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A new approach to fishery waste revalorization to enhance Nile tilapia (*Oreochromis niloticus*) digestion process

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Abstract

The main goal of this research was to analyse in vitro compatibility of Nile tilapia (*Oreochromis niloticus*) digestive proteinases and enzymes recovered from species comprising fishery waste as *Pleoticus muelleri*, *Artemesia longinaris* and *Patagonotothen ramsayi*. It evaluated the use of exogenous enzymes as feed supplements to increase digestion efficiency in tilapia fingerlings and juveniles (3.5 ± 0.11 g and 11.6 ± 1.5 g, respectively). We successfully have obtained proteinases from fishery waste as source of exogenous enzymes. *P. muelleri* and *A. longinaris* enzymes had more activity of acid and alkaline proteinases than *P. ramsayi*. SDS-PAGE gels demonstrated that Nile tilapia digestive proteinases keep their activity when combined with each exogenous proteinase. Exogenous enzymes varied in their ability to enhance hydrolysis of different feed ingredients. *P. muelleri* by-products are the best candidates to be employed as feed supplements for tilapia juveniles. Enzymes from this by-product did not affect the activity and integrity of fish digestive enzymes, improved the hydrolysis of different protein sources (fish meal, squid meal, shrimp meal and soybean meal), and maintained its activity after being exposed to high temperatures and acid pHs. Our findings are applicable to other places where *O. niloticus* is raised utilizing local fishery waste, and also to different cultured species.

KEYWORDS

digestive proteinase, exogenous enzyme, feed supplement, fishery by-product, fishery discard, *Oreochromis niloticus*

1 | INTRODUCTION

Fisheries play an important role in many human societies. Nowadays, an integrated and sustainable exploitation of fishery resources is a must as only half of the catch is used for human consumption. Therefore, fisheries' management is facing severe environmental problems worldwide because not all that is obtained from the oceans is adequately exploited. Some issues include waste management as big amounts of by-products (heads, exoskeletons, bones, guts, skin, etcetera) are generated by fish processing industry (Ferraro et al., 2010), and also a huge number of non-target species are continuously captured (known as bycatch) for then discarded (Góngora, Bovcon, & Cochia, 2009). These large quantities of waste create serious pollution and disposal problems in both developed and developing countries (Kristinsson, 2006) so its proper utilization should become a priority for many countries (Penven, Perez-Galvez, & Bergé, 2013). Fortunately, fishery waste contains valuable proteins, lipids, polysaccharides, minerals and other bioactive compounds holding exclusive features, some of which are a result of specific environmental factors prevailing in the oceans (Shahidi, 1997). For example, proteases of marine organisms have a diversity of catalytic characteristics as activity and/or stability over wide domains of pH and temperature, besides certain substrate specificities (Blanco, Sotelo, Chapela, & Pérez-Martín, 2007). Therefore, fishery discards and by-products can be exploited as a basis to derive other products like human supplements and biochemical, plant fertilizers, aquaculture ingredients and supplements, livestock feeds, industrial

ingredients, among others (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011).

In Southwest Atlantic waters, fishery industry represents an important economic activity where hake *Merluccius hubbsi* (Merlucciidae) and red shrimp *Pleoticus muelleri* (Solenoceridae) are some of the most exploited species (Gongora, Gonzalez Zevallos, Pettovello, & Mendia, 2012). During the first semester of 2016, hake and red shrimp total landings were 112,753 and 35,002.7 tons, respectively (MINAGRI, www.minagri.gob.ar/site/pesca/pesca_maritima/02- desembarques/lectura.php?imp=1&tabla=especie_flota_2016). Like many others around the world, both fisheries produce enormous volumes of bycatch discards (including species as *Artemesia longinaris* (Penaeidae), *Patagonotothen ramsayi* (Nototheniidae) and even *M. hubbsi* and *P. muelleri*) and by-products from their processing (Bovcon, Góngora, Marinao, & González-Zevallos, 2013; Kandra, Challa, & Jyothi, 2012). Previous research has reported the presence of proteolytic activity in enzyme extracts of *P. muelleri* and *A. longinaris* (Fernández Gimenez, García-Carreño, Navarrete del Toro, & Fenucci, 2001; Fernández Gimenez, García-Carreo, Navarrete del Toro, & Fenucci, 2002). These enzymes show catalytic properties and functional characteristics which makes them valuable targets for a wide variety of biotechnological applications.

Nile tilapia (*Oreochromis niloticus*, Cichlidae) are tropical cichlid teleosts native to Africa and the Middle East (Trewavas, 1983). They are the second most farmed fish worldwide, and their production has quadrupled over the past decade due to ease of aquaculture, marketability and stable market prices (Wang & Lu, 2015). Digestive enzymes are among the most prevailing factors influencing food utilization in fish (Jun-sheng, Jian-lin, & Ting-ting, 2006). Organisms ingest their food as large polymeric compounds that need to be broken down and where proteases carry out an important role owing to their ability to degrade proteins (Murado, González, & Vázquez, 2009). However, to date, no organism has been found in which protein digestion is fully efficient, and this varies according to the protein source supplied (Sacristán et al., 2015; Smith, Lee, Lawrence, & Strawn, 1985; Xiang-He, Ji-Dan, Jiang-Hong, & Kun, 2013). As nutrition is the most expensive factor in the aquaculture industry (representing over 50% of operating costs), a proper feeding management become an essential tool for successful tilapia culture practices (El-Sayed, 2006).

In recent years, the use of exogenous enzymes has gained attention due to their capability to increase the effectiveness of digestion by helping to break down antinutritional factors (e.g., phytase) or by improving the digestibility of many ingredients (Mireles-Arriaga, Espinosa-Ayala, Hernández-García, & Márquez-Molina, 2015). According to some recent studies with Nile tilapia, supply exogenous enzymes within the diet improves animal performance by enhancing nutrient digestibility (Adeoye, Jaramillo-Torres, Fox, Merrifield, & Davies, 2016; Li, Li, & Wu, 2009; Lin, Mai, & Tan, 2007). However, these studies worked with commercial exogenous enzymes, which are generally expensive and designed for farmed animals. Therefore, proteinases extracted from marine by-products and discards could be goods of great interest, and using them in aquaculture production could be a promising strategy to improve Nile tilapia digestibility and feed efficiency.

Additionally, distribution and concentration of intestinal enzymes differ with intestinal morphology and feeding habits. Changes in intestinal morphology and physiology are more evident in early stages of development, and thus, fish capability to use feedstuff varies at different growth stages (Jun-sheng et al., 2006; Tengjaroenkul, Smith, Caceci, & Smith, 2000). The purpose of this study was to evaluate the in vitro interaction between exogenous multi-enzyme extracts obtained from fisheries by-products (*Pleoticus muelleri* and *Artemesia longinaris*) and discards (*Patagonotothen ramsayi*) with enzymes of *Oreochromis niloticus* fingerlings and juveniles. In addition, stability of exogenous enzymes and their contribution to the hydrolysis of different raw ingredients were estimated to assess their potential as nutritional supplement sources for Nile tilapia.

