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Digestive proteinases from the marine fish processing wastes of the South-West Atlantic Ocean: Their partial characterization and comparison

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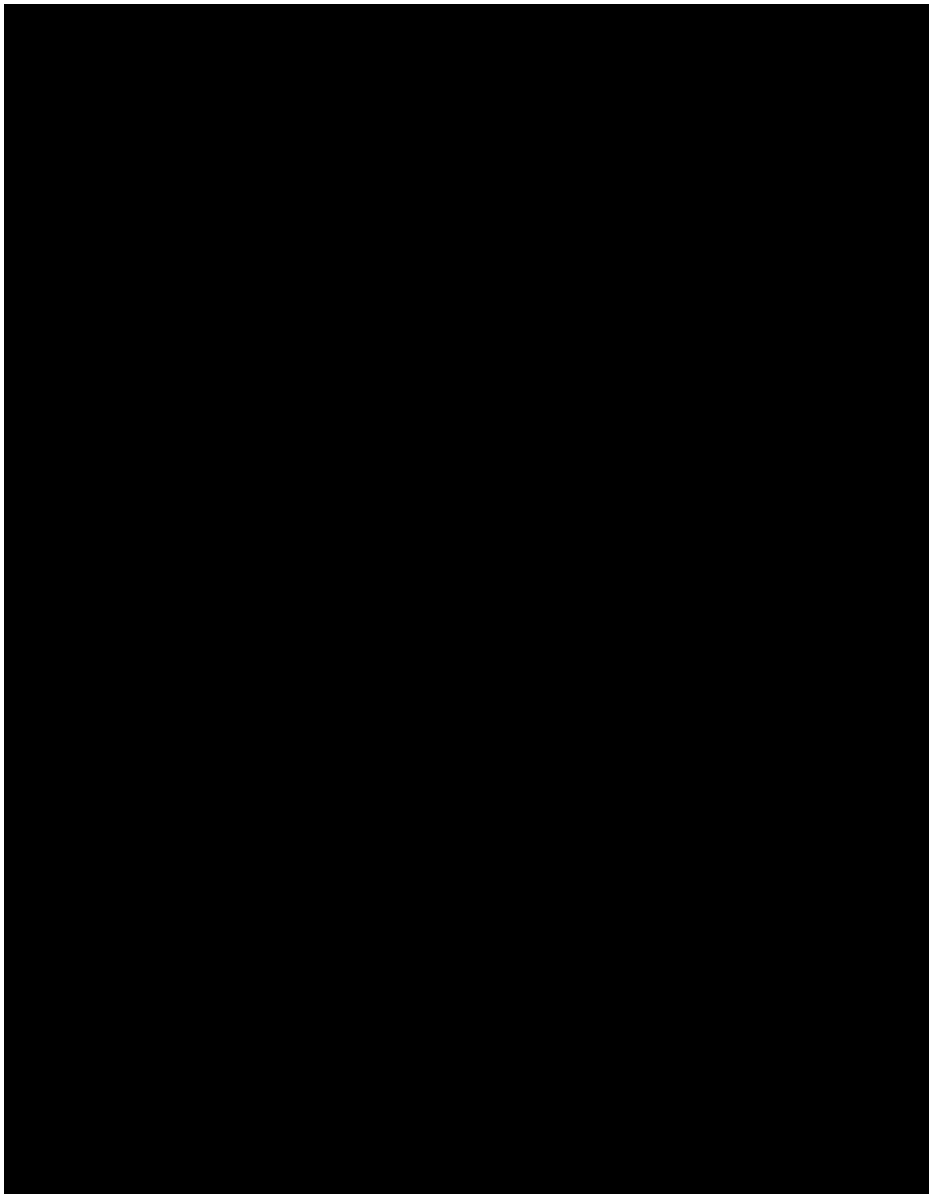

1 | INTRODUCTION

Fish and fishery products are one of the most commercialized foods in the world and represent a valuable resource of nutrients for diversified and healthy diets. Total global fish production reached an all-time high of 179 million tonnes in 2018 and it is expected to reach 201 million tonnes in 2030 (FAO, 2020). However, 70% of fish used in industrial processing is discarded as processing leftovers, generating large amounts of wastes and other by-products (Olsen *et al*., 2014). This solid waste includes muscle (15%–20%), viscera (12%–18%), spines (9%–15%), heads (9%–12%), skin and fins (1%–3%), and scales (Sahu *et al*., 2016). Although a fraction of this waste is used in the elaboration of fishmeal (Mo *et al*., 2018), almost all of it is not used and is directly discarded on open-air dumps or water sources (Pereira & Fernandez Gimenez, 2016). Fishery by-products and wastes extracted from inexpensive marine feedstock may have an even higher value than the starting material, therefore it is necessary to identify techno-economic viability to recycle these wastes (Oliveira *et al*., 2017).

There are some studies about commercial applications for enzymes recovered from fish by-products (Kim & Dewapriya, 2014) where the development of new and improved technologies have allowed these enzymes to be used more efficiently in several industrial processes, such as the production of feed, silage, fertilizers, biodiesel and biogas, and cosmetics (FAO, 2020). These enzymes can catalyse reactions without additional thermal aid and can be easily inactivated by mild heat, reducing manufacturing cost and processing times (Kuddus, 2018). These natural products are more accepted than the corresponding synthetic ones because they are considered safer by consumers (Ferraro *et al*., 2013).

Proteases are the most widely used group of enzymes in industrial bioprocesses, which is why they represent about 40%–65% of the total commercial enzyme preparations (Atta *et al*., 2017). In this sense, fish viscera are one of the most important wastes because they contain digestive enzymes, especially aspartic proteinases (pepsin) in the stomach and serine proteinases (trypsin, chymotrypsin, collagenase and elastase) in the intestine and pyloric ceca (Klomklao *et al*., 2011; Vannabun *et al*., 2014). These digestive proteinases have unique properties compared to their counterpart enzymes from land animals. Some of the distinctive features of the proteinases include greater stability over a wide range of pH, and higher catalytic efficiency and thermostability at low temperatures (Aspevik *et al*., 2017).

