Latin American Journal of Aquatic Research, 52(1): 47-58, 2024
DOI: 10.3856/vol52-issue1-fulltext-3037

Research Article

Evaluation of protein and lipid ingredients through *in vitro* digestibility for tropical gar (*Atractosteus tropicus*) juveniles

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ABSTRACT. Tropical gar (*Atractosteus tropicus*) is an ecologically, culturally, economically, and scientifically important species. Therefore, it is essential to know the available resources to design feeds for the culture of this species. This study evaluated the *in vitro* digestibility of meals and oils commonly used in aquaculture, using crude extract of the stomach and intestines of *A. tropicus* juveniles. We used the pH stat titration system to determine the degree of hydrolysis (DH%), the concentration of amino acids released (mg g of meal^{-1}), and the speed of hydrolysis (SH%) of various animal and vegetable ingredients. The relative digestibility of each protein ingredient was calculated using bovine hemoglobin for acid digestion and Hammersten bovine casein for the alkaline phase as reference ingredients (100% digestibility). For lipid ingredients, fish oil was used as a reference. The highest relative digestibility for protein ingredients was observed with fish, crustaceans, beef, poultry meat and offal, and beef blood meals. The best lipid SH was observed with soybean lecithin and cod liver, soybean, olive, maize, and canola oils. It is possible to use a wide variety of terrestrial animal proteins and vegetable oils for manufacturing a diet for *A. tropicus* rearing.

Keywords: *Atractosteus tropicus*; tropical gar; pH-stat; proteases; lipases; *in vitro* digestibility

INTRODUCTION

Tropical gar (*Atractosteus tropicus* Gill, 1863) is one of the seven ancestral species from the family Lepisosteidae. It inhabits swamps, rivers, and coastal lagoons from Mexico to Central America (Bussing 2002, Miller et al. 2009). It is a species with ecological, cultural, and economic importance. In recent years, its usefulness was highlighted in genetic, physiological, and biomedical studies because lepisosteids have a basal genome without a third whole genome duplication (3R WGD) typical of teleosts, allowing research without sub or neofunctionalization of genes (Amores et al. 2011, Braasch et al. 2016). The species has been described as carnivorous, consuming mainly fish and small crustaceans (Reséndez & Salvadores 1983, Mora-Jamett et al. 1997).

In Mexico, the pressure of overfishing reduced wild populations of *A. tropicus*, and, therefore, investigations were initiated to close its culture cycle and carry it out on a pilot scale (Márquez-Couturier et al. 2015). In nutrition, there are studies regarding their nutritional requirements (Márquez-Couturier et al. 2006, Huerta-Ortiz et al. 2018), digestive physiology (Guerrero-

Associated editor: Crisantema Hernández

Zárate et al. 2014, Frías-Quintana et al. 2015), carbohydrate inclusion and its effect on intermediary metabolism (Frías-Quintana et al. 2016, 2017, Guerrero-Zárate et al. 2019), use of prebiotics in their diets (Nájera-Arzola et al. 2018, Nieves-Rodríguez et al. 2018), and expression of genes involved in lipid metabolism and trypsin synthesis (Jiménez-Martínez et al. 2019, Jesús-De la Cruz et al. 2020, De la Cruz-Alvarado et al. 2021).

In fish, the various stages of development are known to involve changes in the digestion capacity, assimilation, and utilization of nutrients. In *A. tropicus*, some studies of digestive physiology have revealed differences in digestive capacity between the larval and juvenile stages, marked by the increase in protease activity during the juvenile stage, as well as the appearance of five alkaline proteolytic isoforms, concerning the three bands found during the larval period. Moreover, an increase in lipase activity has been detected from yolk absorption and live prey feeding, gradually decreasing until the juvenile stage is reached (Guerrero-Zárate et al. 2014, Frías-Quintana et al. 2015). These points towards an increase in the digestive capacity of juveniles and, therefore, better use and possible diversification of ingredients in the formulation of their diets.

The studies carried out so far in *A. tropicus* have contributed to the formulation of a specific food for the species during the larval period (Frías-Quintana et al. 2010, Saenz de Rodrigáñez et al. 2018); however, in the juvenile stage, the appropriate ingredients have not yet been identified to formulate specific diets for this stage.

