Potential of agricultural and fishery wastes as sustainable feed ingredients for Nile tilapia (Oreochromis niloticus)

Clara Liebana (claraliebana166@gmail.com)
Instituto de Investigaciones Marinas y Costeras (IIMyC), UNMdP-CONICET
Yamila Eliana Rodriguez
Instituto de Investigaciones Marinas y Costeras (IIMyC), UNMdP-CONICET
Analía Verónica Fernández-Gimenez
Instituto de Investigaciones Marinas y Costeras (IIMyC), UNMdP-CONICET
Federico Alcides Cecchi
Universidad Tecnológica Nacional (UTN), Facultad Regional Mar del Plata
Nair de los Ángeles Pereira
Instituto de Investigaciones Marinas y Costeras (IIMyC), UNMdP-CONICET

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Abstract

Soybean meal (SBM) is the most widely employed vegetable ingredient in aquafeeds, but its rising price and the negative environmental impacts of its cultivation open research efforts for its replacement. More sustainable aquaculture may be achieved by utilizing agricultural and fishery by-products; thus, this study aims to incorporate pea meal (PM) and shrimp protein hydrolysate (SH) as protein ingredients in tilapia juveniles' feed. The presence of protease inhibitors and the *in vitro* digestion were evaluated for raw (PM0) and extruded pea meals (PM150), while SH characteristics were studied as well. The nutritional quality of alternative ingredients was assessed to formulate a diet that meets tilapia juveniles' requirements, and its effects on growth were evaluated. Extrusion significantly reduced the PM inhibitory effect on tilapia proteases (from 5.4–1.9%). *In vitro* protein digestion of PM150 (7.8 ± 1.35%) did not differ from the control treatment with fish meal (FM; 11.8 ± 1.94%). Additionally, SH presented 41.6 ± 2.91% of DPPH radical scavenging activity. Tilapias fed with a diet where SBM was totally replaced by the dietary inclusion of 25% PM150 and 10% SH, presented comparable growth indicators to those exposed to the control diet. Extrusion reduced PM proteolytic inhibitory effect and improved protein digestibility. SH presented antioxidant effect, as well as, provided flavor enhancers and essential amino acids to the diet. It is feasible to achieve a sustainable dietary formulation for juvenile tilapia using the evaluated by-products without affecting its growth performance.

1. Introduction

Aquaculture is the fastest-growing farmed food sector and will soon become the main fish and shellfish source for human consumption. The over-demand and rising prices of fish meal (FM), a primary dietary protein source of aquafeeds, have generated the need to find alternative protein sources (El-Sayed et al. 2000; Khieokhajonkhet et al. 2021). This demand was mostly covered by soybean meal (SBM), which has become the most widely used protein source to replace FM for finfish diets (Khieokhajonkhet et al. 2021). However, the high consumption of SBM caused a dramatic price jump from $190 per metric ton to over $601 per metric ton from 2002 to 2022 (IndexMundi 2022). Thus, after FM, SBM has become the most expensive ingredient in fish feed (Pradhan et al. 2020). Also, its cultivation is related to an increase in the carbon footprint because of its importation from producing countries to consuming countries (Tallentire et al. 2018). Furthermore, SBM has some nutritional problems given to its amino acid imbalance and its high content of antinutritional factors, which limit nutrients utilization (Qi et al. 2021). Therefore, it is vital to explore appropriate protein sources that promote SBM replacement.

Many potential alternative ingredients from animal and plant origin can be used in aquafeeds. Particularly, pea *Pisum sativum* is commonly used in agricultural countries as a food source and is considered one of the most important legumes in the world (Magbanua and Ragaza 2022). Pea meal (PM) produced by broken is considered a by-product of low commercial value and is rarely used in human diets. However, is a good protein source because it has low fat, is gluten-free, and is rich in minerals and vitamins (Ma et al. 2017). Tilapia *Oreochromis niloticus*, one of the most farmed fish in the world, can tolerate high levels of plant dietary protein due to their omnivorous habits (Schulz et al. 2007).