Argentina has 4700 km of coastline on the Argentine Sea in the Southern Atlantic Ocean, making fishing a significant economic activity with various fishing resources, being the demersal resources one of the most important ones. The Argentine hake *Merluccius hubbsi* (Marini 1933) is one of the main fishing resources, with around 417 mil tonnes captured in 2018 representing 50% of the total catches (Allega *et al*., 2019). In the same year the Brazilian flathead *Percophis brasiliensis* (Quoy & Gaimard 1825) and the Stripped weakfish *Cynoscion guatucupa* (Cuvier 1830) captures were 6538 and 8814 t, respectively (Prosdocimi *et al*., 2019). These species are actively fished as they are commercialized in the international market as frozen fillet, headless and gutted, supplying Mar del Plata city (Argentina) as the main port of landing (Irusta *et al*., 2016). However, discards of these species represent rising volumes of by-products that have emerged from fishery processing and have serious environmental security implications (Pereira and Fernández-Gimenez, 2016). *P. brasiliensis*, *C. guatucupa* and Brazilian codling *Urophycis brasiliensis* (Kaup 1858) are the by-catch of the targeted species, such as *M. hubbsi* (Bovcon *et al*., 2013). So far, the characteristics of the digestive enzymes of these species and their potential application have not been studied, therefore their study is novel and important since they are part of the main fishery resources of the Argentinean Sea. When they are marketed in fillet format, a large amount of waste is generated. Thus, it is important to know the physiology of these species through the characterization of their enzymes to revalue, power market and ensure their profitable utilization at an industrial scale with the main purpose of contributing to the reduction of environmental pollution caused by the accumulation of this waste. To achieve this, a description of operational parameters and biochemical characteristics of the enzymes recovered from fish wastes are necessary. Therefore, the characterization of enzymes is crucial for a correct process design and useful to understand the types of enzyme, modes of action and activity values. The effect of pH and temperature on the enzymatic activity is evaluated with the affinity for specific substrates in most studies (Martínez-Cárdenas *et al*., 2017). Until now, there have been no comparative studies evaluating the potential of the digestive enzymes present in fish wastes from actively commercialized species.

Although the activity of digestive enzymes of various fish species such as *Sardinops sagax caerulea* (Castillo-Yañez *et al*., 2004), *Sardinella aurita* (Khaled *et al*., 2011), *Salaria basilisca* (Ktari *et al*., 2012), *Pangasianodon gigas* (Vannabun *et al*., 2014), *Raja Clavata* (Lassoued *et al*., 2015), *Liza aurata* (Bkhairia *et al*., 2016), *Scomberomorus guttatus* (Rengasamy *et al*., 2016), *Seriola dumerili* (Oliveira *et al*., 2017), *Prochilodus lineatus* (Gomez *et al*., 2018), *Sardinella longiceps* (Ramkumar *et al*., 2018) and *Bagre panamensis* (Rios-Herrera *et al*., 2019) have been extensively reported, a small number of researchers have focused on the digestive enzymes of Argentine marine fish species. In this sense, only trypsin from the viscera and heads of *M. hubbsi* (Lamas *et al*., 2015) and *Engraulis anchoita* (Lamas *et al*., 2017) have been characterized.

The aim of this study was to characterize and compare specific activities of acid and alkaline proteinases under different pH and temperature conditions, and the yield of viscera waste generated during the processing of *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis*, and *C. guatucupa*.

2 | MATERIALS AND METHODS

2.1 | Fish samples

Specimens of *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis* and *C. guatucupa* were fished off the coast of Mar del Plata (38°04′S, 57°30′W) (Figure 1) by a commercial fleet, and the stomach, small intestine and pyloric ceca were immediately extracted and kept on ice until docking.

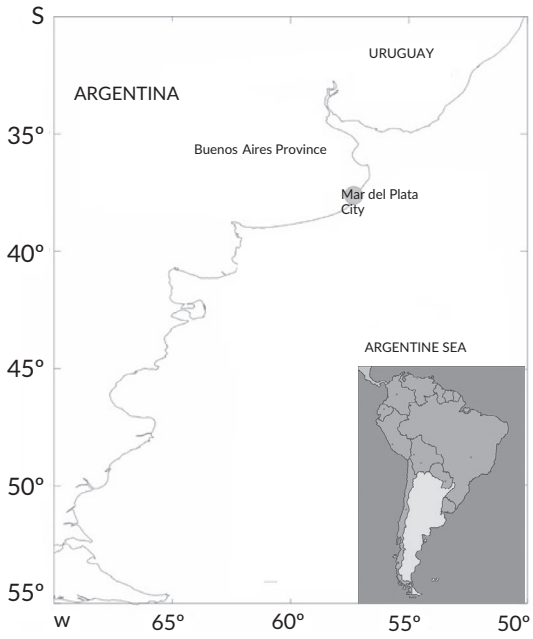

FIGURE 1 Map showing the sampling zone along the coastline of Mar del Plata city (Argentina)

Viscera of four adult fishes of each species were used in this study. All samples were placed in polystyrene boxes containing ice and transported to the research laboratory in Mar del Plata National University within 30 min for further processing. They were rinsed with cold distilled water, and then stored in polyethylene bags at −20°C for further use. Viscera were treated as four samples per species per organ (stomach and intestine-ceca). The biological parameters of the specimens used of each species and the stomach and intestine-ceca weight used for digestive extracts are presented in Table 1.

2.2 | Preparation of crude extracts

Stomach and intestine-ceca were minced and crushed separately in a glass-Teflon tissue homogenizer with distilled water for intestine-ceca, and with water adjusted to pH 2 using 0.1 N HCl in stomachs (1:4 w/v). Undigested food in the digestive tract was removed. The resulting materials were centrifuged at 10,000*g* for 30 min at 4°C (Presvac EPF 12R). The supernatants were separated and stored until used as crude extracts for all the enzymatic assays. Enzymatic extracts from intestine-ceca were frozen at −20°C. Extracts from stomachs were maintained overnight at 4°C to activate the pepsinogen and then at −20°C until use.

2.3 | Enzymatic assays

The soluble protein content of the extracts was determined by the Bradford (1976) method using bovine serum albumin (Sigma A9647) as the standard protein, expressed as milligrams of protein per millilitre of crude extract.

Acid proteinase activity was determined at pH 2 using stomach samples, according to Anson (1938). Shortly after, 5 μl of each crude extract was mixed with 250 μl of universal buffer (57 mM boric acid, 36 mM citric acid, 38 mM monobasic sodium phosphate and 1 N sodium hydroxide) pH 2 (Stauffer, 1989) in Eppendorf tubes and 250 μl of 0.5% w/v haemoglobin (Sigma H2625) as the substrate dissolved in universal buffer pH 2. The mixture was incubated for 30 min at 25°C. The reaction was stopped by adding 250 μl of 20% w/v trichloroacetic acid (TCA). Next, all tubes were centrifuged at 10,000*g* for 5 min and absorbance of the supernatants was measured at 280 nm in a spectrophotometer (SPECTROstar Nano BMG LABTECH, Germany).

