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Exogenous enzymes in aquaculture: Alginate and alginate-bentonite microcapsules for the intestinal delivery of shrimp proteases to Nile tilapia

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ABSTRACT

Shrimp processing waste holds digestive proteases with a great potential to be used as feed supplement for Nile tilapia *Oreochromis niloticus*. In the present work, we characterize both sodium alginate (A) and sodium alginate-bentonite (AB) microcapsules to entrap enzymes obtained from *Pleoticus muelleri* processing waste. Also, we evaluate these encapsulation methods as a strategy to improve intestinal delivery of exogenous enzymes in *O. niloticus* in order to enhance their digestion process. The effects of different storage methods, *in vitro* simulation of gastric pH conditions and exposition to 40 °C were studied. In order to evaluate if microencapsulated shrimp enzymes are active when they reach fish gut, animals were exposed to three treatments: (1) fast, (2) diet and, (3) AB capsules + diet. Alginate capsules were more affected by different storage methods than alginate-bentonite ones. SEM images showed a correlation between decreased enzyme activity and capsule microstructure changes. The best method to store the AB beads is at −20 °C. After incubating for 7 h at 40 °C, we observed a notorious reduction in the enzyme activities of both microcapsules. On the other hand, at pH 3 both microcapsules prevented enzyme irreversible denaturalization and kept 100% of their activity. The overall results indicate that AB capsules are better vehicles to deliver shrimp enzymes in Nile tilapia. In the bioassay, we observed that when fish were fed with treatment 3, the alkaline protease activity in their intestines was 27% higher than that of the diet fish group. Thus, encapsulated shrimp enzymes have a great potential to be used as a feed supplement in fish nutrition. Further trials involving grow bioassays are needed to verify if this shrimp enzyme contained in AB capsules improve fish digestion, feed conversion, body weight and survival rate.

1. Introduction

Fish aquaculture is an important source of protein for human diet. Traditional aquaculture production systems have changed towards modern intensive and super-intensive systems in order to cover the market demands of fish (Pauly and Zeller, 2017). This practice requires great amounts of fish diet that usually contains high levels of fishmeal, which increase the production costs > 40%. Therefore, this has a direct impact on cost-effectiveness of fish aquaculture (Little et al., 2016; Tveterås, 2002). Nile tilapia (*Oreochromis niloticus*) is one of the most farmed fresh-water species, and it is widely distributed around the world. Tilapia are being cultured in many tropical and subtropical regions due to several features of this species, such as resistance to diseases, low oxygen tolerance and their capacity to feed on a wide range of nutrient sources (Ogello et al., 2014; Zhao et al., 2010). Cultured tilapia species have increased, being more than 20 species farmed around the world; hence, an adequate feeding strategy is essential to achieve a reliable tilapia farming (El-Sayed, 2006). Since the past decade, many research efforts in aquaculture nutrition have focused on the replacement and supplementation of fishmeal based fed with other nutrients sources as plant-based meals and other co-products (Castillo and Gatlin, 2015; Mahmoud et al., 2014; Moesch et al., 2016). Tilapia obtains essential nutrients like amino acids, simple carbohydrates, *etc.* by metabolizing large polymeric compounds using digestive enzymes (*e.g.*, proteases, carbohydrases, and lipases); however, it is known that not all feed nutrient sources are efficiently digested. For instance, antinutritional factors (*e.g.*, phytin, protease inhibitors) could be present in feeds and affect fish growth (El-Sayed, 1999; Francis et al., 2001).

In the past decade, the supplementation of animal diets with exogenous enzymes has substantially increased. This new approach has

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favored complex polymeric compounds digestibility, hence improving the utilization of dietary nutrients, energy conversion, and reducing anti-nutritional factors during food-absorption processes (Farhangi and Carter, 2007). More recently, the use of exogenous enzymes in diets for several fish species – like rainbow trout (Rodehutscord and Pfeffer, 1995), catfish (Jackson et al., 1996), and salmon (ali Zamini et al., 2014; Refstie et al., 1999) – has been reported. Tilapia farming has not been an exception to advances indicating that enzyme supplementation is an excellent alternative to enhance nutritional properties of fish diets (Castillo and Gatlin, 2015; Mahmoud et al., 2014). Li et al. (2009) reported that supplementation with non-starch polysaccharides (NSPs) enzyme and phytase increase the enzymatic activity of amylase at both hepatopancreas and intestine of tilapia. Other authors have reported that added xylanase, β-glucanase, and cellulose can hydrolyze high molecular weight polysaccharides from NSPs fish diets and promote the reduction of gut viscosity, which increases NSPs digestion and modulates the intestinal microbiota (Adeoye et al., 2016; Yigit and Olmez, 2011). Furthermore, protease supplementation in aquaculture has been carried out in order to improve protein digestibility in fish species as Gibel carp (*Carassius auratus gibelio*) (Liu et al., 2017), Caspian salmon (*Salmo trutta caspius*) (ali Zamini et al., 2014) and tilapia species (Adeoye et al., 2016; Lin et al., 2007). These approaches have demonstrated that the addition of exogenous enzymes to aquafeeds enhance protein and carbohydrate digestibility, and thus, improve nutritional markers as feed conversion, body weight and the survival rate in fish aquaculture.