Although PM is a potential protein source for tilapia feed, it presents some disadvantages, such as low palatability, amino acid imbalance, and the presence of antinutritional compounds that could affect protein digestibility (Ribéreau et al. 2017). This could be solved by exposing PM to an extrusion treatment, that deactivates its antinutritional factors and, in second place, improves its palatability and functional properties (Ma et al. 2017; Magbanua and Ragaza 2022; Ribéreau et al. 2017). Shrimp protein hydrolysate (SH) represents an interesting feed supplement in formulated diets containing PM, as it can provide essential amino acid and enhances palatability (Pereira et al. 2021). Dietary supplementation of plant-based diets with SH has shown improvements in fish growth, feed utilization performances, digestibility, immunity, intestine morphology, and disease resistance (Gisbert et al. 2018; Li et al. 2021). Shrimp *Pleoticus muelleri p*rocessing generates tons of head wastes, which are usually discarded causing negative environmental effects (Pereira et al. 2021). These residues can be revalued through the elaboration of protein hydrolysates by autolysis, producing a protein ingredient that usually has a good amino acid profile (Pereira et al. 2021).

This study aims to formulate a balanced feed, that covers *O. niloticus* juveniles' nutritional requirements, by replacing SBM with PM and SH, both ingredients recovered from food processing wastes. Through *in vitro* assays, we first evaluated extrusion treatment effects on PM protease inhibitors and digestibility. Subsequently, SH characteristics and the effects of PM and SH dietary inclusion on tilapia performance by a feeding trial were evaluated.

2. Materials And Methods

2.1 Tilapia enzyme extracts

A fish farm from 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 as a substrate solution 0.5% (w/v) bovine hemoglobin (Sigma H 2625) in 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 carried out by García-Carreño (1992) and adapted by González-Zamorano et al. (2013) was employed 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 in the microplate spectrophotometer setting the wavelength 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) where bovine serum albumin (Sigma A9647, St. Louis, MO) was used as the standard. Specific activity was expressed as the activity unit per mg of soluble protein (U/mg protein).

2.2 Protease inhibition assay

The inhibitory effect of PM on tilapia alkaline proteases was evaluated following an adaptation of El-Sayed et al. (2000) methodology. For this, pea *Pisum sativum* waste was provided by a regional producer (Buenos Aires, Argentina). The legume seeds were grinded to obtain a meal (PM0). One fraction of this PM was next extruded at 150°C for 15 sec with the aim of inactivating the inhibition factors (PM150). Commercial FM was obtained from Coomarpes Ltda. (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, for the alkaline protease inhibition assay, 5 µL of each supernatant was mixed with 250 µL of 50 mM Tris-HCl buffer at pH 8. Immediately, 5 µL of IE was added to the reaction tubes that were next incubated for 60 min at 25°C under continuous agitation. Finally, alkaline protease activity was determined as detailed in section 2.1. To determine the inhibition rate, the alkaline proteolytic activity of IE in absence of meals was set as 100%. All assays were run in triplicate.

2.3 In vitro digestibility of the 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) methodology. Tilapia digestion includes an acid phase in the stomach, followed by an alkaline one carried out at the intestine level; thus, determining the hydrolysis degree in both conditions becomes essential. The assessments were carried out 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), obtaining 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 Ml SE were added to every solution, and then, 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 the addition of 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 the addition of 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 proposed by 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; and F_{pH} is 1.08 (correction factor for pH 2.0 at 25°C). On the other hand, 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 [7.6–9.2meqv/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.

2.4 Preparation of shrimp hydrolysate

SH was elaborated through enzyme autolysis using *Pleoticus muelleri* processing waste, composed of cephalothoraces, based on the method described by Leal-Goncaves et al. (2010). The raw material was grounded in distilled water (1:1 v/v) and next submitted to digestion in a jacketed stirred reactor connected to a thermostated 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.

2.5 Shrimp hydrolysate characterization

The HD of SH was estimated according to the methodology proposed by Baek and Cadwallader (1995). For this, 500 µL of HD were mixed with 1000 µL of 0.3M TCA, incubated at room temperature for 20 min and next 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 30ºC 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 hydrolysis reaction (initial time), and used as 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 by 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 scavenging effect (SE), was calculated as follows: $SE\% = (1 - (Abs_{sample} / Abs_{control})) \times 100$. Where $Abs_{control}$ is the absorbance without sample and Abs_{sample} is the absorbance with sample.

2.6 Proximate and amino acid analyses

PM150 and SH 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 conducted at the “Laboratorio Fares Taie” (Mar del Plata, Argentina).