Alkaline proteinase activity on intestine-ceca samples was measured using a similar protocol, using 0.5% w/v azocasein (Sigma A2765) as the substrate dissolved in universal buffer pH 8. In this case, the absorbance of the supernatant was measured at 366 nm, according to García-Carreño (1992).

One unit of acid and alkaline proteinase activity (U) was defined as the change in the absorbance per minute in the crude extract under the assay conditions. Total activity (TA) was expressed as units of enzymes per millilitre of crude extract (U ml^{-1}). The specific activities (SA) were expressed as units per milligram of protein (U mg $protein^{-1}$). The viscera yield (Y) was calculated as units per gram of tissue (U g $tissue^{-1}$). All assays were performed in triplicate. Trichloroacetic acid was added in negative control treatments before supplying the substrate.

To determine the optimum pH, the proteinase activity was assayed at pH 2, 3 and 4 for stomach samples and pH 7, 8, 9.5 and 11.5 for intestine-ceca samples. The universal buffer was used in all samples. The substrate (azocasein or haemoglobin) was dissolved in each of the pH buffer solutions, and the protocol was followed as described above. The effect of pH on the stability of proteinases was evaluated by pre-incubating a mix of 5 μl of crude extract and 250 μl of adequate buffer during 30, 60 and 150 min at 25°C. The

TABLE 1 Biological parameters of *Merluccius hubbsi*, *Percophis brasiliensis*, *Urophycis brasiliensis* and *Cynoscion guatucupa*

Species	Total length (cm)	Standard length (cm)	Total weight (g)	Stomach weight (g)	Intestine-ceca weight (g)
Merluccius hubbsi	50.67 ± 4.51	44.33 ± 4.51	816.66 ± 98.32	11.63 ± 1.16	5.69 ± 1.20
Percophis brasiliensis	47.67 ± 3.33	42.00 ± 5.29	620.50 ± 53.04	5.27 ± 0.45	4.13 ± 0.33
Urophycis brasiliensis	23.37 ± 1.03	20.50 ± 1.29	111.00 ± 13.19	1.66 ± 0.07	2.00 ± 0.08
Cynoscion guatucupa	24.87 ± 1.11	22.37 ± 0.48	117.00 ± 12.66	2.62 ± 0.11	1.26 ± 0.61

Note: Values indicate means and standard error ($n = 4$).

corresponding substrate solution was added after the pre-incubation while the TCA was added before the substrate in the control treatments. The protocol was followed as described above.

To determine the optimum temperature, the enzyme activities at pH 2 for stomach samples and pH 8 for intestine-ceca samples were assayed at 10, 30, 50 and 70°C. The universal buffer was used in all samples. The substrate (azocasein or haemoglobin) was dissolved in each of the pH buffer solutions following the protocol described above. Thermal stability was evaluated by pre-incubating a mix of 5 μl of crude extract and 250 μl of adequate buffer during 30, 60 and 150 min at the above-mentioned temperatures. The corresponding substrate solution was added in the control treatments after the pre-incubation, while the TCA was added before the substrate. The mixture was incubated 30 min at each temperature (10, 30, 50 and 70°C) following the protocol described above.

In the cases where the enzymes still had activity at 70°C for 150 min, a denaturation assay was performed. First, enzymatic activity was determined in universal buffer at pH 2 or pH 8, for stomach or intestine-ceca, respectively. Second, all the tubes were pre-incubated at 80 and 90°C for 5, 10 and 20 min. Finally, the corresponding substrate solution was added and TCA was added previously to the substrate in the control treatments. The protocol was followed as described above. Residual enzyme activity was calculated as percentage (%) and the specific activity measured without pre-incubation was considered as the treatment with 100% activity at pH 2 for acid proteinases and pH 8 for alkaline proteinases at 25°C. Enzymes were considered denatured when the residual enzymatic activity was less than 5%.

2.4 | Statistics

After testing data normality and homogeneity of variance, differences of total activity, viscera yield, specific activity and soluble protein content from stomachs and intestine-ceca crude extract for each species were analysed by one-way ANOVA followed by a Tukey's multicomparison test to compare the significant differences among the means.

To explore the factors that affect enzyme activity, we conducted a generalized linear mixed models (GLMM) with gamma error distribution (since data sets did not find a normal distribution). The dependent variable was the specific activity (SA) and the fixed factors were pH treatment (levels 2, 3 and 4 for acid proteinases and 7, 8, 9.5 and 11.5 for alkaline proteinases) or temperature treatment (levels 10, 30, 50 and 70°C), incubation time (levels 0, 30, 60 and 150 min), species (levels *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis* and *C. guatucupa*) and their interactions. Samples (1–4) were included as a random effect to account for repeated measurements on the same crude extract from an individual fish. This model was first compared with a GLM model (without the random effect) to evaluate the relevance of the random factor, then the Akaike information criterion corrected for a small sample size (AICc) was used to contrast models with different combinations of independent variables. The best model was the one that featured the lowest AIC value and the highest Akaike weights (wi, the probability of a model to be the best). The significance of the explanatory variable (s) was tested using a deviance analysis test (Fisher's F test). When significant differences in any factor were found, Tukey's tests were applied to the constructed model to make *post hoc* multiple comparisons and detect which level differed.

All statistical analyses were carried out using R software version 3.6.1 (R Development Core Team, 2019). The generalized linear mixed models were conducted with the package glmmADMB model selection and calculation of the coefficient of determination with the package MuMIn, and multiple comparisons for the best model with the package multcomp. Results are presented as mean ± standard error (S.E.), and for null hypothesis testing, statistical tests were considered significant at $\alpha = 0.05$.