Nevertheless, some restraints on their utility can be found; for example, exogenous enzymes could be damaged by the action of endogenous proteases and other physicochemical factors (*e.g.*, pH) present in fish's digestive system. In addition, industrial processes during diet production, such as extrusion, pelletizing and heat drying, could affect exogenous enzymes structural integrity and therefore decrease its functionality. To deal these problems, many polymeric materials and lipids delivery systems have been developed to potentiate the efficiency of exogenous enzymes in both medical and food industries (Kumari et al., 2013; Yoon et al., 2001). Numerous techniques are effective to proper enzyme immobilization, such as microencapsulation in a gel matrix using polymeric compounds as alginate (Yoon et al., 2001), chitosan (DeGroot and Neufeld, 2001) and xylans (Chimphango et al., 2012). Briefly, microencapsulation comprises the entrapment of a substance (enzyme) into a particle, and as a result, the polymeric matrix protects the active molecule from external factors that affect its integrity (Chang and Prakash, 1999; Lovett and Felder, 1990). In fact, several microencapsulation methods have been developed. Chemical microencapsulation comprises changes in solvent properties or a chemical reaction that promotes the complexation of the material, generally polyelectrolytes as alginate and chitosan (Yoon et al., 2001). Alginate is extensively used to microencapsulate a diversity of substances; such as probiotic bacteria (Rosas-Ledesma et al., 2012), bioactive molecules (Ferrándiz et al., 2016; Yoo et al., 2006), and enzymes as glucose oxidase (Blandino et al., 2000), β-galactosidase (Taqieddin and Amiji, 2004), yeast invertase (Tanriseven and Doan, 2001), lipases and proteases (Mong Thu and Krasaekoopt, 2016).

Enzyme encapsulation has been used in food technology processes as dairy and meat fermentations and metabolite production (Gibbs et al., 1999; Nedovic et al., 2011). In fish aquaculture, alginate-calcium microcapsules have been proposed as an effective and low-cost method to deliver bioactive compounds as *Aeromonas hydrophila* (Rodrigues et al., 2006), bovine serum albumin (Polk et al., 1994), and nucleic acids vaccines (Nácher-Vázquez et al., 2015; Tian et al., 2008). However, reports of enzyme encapsulation in fish aquaculture are limited and, owing to their biochemical characteristics, enzyme retention in alginate microcapsules can be challenging. These biomolecules are water-soluble and unstable, losing their activity quickly during the encapsulation procedure. Bentonite – clay from volcanic ash with excellent ion exchanging properties (Adamis and Williams, 2005) – has been widely used in animal feeds production because of its binding and lubricating properties (Mumpton and Fishman, 1977); but also, this clay might improve enzyme encapsulation efficiency. Previous research observed that when 1% of bentonite was added to an alginate solution, the enzyme loss was significantly reduced and did not affect the enzyme activity (Dashevsky, 1998).

As mentioned above, exogenous enzyme supplementation has become the most usual strategy in aquaculture nutrition industry to improve fish feed. Most of these reports comprise the use of commercial enzymes that increase the food cost and therefore affects the fish marketing. As an alternative, the use of an enzyme-rich extract from marine fishery by-products has gained interest to improve fish feed digestibility in tilapia farming. Recently, Rodriguez et al. (2017) have reported the *in vitro* interaction of *O. niloticus* digestive proteases with enzyme extracts obtained from different fishery waste (*Pleoticus muelleri*, *Artemesia longinaris* and *Patagonotothen ramsayi*). The authors found that these enzyme extracts did not affect the function of tilapia enzymes, and indeed improve the digestibility of several fish-feed meal sources (fish, squid, shrimp and soybean meal). These findings suggest that enzymes recovered from fishery by-products could be supplemented in fish food (Rodriguez et al., 2017). In the present work, we characterize both alginate and alginate-bentonite microcapsules to entrap exogenous enzyme obtained from *P. muelleri* fishery by-products. Also, we evaluate these encapsulation methods as a strategy to improve intestinal delivery of exogenous enzymes in Nile tilapia in order to enhance its digestion process.

2. Material and methods

Animal care and experimental protocols were approved by the Institutional Animal Welfare & Ethical Review Committee at Mar del Plata National University (RD 200/15).

2.1. Microencapsulation of shrimp enzymes

2.1.1. Enzyme extract

Samples of shrimp *Pleoticus muelleri* processing waste were provided by manufacturing plants from Mar del Plata, Argentina (38° 02′ S, 57° 30′ W). All samples were immediately frozen and transported to the laboratory. After thawing, the cephalothoraxes of three shrimp were removed. Subsequently, using a glass-Teflon tissue homogenizer, each sample was separately crushed in ice-cold distilled water (1:2 w/v). The resulting material was then centrifuged at 10,000*g* for 30 min at 4 °C (Presvac EPF 12R). The supernatants obtained (shrimp enzyme extracts-SE) were frozen at −20 °C and stored until use.

2.1.2. Enzyme encapsulation

Microcapsules were elaborated according to an adaptation of Dashevsky (1998) methodology. Alginate (sodium salt of alginic acid for feed purposes; Química Industrial Kubo S.A., Mar del Plata, Argentina) at the concentration of 1.5% (w/v) and alginate-bentonite (Química Industrial Kubo S.A., Mar del Plata, Argentina) at 1.5:1.0% (w/v) were used to encapsulate the shrimp enzyme extracts (three replicates). The two polymers were prepared in distilled water. Shrimp enzymes (SE) were encapsulated in the mentioned hydro polymers by mixing 8 mL of enzyme extract in 12 mL of each polymer solution; these volumes were found to be the most appropriate since the microcapsules obtained had similar alkaline protease activity than the crude shrimp enzyme extract (alginate: 0.4 ± 0.17 U mL^{-1}; alginate-bentonite: 0.4 ± 0.21 U mL^{-1}; SE: 0.4 ± 0.08 U mL^{-1}). Then, the solution obtained was stirred to suspend the bioactive material in the polymer. The cationic solution for gelling was 1 M $CaCl_2$. Next, using a pressure system, the polymer-enzyme solutions were extruded through a syringe with a needle, 0.8 mm diameter. When droplets were dropped into the cationic solution constantly magnetically stirred, beads were immediately formed. Consequently, two different shrimp enzymes

microcapsules were obtained for the following assays, the sodium alginate capsules (A) and the alginate-bentonite (AB) ones.