2 | MATERIAL AND METHODS

All investigation complied with the Mar del Plata National University Animal Welfare & Ethical Review Committee (RD 200/15).

2.1 | Exogenous proteinases feed supplements

Samples of two local fishery by-products (*Pleoticus muelleri* (*Pm*) and *Artemesia longinaris* (*Al*)) were provided by fishing trawlers and manufacturing plants from Mar del Plata, Argentina (38° 02′ S, 57° 30′ W). *Patagonotothen ramsayi* (*Pr*) samples were taken from bycatch of local hake fisheries. All samples were frozen on board and transported to the laboratory. After thawing, the cephalothorax of ten *P. muelleri* (*Pm*) and ten *A. longinaris* (*Al*) and the head and digestive system of five *P. ramsayi* (*Pr*) were removed. Then, using a glass-Teflon tissue homogenizer, each sample was separately crushed in ice-cold 100 mM Glycine-HCl pH 3 and 100 mM Tris-HCl pH 8 buffers containing 150 mM NaCl, to maintain appropriate conditions for neutral and alkaline proteinases, respectively. The resulting material was centrifuged at 10,000 *g* for 30 min at 4°C (Presvac EPF 12R). The supernatants (exogenous crude enzyme extracts) were frozen at −20°C and stored until used.

2.2 | Digestive proteinases of tilapia

Forty Nile tilapia *Oreochromis niloticus* were obtained from a local producer (Buenos Aires, Argentina). The fish were divided into two growing stages: fingerlings and juveniles, with average body weights being 3.5 ± 0.11 g and 11.6 ± 1.5 g, respectively. Immediately upon collection (24 hr after feeding), fish were anesthetized on ice, and killed. Body cavity of each fish was opened, and the entire alimentary tract was quickly removed and placed on ice. Next, stomach and intestine were dissected and homogenized (30 mg tissue/ml) in ice-cold 100 mM Glycine-HCl pH 3 and 100 mM Tris-HCl pH 8 buffers, respectively, containing 150 mM NaCl, using a tissue homogenizer. The resulting preparations were centrifuged at 10,000 *g* for 30 min at 4°C. Supernatants (crude enzyme extracts) were frozen at −20°C and used in further assays (Santos et al., 2013). Each stomach enzyme extract of

fingerlings (*OnF*) and juveniles (*OnJ*) was used in all assays carried out in acid conditions (pH 3), while their intestine enzyme extracts were employed in alkaline condition assays (pH 8).

2.3 | Determination of protein content

Soluble protein content was determined according to Bradford (1976) on *OnF* and *OnJ* crude enzyme extracts and also on exogenous enzyme extracts obtained from *Pm*, *Al* and *Pr*. Bovine serum albumin (Sigma A9647) was employed as the standard.

2.4 | Enzymatic activity

Acid proteinase activity at pH 3 was determined in a substrate solution containing 0.5% (w/v) bovine haemoglobin (Sigma H2625) in 200 mM Glycine-HCl buffer according to the method originally described by Anson (1938) and then modified by Celis-Guerrero, García-Carreño, and Navarrete Del Toro (2004). One millilitre of the substrate solution was mixed in a reaction tube with 20 µl of each enzyme extract (*OnF*, *OnJ*, *Pm*, *Al*, *Pr*) and then incubated for 10 min at 25°C. The reaction was stopped by adding 500 µl of 20% (w/v) trichloroacetic acid (TCA) and cooling on ice for 10 min. Then, tubes were centrifuged for 5 min at 10,000 *g* and absorbance of the supernatants was measured at 280 nm against distilled water using a Diode Array Spectrophotometer (Shimadzu UV-2102). For blanks, TCA solution was added before the substrate was supplied. All assays were run in triplicate. Total proteinase activity was expressed as units per ml of enzyme extract (U/ml = Abs 280 × ml total/0.051 × min × ml enzyme, where 0.051 represents the molar extinction coefficient of Tyrosine).

Alkaline proteinase activity at pH 8 was assayed using 0.5% (w/v) azocasein (Sigma A 2765) as substrate in 50 mM Tris-HCl buffer, according to García-Carreño (1992). Five microlitres of each enzyme extract was mixed with 250 µl of 50 mM Tris-HCl buffer. Substrate solution (250 µl) was added, and the mixture was next incubated for 20 min at 25°C. The reaction was stopped by the adding 250 µl of 20% (w/v) TCA and cooling on ice for 5 min. Then, tubes were centrifuged for 5 min at 10,000 *g* and absorbance of the supernatants was measured at 440 nm against distilled water in an Epoch BioTek Microplate Spectrophotometer (Gen5™ Software). For blanks, TCA solution was added before the substrate was supplied. All assays were run in triplicate. Total proteinase activity was expressed as change in absorbance per min per ml of enzyme extract (U/ml = Abs 440/min × ml).

2.5 | Activity staining

Activity staining was used to detect proteolytic activity once SDS-PAGE electrophoresis was performed. Method of García-Carreño, Dimes, and Haard (1993) was employed to assess tilapia alkaline proteinases activity in presence of exogenous enzymes, while for acid proteinases, the activity was determined according to an adaptation of Cardenas-Lopez and Haard (2009) method. First, 125 mU/ml of each enzymatic extracts (*OnF*, *OnJ*, *Pm*, *Al*, *Pr*) and their mixtures (*OnF* + *Pm*, *OnF* + *Al*, *OnF* + *Pr*, *OnJ* + *Pm*, *OnJ* + *Al*, *OnJ* + *Pr*) were incubated in proper buffer solutions during 30 min at 25°C. Subsequently, 5 mU/ml from each enzymatic extract or mixture was combined with 1:1 (v:v) 2X sample buffer. Each sample and 6 µl molecular weight protein standard (Sigma SDS7) were loaded on a minivertical gel electrophoresis device (Mini protean tetra cell Bio-Rad). A 12% SDS-PAGE gel was employed for alkaline proteinases, while for acid proteinases, the same gel was used but containing 0.1% casein. Electrophoresis was performed under 30 mA per gel with a constant power supply unit at 4°C. Next, gels for alkaline proteinases were allowed to hydrolyse 3% w/v casein (Sigma C7078), whereas gels containing casein for acid proteinases were washed with cold water, soaked in 1% Triton X for 15 min (two times) in ice, then soaked for 30 min in iced 100 mM sodium phosphate buffer pH 7 containing 1 mM EDTA. The casein gels were washed in cold water later and incubated in 100 mM sodium phosphate buffer pH 6 at 55°C for 2 hr. Finally, both gels were first washed with distilled water and then immersed in a solution containing 40% methanol, 7% acetic acid and 0.05% Coomassie brilliant blue R-250. Following a staining period of 24 hr, gels were destained with a similar solution but without the Coomassie dye.