3 | RESULTS

3.1 | Enzymatic activity, viscera yield and soluble protein

Total activity (U ml^{-1}), specific activity (U mg $protein^{-1}$), viscera yield (U $gtissue^{-1}$) and soluble protein content (mg ml^{-1}) are shown in

TABLE 2 Total activity (TA), specific activity (SA), viscera yield (Y) and soluble protein (SP) of crude extracts of *Merluccius hubbsi*, *Percophis brasiliensis*, *Urophycis brasiliensis* and *Cynoscion guatucupa*

		M. hubbsi	*P. brasiliensis*	*U. brasiliensis*	*C. guatucupa*
Stomach	TA (U ml^{-1})	1.77 ± 0.57	1.43 ± 0.16	1.55 ± 0.53	1.15 ± 0.26
	SA (U mg $protein^{-1}$)	0.50 ± 0.13 ^{a}	1.90 ± 0.46 ^{ab}	7.26 ± 3.04 ^{b}	0.38 ± 0.09 ^{a}
	Y (U g $tissue^{-1}$)	5.31 ± 1.71	4.29 ± 0.49	4.65 ± 1.57	3.45 ± 0.79
	SP (mg ml^{-1})	3.90 ± 1.54 ^{b}	0.87 ± 0.17 ^{a}	0.24 ± 0.09 ^{a}	3.26 ± 0.90 ^{b}
Intestine-ceca	TA (U ml^{-1})	0.75 ± 0.32 ^{a}	3.46 ± 0.35 ^{b}	0.65 ± 0.09 ^{a}	0.25 ± 0.06 ^{a}
	SA (U mg $protein^{-1}$)	0.31 ± 0.13 ^{a}	1.82 ± 0.43 ^{b}	1.40 ± 0.26 ^{b}	0.15 ± 0.04 ^{a}
	Y (U g $tissue^{-1}$)	2.25 ± 1.09 ^{a}	10.38 ± 1.30 ^{b}	1.95 ± 0.29 ^{a}	0.75 ± 0.19 ^{a}
	SP (mg ml^{-1})	2.49 ± 0.34 ^{a}	2.01 ± 0.23 ^{ab}	0.49 ± 0.06 ^{b}	1.79 ± 0.41 ^{ab}

Note: Assays were carried out at 25°C for 30 min, at pH 2 (stomach) or pH 8 (intestine-ceca). Values indicate means and standard error from three replicates ($n = 4$). Different superscript letters in each row indicate significant differences between species (ANOVA, Tukey test, $P < 0.05$).

TABLE 3 Specific proteinase activity at different pH conditions for stomach (a) and intestine-ceca extracts (b) at 25°C from fishery waste of *Merluccius hubbsi*, *Percophis brasiliensis*, *Urophycis brasiliensis* and *Cynoscion guatucupa*

(a)

	Specific activity (U mg protein^{-1}) pH		
Species	**2**	**3**	**4**
M. hubbsi #	0.49 ± 0.14	0.25 ± 0.09	0.11 ± 0.03
P. brasiliensis ‡	1.90 ± 0.46 ^{a}	1.14 ± 0.33 ^{ab}	0.08 ± 0.03 ^{b}
U. brasiliensis §	7.26 ± 3.04 ^{a}	3.48 ± 0.33 ^{a}	0.86 ± 0.43 ^{b}
C. guatucupa #	0.38 ± 0.09	0.26 ± 0.09	0.12 ± 0.04

(b)

	Specific activity (U mg protein^{-1}) pH			
Species	**7**	**8**	**9.5**	**11.5**
M. hubbsi #	0.23 ± 0.10	0.31 ± 0.13	0.24 ± 0.12	0.62 ± 0.09
P. brasiliensis ‡	1.41 ± 0.32	1.82 ± 0.44	1.73 ± 0.58	2.67 ± 0.46
U. brasiliensis ‡	1.57 ± 0.26	1.40 ± 0.27	1.83 ± 0.44	1.58 ± 0.43
C. guatucupa #	0.15 ± 0.04 ^{a}	0.15 ± 0.04 ^{a}	0.19 ± 0.07 ^{ab}	0.56 ± 0.15 ^{b}

Note: Different small superscript letters in the same row indicate significant differences among different pH conditions for individual species. Different symbols (#, ‡, §) indicate significant differences among different species at different pH conditions (generalized linear mixed model, Tukey test, $P < 0.05$). Values indicate means and standard error from three replicates ($n = 4$).

Table 2. In the present work, total enzymatic activity in the intestine-ceca crude extracts was significantly different among the species and exhibited the highest value for *P. brasiliensis* (3.46 ± 0.35 U ml^{-1}). Specific enzymatic activity in the stomach crude extracts was significantly higher for *U. brasiliensis* (7.26 ± 3.04 U ml^{-1}) compared with the other samples (ANOVA, $P < 0.05$). For intestine-ceca crude extracts, specific enzymatic activity was significantly different between species (ANOVA, $P < 0.001$) and evidenced the highest activities for *P. brasiliensis* and *U. brasiliensis* (1.82 ± 0.43 U mg protein^{-1} and 1.40 ± 0.26 U mg protein^{-1}, respectively). The four species presented similar viscera yield for stomach proteinases, but the yield was higher in the *P. brasiliensis* extracts of intestinal proteinases (10.39 ± 1.30 U g tissue^{-1}). Soluble protein contents showed significant differences among the enzyme extracts studied (ANOVA, $P < 0.05$). In the stomach, *M. hubbsi* and *C. guatucupa* crude extracts evidenced the highest values (3.90 ± 1.54 mg ml^{-1} and 3.26 ± 0.90 mg ml^{-1}, respectively) while in intestine-ceca, only *M. hubbsi* crude extracts evidenced the highest value (2.49 ± 0.34 mg ml^{-1}).

3.2 | Optimum pH and stability of proteinases

Considering acid proteinases, the model that best fitted the data (wi ≥ 0.05) contained the variables species and pH treatment, and their interaction (see Supporting Information Table A1a). The predictor variable incubation time did not have a significant effect on the specific activity.

Table 3a shows the stomach proteinase activity of the crude extract as a function of the different pH conditions without pre-incubation. The optimum pH for *P. brasiliensis* enzymes was pH 2 and for *U. brasiliensis* enzymes was pH 2–3. Stomach enzymes of *M. hubbsi* and *C. guatacupa* had similar activity values for all tested pH with a higher activity at pH 2. The lowest specific activity was evidenced on *M. hubbsi* and *C. guatucupa* enzymes without significant differences between them, whereas *P. brasiliensis* and *U. brasiliensis* enzymes showed the highest specific activity with significant differences between them. The specific activity values were higher for *U. brasiliensis* enzymes than for *P. brasiliensis* enzymes.