Throughout all the assays of this research, microcapsule stability was evaluated by quantifying their enzyme activity because if the capsules result deteriorated by the treatment performed, the enzymes that they hold will be lost or denaturalized. Consequently, the alkaline protease activity was assessed by spectrophotometry, according to the method reported by García-Carreño (1992) by using 0.5% (w/v) azocasein (Sigma A 2765) as a substrate in 50 mM Tris-HCl buffer pH 8. For this, 250 μL azocasein solution was reacted with immobilized (3 A or 3 AB capsules) or free shrimp enzymes (5 μL of SE) at room temperature for 30 min. The wavelength for the readings was 366 nm. All assays were run in triplicate. Total protease activity was calculated.

2.2. Characterization of capsules containing shrimp enzymes

2.2.1. Storage

First, separate 5 g batches of A and AB shrimp capsules were stored at 4 °C during 264 h; then, subsamples were taken at: 0, 24, 48, 96, 168 and 264 h. Secondly, 2 g of batches of A and AB capsules were exposed to freeze-drying for 24 h in a MICRO-Modulyo Freeze Dryer-1.5Liter (Thermo Electron Corporation). Additionally, 2 g of each microcapsule class was subjected to freezing at −20 °C and −80 °C over 48 h. Two control trials were performed at each condition: shrimp enzyme extract not encapsulated (SE) and A and AB capsules freshly prepared and containing shrimp enzymes. After each storage time, samples were thawed, and then, their enzyme activity was analyzed.

2.2.2. Microstructure of capsules exposed to different storage conditions

A detailed observation of the external surface of A and AB microencapsulates exposed to different treatments was done through Scanning Electron Microscopy (SEM). The treatments were: capsules freshly made (as control); 3 and 7 days at 4 °C; 2 days at −20 °C and −80 °C. Samples were previously metalized with Ag/Pd in a Denton Vacuum Desk II metallizer. The analyses were carried out with a Jeol JSM 6460LV microscope in the *Laboratorio de Microscopía Electrónica at the Universidad Nacional de Mar del Plata, Argentina*.

2.3. Microcapsules for enzyme delivery in Nile tilapia

2.3.1. Simulated gastric pH conditions

Encapsulated shrimp enzymes need to pass through tilapia stomach in order to reach the intestine, where they would help with digestion. Alkaline protease stability of shrimp enzymes contained in A and AB microcapsules was tested after their exposition to fish digestive pH conditions. Both acidic and neutral extracapsular solutions were employed to imitate the stomach and intestinal regions, respectively. An adaptation of Rodriguez et al. (2017) methodology was utilized to simulate the acid secretion cycle of Nile tilapia. First, A and AB microcapsules were separately exposed to abrupt or gradual pH drop. In the abrupt treatment, 60 capsules were immersed during 1 h in 10 mL of 200 mM Glycine-HCl buffers at different pH: 3, 2.5, 2 or 1.5; while in the gradual pH variation treatment, capsules were successively transferred through every mentioned buffer every 1 h, and subsamples were taken in each pH shift. This different pH variation pretended to reproduce the several pH scenarios that capsules can encounter when reaching the stomach, and also the time length that they spent stored in this organ (Moriarty, 1973). After acidic incubation, 3 capsules of each subsample were placed in 250 μL of 50 mM Tris-HCl pH 7 and incubated for 1 h, simulating the intestinal conditions. All incubations were run at 28 °C, the Nile tilapia growth temperature. Capsules not exposed to acidic conditions (incubated 1 h at pH 7) were used as the control treatment. Six replicates of each treatment were performed. Finally, alkaline protease activity was assayed as described before.

2.3.2. Supply method

In this study, A and AB microcapsules are evaluated to deliver shrimp enzymes to farm tilapia. If microencapsulates are pretended to be included in fish diets, it is important to analyze first if they can withstand feed drying temperatures. A previous study has demonstrated that, when incubated for 7 h at 40 °C, proteases extracted from *P. muelleri* fishery waste are stable, and would remain active after the feed drying process (Rodriguez et al., 2017). Thus, the stability of microcapsules exposed to sun drying temperature was evaluated (farmers regularly use the sun to dry their feed). For this, 10 capsules of each material (A and AB) were separately exposed to 40 °C (drying temperature utilized by low-budget producers) during a 7 h period in a digital dry bath (Numak MiniT). After incubation, protease activity at pH 8 of all treatments was assayed as explained in Section 2.1.2. Assays were run in triplicate. Control treatments of both microcapsules types were those at the initial time without incubation.

Additionally, microcapsules can be provided directly to the fish without being included in formulated feed; however, first, it is necessary to analyze if the fish consume these items. Thus, before starting the bioassay with tilapia juveniles, microcapsules consumption was checked.

2.3.3. Experimental fish and feeds

A previous assay was performed with Nile tilapia *Oreochromis niloticus* to establish the proper dissection time, verify if the microcapsules were present in the digestive tract and how was their appearance. The fish employed had the same size than those used in this work. As a result, 3 h after feeding was chosen as the proper dissection time because all specimens presented content in their proximal intestines. Also, the microcapsules were observed in their digestive tracts and, in most cases, capsules appeared broken or were difficult to find in the gut, indicating that they released their content (data not shown).

Nile tilapia *Oreochromis niloticus* (mean weight = 6.0 ± 0.93 g) were obtained from the "Estación de Piscicultura" (Necochea, Buenos Aires, Argentina) and transported to the laboratory (Laboratorio de Fisiología de Organismos Acuáticos y Biotecnología Aplicada, IIMyC). Fish were randomly assigned to three groups of 12 animals, individually weighed and placed into 70-L rectangular plastic aquaria (60 × 35 × 25 cm width × length × height) with dechlorinated tap water at 28 ± 1 °C in closed systems. The photoperiod was 10 h light:14 h darkness. The aquaria were supplied with continuous aeration, and a 50% of total water volume was replaced daily. A reference diet based on fish and soybean meals and containing 32% of protein (Table 1) was formulated (Mahmoud et al., 2014). The pelleted feed was dried in an oven at 40 °C. Fish were hand fed twice a day with the mentioned diet until the beginning of the bioassay (7 days of acclimation), at a rate equal 5% of their body weight. Prior to the trial, fish were starved during 24 h to ensure that their digestive tracts were empty. In order to evaluate if encapsulated shrimp enzymes are active when reaching fish gut, animals were exposed to three different treatments: "Fast", fish were not feed; "Diet", fish were feed with reference diet at a rate equal 5% of their body weight; and, "Enzyme encapsulated + diet", fish were feed with AB capsules and reference diet at a rate equal to 2.5% and 5% of their body weight, respectively. Just one type of microcapsule (AB) was used in this assay, selected according to the *in vitro* results obtained in previous sections. Three hours since the feed ingestion, all fish of each treatment (n = 12) were killed by an overdose of benzocaine diluted in iced water ($\geq 250\ mg\ L^{-1}$). Then, fish were dissected and their digestive tracts removed. Subsequently, over chiller ice packs, each intestine was divided into two equal sections; the proximal one was weighed and utilized for enzyme extracts preparation. Alkaline protease activity at pH 8 was assayed as explained above.