2.6 | Hydrolysis of protein substrates

To evaluate possible synergistic cooperation between proteinases from stomach and intestine of tilapia (*OnF* and *OnJ*) and exogenous enzymes, we evaluated the hydrolysis of haemoglobin and azocasein by each enzymatic extract and their mixtures. Acid and alkaline activities were determined according to the protocol explained in previous section, using an appropriate volume of each enzyme so as to contain 50 mU/ml of activity. Afterwards, the volume was completed up to 500 µl with 200 mM Glycine-HCl buffer pH 3 for acid proteinases or 50 mM Tris-HCl buffer pH 8 for alkaline enzymes. Total proteinase activity (acid and alkaline) was calculated as indicated previously. Hydrolysis displayed by Nile tilapia enzymes was considered as 100% for the analysis.

2.7 | Integrity of alkaline enzymes exposed to stomach conditions

Alkaline exogenous enzymes need to go through tilapia stomach conditions to reach the intestine where they perform their action. Owing that, in this research, we have evaluated the effects of stomach environment on the integrity of each exogenous enzyme extract. Fifty mU/ml of each enzyme extract (*Pm*, *Al* or *Pr*) was separately added to proper buffer solutions (200 mM Glycine-HCl pH 3 for treatments simulating stomach conditions and 50 mM Tris-HCl pH 7 for control assays). Assay procedure is schematized in Figure 1 to simulate the Nile tilapia cycle of acid secretion proposed by Moriarty (1973). In the treatments, pH was manually dropped with a solution of 0.1 N HCl, while control treatments were always run at pH 7. Aliquots of 10 µl were taken at each time mentioned in Figure 1 and then added to 250 µl of buffer Tris-HCl. Next, all samples were incubated at pH 7 for 1 hr. After that, proteinase alkaline activity was assayed as previously

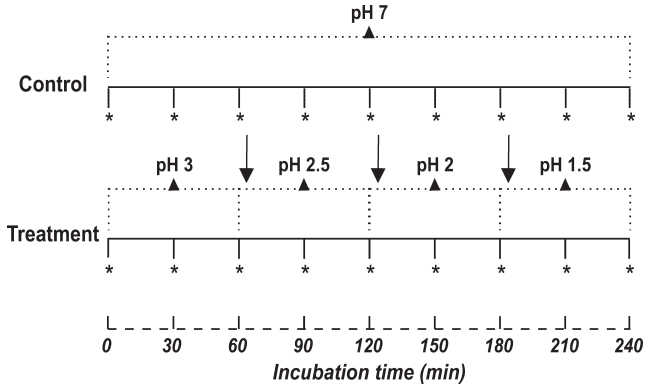

FIGURE 1 Assay procedure to evaluate the integrity of alkaline exogenous enzymes exposed to Nile tilapia stomach conditions. Fifty mU/ml of enzyme extract *Pm*, *Al* or *Pr* was separately added to 200 mM Glycine-HCl pH 3 buffer solution for treatments and to 50 mM Tris-HCl pH 7 buffer solution for control assays. (*) Aliquots of 10 µl were subsequently taken at different incubation time to being then incubated at pH 7. Arrows indicate the moment when pH was manually dropped with 0.1 N HCl in treatments

described. Treatments were run in triplicate and control treatments in duplicate. Temperature for all the assays was 29 ± 1°C simulating usual environment conditions where Nile tilapia habits.

2.8 | Hydrolysis of feed ingredients

If proteinases extracted from fishery waste are intended to improve feed digestion, it will be essential to determine the hydrolysis degree of the protein fraction comprising tilapia's feedstuff. Hence, it was necessary to evaluate which kind of ingredient would result in better hydrolysed to be employed in future feed formulations alongside the proper exogenous enzyme. Four meal types were used to assess their hydrolysis by Nile tilapia enzymes and when combined with each exogenous enzyme. Two commercial ingredients usually employed for Nile tilapia feed formulations were evaluated: fish meal (Salerno®, Argentina) and soybean meal (Bunge®, Argentina). Additionally, two raw ingredients were self-elaborated in the laboratory using alternative protein sources obtained from local fishing fleet. For this, viscera, head and tentacles of *Illex argentinus* and cephalothoraxes of *P. muelleri* were dried separately in an oven-dried at 60°C and then ground to a fine powder, obtaining the meals used in following assays. These raw ingredients and meals were chosen based on their potential as cheap and easily available protein sources.

With the purpose of determining protein content of ingredient soluble part, 1 g of each meal was incubated over 1 hr with 5 ml of distilled water. These incubation tubes were constantly shaken using an Orbital Shaker (TS-2000 A Química Fenix Argentina). When incubation time concluded, tubes were centrifuged for 15 min at 10,000 *g* and supernatants were kept to measure soluble protein concentration by Biuret test, according to Ohnishi and Barr (1978) procedure. For this, 1 ml of Biuret reagent ($C_2H_5N_3O_2$) was added into the supernatants. After waiting 30 min, the absorbance was read at 550 nm against distilled water.

An adaptation of Chong, Hashim, and Ali (2002) methodology was used to assess ingredient hydrolysis by stomach and intestine enzymes of *OnF* and *OnJ* in presence of exogenous enzymes preparations (*Pm*, *Al* and *Pr*). To simulate stomach or intestine conditions, 200 mM Glycine-HCl at pH 3 or 50 mM Tris-HCl at pH 7 was used, respectively. Maximum pH variation of ingredient-enzyme incubation mixture was employed as the criteria to evaluate hydrolysis degree of each ingredient against haemoglobin or casein hydrolysis, for acid or alkaline proteinases, respectively. Three treatments were carried out: (i) ingredient + Nile tilapia enzymes; (ii) ingredient + Nile tilapia enzymes + exogenous enzymes; (iii) ingredient. The last one was developed to discard the occurrence of ingredient auto-hydrolysis. A solution with proper buffer was prepared with each ingredient (10 mg protein per ml) and then wagged for 1 hr in an Orbital Shaker. Then, tubes were centrifuged for 15 min at 10,000 *g* and supernatants were mixed with a volume of enzyme extract containing 50 mU/ml of activity, or a mixture of Nile tilapia enzymes and exogenous enzymes containing 50 mU/ml of each one. Afterwards, incubation of substrate-enzyme mixture was carried out in a water bath at 29 ± 1°C. The magnitude of the pH variation was measured at different incubation times 0, 10, 20, 30, 45, 60 and 90 min. Assays were run in triplicate. Changes in pH values of the ingredients incubated without enzyme addition were used as control. The *Relative protein hydrolysis* (*RPH*) for each ingredient was calculated. The equation can be written as: $RPH = [(I_{max}\,\Delta pH/IT_{max})/(S_{max}\,\Delta pH/ST_{max})] * 100$. Where: $I_{max}\,\Delta pH$, Maximum variation of pH employing ingredient as source of protein; IT_{max}, Time when the maximum pH variation was recorded employing ingredient; $S_{max}\,\Delta pH$, Maximum variation of pH employing casein or haemoglobin as source of protein; ST_{max}, Time when the maximum pH variation was recorded employing casein or haemoglobin.