Figure 2a–d shows the stability of stomach proteinases at the different pH conditions and pre-incubation times. Acid proteinases from all species were highly stable in the range of pH 2–4. During 150 min, the enzymes kept their initial activity without significant differences at different incubation times (see Supporting Information Table A2a).

Considering alkaline proteinases, the model that best fitted the data (wi ≥ 0.05) contains the variables species and pH (see Supporting Information Table A1b). The predictor variable incubation time did not have a significant effect on the specific activity.

Table 3b shows the intestine-ceca proteinase activity of the crude extract as a function of the different pH conditions without pre-incubation. The optimum pH for *C. guatucupa* enzymes was 11.5, while crude extracts of *M. hubbsi* and *P. brasiliensis* had the highest activity at pH 11.5 but without significant differences. For *U. brasiliensis*, the catalytic activity peaked at pH 9.5 without significant differences. The lowest specific activity was evidenced for *M. hubbsi* and *C. guatucupa* enzymes, without significant differences between them, whereas *P. brasiliensis* and *U. brasiliensis* showed the highest specific activity without significant differences between them.

Figure 2e–h shows the stability of intestine-ceca proteinases at the different pH conditions and pre-incubation times. Alkaline proteinases from all species were highly stable in the range of 7–11.5. In the course of 150 min, the enzymes kept their initial activity without significant differences at different incubation times (see Supporting Information Table A2b).

3.3 | Optimum temperature and thermostability

Considering acid proteinases, the model that best fitted the data (wi ≥ 0.05) contains the variables species, temperature treatment, their interaction and incubation time, and their interaction with temperature treatment (see Supporting Information Table A3a).

Table 4a shows the stomach proteinase activity of the crude extract as a function of the different temperature conditions without pre-incubation. *M. hubbsi* and *P. brasiliensis* enzymes activity peaked at 30 and 50°C, respectively, but did not show differences among temperatures assayed, while the optimum temperature for *U. brasiliensis* enzymes was 30–50°C and for *C. guatucupa* enzymes

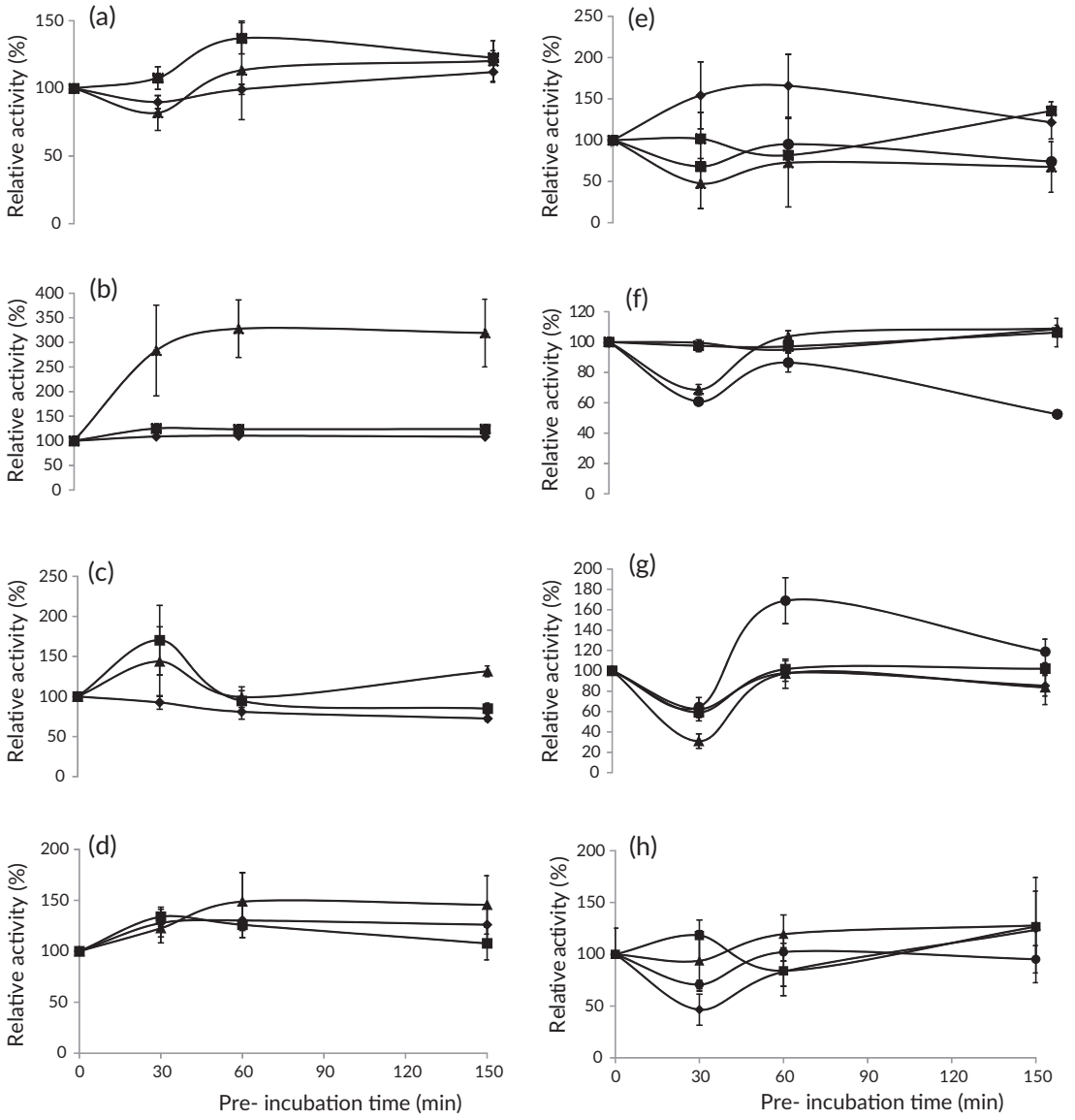

FIGURE 2 pH stability of stomach (left column) and intestine-ceca extracts (right column) at different conditions of pH during 0, 30, 60 and 150 min at 25°C from fishery waste of (a) and (e) *Merluccius hubbsi*, (b) and (f) *Percophis brasiliensis*, (c) and (g) *Urophycis brasiliensis* and (d) and (h) *Cynoscion guatucupa*. —◆— 2; —■— 3; —▲— 4; —◆— pH 7; —■— pH 8; —▲— pH 9.5; —●— pH 11.5

was 50°C. *M. hubbsi* and *C. guatucupa* enzymes evidenced the lowest specific activity without significant differences between them, whereas *P. brasiliensis* and *U. brasiliensis* enzymes showed the highest specific activity without significant differences between them.