Table 1
Composition of the reference diet provided to tilapia *Oreochromis niloticus*.

Ingredients	%
Fish meal (66%*)[a]	20
Soybean meal (28%*)[b]	25
Wheat gluten (84%*)[c]	12
Cornmeal (7%*)[d]	18
Vegetable oil[e]	3
Corn starch	7
Wheat bran	1
Vitamins and minerals premix[f]	14

Each gram of premix contain the following vitamins and minerals: vitamin A, 3.333 IU; vitamin B_1, 1.5 mg; Vitamin B_2, 1.7 mg; Vitamin B_6, 2.2 mg; Vitamin B_{12}, 0.003 mg; Vitamin C, 60 mg; 0.1 mg Vitamin D_3, 300 IU; Vitamin E, 15 mg; calcium pantothenate, 5.5 mg; biotin, 0.2 mg; folic acid, 0.1 mg; nicotinamide, 19 mg; Ca, 62.5; P, 62.5 mg; Mg, 50 mg; Fe, 4.5 mg; Cu, 0.5 mg; Mn, 0.5 mg; Zn, 3.75 mg.
* Determined according to AOAC (1995).
[a] Agustiner®.
[b] Molino Chabas®.
[c] Xantana®.
[d] Prestopronta®.
[e] Natura®.
[f] Supradyn®.

2.4. Data analysis

Since some data set did not find the normality distribution, data analyses were done with Generalized Linear Models (GLM). Models were developed to compare the enzymatic activity of microcapsules or Tilapia intestine (in the case of the bioassay) among different treatments, specifying Gaussian or Gamma family, as appropriate, according to the distribution of each data set. In order to determine the significance of the factor "treatment" on the enzymatic activity (which means, if there is an effect of the treatment), models were contrasted with a null model (without any independent variable) through the Akaike's Information Criteria. When the Akaike's number of a model with the factor "treatment" (m_1) is lower than that of the null model (m_0), indicate significant differences in the enzymatic activity due to such factor. When significant differences were found, and there were more than two levels of treatment, Tukey's tests were applied to the constructed model in order to make post-hoc multiple comparisons and detect which treatments differ in the enzymatic activity. All analyses were done with R.

3. Results

3.1. Storage method

Shrimp enzymes were successfully immobilized in alginate and alginate-bentonite capsules. Alginate capsules produced in this study had diameters from 2.7 to 3 mm, while the alginate-bentonite ones were a bit bigger and had diameters from 2.9 to 3.1 mm. There was no difference in the enzyme activity between treatments, so both capsules had a similar capacity to entrap the shrimp enzyme. The effect of storage at 4 °C in enzyme activity is shown in Fig. 1. Although in the three conditions (SE, A and AB) alkaline protease activity seems to decrease over the time, only alginate microcapsules showed significant differences among the different storage time periods (Table 2). Multiple comparisons indicate that A capsules had significant lower protease activity than the control at 168 and 264 h ($P < 0.05$). On the other hand, significant differences were found during the freeze-drying treatment (Table 2); protease activity significantly decreased ($P < 0.05$) in both microcapsules kinds. Regarding the freezing trial (−20 and −80 °C), the alkaline protease activity of both microcapsule

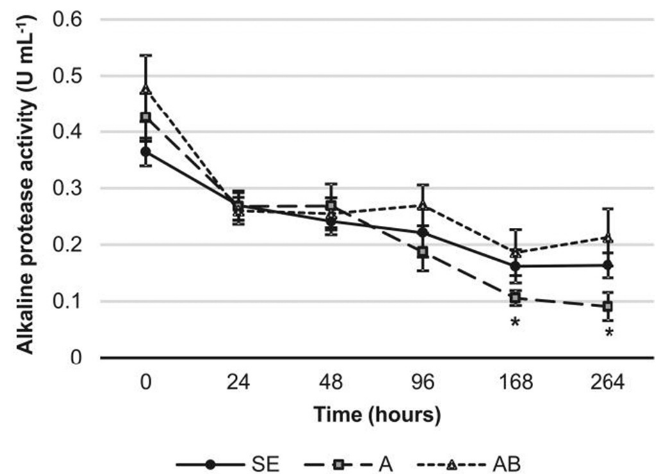

Fig. 1. Alkaline protease activity in different microcapsules exposed to prolonged fridge storage (4 °C). Abbreviations: SE, shrimp enzyme extract not encapsulated; A, alginate microcapsules; AB, alginate-bentonite microcapsules. Means within the same treatment with asterisk (*) are significantly different ($P < 0.05$) from time 0. Error bars display SEM values.

Table 2
Akaike number (AIC) of the null model (m_0, without independent variable) and models with different treatments as the explanatory variable (m_1) to evaluate the effect of treatments on shrimp enzyme extract not encapsulated (SE) and alginate (A) or alginate-bentonite microcapsules (AB) activity. The lower AIC indicates the best-fitted model when comparing m_0 with m_1. In bold: lower AIC of m1, which means the significant effect of the treatment.