2.7 Experimental diets

Two isonitrogenous (33% crude protein) and isocaloric (378 kcal/100 g) diets, referred as Control diet and PM150 + SH diet, were formulated to feed *Oreochromis niloticus* juveniles. Feed formulae were designed according to the nutritional requirements defined for this species by FAO (2022) and NRC (2011). The control fed ingredients were selected based on a formulation routinely used by the “Laboratorio de Acuicultura” (UTN-FRMdP) for Nile tilapia production. PM150 + SH diet was formulated by linear programming using Excel Solver from Windows 2010, in which SBM was totally replaced by 10% of SH and 25% of 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 porcentual formulation and proximate composition of the formulated feeds.

Table 1
Diet formulation and proximate composition of the formulated feeds

	Formulated feeds	
Ingredients (%)	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	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

Table 1

2.8 Fish and experimental conditions

All experimental work involving fish was revised and approved by the Institutional Animal Welfare & Ethical Review Committee at “Universidad Nacional de Mar del Plata” (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) equipped with continuous aeration and a biologic filter. 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 aquaria biomass in three equal rations. Daily feed was adjusted on a weekly basis by batch weighing in a water container following a 24 h deprivation period. Although no significant feed scrap was seen, the aquaria were siphoned once daily and submitted to a 50% water replacement due to feces accumulation and keep water quality. The water temperature was maintained at 28 ± 1ºC and pH 7.5 ± 0.15; additionally, the photoperiod used was a 10 h light/14 h dark cycle. 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 (both counted as viscera) were excised and weighed.

2.9 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; K = 100 × [Final body weight (g)/Total length (cm)^3]; HSI = 100 × [Liver weight (g)/Body weight (g)]; VSI = 100 × [Visceral weight (g)/Body weight (g)].*

2.10 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 assumptions were met, data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's multicomparison 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. Just protease inhibition assay data were analyzed differently through a Student's t-test. Differences are reported as statistically significant when P 0.05.

3. Results

3.1 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 ± 0.03U/mg protein. A low protease inhibition was observed when IE were 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 enzyme extracts activities.

3.3 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 Fig. 1. This experiment 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 with the aim to evaluate the effects of their inclusion in tilapia formulated feeds.

Figura 1

3.3 Shrimp protein hydrolysate characteristics

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

3.4 Proximate composition and amino acid profile

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/100g (88.0 g/100 g on a dry weight basis). The amino acid profiles of these feed ingredients are shown in Table 3. In addition to the data presented in Table 3, the SH presented high quantities of not essential amino acids that are flavor enhancers, such as Glutamic acid (37.8%), Aspartic acid (26.1%), Serine (10.5%), and Threonine (8.6%).

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

Sample	Moisture		Ash	Lipids	Protein	Carbohydrates	Energetic value[1]
PM150	7.5	Wb	6.4	5	33.4	47.7	369.4
		Db	6.9	5.4	36.1	51.6	
SH	91.7	Wb	0.9	0.4	7.3	19.5	N/D
		Db	10.8	4.8	88	234.9	

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

Table 3
Amino acid profile of extruded pea meal and shrimp protein hydrolysate.

Essential amino acid (%)	PM150	SH
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

Table 2

Table 3

3.5 Formulated feed characteristics

A balanced diet including 25% PM150 and 10% SH was obtained, where SBM was 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).

3.6 Growth performance

The fish fed actively on both experimental diets, as well as, mortality during the trial was very low and not affected by dietary treatments. At the end of the trial, there were no differences in growth performance between both groups. 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 4).

Table 4
Growth performance of juvenile tilapia after 40 days of feeding control diet and 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, Final 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.

Table 4

4. Discussion

The present study seeks to identify sustainable ingredients to replace SBM, which has been widely used in the aquafeed industry, but in recent years has increased its global consumption and its price in the international market. We examined two protein sources — PM and SH — because of its potential to ameliorate waste disposal besides providing a valuable source of nutrients for fish. Interestingly, the protease inhibition assay revealed that incubation of tilapia enzymes with PM0 and PM150 resulted in a low reduction of the protease activity measured in the control extracts. However, it was observed that PM extrusion significantly reduced its inhibition effects on tilapia proteases (from 5.4–1.9%). 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 they were exposed to raw and heat-treated SBM. With respect to the *in vitro*

digestion assay, the most interesting finding was that PM0 had a negative effect on the 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 for this might be that the reduction of antinutritional compounds and the partial denaturation of pea proteins caused by extrusion conditions make them more bioavailable (Qi et al. 2021).