2.9 | Temperature stability of exogenous enzymes

Exogenous enzymes need to be stable at some temperatures to withstand formulated feed drying processes. In this study, stability of exogenous enzymes (*Pm*, *Al* and *Pr*) at two drying temperatures during a 7 hr period was evaluated. Samples were placed into a digital dry bath (Numak MiniT) and incubated at 40°C and 60°C. Subsamples of 25 µl were taken at intervals of 1, 2.5, 4, 5.5 and 7 hr. After incubation, proteinase activities at pH 3 and 8 were assayed as explained in previous section. Assays were run in triplicate. Control treatments of all exogenous enzymes were those at initial time without incubation and were set to 100%.

2.10 | Statistics

Data sets were presented as mean and standard deviation (*SD*). After testing data normality and homogeneity of variance, differences among data sets were analysed by ANOVA followed by a Tukey's multicomparison test. Differences are reported as statistically significant when $p < .05$. Arc sine transformation was applied to percentages (Sokal & Rohlf, 1995). Analyses were made using NCSS 8 Software.

3 | RESULTS

3.1 | Protein content and proteinase activity

Soluble protein contents did not show significant differences among the enzyme extracts studied ($p > .05$); values were between 2.78 and 4.69 mg/ml (Table 1). Proteinase activities are illustrated in Table 1 as well. Acid proteinase activity was significantly lower for *Pr* extracts compared with the other ones, while alkaline proteinase activities were higher for tilapia intestine extracts in comparison with all fishery waste enzyme extracts (*Pm*, *Al* and *Pr*) ($p < .05$) (Table 1). No significant differences were found between the two stages of Nile tilapia studied ($p > .05$) (Table 1).

TABLE 1 Soluble protein and enzyme activity of protein extracts recovered from stomach and intestine of *Oreochromis niloticus* and enzyme extracts from different fishery wastes (*Pleoticus muelleri*, *Artemesia longinaris* and *Patagonotothen ramsayi*)

	SP (mg/ml)	AP (U/ml)	AkP (U/ml)
Tilapia			
Fingerlings			
S	2.78 ± 0.475[a]	10.69 ± 0.333[b]	–
I	3.39 ± 0.735[a]	–	1.11 ± 0.022[d]
Juveniles			
S	3.11 ± 0.532[a]	9.64 ± 1.846[b]	–
I	3.33 ± 0.694[a]	–	1.16 ± 0.102[d]
Exogenous enzymes			
Pm	3.75 ± 0.337[a]	9.99 ± 0.857[b]	0.59 ± 0.021[c]
Al	3.67 ± 0.434[a]	12.53 ± 2.315[b]	0.34 ± 0.011[b]
Pr	4.69 ± 0.767[a]	3.69 ± 1.022[a]	0.07 ± 0.007[a]

Values are means and standard deviation of three replicates. Means within the same columns with different superscripts (a–d) are significantly different ($p < .05$).

SP, protein soluble; AP, acid proteinase activity; AkP, alkaline proteinase activity; S, stomach; I, intestine; Pm, *Pleoticus muelleri*; Al, *Artemesia longinaris*; Pr, *Patagonotothen ramsayi*.

3.2 | Activity staining

Protein extracts from *Pm* and *Al* displayed significant acid proteinase activity, while *Pr* just evidenced a few weak bands (Figure 2). When extracts of tilapia fingerlings and exogenous enzymes were incubated together, all *OnF* bands remained active. Additionally, all acid proteinases from *Pm* and *Al* and only one from *Pr* kept their activity. Conversely, tilapia juveniles evidenced just one active acid proteinase band, which was absent just when this extract was incubated with *Al* enzymes (Figure 2).

On the other hand, alkaline zymogram of tilapia intestine enzymes, fishery waste enzymes and their combinations is shown in Figure 3. Nile tilapia fingerlings evidenced five active alkaline proteinases, while the juveniles only showed three active bands. Similarly, exogenous enzymes (*Pm* and *Al*) possessed several alkaline proteinases as previously described by Fernández Gimenez et al. (2001, 2002). When *Pm* and *Al* enzymes were aggregated to the crude enzyme extract, all bands from *OnF* and *OnJ* remained active (Figure 3). In addition, the five staining bands from the red shrimp and three from *A. longinaris* continued actives when they were mixed with both stages of tilapia enzyme extracts. However, we could not confirm alkaline proteinase activity in *P. ramsayi* by SDS-PAGE.

3.3 | Hydrolysis of haemoglobin and azocasein

Substrate hydrolysis performed by Nile tilapia enzymes was considered as 100% for the analysis (Figure 4). Hydrolysis of haemoglobin and azocasein was not significantly increased when exogenous enzymes were added to both enzyme extracts of tilapia ($p > .05$) (Figure 4).

3.4 | Integrity of alkaline enzymes exposed to stomach conditions

The effect that tilapia's stomach enzymes and acid conditions have on the integrity of alkaline exogenous proteinases was evaluated. Enzyme extracts obtained from the three fishery wastes kept their activity at least 150 min under acidic pH conditions (Figure 5). In both

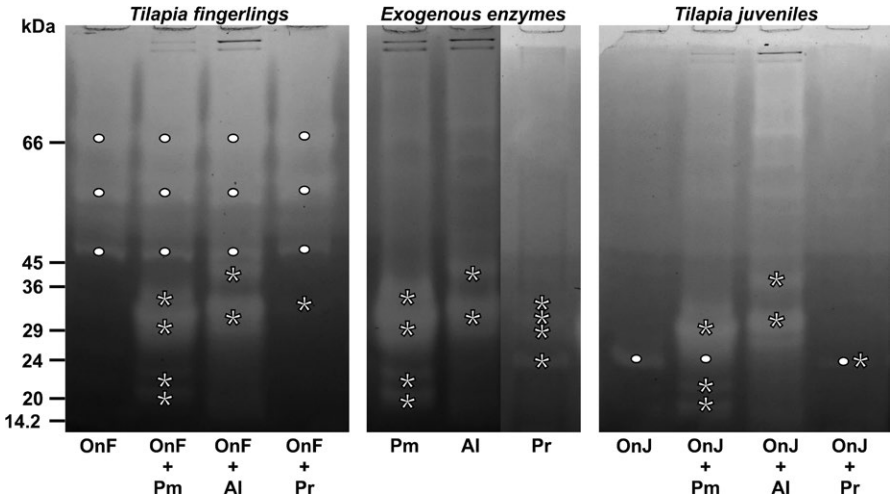

FIGURE 2 Zymogram of enzyme extracts and their mixtures at pH 3. (o) Nile tilapia activity bands. (*) Exogenous enzymes activity bands. OnF, stomach enzyme extract of *Oreochromis niloticus* fingerlings; OnJ, stomach enzyme extract of *O. niloticus* juveniles; Pm, *Pleoticus muelleri* cephalothorax enzyme extract; Al, *Artemesia longinaris* cephalothorax enzyme extract; Pr, *Patagonotothen ramsayi* head and viscera enzyme extract