Figure 3a–d shows the stability of stomach proteinases at different temperature conditions and pre-incubation times. Acid proteinases of *M. hubbsi* were stable during 150 min at 10 and 30°C and at 50 and 70°C lost their initial activity at rates of 71% and 62%, respectively. *P. brasiliensis* and *C. guatucupa* enzymes were stable for 150 min at 10, 30 and 50°C and at 70°C activity was almost null. *U. brasiliensis* enzymes were stable at 10 and 30°C, and maintained a high activity at 30°C, from 11.78 ± 3.88 U mgprotein^{-1} (time 0 min) to 11.6 ± 3.58 U mgprotein^{-1} during 150 min, thus these extracts had the highest activity values over a wide temperature range compared to the other species. However, at 50°C after 30 min the enzymes lost 77% of their initial activity see Supporting Information Table A4a). However, acid proteinases for the other studied species had activity after 150 min, therefore a denaturation assay was performed to determine the temperature where these enzymes lose more than 95% of their initial activity. The results showed that proteinase residual activities for *M. hubbsi*, *P. brasiliensis* and *U. brasiliensis* enzymes after 5, 10 and 20 min of pre-incubation, respectively, were less than 5% at 80°C (data not shown).

Considering alkaline proteinases, the model that best fitted the data (wi ≥ 0.05) contains the variables species, incubation time, their interaction and temperature, and their interaction with incubation time (see Supporting Information Table A3b).

TABLE 4 Specific proteinase activities at different temperature conditions for stomach extracts (a) and intestine-ceca extracts (b) from fishery waste of *Merluccius hubbsi*, *Percophis brasiliensis Urophycis brasiliensis* and *Cynoscion guatucupa*

(a)

	Specific activity (U mg protein^{-1}) Temperature (°C)			
Species	**10**	**30**	**50**	**70**
M. hubbsi #	0.34 ± 0.06	0.65 ± 0.16	0.59 ± 0.14	0.21 ± 0.03
P. brasiliensis ‡	2.06 ± 0.47	3.14 ± 0.75	3.63 ± 0.71	1.35 ± 0.39
U. brasiliensis ‡	6.72 ± 1.42 ^{ab}	11.78 ± 3.88 ^{a}	11.79 ± 4.11 ^{a}	2.42 ± 0.65 ^{b}
C. guatucupa #	0.33 ± 0.12 ^{ab}	0.61 ± 0.18 ^{ab}	0.79 ± 0.16 ^{a}	0.11 ± 0.03 ^{b}

(b)

	Specific activity (U mg protein^{-1}) Temperature (°C)			
Species	**10**	**30**	**50**	**70**
M. hubbsi	0.14 ± 0.06 ^{a}	0.44 ± 0.20 ^{ab}	1.30 ± 0.43 ^{b}	0.14 ± 0.06 ^{a}
P. brasiliensis	0.51 ± 0.17 ^{a}	1.90 ± 0.46 ^{ab}	3.11 ± 0.56 ^{b}	0.96 ± 0.25 ^{a}
U. brasiliensis	0.51 ± 0.24 ^{a}	1.75 ± 0.33 ^{b}	5.05 ± 0.71 ^{c}	0.48 ± 0.08 ^{a}
C. guatucupa	0.20 ± 0.09 ^{a}	0.20 ± 0.07 ^{a}	1.90 ± 0.47 ^{b}	0.34 ± 0.09 ^{ab}

Note: Assays were carried out at pH 2 (stomach) or pH 8 (intestine-ceca). Values indicate means and standard error from three replicates ($n = 4$). Different small superscript letters in the same row indicate significant differences among different temperature conditions at the same incubation time for individual species. Different symbols (#, ‡, §) indicate significant differences among different species at different temperature conditions and time incubation (generalized linear mixed model, Tukey test, $P < 0.05$).

Table 4b shows the intestine-ceca proteinase activity of the crude extract as a function of the different temperature conditions without pre-incubation. Specific activities were similar for enzymes of all species with an optimum temperature at 50°C and the maximum value observed was 5.05 ± 0.71 U mgprotein^{-1} for the crude extract of *U. brasiliensis*.

Figure 3e–h shows the stability of intestine-ceca proteinases at the different temperature conditions and pre-incubation times. The results showed that alkaline proteinases of all species were thermostable at 10 and 30°C for 150 min, without significant differences. After 30 min at 50°C, there was a pronounced and significant loss of specific activity for *M. hubbsi* and *U. brasiliensis* enzymes (90% and 53% of their initial activity, respectively). After 150 min at 30°C, the *C. guatucupa* enzymes doubled their initial activity significantly while the enzymes of the other species maintained it. However, after 150 min at 50°C, *C. guatucupa* enzymes lost 96% of their initial activity. At 70°C, the alkaline proteinase activity was almost null for all studied species, thus the denaturation assay was not necessary. Proteinase residual activity was less than 5% after 30 min for *P. brasiliensis* and *U. brasiliensis* enzymes, and after 150 min for *C. guatucupa* enzymes. *M. hubbsi* enzymes showed low enzyme activity and values did not show significant differences at different incubation times (see Supporting Information Table A4b).

4 | DISCUSSION

This study displays novel results about biomolecules, as proteinases of wastes, from four commercial fish species from the south-west Atlantic Ocean. This is the first research that compares activity and some properties of proteinases recovered from *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis* and *C. guatucupa*.

Previous studies have shown that the digestive enzyme activity is related to the feeding habits of fishes (Gioda *et al*., 2017; Martínez-Cárdenas *et al*., 2017). All species studied in this work are carnivorous. *M. hubbsi* is a generalist and opportunistic predator that feeds mainly on demersal fishes, followed by crustaceans and cephalopods (Alonso *et al*., 2019), while *U. brasiliensis* is a generalist that has shrimp as its main prey, but also feeds on crabs and shellfish (Acuña Plavan *et al*., 2007). In contrast, *C. guatucupa* has a varied diet that changes throughout its development. In the early stages it feeds on small crustaceans and progressively increases its consumption of fish (Viola *et al*., 2018), whereas *P. brasiliensis* feeds mainly on fish and squid (Milessi & Marí, 2012). The digestion of food proteins begins in the stomach where pepsin is the active proteinase and it finishes in the intestine with alkaline proteinase activities such as trypsin and chymotrypsin, resulting in the release of amino acids and peptides (Martínez-Cárdenas *et al*., 2017).