1. Considering the results obtained through the *in vitro* assays, PM150 was selected and supplemented with SH with the aim to evaluate the effects of its inclusion in diets for tilapia. We proposed the SH elaboration by 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 antioxidant effect (Pereira et al. 2021). This result agrees with the findings of Pereira et al. (2021) who found a DPPH radical scavenging activity of 63.06% in *P. muelleri* hydrolysate by autolysis. Regarding chemical analysis, PM150 protein content was 33.44%, while the protein values reported by previous works were between 19 to 35% (Ma et al. 2017; Magbanua and Ragaza 2022). The lipid value obtained 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 among 55 and 91% (Cao et al. 2009; Ruttanapornvareesakul et al. 2005). The amino acid profile of PM150 revealed that it had high values of some essential amino acids like Histidine, Lysine, Phenylalanine, and Threonine, but low content of others, such as Isoleucine, Leucine, Methionine, and Valine. This amino acid imbalance is in accordance with other works (Schulz et al. 2007) who reported limited amounts of lysine and methionine in PM0. On the other hand, in agreement with Pereira et al. (2021), high content of flavor enhancers was observed in SH, such as Glutamic acid, Aspartic acid, Serine, and Threonine. Therefore, SH could be acting as a flavor enhancer, reducing the negative effects of the low palatability of PM150.

The formulated PM150 + SH diet managed to meet the nutritional requirements of tilapia, which it´s known to require 3036 kcal/kg of energy value, 5 to 8% of lipids, and 30–35% of proteins (Wang et al. 2003). 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 necessary to satisfy the nutritional requirements of the species (1.5%) established by the NRC (2011). Nevertheless, more recently, Xiao et al. (2017) indicated that juvenile tilapia requires between 1.15 and 1.27% of dietary valine for optimal growth and maybe this could explain why the PM150 + SH diet didn't 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 the replacement of

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% of pea protein isolate in diets for tilapia without impairments in its growth response, but they found a significant decreasing growth performance at higher inclusion levels.

These authors related this result to the lysine and methionine deficiency in PM. Because of that, we proposed to evaluate the supplementation of PM with SH, an ingredient that can improve the palatability and the amino acid profile of the tilapia aquafeeds.

In recent years, many studies have shown positive results in the total or partial replacement of SBM with various plant ingredients in the diet of different fish species, including tilapia (Fadel et al. 2017; Khieokhajonkhet et al. 2021; Pradhan et al. 2020). In this research, the results of the feeding trial indicate that PM150 and SH can be incorporated (in replacement of SBM) into a feed for tilapia juveniles without affecting their performance. These findings are in line with those of Egerton et al. (2020), who showed that supplementation of a high plant-based diet with 10% of fish protein hydrolysate allowed fish to grow as well as fish fed the control diet. Further experimental investigations are neede12/26/2022d to assess the long-term in vivo effects of PM150 + SH dietary inclusion on tilapia physiology and productive yields. Also, more research using controlled trials is needed to determine the effects of PM150 and SH separately on tilapia performance.

This study set out to evaluate the replacement of SBM with PM and SH as alternative feed ingredients for juvenile tilapia. First, the results revealed that PM extrusion significantly reduced the inhibitory effect on tilapia proteases. Second, *in vitro* protein digestion of PM150, which was performed by tilapia acid and alkaline proteases, did not differ from the control treatment with FM. third, SH presented a high antioxidant effect and provided flavor enhancers and essential amino acids to the tilapia-formulated feed. Finally, tilapias could be fed with a balanced diet where SBM was completely replaced by PM150 and SH. In conclusion, the replacement of SBM with more economical and sustainable ingredients, from agricultural and marine waste sources, could be successfully employed for tilapia nutrition.

Declarations

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Statements & Declarations

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Author contributions Conceived and designed the experiments: CL NAP YER AVFG. Performed the experiments: CL NAP YER FA. Analyzed the data: CL NAP YER. Contributed reagents /materials /analysis tools: AVFG FA. Wrote the paper: CL NAP YER.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Ethics approval All the experiments involving fish was revised and approved by the Institutional Animal Welfare & Ethical Review Committee at “Universidad Nacional de Mar del Plata” (UNMdP, CICUAL 6-2041/19RD 378).

