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Exogenous proteases from seafood processing waste as functional additives in rainbow trout aquaculture

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Abstract

Previous studies about enzyme additives for salmonids employed commercial proteases from bacteria, but research obtaining such enzymes from other organisms are scarce. We aimed to increase digestive protease activity in *Oncorhynchus mykiss*, by analysing potential sources of exogenous proteases generated from different seafood processing waste. To date, there is no information regarding the influences that exogenous enzymes have on the activity and integrity of endogenous enzymes in salmonids. The compatibility and interaction between endogenous and exogenous proteases were evaluated through protein hydrolysis and electrophoresis. We also evaluated the capacity of two types of microcapsules (Alginate–Chitosan and Alginate–Chitosan–Bentonite) to protect the protease activity after being exposed to the gastric environment of trout. The seafood wastes had activities around 0.23–2.53 U/ml at 15°C. Among all the exogenous proteases evaluated, *P. granulosa's* extract showed the best performance, followed by *L. santolla's* extract. Such extracts increased the protein hydrolysis to equal or higher levels than the commercial protease and did not alter the integrity of trout proteases. The optimal catalytic function at low temperatures of these extracts might be linked to the habitat of these arthropods. Alginate–Chitosan–Bentonite microcapsules are recommended in preference to Alginate–Chitosan capsules for delivery of enzymes in salmonids because they ensure the activity of exogenous proteases for longer in acid pH (6 vs. 4 h). This research promotes a re-evaluation of seafood processing wastes through the production of a potentially functional additive that may improve trout protein digestion.

KEYWORDS
exogenous proteases, functional additive, microcapsule, rainbow trout, seafood processing waste

1 | INTRODUCTION

Aquaculture is one of the fastest-growing food production sectors in the world and has diverse environmental impacts. This activity can have a positive environmental effect by reducing human dependence on wild fish, and therefore decrease pressure on natural systems. However, farming aquatic organisms degrades the environment by increasing organic pollution, nutrient enrichment and other types of waste (Cole et al., 2009). Part of this contamination comes from faecal production, since the animal's digestive system is not entirely efficient (Ojha et al., 2019). Further, in aquatic organisms, the digestibility coefficients of formulated feeds are around 5 to 10% lower than natural food (Jobling, 1986). The use of exogenous feed enzymes has gained attention in aquaculture because such functional

additives can improve nutrient digestion, reduce faecal disposal and even feed costs (Abo Norag et al., 2018; Cheng et al., 2004; Córdova-Murueta et al., 2017; González-Zamorano et al., 2013; Maas et al., 2018; Mireles-Arriaga et al., 2015). Aquafeeds are expensive principally because of their high reliance on costly protein ingredients such as fish meal, which are the most important dietary component for fish. However, proteases can be employed to improve protein utilization and reduce the required amount of this nutrient. Exogenous proteases have been supplied to several fish species (as for example *Oreochromis niloticus*, *Salmo trutta caspius* and *Clarias gariepinus*), demonstrating protein digestibility enhancements and improvement of nutritional indicators such as feed conversion and body weight (Adeoye et al., 2016; ali Zamini., 2014; Hassaan et al., 2019; Kemigabo et al., 2019). Rainbow trout (*Oncorhynchus mykiss*) farming has been the focus of numerous advances in protease additives as a viable alternative to enhance nutritional properties of fish diets (Dalsgaard et al., 2012; Drew et al., 2005; Yigit et al., 2018). However, most studies examined the effect of commercial proteases on growth performance and nutrient digestibility. To date, there is no information about the influence, activity and integrity of exogenous enzymes have on the activity and integrity of endogenous enzymes in salmonids.

Finding new protease sources could represent a commercial opportunity, with most available proteases designed for livestock and poultry production (Mireles-Arriaga et al., 2015). In recent years, the interest in seafood processing waste and unutilized catch for the production of biofunctional feed ingredients has increased (Le Gouic et al., 2019). Marine-derived waste components contain enzymes with a wide variety of catalytic features, such as high activities over a wide range of temperature values (Venugopal, 2016). Such plasticity facilitates the adaptation of fish and invertebrates to the variable conditions of the marine environment, including even extreme, polar habitats. Consequently, enzymes extracted from seafood processing waste could be valuable for the aquaculture food industry as enzyme additives. Rodriguez et al. (2017), Rodriguez et al., (2019) successfully extracted exogenous proteases from different fishery wastes and showed the potential for some proteases to be exploited as feed additives for aquaculture. However, such research aimed to improve the digestion of tropical species as Nile tilapia (*Oreochromis niloticus*) and Australian redclaw crayfish (*Cherax quadricarinatus*) (Meade et al., 2002; Trewavas, 1983). Rainbow trout require water temperatures ranging from 13 to 18°C for optimal growth (Hardy, 2002), so a suitable enzyme additive should have high proteolytic activity at low temperatures. Therefore, it is important to evaluate processing wastes from a variety of marine/aquatic taxa to find the best source of low-temperature proteases to enhance protein hydrolysis.

Despite clear benefits, there are also some problems in the use of marine-derived enzymes; because such biomolecules are water-soluble and are very sensitive to the gastric conditions of farmed fish. To overcome these problems, an effective delivery system needs to be developed to ensure the efficiency of enzyme additives. Microencapsulation may represent one possible technique for a variety of substrates, such as alginate and chitosan (DeGroot & Neufeld, 2001; Mong Thu & Krasaekoopt, 2016; Yoon et al., 2001). However, reports of enzyme microencapsulation for aquaculture are scarce, perhaps because enzyme retention can be challenging.

As mentioned above, there are several previous works about the effects of enzyme additives in salmonids; however, all of them employed commercial proteases from microorganisms. The present study proposes a different approach because it intends to obtain such enzymes from seafood processing remains. This sustainable feed additive may improve protein digestion in salmonids, and it would also reduce the negative environmental impacts associated with fisheries and fish farming. Also, previous research about exogenous proteases in salmonids studied the *in vivo* effects, as growth parameters and digestibility. Nonetheless, there is no information regarding the influences that exogenous enzymes have on the activity and integrity of endogenous enzymes in this taxon. Thus, in this study, a variety of seafood processing waste was evaluated to find the best source of low-temperature proteases. Here, we aimed to find the most suitable potential exogenous enzyme to be used as a feed additive to improve the digestion process of *O. mykiss*. To achieve this goal, we assessed the effects of mixing *in vitro* exogenous multienzyme extracts obtained from different crustaceans (*Pleoticus muelleri*, *Paralomis granulosa*, *Lithodes santolla*, *Munida gregaria*) and fish (*Engraulis anchoita*, *Merluccius hubbsi* and *Brevoortia aurea*) processing wastes with enzymes of rainbow trout. In order to design a vehicle that can withstand gastric conditions and deliver exogenous enzymes into the intestine of the fish, we also evaluated two different types of microcapsules: alginate–chitosan (AC) and alginate–chitosan–bentonite (ACB).