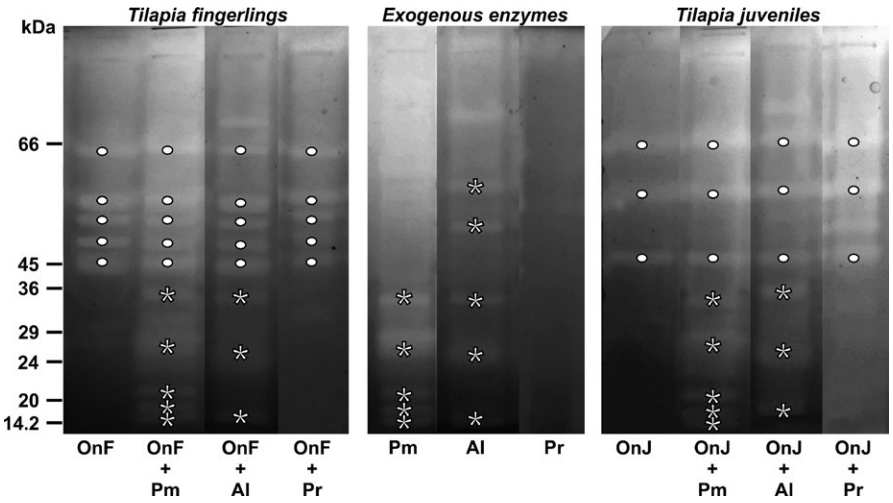

FIGURE 3 Zymogram of enzyme extracts and their mixtures at pH 8. (o) Tilapia activity bands. (*) Exogenous enzymes activity bands. OnF, intestinal enzyme extract of *Oreochromis niloticus* fingerlings; OnJ, intestinal enzyme extract of *O. niloticus* juveniles; Pm, *Pleoticus muelleri* cephalothorax enzyme extract; Al, *Artemesia longinaris* cephalothorax enzyme extract; Pr, *Patagonotothen ramsayi* head and viscera enzyme extract

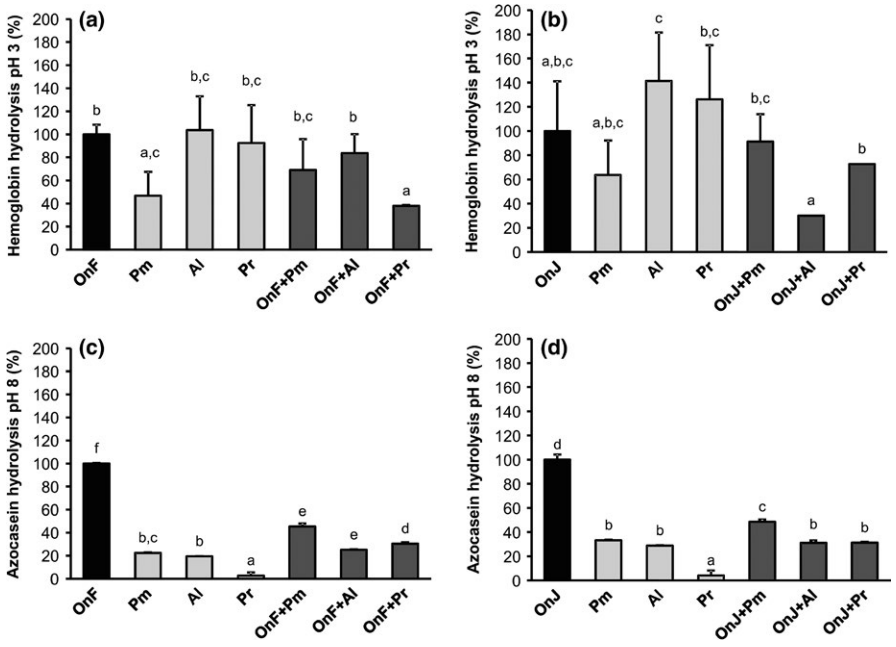

FIGURE 4 Substrate hydrolysis percentages displayed by digestive enzymes and their mixtures at pH 3 and 8. (a) Haemoglobin hydrolysis at pH 3 of Nile tilapia fingerlings, exogenous enzymes and its mixtures. (b) Haemoglobin hydrolysis at pH 3 of Nile tilapia juveniles, exogenous enzymes and its mixtures. (c) Azocasein hydrolysis at pH 8 of Nile tilapia fingerlings, exogenous enzymes and its mixtures. (d) Azocasein hydrolysis at pH 8 of tilapia juveniles, exogenous enzymes and its mixtures. Means within the same graphic with different superscripts are significantly different ($p < .05$). Error bars display standard deviation values. The proper tilapia enzyme extract represents untreated samples and was set to 100%. OnF, enzyme extract of *Oreochromis niloticus* fingerlings; OnJ, enzyme extract of *O. niloticus* juveniles; Pm, *Pleoticus muelleri* cephalothorax enzyme extract; Al, *Artemesia longinaris* cephalothorax enzyme extract; Pr, *Patagonotothen ramsayi* head and viscera enzyme extract; OnF + Pm, OnF + Al, OnF + Pr, OnJ + Pm, OnJ + Al and OnJ + Pr, mixtures of enzymes

incubation treatments, exogenous enzymes showed their maximum activity on the incubation time period from 90 to 120 min. Also, exogenous enzymes tolerated acid pH environments ranging from three to two (Figure 5).