Treatment	SE	A	AB
4 °C (different times)	m_0 = −26.7 m_1 = −17.2	**m_0 = −15.3** **m_1 = −20.9**	m_0 = −8.1 m_1 = 5.3
Freezed-dried (yes or not)		**m_0 = 20.2** **m_1 = 16.3**	**m_0 = 25.8** **m_1 = 3.9**
Freezer (−20 °C, −80 °C or none)	m_0 = −18.2 m_1 = −11.6	**m_0 = −31.5** **m_1 = −50.3**	**m_0 = −8.8** **m_1 = −11.1**
pH (gradual-7, 3, 2.5, 2, 1.5)		**m_0 = 34.3** **m_1 = 0.56**	**m_0 = 69.9** **m_1 = 24.9**
pH (abrupt- 3, 2.5, 2, 1.5)		**m_0 = 35.1** **m_1 = 0.39**	**m_0 = 70.1** **m_1 = 27.4**
40 °C (yes or not)		**m_0 = 3.1** **m_1 = −3.7**	**m_0 = 2.7** **m_1 = −16.7**

kinds presented some differences with the control treatment (Table 2). Multiple comparison ($P < 0.05$) indicate that A microcapsules had significantly lower activity after being frozen at −20 °C and −80 °C; meanwhile, AB microencapsulate just showed lower protease activity than its control at −80 °C (Fig. 2). SE activity was not affected by any freeze treatment (Fig. 2).

3.2. Microstructure of capsules

Scanning electron micrographs of A and AB capsules and their surface morphology are shown in Fig. 3. Freshly made A and AB capsules were not sphere-shaped and appeared misshapen and abraded (Fig. 3a, b). The metallization technique employed seems to affect capsules shape; however, they were used successfully to provide valued microstructural information. The beads were about 850–1000 μm in size and had a relatively rough surface with wrinkles. A marked difference was observed between the microstructure of A and AB microcapsules. The surface of A capsules revealed a highly porous morphology with the presence of channels (Fig. 3c). The addition of bentonite had dramatically modified the capsule surface, which is evident in Fig. 3d. These capsules were more spherical, had a relatively smoother surface, and the porous structures that presented the alginate capsules seem to be filled with bentonite. It was observed that keeping

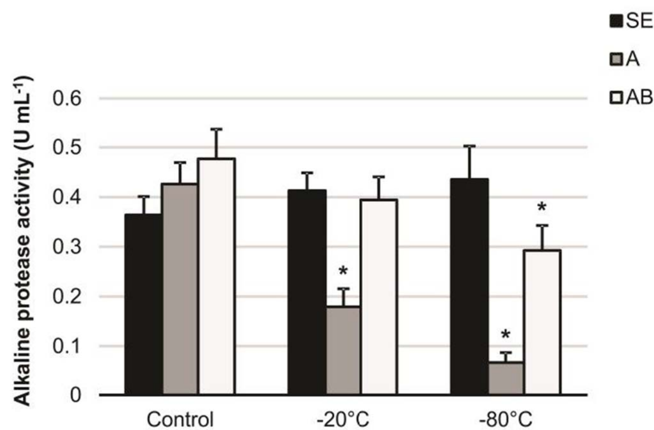

Fig. 2. Alkaline protease activity in different microcapsules exposed to two freezing temperatures (−20 °C and −80 °C). Abbreviations: SE, shrimp enzyme extract not encapsulated; A, alginate microcapsules; AB, alginate-bentonite microcapsules. Means with asterisks (*) are significantly different ($P < 0.05$) from the corresponding control treatment. Error bars display SEM values.

the capsules at 4 °C throughout 3 days did not change the microstructure of any capsules types. However, when they were stored during 7 days, the A capsules presented changes in their surface that are visible with the higher magnification (Fig. 3e), while AB capsules microstructure remained similar. Thus, prolonged storage at 4 °C caused the appearance of a wavy surface in A capsules. Additionally, freezing at −20 °C had a notorious effect on A capsules shape and surface as well (Fig. 3f). On the other side, when frozen at −80 °C, both class of capsules suffered notorious changes on their surface and appeared extremely wrinkled and abraded (Fig. 3g, h).

3.3. Integrity of encapsulated enzymes exposed to digestive pH conditions

The effect that tilapia's stomach acidic conditions have on the integrity of microcapsules was evaluated. Significant differences were found in both treatments, gradual and abrupt exposures to pH drop (Table 2). Moreover, the enzyme activity of A and AB capsules decreased significantly at pH 2.5, 2 and 1.5 compared to the alkaline control ($P < 0.05$; Fig.4a, b). However, when both microcapsules were exposed to pH 3 (both abrupt and gradual ways), alkaline protease activity remained as active as the control ($P > 0.05$; Fig. 4a, b).

3.4. Temperature stability of microcapsules

Significant differences were found between the control and the drying temperatures for both microcapsules (Table 2). The exposition to 40 °C during 7 h caused a significant reduction of the enzyme activity of A and AB microcapsules ($P < 0.05$; Fig. 5).

3.5. Alkaline protease activity in fish gut

Before the bioassay, it was observed that, when offered together, fish consumed the microcapsules as much as the feed provided (data not shown). Due to the positive results obtained in the previous assays, AB capsules were selected to analyze their activity in the fish intestine. There were significant differences in the activity of gut alkaline proteases obtained from fish of different treatments (GLM: $AICm_0 = 262.9$; $AICm_1 = 209.2$). Enzyme activity in the fish gut was higher in fed animals (“Diet” and “Enzyme encapsulated + diet” treatments) than in fasted animals (Fig. 6). Additionally, the tilapia that consumed shrimp enzyme encapsulated had significantly more alkaline protease activity in their gut than those fish fed with the control diet (Fig. 6).