2 | MATERIALS AND METHODS

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as we used frozen commercial samples from a fish farm and seafood processing plants.

2.1 | Rainbow trout endogenous enzymes

A fish farm from Junín de los Andes (Neuquén, Argentina) provided the viscera from *O. mykiss*. Two different fish sizes were utilized because previous works demonstrated that exogenous enzymes could exert varied effects depending on the organism's weight (Rodriguez et al., 2017,2019). Stomachs and intestines with pyloric caecas of ten fish weighing 21.1 ± 3.02 g (*Om1*, $n = 5$) and 58.3 ± 5.73 g (*Om2*, $n = 5$) were sampled. These sizes were selected because during trout culture fish are classified and, to obtain optimal growth, organisms with such weights are raised separately (FAO, 2009). The dissection of these organs was made in fed animals to ensure the presence of proteolytic activity. Samples were freeze-dried (−80°C) and then transported to the Laboratory of

'Fisiología de Organismos Acuáticos y Biotecnología Aplicada' (IIMyC, Mar del Plata, Argentina). Dried organs were re-suspended in distilled water. Then, the stomach and intestine with pyloric caeca (1:3 w/v) were separately homogenized in ice-cold water at pH 2 or pH 8 adjusted with 0.1 M HCl and 0.1 M NaOH respectively. These preparations were centrifuged (10,000 *g* for 30 min at 4°C) and the resulting supernatants (crude endogenous enzyme extracts) were stored at −20°C.

2.2 | Exogenous enzymes

Samples of *P. muelleri* (*Pm*), *P. granulosa* (*Pg*), *L. santolla* (*Ls*), *M. gregaria* (*Mg*), *E. anchoita* (*Ea*), *M. hubbsi* (*Mh*) and *B. aurea* (*Ba*) were supplied by seafood processing plants and artisanal fishers from Mar del Plata, Mar Chiquita and Ushuaia, Argentina. These samples were frozen and transported to the laboratory. After thawing, the cephalothoraxes of *Pm*, the midgut glands of *Pg*, *Ls* and *Mg*, the intestines (and pyloric caeca when present) of *Ea*, *Mh* and *Ba* were utilized to prepare the different enzyme extracts. Pools of three individuals per sample (six in the case of *Mg* due to the small body size) were separately homogenized using a Teflon-glass tissue homogenizer in ice-cold distilled water (1:3 w/v and 1:2 w/v, for crustacean and fish samples respectively). Next, the homogenates were centrifuged at 10,000 *g* for 30 min at 4°C (Presvac EPF 12R, San Martín, Argentina). The supernatants (crude exogenous enzyme extracts) were frozen and stored at −20°C until use. These extracts contain enzymes but also other proteins. The commercial protease (CP) RONOZYME®ProAct (DSM, Heerlen, Netherlands) was used as a positive control.

2.3 | Determination of protein concentration

Soluble protein concentration was determined according to Bradford (1976) and bovine Serum Albumin (Sigma A9647) was used as the standard.

2.4 | Enzymatic activity

An adaptation of García-Carreño (1992) methodology was employed to determine the alkaline protease activity in the rainbow trout extracts (*Om1* and *Om2*) and exogenous enzymes extracts. For this, 0.5% (w/v) azocasein (Sigma A 2765) was used as the substrate in 50 mM Tris-HCl buffer at pH 7.5. The incubation was performed at 15°C, in order to simulate the environmental conditions where this species is cultivated. The readings were performed in a Microplate Spectrophotometer with Gen5™ Software (Epoch BioTek). The wavelength was 366 nm. All assays were run in duplicate. Total protease units of activity were expressed as change in absorbance per minute per ml of enzyme extract ($U = Abs\ 366 / min \times ml$).

2.5 | Electrophoresis

The assessment of proteins present in the different enzyme extracts was done by SDS-PAGE according to Laemmli (1970). For this, one volume of each enzyme extract (containing 20 µg of protein) was mixed with one volume of 2 × sample buffer and then loaded on a minivertical gel electrophoresis device (Mini protean tetra cell Bio-Rad). In addition, 6 µl molecular weight standard was mixed with sample buffer and then loaded into the plate. Electrophoresis was carried under 30 mA per gel with a constant power supply unit at 4°C. After electrophoresis, gels were stained as described by Rodriguez et al. (2019).

2.6 | Enzyme interaction

The most suitable exogenous enzyme is that which increases protein hydrolysis without affecting fish endogenous proteases. In this work, we evaluate the contribution of each exogenous enzyme to protein hydrolysis, and also, if these enzymes exert a negative effect in rainbow trout digestive proteases.

First, 5 µl of intestine + pyloric caeca extracts (*Om1* or *Om2*) were separately combined with 5 µl of each exogenous enzyme (*Pm*, *Pg*, *Ls*, *Mg*, *Ea*, *Mh*, *Ba*). Activities were determined according to the protocol previously described. The activities of rainbow trout extracts were set as the control while the market protease (*CP*) was used as a positive control of the synergistic activity.

Secondly, an adaptation of García-Carreño et al. (1993) method was used to analyse the integrity of digestive enzymes from each rainbow trout size when mixed with the exogenous enzymes. For this, 5 µl of rainbow trout enzyme preparation (*Om1* or *Om2*) and its mixture with 5 µl of each exogenous enzymes (*Pm*, *Pg*, *Ls*, *Mg*, *Ea*, *Mh*, *Ba*) were incubated in 10 µl of 50 mM Tris-HCl buffer at pH 7.5 at 15°C for 1 min. Subsequently, each rainbow trout extract or mixture was combined with 1:1 (v/v) 2 × sample buffer and then separated by electrophoresis as described above. When the electrophoresis ended, gels were washed in cold water. Next, gels were washed in cold water and incubated for 90 min in a 3% (v/w) casein solution (Sigma C7078) prepared with the same Tris-HCl buffer. Finally, the gels were washed with distilled water and stained in the same way that protein electrophoresis.