3.5 | Hydrolysis of feed ingredients

The magnitude of pH variation represents a sign of substrate hydrolysis degree when the enzyme extract and the feed ingredient are combined. In the present study, results showed that the enzyme combinations varied in their ability to hydrolyse each ingredient. Acid hydrolysis was improved by exogenous enzymes only when squid meal was employed and just for *OnF* enzyme extracts ($p < .05$) (Table 2a). On the other hand, in most cases, exogenous enzymes have enhanced the hydrolysis of feed ingredients in intestine environmental conditions, where increments were more evident when these enzymes were added to *OnJ* extracts than to the *OnF* ones (Table 2b). Additionally, when *OnJ* was mixed with *Pm*, the alkaline

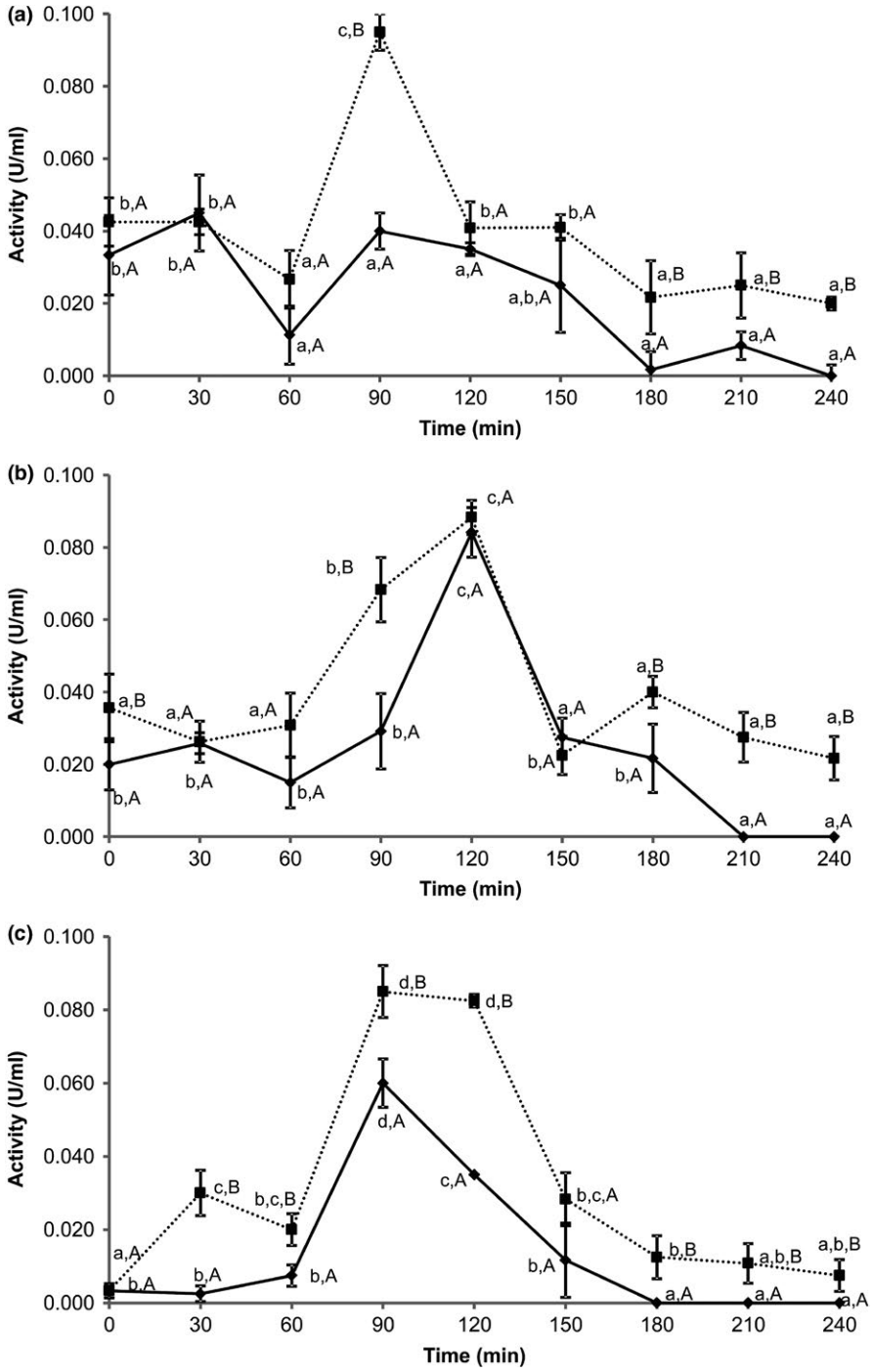

FIGURE 5 Activity of exogenous alkaline enzymes exposed to tilapia stomach conditions during 240 min (a) *Pleoticus muelleri* cephalothorax enzymes. (b) *Artemesia longinaris* cephalothorax enzymes. (c) *Patagonotothen ramsayi* head and viscera enzymes. Solid lines represent treatments carried out in acidic conditions, and dashed lines indicate control assays in alkaline conditions. Means within the same treatment with different superscripts (a–d) are significantly different ($p < .05$). Means within the same time with different superscripts (A–B) are significantly different ($p < .05$). Error bars display standard deviation values

hydrolysis of all the ingredients assayed was significantly higher ($p < .05$) (Table 2b).

3.6 | Temperature stability of exogenous enzymes

The tolerances of exogenous enzymes to different temperatures are shown in Figure 6. In general, acidic enzymes were less stable than alkaline ones. At 40°C, *Pm* acid enzymes had only 50% of residual activity after 5.5 hr, while *Al* and *Pr* had no signs of activity after incubation times of 4 and 2.5 hr, respectively (Figure 6a). On the other hand, alkaline proteinase activity of *Pm* and *Al* remained stable for 7 hr at 40°C; however, after 2.5 hr, the same activity was not registered for *Pr* (Figure 6b). By contrast, at 60°C, enzyme activities quickly disappeared for the three exogenous enzymes extracts (Figure 6).

4 | DISCUSSION

One of the most urgent threats to the world's remaining fish stocks is the indiscriminate capture and discard of non-target organisms (by-catch) (Davies, Cripps, Nickson, & Porter, 2009) and the rising volumes of by-products discharged by fishery processing, creating a serious

TABLE 2 Relative protein hydrolysis (RPH) of feed ingredients for *Oreochromis niloticus* fingerlings and juveniles and adding exogenous enzymes extracted from *Pleoticus muelleri*, *Artemesia longinaris* and *Patagonotothen ramsayi*. (a) In tilapia stomach acidic conditions. (b) In tilapia intestine alkaline conditions

