PM0. On the other hand, in agreement with Pereira et al. (2021), a high content of amino acids flavor enhancers was observed in SH, such as Glutamic acid, Aspartic acid, Serine, and Threonine.

The formulated PM150+SH diet successfully met the nutritional requirements of tilapia, which is known to require an energy value of 3036 kcal/kg, 5-8% lipids, and 30-35% protein (FAO, 2022). Also, the proposed formulation supplied most of the essential amino acids necessary for tilapia nutrition. However, the valine content in the diet (1.3%) was lower than the necessary amount to meet the nutritional requirements of the species (1.5%) established by the NRC (2011). Nevertheless, more recently, Xiao et al. (2017) indicated that juvenile tilapia require between 1.15 and 1.27% of dietary valine for optimal growth, which may explain why the PM150+SH diet did not have any negative effects on tilapia growth.

There are only a few studies that evaluated pea proteins as an ingredient for tilapia aquafeeds (Magbanua and Ragaza, 2022). Moreover, the available studies have only focused on replacing FM, while the replacement of SBM has not been studied yet. It has been demonstrated that dietary inclusion levels of PM around 10-50% did not impair tilapia performance (Magbanua and Ragaza, 2022). Abushweka (2018) found that, even though the dietary inclusion of PM had the highest food conversion and protein efficiency index compared to other tested protein ingredients, its low palatability negatively affected tilapia growth. Besides its low palatability, another factor that could reduce fish growth may be the amino acid imbalance. Schulz et al. (2007) included up to 15% pea protein isolate in diets for tilapia without impairing its growth response, but they found a significantly decreased growth performance at higher inclusion levels. These authors related this result to the lysine and methionine deficiency in PM. Therefore, we proposed evaluating the

supplementation of PM with SH, an ingredient that could enhance the amino acid profile and palatability of tilapia aquafeed. However, even though it has been demonstrated that SH presents a high content of amino acids related to an improvement in palatability, it is necessary to evaluate feed consumption in experimental diets to test its flavor-enhancing effects.

In recent years, many studies have shown positive results in the total or partial replacement of SBM with various plant ingredients in the diets of different fish species, including tilapia (Fadel et al., 2017; Pradhan et al., 2020; Khieokhajonkhet et al., 2021). In this research, the results of the preliminary feeding trial indicate that PM150 and SH could be incorporated — in replacement of SBM — into a feed for tilapia juveniles without affecting their growth. These findings are in line with those of Egerton et al. (2020), who showed that supplementation of a high plant-based diet with 10% fish protein hydrolysate allowed fish to grow as well as fish fed the control diet. However, further experimental investigations are needed to assess the long-term *in vivo* effects of PM150+SH dietary inclusion on tilapia physiology, productive yields, and diet digestibility. Additionally, further research using controlled trials is necessary to determine the effects of PM150 and SH separately or in varying combination levels on tilapia performance.

Conclusions

This study evaluated PM and SH as alternative feed ingredients for tilapia juveniles in the replacement of SBM. First, the results revealed that PM extrusion significantly reduced the *in vitro* inhibitory effect on tilapia proteases. Second, *in vitro* protein digestion of PM150, performed by tilapia acid and alkaline proteases, did not differ from the control treatment with FM. Third, SH exhibited antioxidant properties and served as a valuable source of essential amino acids for the tilapia diet. Finally, the results indicated that tilapia could be nourished effectively with a well-balanced diet in which PM150 and SH entirely replaced SBM. In conclusion, this study demonstrates that cost-effective and sustainable ingredients derived from agricultural and marine waste sources could be successfully employed for tilapia nutrition.

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