2.7 | Exogenous enzymes encapsulation procedure

Two types of microcapsules were elaborated: alginate–chitosan (AC) and alginate–chitosan–bentonite (ACB) microcapsules to entrap the exogenous enzymes obtained from fishery processing waste. To evaluate the behaviour of these microcapsules under in vitro digestion, just one exogenous enzyme extract was selected for its encapsulation based on the results obtained in the prior assays. The microencapsulating procedure outlined by Vandenberg et al. (2001) was modified to make it more suitable for enzymes crude extracts.

For the basal encapsulation protocol, 1.5% (w/v) alginate (sodium salt of alginic acid for feed purposes; Química Industrial Kubo S.A.) was dissolved in distilled water. The exogenous enzyme added at 40% loading rate. Chitosan (INTI, Mar del Plata, Argentina) was dissolved in 0.2% (w/v) acetic acid and the pH was adjusted to 7 with 2 M NaOH. Next, $CaCl_2$ was added to obtain a final concentration of 1.5% (w/v). Then, using a pressure system, the alginate-enzyme solution was extruded drop-wise into 50 ml chitosan-calcium chloride solution through a syringe with a needle, 0.8 mm diameter. The microcapsules were formed immediately and were allowed to react for 30 min. Microcapsules were removed from the encapsulation medium via filtration and rinsed twice with distilled water. Finally, all AC microcapsules were then washed twice with acetone and dried under fume hood at 4°C in order to avoid enzyme denaturalization. ACB microcapsules were elaborated in the same way, but 1% (p/v) bentonite (Química Industrial Kubo S.A.) was added to the initial alginate solution.

2.8 | Stability of free and encapsulated exogenous enzyme under simulated gastric conditions

Exogenous enzymes need to go through the fish stomach to reach the intestine where they would act. In this research, we evaluate the effect of the stomach environment on the activity of free exogenous enzyme (FE), and when the same enzyme was immobilized in AC or ACB microcapsules. First, FE (5 µl), AC (3 microcapsules) and ACB (3 microcapsules) were placed into 50 µl of 200 mM buffer Glycine-HCl pH 2 and then incubated for 24 hr at 15°C. These conditions pretended to reproduce pH, temperature and length of trout gastric digestion (Windell & Norris, 1969). Also, in order to study if the stomach pepsin affects the integrity of the FE extract selected, another treatment included the addition of 5 µl of trout stomach enzyme extract in replacement of an equivalent buffer volume.

For all the treatments, samples (3 replicates) were taken at 0, 0.5, 2, 4, 6 and 24 hr. Subsequently, 500 µl of 50 mM Tris-HCl pH 7.5 was added and incubated for 1 hr at 15°C, simulating the intestinal conditions. The free enzyme not exposed to acidic conditions (incubated 24 hr at pH 7.5, 15°C) was used as the control treatment. Finally, alkaline protease activity was assayed as described before.

2.9 | Statistical analysis

Datasets are presented as the mean and standard error of the mean (SEM). Differences in the total protease activity (response variable) between digestive extracts from *Om1* and *Om2*, among different exogenous enzyme extracts, and enzyme combinations were separately evaluated with generalized linear mixed models (GLMM) (Zuur et al., 2009). In all cases, the treatment repetition was included as a random effect and, when needed, the variance was structured.

On the other hand, generalized linear models (GLM) were developed to compare the enzymatic activity of free exogenous enzyme (FE) and different microcapsules (AC, ACB) among different pH and incubation times in the gastric simulation assay. Data presented Gaussian distribution. In order to determine the significance of the different factors ('treatment', and the interaction 'treatment: time') on the enzymatic activity, models (named 'm1' and 'm2' respectively) were contrasted with a null model identified as 'm0' (without any independent variable).

In all cases, the goodness of fit of the model was evaluated throughout chi-squared test (Faraway, 2006) while all the formulated models were compared through Akaike's Information Criterion (AIC) (Akaike, 1973). This method is based on the construction of different models with the biologically pertinent variables combination and the selection of the best model among them. Such best model describing the data under analysis is that which presents the lowest AIC. Once the model was selected, assumptions were tested trough the graphical analysis of the residuals and its adjustment was analysed by comparing the residual deviance with the degrees of freedom. Subsequently, the significance of the explanatory variable (s) was tested using a deviance analysis test (Fisher's *F* Test). When significant differences were found, Tukey's tests were applied in order to make post hoc multiple comparisons and detect which treatments differ in the enzymatic activity. All analyses were done with Open Access Software R (Core Team R Development, 2011).

3 | RESULTS

3.1 | Protein content and enzymatic activity

Table 1 shows soluble protein contents and alkaline protease activities present in the intestine + pyloric caeca extracts obtained from the different sizes of *O. mykiss* studied. Soluble protein values were

TABLE 1 Soluble protein and alkaline protease activity of protein extracts recovered from the intestine and pyloric caeca of rainbow trout *O. mykiss*

Rainbow trout	Soluble Protein (mg/ml)	Total Activity (U/ml)
Intestine + pyloric caeca		
Om1	1.09 ± 0.134[b]	2.05 ± 0.246[a]
Om2	0.71 ± 0.070[a]	2.38 ± 0.425[a]
Statistical analysis		
AIC m_0	24.6	37.1
AIC m_1	21.3	38.2
p-value	0.0119	0.4686

Note: Values are means and SEM of five replicates. [a–b]Means within a column with different superscript letters are significantly different ($p < 0.05$). Abbreviations: *Om1*: intestine + pyloric caeca extract of *O. mykiss* weighing 21.1 ± 3.02 g; *Om2*: intestine + pyloric caeca extract of *O. mykiss* weighing 58.3 ± 5.73 g; AIC m_0, Akaike number obtained of the null model (without independent variable); AIC m_1, Akaike number of the model with the fish size (*Om1* and *Om2*) as the explanatory variable.

higher for *Om1*, but total protease activities did not differ between the two sizes (Table 1).

Moreover, there were significant differences in the protein content of the different exogenous enzyme sources evaluated (Table 2). When the proteolytic activities of the same extracts were compared, significant differences were also found (Table 2). The *Ba* crude extracts had the highest enzyme activity, even higher than that of the enzyme additive sold in the market (Table 2).