Implementing apparent digestibility studies (*in vivo*) poses challenges such as difficulty collecting feces, leaching of nutrients, and the slow growth of organisms, which is complicated and expensive due to the time and space required. An alternative is *in vitro* systems, which simulate *in vivo* digestion using active enzyme extracts from different sections of the digestive tract of the studied species. Among the most used techniques is titration by adjusting the pH using the pH-stat system (Moyano et al. 2015). In the case of protein ingredients, this allows us to determine the degree of hydrolysis (DH) of an ingredient or diet and to calculate the number of peptidic bonds hydrolyzed by proteases, and with this, make a quantitative approximation of the digestive capacity of the studied organism.

Due to the role of proteins in tissue formation for growth, most research on digestibility *in vitro* in crustaceans and fish focuses on meals (Ezquerra et al. 1997, Lemos & Nunes 2008, Moyano et al. 2015). However, lipids also have the same challenge given their essentiality in multiple functions, hormonal, metabolic, and energy, as well as their participation in the structure and function of cell membranes. Therefore, it was proposed that the pH-stat method can be used to calculate the hydrolysis speed of lipids based on NaOH consumption using lipases from crude hepatopancreatic multi-enzymatic extracts of shrimp or other studied organisms (Nolasco et al. 2006). This method has already been reported in freshwater fish such as the three-spot cichlid (*Amphilophus trimaculatus*) (Toledo-Solís et al. 2020). Furthermore, many studies have not appropriately accomplished the requirements needed for an accurate simulation of the *in vivo* digestion process because only the intestinal stage of the digestion but not the gastric phase is simulated; such an approach may be suitable in the case of decapod crustaceans, but incorrect for many fish species with a functional stomach (Moyano et al. 2015). Therefore, the objective of the present study was to evaluate the *in vitro* digestibility of 14 meals and 12 oils, both from different animal and vegetable sources, commonly used in the aquafeed formulation using multienzyme extracts of the stomach and intestines from *A. tropicus* juveniles.

MATERIALS AND METHODS

Experimental fish

The multienzyme extracts were elaborated using *A. tropicus* juveniles from the Laboratorio de Fisiología en Recursos Acuáticos, División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco, Mexico. During larviculture, fish were fed following the scheme proposed by Márquez-Couturier et al. (2006). Once adapted to the balanced pelletized feed, fish were supplied three times daily with commercial trout feed (Silver Cup, El Pedregal, Edo. Mex., México, 45% protein, and 16% lipid). When fish reached an average weight of 73.6 ± 22.7 g, a random sample was taken (30 fish). These were kept in starvation for 24 h and were subsequently anesthetized with clove oil (0.1 mL L^{-1}). The fish were quickly slaughtered by decapitation by trained personnel according to the NOM-062-ZOO-1999, Mexico, and then were rapidly dissected on ice to obtain stomach and intestines with pyloric caeca samples. The dissected organs were frozen at -80ºC until use.

Gastro-intestinal extract preparation and determination of enzyme activity

The organs were homogenized with distilled water at 0.5 g of tissue (wet weight) per mL of distilled water.

The mixture was centrifuged at 16,000 *g* for 15 min at 4°C. Once the supernatants were obtained, the pH was adjusted to 3 with HCl 0.1 N for the stomach extracts and 8 with NaOH 0.1 N for the intestine extracts, and they were divided into 100 µL aliquots. The extracts were kept at -80ºC until use. The activity of acid proteases was determined using 1% hemoglobin bovine (USBiological) in a glycine-HCl 100 mmol L^{-1} buffer at pH 2 and 25°C following the method of (Anson 1938). The activity of the alkaline proteases was evaluated following the method of (Walter 1984) using 1% casein (Research Organics) in a Tris-HCl 100 mmol L^{-1}, $CaCl_2$ 10 mmol L^{-1} buffer at pH 9 and 25°C. One unit of enzyme activity was defined as 1 µg of tyrosine liberated per minute at 280 nm with a molar extinction coefficient (MEC) of 0.008 mL μg^{-1} cm^{-1} (Moyano et al. 1996). Lipase activity was measured according to Versaw et al. (1989) using ß-naphthyl caprylate 100 mmol L^{-1} as substrate at pH 7.5 and 35°C; one unit of enzyme activity was defined as 1 µg of naphthol released per minute at 540 nm with a MEC of 0.02 M^{-1} cm^{-1} (Frías-Quintana et al. 2017). All enzyme activities were expressed per mg of protein. Soluble protein concentration was determined according to Bradford (1976) using a standard curve with bovine serum albumin (BSA). All assays were performed in triplicate.