4. Discussion

In the aquaculture industry, exogenous enzymes have been gaining importance over the last few years. Previous works have demonstrated that supplementing diets with enzymes lead to better utilization of nutrients improving growth performance (ali Zamini et al., 2014; Castillo and Gatlin, 2015; Adeoye et al., 2016). Additionally, it has been proved that shrimp processing waste holds digestive proteases with a great potential to be used as feed supplement for Nile tilapia (Rodriguez et al., 2017). However, before intending them as exogenous enzymes for monogastric species, it is necessary to immobilize such proteases and find the best carrier to ensure their effective performance. Enzymes can be immobilized by various techniques (Norouzian, 2003). Alginate has long been employed as the encapsulation material for oral delivery of proteins because it is a hydrogel reported to be innocuous and biodegradable when supplied orally (de Araújo Etchepare et al., 2015). Despite the fact that alginate matrices cross-linked with Ca^{+2} ions are suitable for encapsulation, the resulting gel is sensitive to extreme pH and capsules are unstable under the physiological environment leading to enzyme loss (Taqieddin and Amiji, 2004). Previous work demonstrated that bentonite could be used to prevent enzyme loss during encapsulation (Dashevsky, 1998); but, to our knowledge, there is no preceding research addressing the oral delivery of enzymes through alginate-bentonite capsules. In this study, enzymes extracted from shrimp processing waste were efficiently encapsulated within calcium alginate-based beads, with and without bentonite, by using the injection-gelation method. There were no differences in the amount of enzyme entrapped by both capsules materials. However, alginate and alginate-bentonite microcapsules displayed differential responses to the numerous tests that we performed, and this was revealed by the quantification of their enzyme activities.

Different storage methods were evaluated in order to find the best that ensure the maximum enzyme activity; also, to complement the enzyme activity data, the surface microstructure of capsules subjected to the different storage assays was studied. In general, alginate beads were more affected by the different storage methods. Such capsules showed a significant reduction of enzyme activity after their exposition to all storage methods, whereas alginate-bentonite capsules showed a decrease in protease activity only when exposed to the freeze-drying process and −80 °C. However, there was a reduction tendency in enzyme activity when AB beads were stored at 4 °C. Mong Thu and Krasaekoopt (2016) have reported, through the quantification of protease and lipase activities, the stability of microcapsules exposed to 4 °C during ten weeks. However, these authors used chitosan, xanthan gum, and maltodextrin as copolymers of alginate, which could explain their differences with our results. Scocca et al. (2007) stated that it is necessary to add a cryoprotectant to the capsule formulation in order to protect the enzyme activity from the freeze-dried process. However, other authors have subjected alginate capsules to freeze-drying without experimenting activity loss of pure enzymes entrapped (Won et al., 2005). Betigeri and Neau (2002) suggested that freeze-drying could lead to an enzyme activity reduction depending on beads water content because the freezing of water would take out the enzymes remaining on capsule surface. Since in this study we did not work with pure enzymes extract, it is probable that shrimp enzyme extracts had higher water content and, therefore, the freezing process affected the microcapsules enzyme activities considerably. Moreover, SEM images of this study showed a correlation between decreased enzyme activity and capsule microstructure changes. Thus, it is probable that the observed alterations in beads external surface may have led to a leakage of enzymes. Bentonite clearly contributed to reducing this leak. In addition, results indicate that storage at −20 °C is the best method to preserve the alginate-bentonite beads.

Moreover, we assayed if both microcapsules types can be included in fish feed formulations, since a limiting factor to transfer this technology could be their permanence at high temperatures for several