Also, the electrophoresis of rainbow trout extracts showed that the molecular weight of the proteins present in both sizes studied ranged from 18 to 75 kDa. On the other side, the different extracts obtained from fish processing waste hold different protein profiles that range from 15.5 to 72.8 kDa.

3.2 | Enzymes' interaction

The interaction between *Om1*'s digestive enzymes and the different exogenous enzymes is shown in Figure 1. There were significant differences in azocasein hydrolysis between the different enzyme combinations analysed (Figure 1a; GLMM: AICm0 = −17.5, AICm1 = −115.8; *p*-value = 2.2^{-16}). The hydrolysis was enhanced by the market protease (CP), and also, by the extracts obtained from *Pg*, *Ba* and *Ls*. Moreover, the exogenous proteases obtained from *Pg* had even more activity than the commercial protease. However, when the *Om1* enzyme preparation was mixed with the extracts recovered from *Mh*, its enzyme activity significantly declined; while its activity did not significantly change when *Om1* was combined with *Mg* and *Ea* enzymes (Figure 1a).

The alkaline protease activities are also shown in the SDS-PAGE gels that have hydrolysed the casein substrate after running (Figure 1b). The extracts of *Om1* had seven alkaline proteases in the rage of 27.5 and 63.4 kDa. When *Pm*, *Pg*, *Ls*, *Mg* and *Ea* crude extracts were added to the endogenous enzymes, all the *Om1* bands remained active (Figure 1b). Furthermore, such exogenous enzymes added some proteases to the *Om1* extracts (*Pm*: 21.7 and 19.9 kDa; *Pg*: 16.6, 18.2 and 22.8 kDa; *Ls*: 17.8, 20.2 and 22.1 kDa; *Mg*: 18.2 and 22.7 kDa; *Ea*: 34.9 kDa). Conversely, when *Mh* and *Ba* were combined with rainbow trout extracts, the band from *Om1* weighing 35.7 kDa disappeared or lost intensity (Figure 1b).

On the other hand, when *Om2*'s digestive enzymes were combined with the exogenous enzyme preparations, there were significant differences in the total protease activity between the different mixes (Figure 2a; GLMM: AICm0 = −11.6, AICm1 = −48.1; *p*-value=2.2^{-16}). The azocasein hydrolysis was significantly improved by *Pg* and *Ls* extracts, and also by the commercial enzyme; but when *Pm* or *Mh* was added to rainbow trout extracts, the protease activity was declined (Figure 2a). Also, it was observed that *Mg*, *Ea* and *Ba* extracts did not increase or decrease the activity of *Om2* extracts (Figure 2a). The zymogram for *Om2* shows that they possess active proteases ranging from 30.9 to 63.4 kDa (Figure 2b). Most of the exogenous enzymes evaluated did not affect the activity of *Om2* proteases, and moreover, they have some additional active proteases (*Pg*: 16.6 and 18.2; *Ls*: 17.8 and 20.2; *Mg*: 18.2; *Ea*: 34.9 and 30.2; *Ba*: 62.6 and 25.4). However, it seems that *Pm* and *Mh* enzyme extracts reduce the intensity of rainbow trout activity bands (Figure 2b).

TABLE 2 Soluble protein and alkaline protease activity of different exogenous enzymes crude extracts

Exogenous enzymes	Soluble Protein (mg/ml)	Total Activity (U/ml)
Fish processing waste		
Pm	4.04 ± 1.024^{ab}	0.42 ± 0.034^{a}
Pg	3.35 ± 0.394^{ab}	1.33 ± 0.104^{ab}
Ls	3.25 ± 0.644^{ab}	1.55 ± 0.270^{ab}
Mg	1.12 ± 0.254^{ab}	0.73 ± 0.150^{a}
Ea	3.25 ± 0.382^{ab}	0.38 ± 0.034^{a}
Mh	4.51 ± 0.711^{b}	0.23 ± 0.030^{a}
Ba	2.08 ± 0.314^{ab}	2.53 ± 0.030^{b}
Market		
CP	0.35 ± 0.030 ^{a}	1.01 ± 0.017^{ab}
Statistical analysis		
AIC m_0	165.8	27.9
AIC m_1	147.7	12.8
p-value	0.0011	1.059^{-08}

Note: Values are means and SEM of three replicates. $^{a-b}$Means within a column with different superscript letters are significantly different ($p < 0.05$). Abbreviations: *Pm*: *P. muelleri* cephalothorax extract; *Pg*: *P. granulosa* midgut gland extract; *Ls*: *L. santolla* midgut gland extract; *Mg*: *M. gregaria* midgut gland extract; *Ea*: *E. anchoita* intestine extract; *Mh*: *M. hubbsi* intestine extract; *Ba*: *B. aurea* intestine and pyloric caeca extract; and commercial protease RONOZYME®ProAct (*CP*); AIC m_0, Akaike number of the null model (without independent variable); AIC m_1, Akaike number of the model with the type of exogenous enzyme as the explanatory variable.

3.3 | Stability of free and encapsulated exogenous enzyme under simulated gastric conditions

The extract obtained from *P. granulosa* was encapsulated to evaluate AC and ACB microcapsules performance under simulated gastric conditions. Such enzyme preparation was selected because it contributes to the activity of rainbow trout without affecting the integrity of fish digestive proteases, and also, presented the highest synergistic effect (Figures 1 and 2); however, these encapsulation materials can be utilized to immobilize any of the extracts studied. First, Table 3 shows that trout stomach enzymes did not affect the activity of *Pg* free extract (FE); however, in both treatments, there is a significant reduction in total activity after 30 min of exposure to pH 2.