Experimental ingredients

For this study, a total of 14 meals and 12 oils, both from different animal and vegetable sources, were evaluated. These ingredients were obtained from different commercial suppliers (Table 1).

Determination of the degree of hydrolysis (DH)

The degree of hydrolysis was determined using 5 mL of an aqueous solution containing each meal with a concentration of 8 mg of crude protein per mL, pH 3.5 for the acid phase (Saunderset et al. 1973), modified by Dimes & Haard (1994). In the pH stat titration system (Titrino 918, Methrom, Suiza), 50 U mL^{-1} of protease activity from the crude stomach extract was added to an aqueous solution, keeping in the mixture continuously stirring at 37°C for 15 min. For the alkaline phase, the pH of the aqueous solution was adjusted to 8 with NaOH (0.1 N). Then, 10 U mL^{-1} of crude intestine extract was added to the pH system, keeping it under continuous stirring for 45 min at 37ºC. In both cases, the amount of hydrochloric acid (HCl, 0.1 N) and sodium hydroxide (NaOH, 0.1 N) used were recorded.

The DH was calculated from the volume of HCl necessary to maintain the pH at 3.0 or NaOH to maintain the pH at 8.0 once the digestion was complete. The ingredients' auto-hydrolysis level was determined in the absence of the enzyme extract, which was replaced by an equivalent volume of distilled water. All tests were performed in triplicate. DH was expressed as ratio of hydrolyzed peptide bonds to total protein peptide bonds by the following equation: $DH = b \times Nb / (Mp \times \alpha \times h_{tot}) \times 100$, where DH: degree of hydrolysis; b (mL): consumption of HCl or NaOH; Nb: normality of the titrants; α: constant of group disassociation α-NH_2 (1.4); Mp (g): mass of protein in the mix of the reaction, and h_{tot}: number of total peptide bonds from the protein substrate (Rutherfurd 2010). The DH values were expressed as relative digestibility using reference DH from the hemoglobin in the acid phase and the casein in the alkaline phase; in both cases, reference ingredients were considered 100% (Alarcón et al. 2002).

Determination of total free amino acids (TFA)

Throughout *in vitro* digestion, samples of the hydrolyzed ingredient were collected every 100 s for the acid phase and every 250 s for the alkaline phase to determine the concentration of amino acids released. The reaction was stopped by adding 20 µL of 12% trichloroacetic acid (TCA). All samples were performed in triplicate and frozen at -80ºC until analysis. Total free amino acid analysis (TFA) is based on the union of the amino terminal part with the reagent *0*-phthaldialdehyde (OPA) based on the technique described by Church et al. (1983). TFA concentration was calculated based on a standard curve made with L-leucine (Sigma-Aldrich, 61-90-5) at increasing concentrations (0-20 µg mL^{-1}). The samples were read in a spectrophotometer at 340 nm. The tests were carried out in triplicate.

Determination of speed of hydrolysis (SH)

The *in vitro* digestion of oils was determined as described by Nolasco et al. (2006). Emulsions were prepared to contain 485 µL of distilled water, 144 µL of the oil to be tested, and 285 µL of extracted bile salts (10 mmol L^{-1} Sodium taurocholate, Sigma-Aldrich CAS: 345909-26-4). The emulsion was adjusted to pH 9.2. Subsequently, 200 µL (approximately 50 U mL of lipolytic activity) of multienzyme extract from the digestive tract was added, and the solution was kept under constant stirring for 15 min at 37°C. The lipase activity was calculated according to Versaw et al. (1989). The amount of alkali (0.1 N NaOH) to maintain the pH at 9 was recorded. The level of oils auto-hydrolysis was determined in the absence of the