Consent to participate All authors consent to participate.

Consent for publication All authors approved the submitted version of this manuscript.

Conflict of interest The authors declare no competing interests.

References

1. Abushweka AAM (2018) Alternative protein sources as a replacement of fish meal in tilapia feeds. Int J Nat Soc Sci **6**:77–90.
2. Adler Nissen J (1986) Enzymic hydrolysis of food proteins. Elsevier appl sci. https://doi.org/http://dx.doi.org/10.1016/0308-8146(87)90169-5
3. Anson ML (1938) The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J Gen Physiol 22(1): 79–89. https://doi.org/10.1085/jgp.22.1.79
4. AOAC (2013) Official Methods of Analysis 938.08. Fish and marine products. Ash of seafood. In *Association of Official Analytical Chemists*. Washington, DC.
5. Baek HH, Cadwallader KR (1995) Enzymatic hydrolysis of crayfish processing by-products. J Food Sci 60(5): 929–935. https://doi.org/10.1111/j.1365-2621.1995.tb06264.x
6. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein– dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3
7. Cao W, Zhang C, Hong P, Ji H, Hao J, Zhang J (2009) Autolysis of shrimp head by gradual temperature and nutritional quality of the resulting hydrolysate. Food Sci Technol 42(1): 244–249.

https://doi.org/10.1016/j.lwt.2008.05.026

8. Diermayr P, Dehne L (1990) Kontrollierte enzymatische proteinhydrolyse im bereich niedriger pH-werte. Z Lebensm Unters Forsch 190(6): 516–520. https://doi.org/10.1007/BF01214073
9. Egerton S, Wan A, Murphy K, Collins F, Ahern G, Sugrue I, Stanton C (2020) Replacing fishmeal with plant protein in Atlantic salmon (*Salmo salar*) diets by supplementation with fish protein hydrolysate. Sci Rep 10(1): 1-16. https://doi.org/10.1038/s41598-020-60325-7
10. El-Sayed AF, Nmartinez I, Moyano FJ (2000) Assessment of the effect of plant inhibitors on digestive proteases of Nile tilapia using *in vitro* assays. Aquac Int 8(5): 403-415. https://doi.org/10.1016/S0044-8486(99)00159-3
11. Fadel AHI, Kamarudin MS, Romano N, Ebrahimi M, Saad CR, Samsudin AA (2017) Carob seed germ meal as a partial soybean meal replacement in the diets of red hybrid tilapia. Egypt J Aquat Res 43(4): 337–343. https://doi.org/10.1016/j.ejar.2017.09.007
12. FAO (2022) Nile Tilapia: Nutritional requirements. Retrieved December 1, 2022, from http://www.fao.org/fishery/affris/species-profiles/nile-tilapia/nutritional-requirements/en/
13. García-Carreño FL (1992) The digestive proteases of langostilla (*Pleuronco desplanipes*, decapoda): their partial characterization, and the effect of feed on their composition. Comp Biochem Physiol B Biochem 103(3): 575–578. https://doi.org/10.1006/abio.1993.1457
14. Gbogouri GA, Linder M, Fanni J, Parmentier M (2004) Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. J Food Sci 69(8): C615–C622. https://doi.org/10.1111/j.1365-2621.2004.tb09909.x
15. Gisbert E, Fournier V, Solovyev M, Skalli A, Andree KB (2018) Diets containing shrimp protein hydrolysates provided protection to European sea bass (*Dicentrarchus labrax*) affected by a *Vibrio Pelagius* natural infection outbreak. Aquaculture 495:136–143. https://doi.org/10.1016/j.aquaculture.2018.04.051
16. González-Zamorano M, Navarrete del Toro MA, García-Carreño FL (2013) Exogenous proteinases as feed supplement for shrimp: *in vitro* evaluation. Aquac Nutr 19(5): 731–740 https://doi.org/10.1111/anu.12020
17. IndexMundi, 2022. Index Mundi Commodity Prices. Retrieved from https://www.indexmundi.com/commodities/?commodity=soybean-meal&months=240 (accessed 3 August 2022).
18. Khieokhajonkhet A, Muichanta S, Aeksiri N, Ruttarattanamongkol K, Rojtinnakorn J, Kaneko G (2021) Evaluation of sacha inchi meal as a novel alternative plant protein ingredient for red hybrid tilapia (*Oreochromis niloticus× O. mossambicus*): growth performance, feed utilization, blood biochemistry, and histological changes. Anim Feed Sci Technol 278: 115004. https://doi.org/10.1016/j.anifeedsci.2021.115004
19. Leal ALG, de Castro PF, de Lima JPV, de Souza Correia E, de Souza Bezerra R (2010) Use of shrimp protein hydrolysate in Nile tilapia (*Oreochromis niloticus, L.*) feeds. Aquac Int 18(4): 635–646. https://doi.org/10.1007/s10499-009-9284-0