The activity of free or immobilized *Pg* extracts under simulated gastric pH conditions of *O. mykiss* is shown in Figure 3. The best model explaining *Pg* proteolytic activity included the interaction between treatment and time (GLM: $AICm_0 = 140.0$; $AICm_1 = 101.6$; $AICm_2 = 61.3$). This indicates that the different ways of delivering

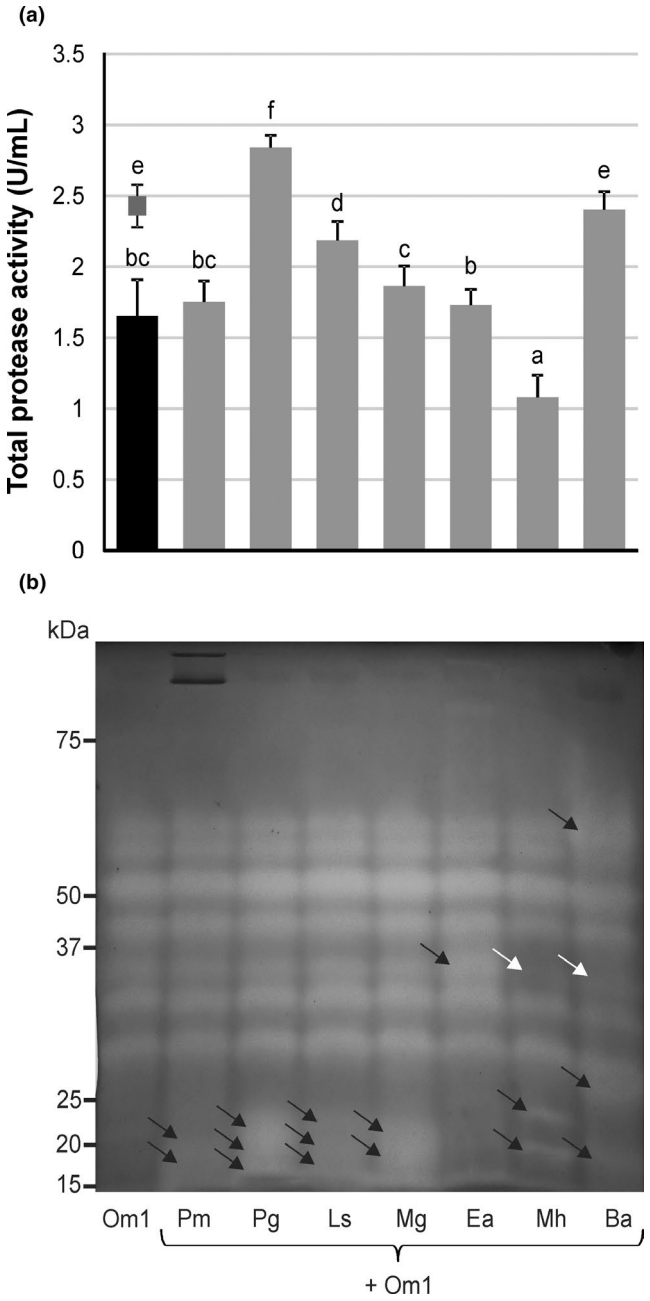

FIGURE 1 Enzyme interaction between digestive enzymes of *Om1* and exogenous enzymes recovered from different fish processing waste. (a) Total protease activity (U/ml) calculated after azocasein hydrolysis at pH 7.5 and 15°C. The black bar shows the mean proteolytic activity of *Om1*'s crude enzyme extracts, grey bars indicate the mean proteolytic activity of *Om1*' crude enzyme extracts combined with the exogenous enzymes recovered from seafood processing waste, and the grey dot shows the mean proteolytic activity of *Om1*'s crude enzyme extracts mixed with the commercial protease RONOZYME®ProAct. $^{a-e}$Means with different superscripts are significantly different ($p < 0.05$). Error bars display SEM values. (b) Zymogram of extract from rainbow trout *Om1* and its mixture with crude enzymes extracts obtained from different seafood processing waste. Black arrows indicate exogenous proteases. White arrows show rainbow trout proteases affected by the addition of exogenous enzymes. Abbreviations: *Ba*, *B. aurea* intestine and pyloric caeca extract; *Ea*, *E. anchoita* intestine extract; *Ls*, *L. santolla* midgut gland extract; *Mg*, *M. gregaria* midgut gland extract; *Mh*, *M. hubbsi* intestine extract; *Om1*, intestine + pyloric caeca extract of *O. mykiss* weighing 21.1 ± 3.02; *Pg*, *P. granulosa* midgut gland extract; *Pm*, *P. muelleri* cephalothorax extract

Pg extracts (FE, AC or ACB) have a different response to the pH conditions assayed, although this response depends on the exposition time length. Multiple comparisons indicate that FE incubated at pH 7.5 did not lose its activity during the entire experiment, and even this activity increased (Figure 3). However, such FE had significantly lower protease activity than the control after 30 min of exposition to gastric conditions (Figure 3). On the other hand, enzyme encapsulation had a positive effect on maintaining the proteolytic activity of *Pg* enzymes. Multiple comparisons indicate that the *Pg* extract contained in the AC microcapsules remained active for 4 hr under gastric conditions; meanwhile, when this enzyme was immobilized in the ACB matrix, its proteolytic activity lasted for 6 hr in the same conditions (Figure 3). However, the three treatments lost their activity after being incubated for 24 hr in acidic pH at 15°C.

4 | DISCUSSION

The various seafood processing wastes used in this research are of interest as aquafeed additives primarily because they have enzyme activity. The presence of activity makes such wastes an ideal raw material that could be employed to elaborate sustainable feed additives for aquaculture. Thus, this research presents the development of a marine natural product obtained from seafood processing waste with high potential to be used for fish feed purposes. As this product improves protein hydrolysis in salmonids, it could enhance nutrient digestion and thus decrease feed waste. So, it has great potential to contribute to reducing the negative environmental impacts associated with fish farming.

We have demonstrated the potential of all the enzymes studied, despite originating from a variety of taxa and organs. Our results demonstrate that alkaline proteases from the evaluated species have catalytic activities ranging from 0.23 to 2.53 U/ml, but only the intestine and pyloric caeca extracts obtained from Brazilian menhaden *B. aurea* (*Ba*) were significantly higher than the other extracts studied. Diet is usually considered to be the main factor influencing digestive enzyme activities in fish (Fernández et al., 2001), and *Ba* is the only herbivorous species of all the animals tested (Cousseau & Perrotta, 2013). Our findings match previous work showing that herbivorous species have higher alkaline proteolytic activities than carnivorous species (Jónás et al., 1983). This difference could be related to the differences in gastrointestinal anatomy of fish with different feed habits. Since most herbivorous fish have small stomachs, or even this organ is absent, intestinal digestion plays a major role, which may explain the higher alkaline proteolytic activity. On the contrary, carnivorous species—who prey on bigger food items—have conspicuous stomachs where acid digestion is long to pre-digest food before getting into the intestine, so acid proteases play a vital role.