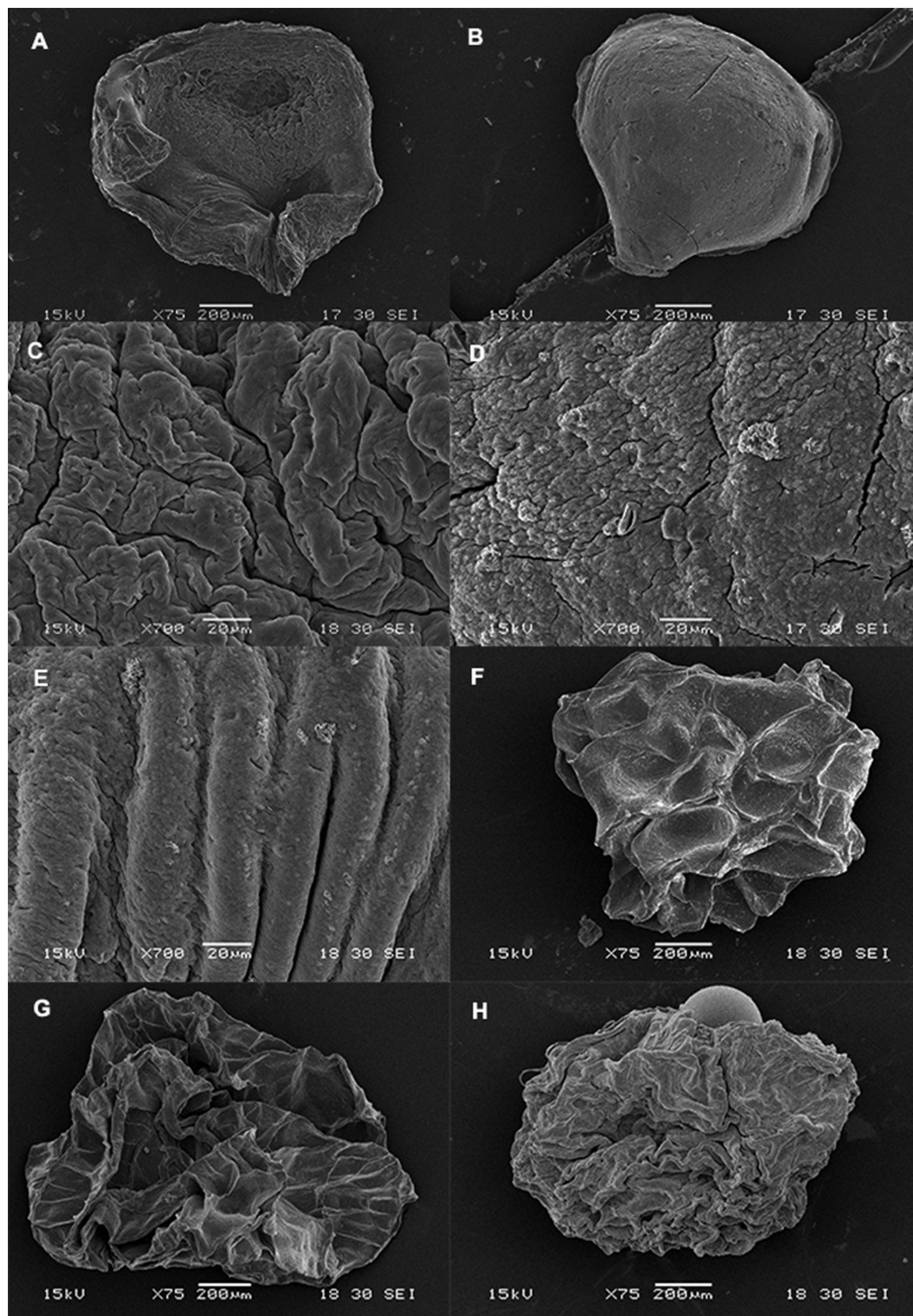

Fig. 3. SEM micrographs of different microcapsules exposed to different treatments. (A) Alginate capsule freshly made. (B) Alginate-bentonite capsule freshly made. (C) Detailed surface morphology of alginate capsule freshly made. (D) Detailed surface morphology of alginate-bentonite capsule freshly made. (E) Surface morphology of alginate capsule after seven days of fridge storage. (F) Alginate capsule after freezing at −20 °C. (G) Alginate capsule after freezing at −80 °C. (H) Alginate-bentonite capsule after freezing at −80 °C.

hours during the food sun drying process. As previously mentioned, in a previous research we demonstrated that alkaline proteases extracted from the same shrimp species are stable through 7 h at 40 °C (Rodriguez et al., 2017). So now we evaluated if microcapsules were also stable under such condition. After incubating for 7 h at 40 °C, we observed a significant reduction in the enzyme activities of both microcapsules class. Accordingly, other authors working with alginate capsules mixed with different polymers found a reduction in the activity of different encapsulated enzymes as a response to high temperature (Arruda and Vitolo, 1999; Bhushan et al., 2008; Taqieddin and Amiji, 2004). Apparently, high temperatures would favor enzyme leakage, so the microcapsules elaborated in this research cannot be included in fish diets.

A basic requirement for oral formulations of enzymes is their resistance to gastric conditions that causes denaturalization and degradation of exogenous proteins. In a recent research work, we evaluated the effect of tilapia's pepsin and stomach pH on the activity of shrimp enzymes; demonstrating that these enzymes can remain active for at least 2.5 h under such conditions (Rodriguez et al., 2017). However, shrimp enzyme activity could be prolonged if they are protected from the low gastric pH levels. For instance, it is known that microcapsule structure is relatively stable at acidic pH and easily

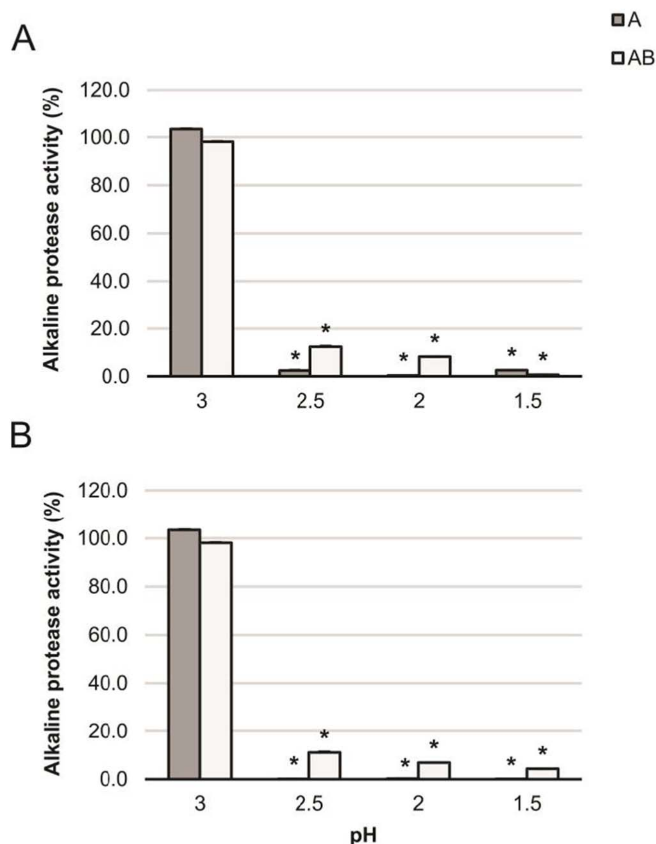

Fig. 4. Percentages of microcapsules alkaline protease activity after being exposed to different scenarios simulating tilapia digestion process. The control treatment (at pH 7) was set as 100%. (A) Gradual pH drop. (B) Abrupt pH drop. Abbreviations: A, alginate microcapsules; AB, alginate + bentonite microcapsules. Means within the same treatment with asterisks (*) are significantly different from the control incubated at pH 7 ($P < 0.05$). Error bars display SEM values.