Enzyme extracts	Fish meal	Squid meal	Shrimp meal	Soybean meal
(a) RPH values at stomach environmental conditions				
OnF	40.00 ± 1.415^{d,B}	15.00 ± 0.069^{c,A}	97.50 ± 3.158^{d,C}	90.00 ± 1.923^{f,C}
OnF + Pm	29.41 ± 0.785^{b,c,A}	79.41 ± 1.218^{f,D}	47.06 ± 1.299^{b,c,B}	61.76 ± 0.722^{d,C}
OnF + Al	5.67 ± 0.087^{a,A}	9.57 ± 0.798^{b,A}	24.11 ± 1.010^{a,C}	17.02 ± 1.530^{a,B}
OnF + Pr	30.18 ± 1.894^{c,A}	59.43 ± 1.88^{e,B}	50.94 ± 3.025^{c,B}	22.64 ± 1.934^{a,b,A}
OnJ	41.51 ± 1.351^{d,B}	19.81 ± 0.641^{d,A}	50.94 ± 2.049^{c,C}	76.42 ± 1.345^{e,D}
OnJ + Pm	27.45 ± 0.669^{b,c,B}	11.76 ± 1.039^{b,c,A}	38.24 ± 1.559^{b,C}	26.47 ± 1.657^{b,c,B}
OnJ + Al	26.67 ± 0.514^{b,c,B}	10.00 ± 0.433^{b,A}	46.41 ± 0.920^{b,c,D}	33.33 ± 2.038^{c,C}
OnJ + Pr	24.56 ± 0.837^{b,C}	2.63 ± 0.029^{a,A}	44.74 ± 0.970^{b,c,D}	15.79 ± 0.745^{a,B}
(b) RPH values at intestine environmental conditions				
OnF	36.11 ± 1.097^{c,C}	25.93 ± 0.693^{c,B}	14.81 ± 0.751^{a,A}	25.93 ± 0.981^{b,B}
OnF + Pm	12.96 ± 0.693^{a,A}	11.11 ± 0.751^{a,A}	38.89 ± 1.559^{b,C}	–
OnF + Al	58.33 ± 0.346^{d,B}	10.10 ± 0.751^{a,A}	–	–
OnF + Pr	15.00 ± 0.635^{a,b,A}	12.50 ± 0.808^{a,A}	240.00 ± 0.837^{f,B}	–
OnJ	17.65 ± 0.895^{b,B}	–	33.33 ± 1.617^{b,C}	10.97 ± 0.767^{a,A}
OnJ + Pm	150.00 ± 0.635^{e,D}	87.50 ± 0.837^{d,C}	46.67 ± 1.212^{c,B}	37.50 ± 0.866^{c,A}
OnJ + Al	15.87 ± 1.039^{a,b,A}	12.70 ± 0.866^{a,A}	100.00 ± 0.924^{d,B}	–
OnJ + Pr	32.81 ± 0.981^{c,B}	18.75 ± 0.462^{b,A}	153.13 ± 1.097^{e,C}	–

Values are means and standard deviation of three replicates. Means within the same columns with different superscripts (a–f) are significantly different ($p < .05$). Means within the same rows with different superscripts (A–D) are significantly different ($p < .05$).
OnF, stomach or intestine enzyme extract of *Oreochromis niloticus* fingerlings; OnJ, stomach or intestine enzyme extract of *O. niloticus* juveniles; Pm, *Pleoticus muelleri* cephalothorax enzyme extract; Al, *Artemesia longinaris* cephalothorax enzyme extract; Pr, *Patagonotothen ramsayi* head and viscera enzyme extract.

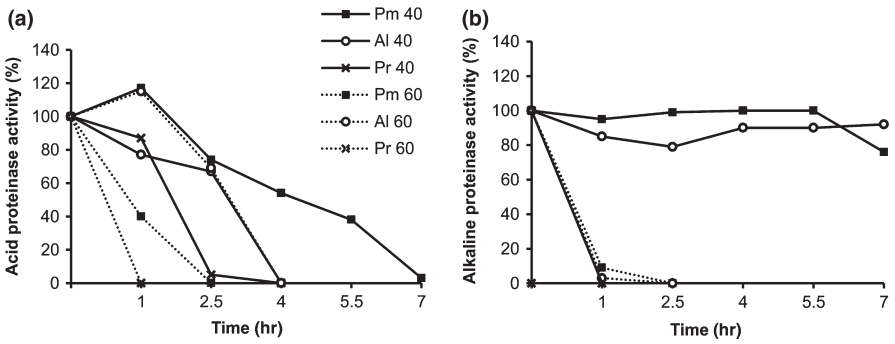

FIGURE 6 Evaluation of exogenous enzymes stability exposed to two drying temperatures of 40°C and 60°C during a 7-hr period. The "control" represents untreated samples at time 0 and was set to 100%. Pm, *Pleoticus muelleri* cephalothorax enzyme extract; Al, *Artemesia longinaris* cephalothorax enzyme extract; Pr, *Patagonotothen ramsayi* viscera and head enzyme extract

environmental problem (Ferraro et al., 2010). We present a novel solution to re-evaluate this vast wasted resource around the world. The data obtained demonstrate that proteinases can be easily recovered from fishery waste and employed as feed supplement in *O. niloticus* diets. Our results describe the great potential of enzymes extracted from fishery discards and by-products to optimize feed protein hydrolysis of cultured animals, and as consequence, improve aquaculture production. In this research, we have verified proteinase activity in the three fishery wastes analysed. In the present study, these proteinases from *P. muelleri*, *A. longinaris* and *P. ramsayi* were successfully extracted, but this idea can be readily extrapolated to other fishery wastes and cultured species as well. It was found that *P. muelleri* and *A. longinaris* by-products showed significantly higher activity of acid and alkaline proteinases than *P. ramsayi*.

In the digestion process, digestive proteinases hydrolyse food proteins. Given the participation of proteinases in food digestion, proteinases have been used as feed supplements to increase the nutritional value of feed. Even though feed supplementation with proteinases or mixed enzymes may have positive effects on animals growth (Singh, Maqsood, Samoon, Phulia, & Danish, 2011; ali Zamini, Kanani, azam Esmaeili, Ramezani, & Zoriezahra, 2014), in some circumstances, inconsistent results in feed trials are described because the enzymes result deactivated by some components in the digestive system of the fed animals (Kazerani & Shahsavani, 2011; Miller, Granzin, Elliott, &