Conversely, Sabapathy and Teo (1993) reported that proteolytic activity is higher in species with carnivorous habits; though feeding

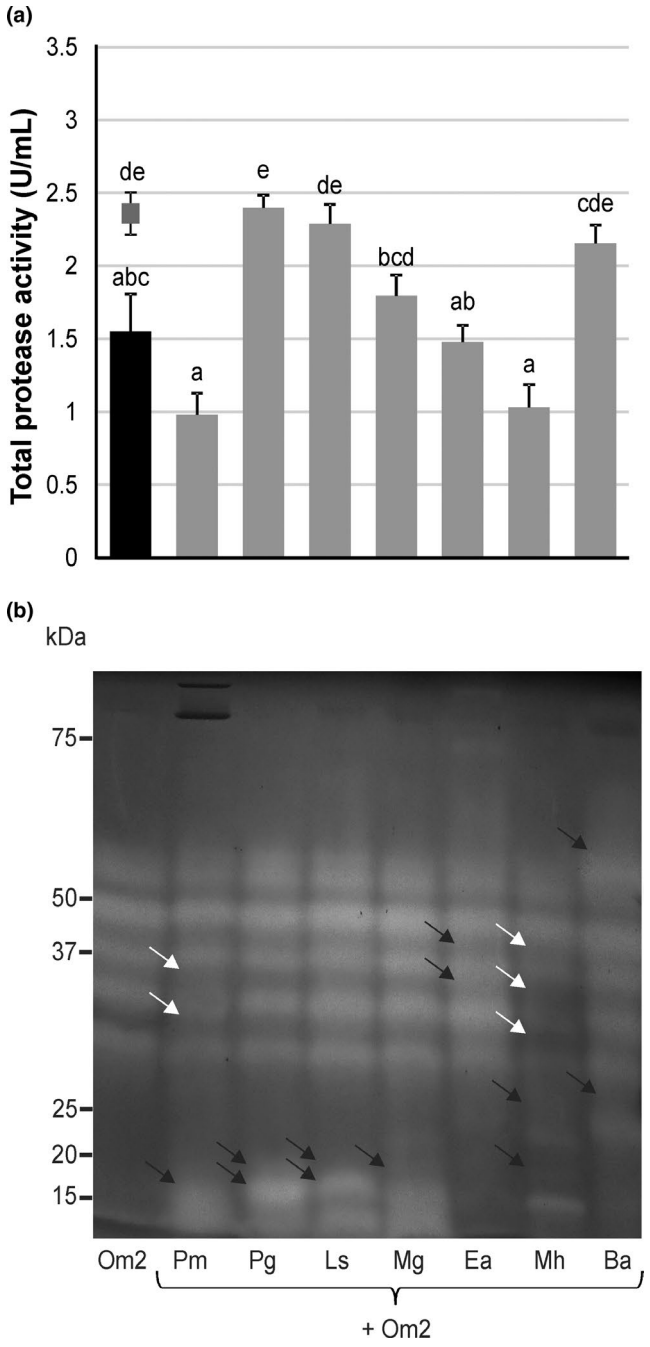

FIGURE 2 Enzyme interaction between digestive enzymes of *Om2* and exogenous enzymes recovered from different fish processing waste. (a) Total protease activity (U/ml) calculated after azocasein hydrolysis at pH 7.5 and 15°C. The black bar shows the mean proteolytic activity of *Om2*' crude enzyme extracts, grey bars indicate the mean proteolytic activity of *Om2*' crude enzyme extracts combined with the exogenous enzymes recovered from seafood processing waste, and the grey dot shows the mean proteolytic activity of *Om2*' crude enzyme extracts mixed with the commercial protease RONOZYME®ProAct. [a–e]Means with different superscripts are significantly different ($p < 0.05$). Error bars display SEM values. (b) Zymogram of extract from rainbow trout *Om2* and its mixture with crude enzymes extracts obtained from different seafood processing waste. Black arrows indicate exogenous proteases. White arrows show rainbow trout proteases affected by the addition of exogenous enzymes. Abbreviations: *Ba*, *B. aurea* intestine and pyloric caeca extract; *Ea*, *E. anchoita* intestine extract; *Ls*, *L. santolla* midgut gland extract; *Mg*, *M. gregaria* midgut gland extract; *Mh*, *M. hubbsi* intestine extract; *Om2*, intestine + pyloric caeca extract of *O. mykiss* weighing 58.3 ± 5.73 g; *Pg*, *P. granulosa* midgut gland extract; *Pm*, *P. muelleri* cephalothorax extract

TABLE 3 Effects of acid pH and trout stomach enzyme extract on the alkaline proteolytic activity of free *P. granulosa* extract

	Total Protease Activity (U/ml)	
Time (hr)	pH 2	pH 2 + trout stomach extract
0	1.66 ± 0.199[a, A]	1.57 ± 0.110[a,A]
0.5	0.69 ± 0.213[a,B]	0.64 ± 0.175[a,B]
2	0.39 ± 0.199[a,B]	0.56 ± 0.067[a,B]
4	0.39 ± 0.042[a,B]	0.44 ± 0.051[a,B]
6	0.50 ± 0.233[a,B]	0.35 ± 0.081[a,B]
24	0.29 ± 0.044[a,B]	0.22 ± 0.085[a,B]
Statistical analysis		
AIC m0		54.7
AIC m1		56.7
AIC m2		6.2
p-value		1.61^{-13}

Note: [a–b]Means within a raw with different superscript letters are significantly different ($p < 0.05$). [A–B]Means within a column with different superscript letters are significantly different ($p < 0.05$). Abbreviations: AIC m_0, Akaike number of the null model (without independent variable); AIC m_1, Akaike number of the model with the treatment as the explanatory variable); AIC m_2, Akaike number of the model with the time as the explanatory variable.

behaviour does not always lead to modulation of digestive enzyme activity (Sabat et al., 1999). Hidalgo et al. (1999) and Chakrabarti et al. (1995) suggest that nutritional habits do not define the concentration of proteolytic enzymes in fish, and many authors have found that phylogeny plays a significant role in influencing digestive enzyme activity (Chan et al., 2004; German et al., 2004). Our data are consistent with this claim because it has been demonstrated that closely related species such as *P. granulosa* (*Pg*) and *L. santolla* (*Ls*) (Anomura: Lithodidae) display similar proteolytic activities.