20. Li S, Dai M, Qiu H, Chen N (2021) Effects of fishmeal replacement with composite mixture of shrimp hydrolysate and plant proteins on growth performance, feed utilization, and target of rapamycin pathway in largemouth bass, *Micropterus salmoides*. Aquaculture 533: 736185. https://doi.org/10.1016/j.aquaculture.2020.736185
21. Ma Z, Boye JI, Xinzhong H (2017) *In vitro* digestibility, protein composition and techno-functional properties of Saskatchewan grown yellow field peas (*Pisum sativum L.*) as affected by processing. Int Food Res 92: 64–78. https://doi.org/10.1016/j.foodres.2016.12.012
22. Magbanua TO, Ragaza JA (2022) Selected dietary plant-based proteins for growth and health response of Nile tilapia *Oreochromis niloticus*. Aquac Fish. https://doi.org/10.1016/j.aaf.2022.04.001
23. NRC (2011) Nutrient requirements of fish and shrimp. The National Academies Press, Washington, DC, 9(3): 220–228.
24. Pereira NDLÁ, Fangio MF, Rodriguez YE, Bonadero MC, Harán NS, Fernández-Gimenez AV (2021) Characterization of liquid protein hydrolysates shrimp industry waste: Analysis of antioxidant and microbiological activity, and shelf life of final product. J Food Process Preserv 00:e15526. https://doi.org/10.1111/jfpp.15526
25. Pradhan C, Divi BG, Dileep N, Peter N, Sankar TV (2020) Replacement of soya bean meal with cashew nut meal as an alternative protein source in the diet of tilapia, *Oreochromis mossambicus*. Aquac Res 51(4): 1660-1672. https://doi.org/10.1111/are.14512
26. Qi M, Zhang G, Ren Z, He Z, Peng H, Zhang D, Ma C (2021) Impact of extrusion temperature on *in vitro* digestibility and pasting properties of pea flour. Plant Foods Hum Nutr 76: 26–30. https://doi.org/10.1007/s11130-020-00869-1
27. Ribéreau S, Aryee ANA, Tanvier S, Han J, Boye JI (2017) Composition, digestibility, and functional properties of yellow pea as affected by processing. J Food Process Preserv 42: 1–9. https://doi.org/10.1111/jfpp.13375
28. Ruttanapornvareesakul Y, Ikeda M, Hara K, Osako K, Kongpun O, Nozaki Y (2005) Effect of shrimp head protein hydrolysates on the state of water and denaturation of fish myofibrils during dehydration. Fish Sci 71: 220–228. https://doi.org/10.1111/j.1444-2906.2005.00951.x
29. Schulz C, Wickert M, Kijora C, Ogunji J, Rennert B (2007) Evaluation of pea protein isolate as alternative protein source in diets for juvenile tilapia (*Oreochromis niloticus*). Aquac Res 38:537–545. https://doi.org/10.1111/j.1365-2109.2007.01699.x
30. Shimada K, Fujikawa K, Yahara K, Nakamura T (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 40(6): 945–948. https://doi.org/10.1021/jf00018a005
31. Tallentire CW, Mackenzie SG, Kyriazakis I (2018) Can novel ingredients replace soybeans and reduce the environmental burdens of European livestock systems in the future? J Clean Prod 187: 338–347. https://doi.org/10.1016/j.jclepro.2018.03.212

32. Wang N, Daun JK, Malcolmson LJ (2003) Relationship between physicochemical and cooking properties, and effects of cooking on antinutrients, of yellow field peas (*Pisum sativum*). J Sci Food Agric 1237:1228–1237. https://doi.org/10.1002/jsfa.1531
33. Xiao W, Li DY, Zhu JL, Zou ZY,Yang H (2017) Dietary valine requirement of juvenile Nile tilapia, *Oreochromis niloticus*. Aquac Nutr 24:315–323. https://doi.org/10.1111/anu.12562
34. Yasumaru F, Lemos D (2014) Species specific *in vitro* protein digestion (pH-stat) for fi sh: method development and application for juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*). Aquaculture 426: 74–84. https://doi.org/10.1016/j.aquaculture.2014.01.012