Norton, 2008). If exogenous enzymes are utilized as feed supplements, it is desired that they would not be inactivated by the physiological conditions of the host digestive system and will keep their activity. A previous study evaluated the effect of non-starch polysaccharides, phytase and citric acid on the activities of endogenous enzymes of tilapia and found that this supplements primarily influence the activity of amylase in the fish digestive tract (Li et al., 2009). Additionally, González-Zamorano, Navarrete del Toro, and García-Carreño (2013) evaluated, by in vitro assays, the supplementation of exogenous enzymes for whiteleg shrimp diets. Their results showed that digestive enzymes from the host could hydrolyse exogenous enzymes. Thus, it becomes essential to guarantee that exogenous enzymes do not inactivate or affect the enzymes of fed animals, and vice versa; that is why enzymes combinations should be tested in vitro before intending them as proteinase supplements in feeds to guarantee that they will contribute to the hydrolysis of food proteins (González-Zamorano et al., 2013). Consequently, our results describe for the first time the in vitro interaction between exogenous proteinases and Nile tilapia endogenous enzymes. This research demonstrated, through activity gels, that tilapia digestive proteinases and most exogenous enzymes kept their activity when combined with every exogenous proteinase tested. Thus, such enzymes recovered from fishery waste become great candidates to be employed as feed supplement. Alternatively, it was evaluated if there is any synergistic cooperation between proteinases from stomach or intestine of tilapia (*OnF* and *OnJ*) and exogenous enzymes. To accomplish this, we quantified the enzyme activities after both extracts (each enzymatic extract and their mixtures) were allowed to hydrolyse azocasein or haemoglobin. In every case, we found that the addition of exogenous enzymes reduced, at different scales, the activity of Nile tilapia enzymes. This result could lead to erroneous conclusions as these enzymes (extracted from *P. muelleri*, *A. longinaris* and *P. ramsayi*) probably induce an inhibition of endogenous digestive enzymes or might have a better substrate affinity than tilapia enzymes. Although casein and haemoglobin are widely employed as substrate for activity evaluation, they are not habitually used in feed formulations for commercial aquaculture where protein fraction represents the most expensive dietary source.

Protein requirements by *O. niloticus* have been extensively studied and established that during the early stages, Nile tilapia need about 30–40 g kg^{-1} dietary protein for maximum growth performance (El-Sayed, 2006). In this research, we assessed if exogenous enzymes extracted from fishery waste can improve the hydrolysis of several ingredients frequently utilized in feed formulations. Effects of exogenous enzymes can be variable and are dependent on many factors such as the quality and type of the ingredient. Our results showed that exogenous enzymes improved ingredient hydrolysis, mainly in alkaline conditions, cooperating with the intestinal enzymes of Nile tilapia. In addition, these exogenous enzymes seem to cooperate better with intestinal enzymes extracted from juvenile stages. When *Pm*, *Al* and *Pr* enzymes were added, the ingredients better hydrolysed were found to be the animal-based ones (fish meal, shrimp meal and squid meal). This finding might be linked to a shift in tilapia feed habits as they grow, who gradually pass from carnivorous habits to herbivore–omnivore ones (El-Sayed, 2006). Therefore, the addition of exogenous enzymes from carnivorous species such as *Pm*, *Al* or *Pr* could increase digestion efficiency of animal-based ingredients. Moreover, when exogenous enzymes of *Pm* were mixed with intestinal enzymes of Nile tilapia, the hydrolysis of all ingredient sources (including soybean meal) increased; thus, alkaline enzymes of red shrimp by-products (*Pm*) become in the best candidates to be employed as feed supplements for Nile tilapia culture.

Even though the Nile tilapia stomach is small and sac-like, it has a gastric function; reaching low pH values and containing pepsinogen in its walls (Bowen, 1982; Moriarty, 1973). In this species, digestion of protein and polypeptides begins by the action of pepsin (a non-specific endoproteinase) in the stomach, where the acid denatures proteins and makes them more available to enzymatic breakdown and nutrient release (Rust, 2002). Afterwards, digestion is continued into the intestine by trypsin and chymotrypsin secreted by the pancreas (Tengjaroenkul, Smith, Smith, & Chatreewongsin, 2002). Thus, if the alkaline enzymes recovered from fishery waste are used as a feed supplement, it will be essential to ensure that they will subsist to stomach conditions and will successfully reach gut to help aid digestion. The results showed that all exogenous enzymes studied tolerate at least 150 min within an acid environment with stomach enzymes of Nile tilapia. Moriarty (1973) determined that *O. niloticus* have a diurnal cycle of acid secretion in their stomach which closely follows its feeding pattern where the concentration of acid gradually increases during the day (from pH 7.0–5.0 in their fasting period at late-night, to pH 2.0–1.5 when feeding during the morning). Additionally, he concluded that food which is retained in the stomach during the day and subjected to pH values of around 1.5–1.4 will be more fully digested in the intestine than the material that passes through more quickly. In consequence, we suggest that first food of the day will be better digested if is supplemented with exogenous enzymes, and also these enzymes will have less chance of losing their activity because they will not have to go through very low pH conditions due to the cycle of acid secretion.

On the other hand, the animal feeds elaboration process habitually involves a drying step, and as a result, it becomes essential to study the effect of food drying temperatures on the stability of exogenous enzymes. Thus, it is important to make sure that enzymes supplemented will not be irreversibly denaturalized during this drying process. *P. muelleri* and *A. longinaris* exogenous enzymes remained stable for enough time to tolerate a drying process; consequently, *Pm* and *Al* by-products can be used as feed supplements without losing their activity. Conversely, *Pr* enzyme extract quickly lost its activity at high temperatures. This may be due to the Antarctic and subantarctic distribution of *P. ramsayi*. However, this species has great potential to be employed in other biotechnological applications because it holds a number of biochemical and physiological specializations that are considered to be cold adaptations, as production of antifreeze glycopeptide compounds (Cheng & Detrich, 2007).

The use of in vitro synergy assays allowed us to easily determine whether enzymes display cooperation in hydrolysis of several ingredients before intending them as exogenous proteinases in in vivo

trials which may be complex, labour-intensive, time-consuming and very expensive. In the present study, we demonstrated that mixing different enzyme systems can break down protein substrates more rapidly and efficiently than either system alone. We have addressed not only a new feed supplement for tilapia cultures but also a method of repurposing fishery waste through an original application. In this research, we successfully extracted proteinases from by-products (*P. muelleri* and *A. longinaris*) and bycatch (*P. ramsayi*) through an easy procedure. The data obtained suggest that exogenous enzymes obtained from *P. muelleri* by-products are the best candidates to be employed as feed supplements for Nile tilapia. This is due to several properties of red shrimp enzymes: they do not affect the activity and integrity of fish digestive enzymes, they improve the hydrolysis of several ingredients, and they maintain their activity after being exposed to high temperatures and acidic pHs. Also, as we mentioned above, right after tilapia start feeding in the morning, pepsins are not completely active yet and stomach pH is still dropping, so the first food consumed is not well digested. Thus, digestion of first feed will be improved by the supplementation of exogenous enzymes. Additionally, *P. muelleri* exogenous enzymes seem to have a better performance in juveniles than in fingerlings. In consequence, supplementing feed formulation with exogenous enzymes best suited to the Nile tilapia digestive enzymes at given stages of growth could result in increased income to the producers by improving the growth and health of the fish, as well as by decreasing feed waste. As a result, fishery industry waste holds a great biotechnological potential as source of proteinases. This study promotes a revaluation of this wasted resource, reducing negative environmental impact effects and promoting environmental sustainability. However, in vitro results might not be the same compared with in vivo ones, so the scope of this research should be expanded in the future by testing feeds employing *P. muelleri* by-products as source of exogenous enzymes in feeding trials for *O. niloticus*.

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