Studies on the application of exogenous proteases to improve growth performance and nutrient utilization of *O. mykiss* have produced mixed results. For instance, Drew et al. (2005) added a commercial protease to a diet containing a mixture of rapeseed and pea, and found an improvement in the apparent nutrient digestibility and feed efficiency. Ogunkoya et al. (2006) added different levels of a commercial enzyme mix to rainbow trout diets containing soybean meal (up to 200 g/kg) and showed no effects on growth or feed efficiency, but did record small effects on apparent nutrient digestibility. Farhangi and Carter (2007) formulated a de-hulled lupin-based diet containing commercial protease and tested the effects both in isolation and in combination with carbohydrases. No effects on the performance of rainbow trout were detected, but the mixed enzyme

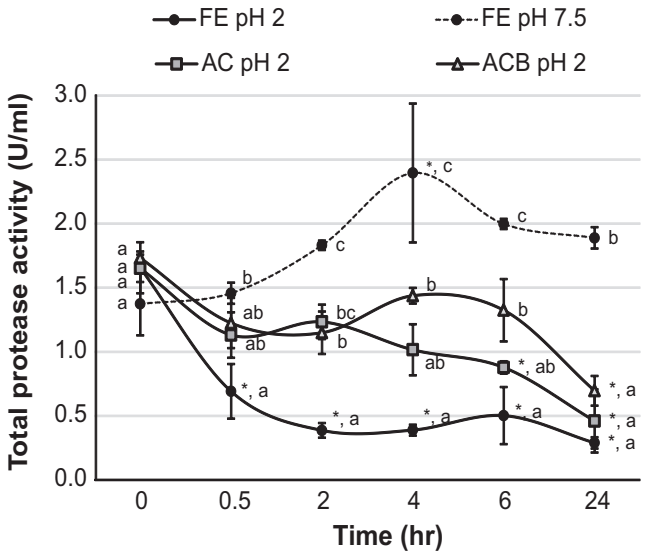

FIGURE 3 Protease activity of free and encapsulated *P. granulosa* extract after being exposed to different scenarios simulating rainbow trout digestion. The control treatment was carried under alkaline conditions (pH 7.5). [a–c]Means with different superscripts indicate significant differences between the different treatments at the same sampling time. *Means within the same treatment with asterisks significantly differ from the activity obtained at the initial time. Error bars display SEM values. Abbreviations: AC, Alginate–Chitosan microcapsules; ACB, Alginate–Chitosan–Bentonite microcapsules; FE, Free enzyme extract

significantly enhanced the protein efficiency ratio. More recently, Dalsgaard et al. (2016) and Yigit et al. (2018) studied the effects on growth parameters and nutrient digestibility of protease supplementation in a soybean meal-based diet in trout. Both works found that there were no differences in growth parameters and feed conversion ratio (FCR) with the addition of protease to the diet; though the enzyme improved the apparent digestibility of protein. Nevertheless, all these previous works utilized commercial proteases from bacteria while we obtained the enzymes from different fish processing waste. Thus, our paper presents an innovative first approach in the application of exogenous proteases for salmonid nutrition.

All the extracts studied have equal or more proteolytic activity than the commercial enzyme utilized as a control, making them good potential candidates to be used as exogenous enzymes. However, the detection of proteolytic activity does not necessarily guarantee a synergistic effect because exogenous enzymes may interact in numerous ways with the digestive enzymes of the fed animals (González-Zamorano et al., 2013). As bioassays are laborious and expensive, the study of *in vitro* enzyme compatibility becomes a valuable tool to determine the most suitable seafood processing waste for rainbow trout digestion. The results show that some extracts—like those obtained from the squat lobster *Mg* and Argentine anchovy *Ea*—did not modify trout protease activity and may therefore not be useful as exogenous enzyme sources. Also, when intestine extracts were obtained from Argentine hake *Mh* and combined with those of *O. mykiss*, the total proteolytic activity was reduced, indicating that hake enzymes may inhibit rainbow trout digestive enzymes. These results are congruous with the zymograms in which some alkaline enzymes from rainbow trout disappeared or lost their activity when the extracts of *Om1* or *Om2* were mixed with *Mh* enzymes. This suggests that hake processing remains are unlikely to be a good source of exogenous enzymes for rainbow trout culture. Similarly, Brazilian menhaden *Ba* is also unlikely to be suitable because its enzymes affected the integrity of *Om1* enzymes. Zymogram revealed that the *Ba* extract shut down a band (MW: 35.6 kDa) of the farmed fish, even when this extract increased the proteolytic activity of their endogenous enzymes. Argentine red shrimp extracts (*Pm*) affected the proteolytic activity of *Om2*'s enzymes. This effect may occur because the shrimp extract attenuates two activity bands belonging to the *Om2*'s endogenous enzymes, weighing around 31 and 35.7 kDa. This is in contrast with the result of Rodriguez et al. (2017), where *Pm* enzymes did not affect the activity and integrity of *Oreochromis niloticus* digestive enzymes, and even improved the hydrolysis of different protein sources. The deleterious effect of shrimp enzymes we observed for trout supports our hypothesis that not every enzyme is compatible with all fish species. The most likely explanation is that the catalytic output may differ among species and individual characteristics such as weight, age and farming system (Yasumaru & Lemos, 2014). Here, we provide strong evidence that exogenous enzymes from fish processing waste have to be tested in vitro to find the extract which is compatible with the endogenous enzymes. Moreover, enzyme additives may be species specific and will not necessarily be effective in all fish species.