Figures

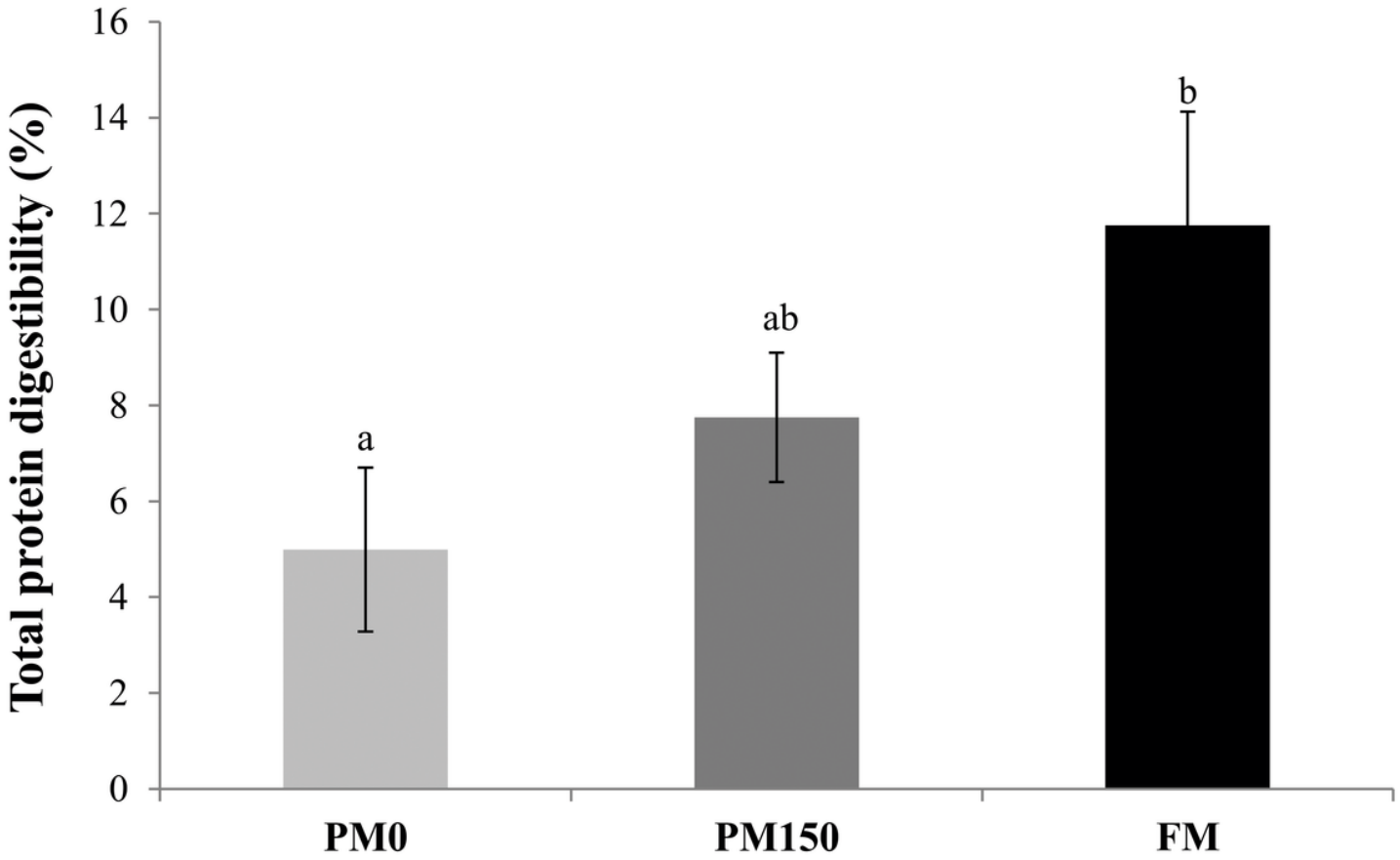

Figure 1

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