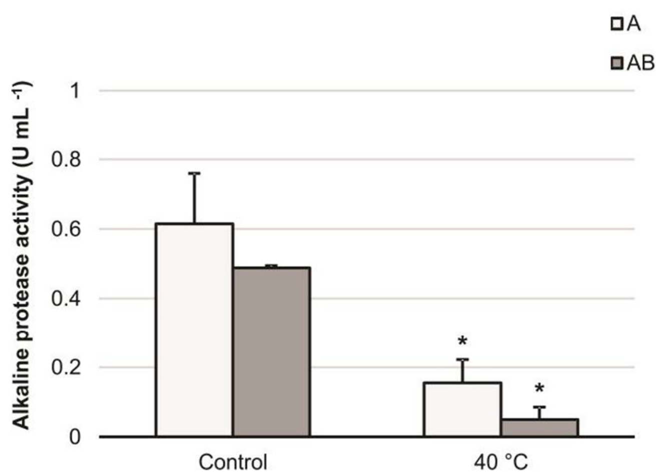

Fig. 5. Evaluation of microcapsules stability (enzyme activity) exposed to 40 °C during a 7-h period. Abbreviations: A, alginate microcapsules; AB, alginate-bentonite microcapsules. Means with asterisks (*) are significantly different from the control treatment without incubation ($P < 0.05$). Error bars display SEM values.

swollen and broken under alkaline conditions (Yoo et al., 2006), making them good enzyme carriers. In this study, we evaluated the protease activity of alginate and alginate-bentonite capsules after being exposed to the Nile tilapia gastrointestinal pH conditions. When tilapias' stomach is empty, its pH values range from 5 to 7 and it becomes progressively more acid with food digestion, reaching pH 3 after 4 to

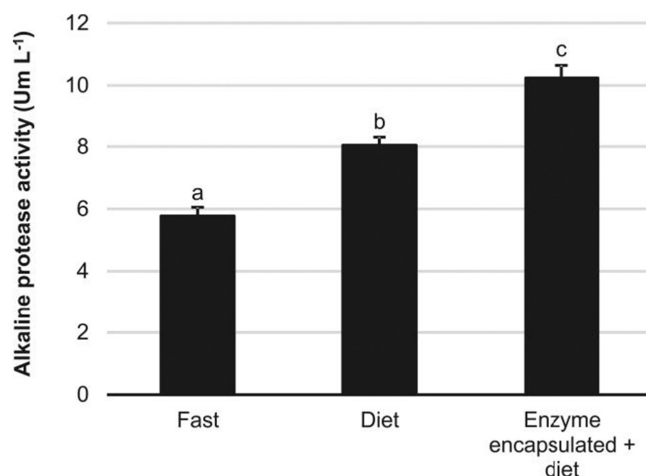

Fig. 6. Alkaline protease activities of Nile tilapia intestines exposed to the following treatments: fast (fish were not fed), diet (fish were feed with reference diet), and enzyme encapsulates + diet (fish were feed with alginate-bentonite microcapsules and reference diet). Means with different letters (a–c) are significantly different ($P < 0.05$). Error bars display SEM values.

6 h of the first ingestion and then it keeps going down (Moriarty, 1973). We found that at pH 3 capsules prevent enzyme irreversible denaturalization and kept 100% of their activity, but enzymes contained in both types of microcapsules were severely affected by pH values below 3. Therefore, the supply of alginate-bentonite encapsulated shrimp proteases to Nile tilapia should be done with the first meal of the day to ensure that microcapsules will not be exposed to pH values below 3. Scocca et al. (2007) reported no effects of low pH (1.2) over enzymes contained in alginate capsules, but the capsules were protected with an enteric polymer. Further studies incorporating this additive to the formulation presented here would improve the pH stability. Moreover, tilapia's stomach enzymes could affect microcapsules integrity, so this factor should also be assessed in the future.

Additionally, it is important to correlate the *in vitro* simulation with a bioassay owed to the impossibility of simulating the exact fish physiology. Previous research has demonstrated that tilapia fed with diet supplemented with commercial exogenous enzymes exhibited superior growth performance in comparison with those fish fed with a control diet (Adeoye et al., 2016). In this sense, we observed that when fish were fed with reference diet and microcapsules containing shrimp enzymes, the alkaline protease activity in their intestines was 27% higher than fish that just consumed the control diet. Such result indicates that alginate-bentonite capsules are good vehicles to deliver shrimp enzymes in Nile tilapia. Also, the ingestion of such clay might provide many other benefits for the fish. It has been reported that the inclusion of bentonite in feeds improves fish growth (Eya et al., 2008; Smith, 1980) and, moreover, its supplementation blocks intestinal absorption of mycotoxins, thus reducing their negative effects on fish health (Ellis, 2000; Winfree and Allred, 1992). Further trials involving growth bioassays are needed to demonstrate if this shrimp enzyme contained in alginate-bentonite capsules improves fish digestion and growth.

Finally, fishery wastes have increased in the last years, becoming a global concern (Caruso, 2016). In this work, we propose to reevaluate a processing waste of a valuable fishery activity in South Atlantic Ocean as the shrimp heads are. However, in order to obtain the shrimp enzyme extract used in our assays we peel its exoskeleton. These shells were not used in this research and were discarded; but they possess useful chemicals as, for example, chitin — a polymer which its water-soluble derivative is chitosan (Yan and Chen, 2015). Previous studies have developed alginate-chitosan core-shell microcapsules for oral delivery of enzymes (Lin et al., 2005; Polk et al., 1994; Taqieddin and Amiji, 2004). Hence, it would be interesting to develop the microencapsulation of shrimp enzymes using alginate, bentonite, and chitosan,

promoting the total utilization of *P. muelleri* processing waste.

5. Conclusions

Immobilization of shrimp enzymes in alginate alone presented good encapsulation efficiency but lost the enzyme when exposed to a variety of conditions. The addition of bentonite to the gelling solution was successful in raising the capsule performance under different storage methods assayed. This could be due to a better retention of the enzyme by possibly blocking the alginate pores or because of the bonds formed between this clay and shrimp enzymes. Thus, bentonite contributed in making the capsules more resistant to the different tested conditions. It was determined that the best storage method for these capsules is at −20 °C where they conserved a high percentage of enzyme activity. Additionally, we observed that when fish ate the reference diet and alginate-bentonite capsules containing shrimp enzymes the enzyme activity in their intestines was 27% higher than fish that just consumed the reference diet. We have addressed not only the characterization of these microcapsules but also their potential to deliver exogenous enzymes to Nile tilapia. Our results indicate that alginate-bentonite capsules could be good vehicles to deliver exogenous shrimp enzymes for fish culture.

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