We found that extracts obtained from 'false king crab' or 'stone crab' *P. granulosa* (*Pg*), followed by southern king crab *L. santolla* (*Ls*), are the best candidates to be used as a feed additive for both rainbow trout sizes studied. Such extracts increased the protein hydrolysis to equal or higher levels than the commercial protease, and did not alter the activity zones of trout proteases. Our results indicate that these extracts do not hydrolyse or inhibit the endogenous proteases. Lithodids are among the largest arthropods and have therefore received great attention from fisheries operating at high latitudes in both hemispheres (Calcagno et al., 2005). Both species are frequently caught together, in the same trap or in traps arranged in very close places (Lovrich & Vinuesa, 2016; Lovrich, 1997). In Argentina, during 2019 it was registered a total landing of 2,139 tons (Ministerio de Agroindustria, 2019). In particular, *Pg* has been a commercial target in Argentina and Chile since the early 1970 s (Comoglio & Amin, 1999). This species is caught at depths of up to 50 m and inhabits the Pacific Ocean from Paso Tenaún (Chile) to Cape Horn (Argentina), and the Atlantic Ocean from 56°S to the Golfo San Jorge, including the Malvinas Islands (Macpherson, 1988). Since the early 1980 s, after declining landings of *L. santolla*, local fisheries have partially or totally switched to *P. granulosa* exploitation (Calcagno et al., 2005). In 2017, the landings of this crab in the Magellanic region (Chile) were about 5.935,6 t (IFOP, 2018). In Argentina, its landings are smaller and come almost exclusively from the Beagle Channel (Wyngaard et al., 2016). The best meat derives from the upper section of their

claws and walking legs, so during the processing the other body parts are often discarded. The unexploited carapace of both lithodids has a conspicuous midgut gland that, owing to its digestive function, holds a high proteolytic activity (Galgani & Nagayama, 1987), and is a good source of exogenous enzymes.

We conducted all assays at 15°C because this temperature is part of the optimal range in which *O. mykiss* grows and survives (Molony, 2001). As an ectothermic animal, trout digestion takes place at low temperatures. We found lithodid alkaline proteases not only had a synergistic effect on trout digestive enzymes but also displayed high activities at 15°C. Both southern and false king crabs live in cold-temperate waters of Subantarctic origin, ranging from 4 to 15°C (Wyngaard et al., 2016). Thus, the catalytic function of *Pg* and *Ls* enzymes at low temperatures might be linked to the habitat of these arthropods, and this might explain its synergistic effect. Two factors stand out as particularly relevant. First, in the Subantarctic region, the location of *O. mykiss* farms and the exploitation of these crustaceans overlap, which makes this technology highly transferable in the short term. Second, the fact that both species exerted a synergistic effect on trout endogenous enzyme activity makes it possible to ensure the continuity of the additive regardless of the abundance of each resource. Future experiments should therefore evaluate the in vitro compatibility of both exogenous extracts to obtain a proper formulation containing the proteases of both lithodids and, subsequently, test their effectiveness in trout growth through in vivo trials.

However, some limitations must be overcome to utilize processing wastes as feed additives. For example, exogenous enzymes may be damaged by stomach pepsin and/or low pH present in fish's digestive systems. In species with true stomachs such as salmonids, pepsinogen and hydrochloric acid are secreted by oxynticopeptic cells into the lumen when food is present (Lovell, 2002). This acid activates pepsin and denatures food proteins, but also makes enzymes additives unavailable to contribute to the alkaline digestion at the intestine level. We did not observe an effect of stomach pepsin on *Pg*'s alkaline proteases activity, though its exposition of just 30 min at pH 2 caused a significant reduction in exogenous enzyme activity. This suggests that low pH exerts a more deleterious effect in the enzymes obtained from seafood than trout pepsin. Thus, before utilizing exogenous enzymes as a feed additive for monogastric species, it is necessary to immobilize such extracts and find the best carrier to ensure their effective performance. Various techniques are employed for the immobilization of enzymes (Norouzian, 2003). One of the most popular is encapsulation in a hydrogel by using polymeric compounds such as alginate, which combined with Ca^{+2} ions form a cross-linked matrix that is innocuous and biodegradable (de Araújo Etchepare et al., 2015; Blandino et al., 2001; Tanriseven & Doan, 2001; Yoo et al., 2006). However, there are certain disadvantages that limit the use of alginate delivery systems. For instance, acidic pH causes the hydrolysis of glycosidic bonds resulting in its depolymerization (Haung & Larsen, 1963). Rodriguez et al. (2018) demonstrated that alginate–bentonite microcapsules are better vehicles than classical alginate capsules because they were more resistant to different assay storage conditions, and enzyme activity was 27% higher in fish intestines following consumption of the alginate–bentonite microcapsules compared to the control treatment without exogenous enzyme. However, that work examined digestion in an omnivorous fish like Nile tilapia (El-Sayed, 2006). Tilapias have a smaller stomach with shorter acid digestion length and larger intestines than carnivorous fish such as *O. mykiss*. It is therefore necessary to test other carriers that could be more resistant to long periods of acidic exposition. Chitosan is also a non-toxic and biodegradable polysaccharide derived from chitin and is employed for delivering bioactive compounds in aquaculture (Masoomi Dezfooli et al., 2018). Here, we demonstrated that alginate–chitosan–bentonite microcapsules (ACB) ensure the activity of stone crab enzymes for at least 6 hr in acid pH, while the alginate–chitosan (AC) microcapsules ensure enzyme protection for just 4 hr. This suggests that ACB carriers could be more effective in delivering enzymes in salmonids. To the best of our knowledge, we present an innovative combination of compounds for encapsulation which has not previously been reported and can be used to deliver both *Pg* proteases, as well as other commercially available enzymes.

In conclusion, this research promotes a re-evaluation of unutilized seafood processing wastes, thus potentially reducing the environmental impact of fisheries. We found exogenous proteases could enhance rainbow trout proteolytic activity, potentially leading to reduced production costs and decreased waste. Among all the seafood wastes evaluated, the protease extracts obtained from the two lithodids species (*P. granulosa* and *L. santolla*) showed the most potential for use as feed additives to enhance the digestive process of rainbow trout. The mixed results of enzymes on host animals reinforce the importance of screening and analysing activity from several marine enzyme sources. Further, it is important to correlate all in vitro observations alongside experiments on living fish due to complications in simulating their exact physiology. Further, trials including bioassays are needed to demonstrate if exogenous enzymes immobilized in ACB microcapsules improve *O. mykiss* digestion and growth. While research using a wider range of sources and greater replication is necessary, our results represent an important first step in the more generalized use of an environmentally and economically efficient technology.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Rodriguez Y. E. has realized the following tasks: conceptualization, methodology, investigation, validation, formal analysis, written the original draft preparation and its visualization. Pereira N. A. performed the next jobs: methodology, investigation, validation, resources, reviewed, and edited the manuscript. Laitano M. V. helped with methodology design, investigation, formal analysis, and also reviewed and edited the manuscript. Moreno P. provided resources and also reviewed and edited the draft. Fernández-Gimenez A.V. was in charge of the conceptualization, supervision, project administration, funding acquisition and also contributed to the draft writing.

DATA AVAILABILITY STATEMENT

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

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