In the present study, the optimum pH for enzymes of stomach extracts from *P. brasiliensis* was pH 2. Similar results were found in viscera of Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yañez *et al*., 2004) and Sábalo (*Prochilodus lineatus)* (Gomez *et al*., 2018). Moreover, *U. brasiliensis* kept high activity at pH 3 as well, whereas *M. hubbsi* and *C. guatucupa* kept high activity at pH 3 and 4. Similar results were found in the viscera of Farmed giant catfish (*Pangasianodon gigas*) (Vannabun *et al*., 2014) and Golden grey mullet (*Liza aurata*) (Bkhairia *et al*., 2016).

On the other hand, the catalytic activities from intestine-ceca crude extracts of *M. hubbsi*, *P. brasiliensis*, and *U. brasiliensis* were

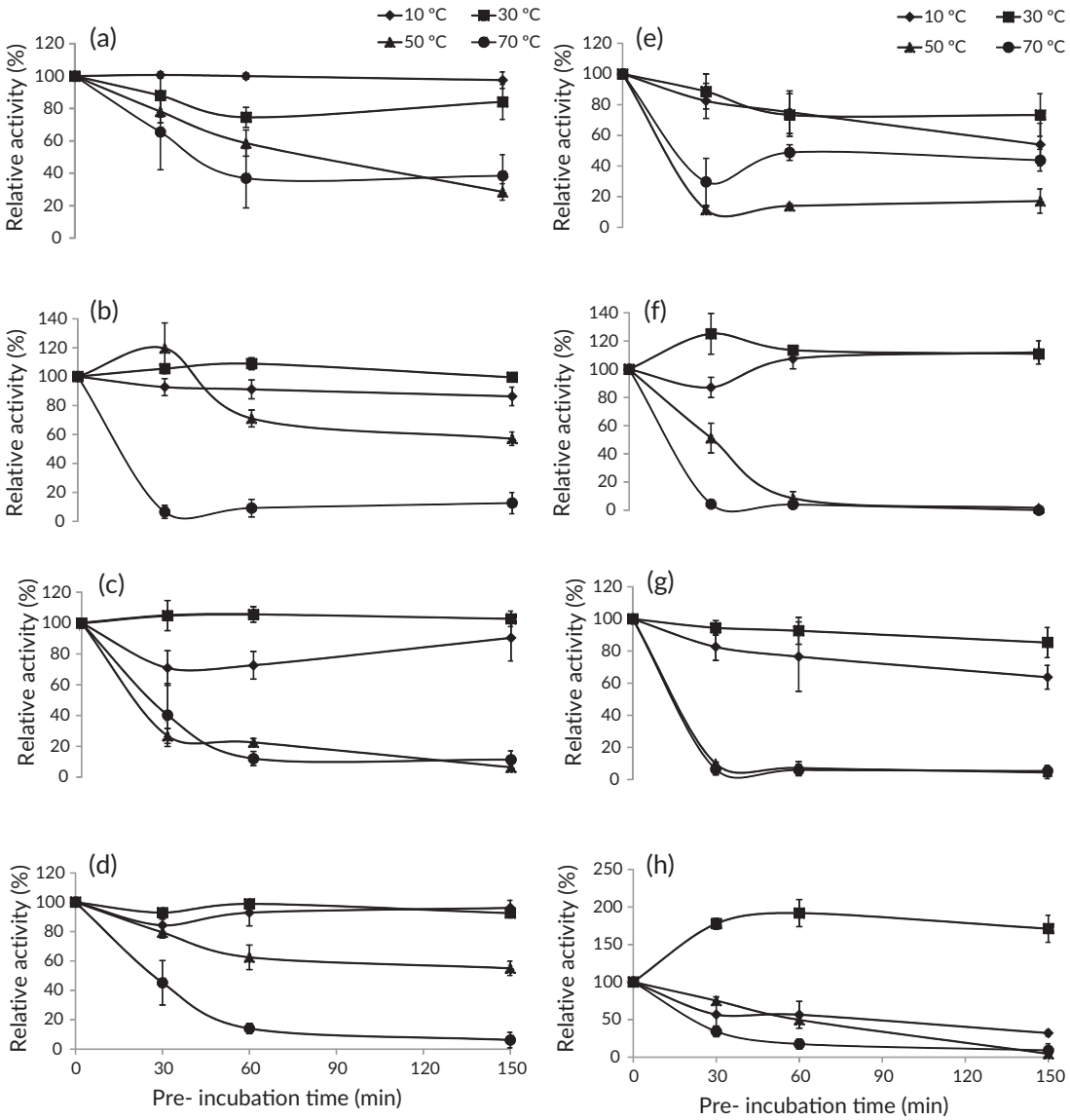

FIGURE 3 Temperature stability of stomach extracts (left column) at pH 2 and intestine-ceca extracts (right column) at pH 8 during 0, 30, 60 and 150 min at 25°C from fishery waste of (a) and (e) *Merluccius hubbsi*, (b) and (f) *Percophis brasiliensis*, (c) and (g) *Urophycis brasiliensis* and (d) and (h) *Cynoscion guatucupa*. —◆— 10°C; —■— 30°C; —▲— 50°C; —●— 70°C; —◆— 10°C; —■— 30°C; —▲— 50°C; —●— 70°C

almost constant over a wide pH range from 7 to 11.5 and only enzymes of *C. guatucupa* evidenced an optimum pH at 11.5. Similar results were reported for Seer fish (*Scomberomorus guttatus*) (Rengasamy *et al*., 2016), Farmed giant catfish (*P. gigas*) (Vannabun *et al*., 2014), Chihuil sea catfish (*Bagre panamensis*) (Rios-Herrera *et al*., 2019), Indian oil sardine (*Sardinella longiceps*) (Ramkumar *et al*., 2018) and Greater amberjack (*Seriola dumerili*) (Oliveira *et al*., 2017).

The effect of temperature on enzyme activity in this work for all the species studied demonstrated that acid proteinases had the highest activity at 30 and 50°C and their catalytic activities were stable at 10 and 30°C for 150 min. Similar results were reported for *Sardinella aurita* (Khaled *et al*., 2011), *S. sagax* (Castillo-Yañez *et al*., 2004), *P. lineatus* (Gomez *et al*., 2018) and *P. gigas* (Vannabun *et al*., 2014) where optimum temperatures were between 33 and 55°C. *C. guatucupa* enzymes were inactivated at 70°C while *M. hubbsi*, *P. brasiliensis* and *U. brasiliensis* enzymes showed residual activities less than 5% at 80°C. High temperatures can cause the loss of catalytic activity because the molecular structure of the protein is unfolded and, consequently, inactivation of the enzymes occurs (Klomklao *et al*., 2011).

Alkaline proteinases of all species were more sensitive to changes in temperature conditions than acid proteinases. These alkaline enzymes evidenced the highest catalytic activity at 50°C and were stable at 10 and 30°C. These values correlate to what was found for the digestive proteinases in *S. guttatus* (Rengasamy *et al*., 2016), *P. gigas* (Vannabun *et al*., 2014), *B. panamensis* (Rios-Herrera *et al*., 2019), *S. longiceps* (Ramkumar *et al*., 2018), *S. dumerili* (Oliveira

et al., 2017) and *M. hubbs*i (Lamas *et al*., 2015), where the optimum temperatures were between 45 and 60°C. However, in those previous studies, alkaline proteinases were stable at higher temperatures than in this research. These differences may occur because fish viscera recovered from tropical water species are used for most studies on digestive proteinases, such as Seer fish, Farmed giant catfish, and Indian oil sardine. The four commercial species selected in the present study inhabit cold waters of the Argentine Sea in the south-western Atlantic Ocean (23–55 °S), where water temperatures are usually below 20°C (Allega *et al*., 2019).

Optimum temperature and stability of fish enzymes are associated with the habitat characteristics (Nalinanon *et al*., 2008). In this sense, enzymes of the species studied in this work were stable at low temperatures (*e.g*., 10°C; Figure 3). However, all the tested enzymes also showed high activity at high temperatures (30–50°C). These ectothermic species can adopt different strategies according to the characteristics of the habitat to maintain a normal level of proteolysis at temperatures considerably lower than the corresponding to endothermic species. One of those strategies is to increase enzyme production to compensate for reduced kinetic efficiency (Brier *et al*., 2007). Although acid and alkaline proteinases showed stability at 10°C, the enzyme activity at this temperature showed low values.

In recent years, the interest in finding new thermostable enzymes has grown mainly because various industrial processes occur at high temperatures using enzymes from mesophilic sources. Thermostable enzymes have certain benefits for industrial processes, such as high reaction rate, longer half-life and lower denaturation temperature, allowing energy to be saved in the process. Fish thermostable proteases are widely used in food, pharmaceutical, laundry industries, leather treatment and bioremediation processes (Bougatef, 2013). Alkaline proteinases from different species such as *Sardinella longiceps* (Ramkumar *et al*., 2018), *Raja Clavata* (Lassoued *et al*., 2015), *Liza aurata* (Bkhairia *et al*., 2015), *Salaria basilisca* (Ktari *et al*., 2012) and *Engraulis anchoita* (Lamas *et al*., 2017) have been studied for detergent formula. These enzymes were stable at 30 and 40°C for 1 h and kept high percentages of their initial activity in the presence of different commercial detergents such as Axion, New Det, Dixan and Ariel. At the same time, these enzymes were generally highly stable in the same pH range as laundry detergents (4.0 to 10.0), with maximum activity at high pH values such as 10.5 (Bougatef, 2013). In the present study, the alkaline enzymes recovered from *M. hubbsi*, *C. guatucupa*, *P. brasiliensis* and *C. guatucupa* were stable for 150 min at 30°C with a low denaturation temperature; their optimum pH was 11.5 and they displayed catalytic activity from pH 7 to 11.5. As a result, these enzymes could be useful for potential application as detergent additives. However, it is necessary to perform further studies evaluating the stability of these enzymes in the presence of the nonionic (Tween 20 and Tween 80) and ionic surfactants (saponin and sodium choleate) present in detergents.

Furthermore, acid proteinases from fishes have been proposed to replace conventional milk-clotting enzymes. Pepsin from *Bagre panamensis* exhibited milk coagulant activity, with maximum activity at 45°C. These enzymes were active at 50°C for 120 min and remained highly active at pH 2–3 (Osuna-Ruiz *et al*., 2019). In the present study, the acid enzymes recovered from *M. hubbsi*, *P. brasiliensis* and *C. guatucupa* kept catalytic activity at 50°C, pH 2–3 for 150 min, making viscera of these species useful as a potential source of milk-clotting proteinases for cheese making. Additionally, pepsins from marine fish have been used on the collagen present in discarded fish skins by means of an acid hydrolysis. The peptides obtained can be applied in many industries, such as he cosmetics, pharmaceutical and food industries (Bkhairia *et al*., 2016; Nalinanon *et al*., 2008).

Approximately 125 million tons of by-products (70% of 179 million tonnes; FAO, 2020) are produced during fish processing and viscera waste represents about 12%–18%. These large amounts of viscera, which are discarded during filleting when fish are not marketed as fresh or frozen, are recognized to be an important source of digestive enzymes, making the estimation of viscera yield valuable information for the biotechnological valorization of waste. In this work, viscera yield values and specific activity are useful parameters to decide with which enzymes and species we can continue our investigations, focusing on the generation of high added-value products and their potential incorporation in different industrial processes. For stomach extracts, the viscera yield was similar for all species while for intestine-ceca extracts the viscera yield was higher for *P. brasiliensis* compared to the other species. Moreover, *P. brasiliensis* and *U. brasiliensis* enzymes showed the highest values of specific activity, making them an interesting target to continue our work.

In conclusion, the viscera of *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis* and *C. guatucupa* are an important source of acid and alkaline proteinases with potential biotechnological uses. These proteolytic enzymes are stable in a wide range of pH and moderate temperatures, and are inactivated at relatively low temperatures, therefore they could be used as biocatalysts in different industries, reducing manufacturing costs and contributing to the reduction of environmental pollution.

Future research regarding partial purification and immobilization of these enzymes, scale-up studies and techno-economic evaluation is needed. Improved utilization of the resource not only could provide several and novel useful higher-value products, but also improve economic returns to the fishing industry without increasing fishing overexploitation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS

I.S.F.: conceptualization, methodology, validation, formal analysis, investigation, writing – original draft, visualization. L.A.B.: methodology, formal analysis, writing – review and editing. N.A.P.: methodology, writing – review and editing. E.C.: methodology, writing – review

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SUPPORTING INFORMATION

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