Table 1. Protein and oil ingredients evaluated by pH-stat method for *A. tropicus* juveniles. [1]USBiological N°Cat H1850; [2]Research Organics N°Cat 10826; [3]Alimentos Pedregal S.A. de C.V.; [4]Proteínas Marinas y Agropecuarias S.A. de C.V. (PROTMAGRO), Guadalajara, Jal., Mex.; [5]CCP Norway; [6]Rastro de Texcoco, Edo. de Mex., Mex.; [7]Consorcio Súper en Guadalajara, Jal., Mex.; [8]GALMEX, S.A., Villahermosa, Tab., Mex.; [9]Paris Pharmacy, Villahermosa, Tab., Mex.; [10]Maceite, Promotora de Productos y Mercados Mexicanos S.A. de C.V., Guadalajara, Jal., Mex.; [11]Canoil, Aceites, Grasas y Derivados S.A. de C.V., Guadalajara, Jal., Mex.; [12]Ybarra Comercial de México, S.A. de C.V. Mex., D.F.; [13]Industrial Patrona S.A. de C.V, Córdoba, Ver, Mex.; [14]Oleico, San Luis Potosí, SLP, Mex.; [15]Nutrioli, RAGASA, S.A de C.V, Monterrey, NL, Mex.

Protein ingredients	Abbreviation	Protein (%)	Lipid ingredients	Abbreviation
Hemoglobin[1]	Hb	90	Fish oil[4]	Fish
Casein[2]	Cas	90	Sardine oil[4]	Sard
Fish meal[3]	FM	64	Cod liver oil[9]	Cod
Fish meal (Aqua)[4]	FMaqua	56	Beef tallow oil[4]	Beef
Hydrolyzed fish[5]	HFM	72	Maize oil[9]	Maiz
Beef blood meal (PROTMAGRO)[4]	BBProt	72	Canola oil[11]	Can
Beef blood meal (Texcoco)[6]	BBTex	64	Olive oil[12]	Oliv
Poultry blood meal[4]	PBM	82	Sunflower oil[13]	Sunf
Chicken meal[4]	CkM	65	Safflower oil[14]	Saff
Meat and viscera chicken meal[4]	mvCkM	60	Soy oil[15]	Soy
Meat bovine meal[7]	mBM	49	Soy lecithin[9]	SoyLec
Meat porcine meal[7]	mPM	54	Salmon oil[4]	Salm
Squid meal[4]	SqM	75		
Crab meal[4]	CM	30		
Shrimp meal[4]	ShM	28		
Soybean paste[8]	SP	49		

enzyme extract, which was replaced by an equivalent volume of distilled water. All tests were performed in triplicate. The hydrolysis rate of each ingredient was determined using the following equation: SH = (P - T) × (Cs) × 1000 / Venz × Cprot, where SH: speed of hydrolysis; P (mL min^{-1}): consumption of NaOH; T (mL min^{-1}): consumption of NaOH of the control lipid; Cs (M): NaOH concentration; Venz (mL): enzyme volume, and Cprot: protein concentration (enzyme) in mg mL^{-1} × 1000 (correction factor) (Nolasco et al. 2006). The SH values were expressed as relative digestibility using fish oil as a reference ingredient.

Statistical analysis

DH and TFA values were transformed to arcsine and analyzed using ANOVA, and assumptions of normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test) were verified. Where significant differences were found, the Tukey test was applied. The relationship between DH and TFA was examined using the Spearman correlation test in the acid and alkaline phases. SH values were analyzed using the Kruskal-Wallis test for multiple comparisons by applying the method of Conover. All analyses were performed with the statistical software Statistica™ v.8.0 (Statsoft, Inc., Tulsa, OK) using an alpha 0.05. The graphics were made with SigmaPlot v.10.0 (Systat Software, Inc., Germany).

RESULTS

In vitro digestibility of protein ingredients

In the present study, DH values for the acid phase were <7.29%, with the control value of hemoglobin 2.87%. The meals that had a higher DH value than the control in the acid phase were shrimp (ShM), fish (FM), meat beef (mBM), fish aqua (FMaqua), and crab (CM) (Fig. 1). In the alkaline phase, the DH range was <2.29% and the control value (Cas) was 0.67%. The meals with higher DH than the control were meat and viscera of chicken (mvCkM), FMaqua, beef blood (BBTex), FM, meat beef (mBM), soybean paste (SP), chicken blood (PBM), beef blood (BBProt) and meat porcine (mPM) (Fig. 1). Finally, the ingredients with the lowest DH values in both phases were squid meal (SqM), chicken meal (CkM) and fish hydrolysate meal (HFM) (see the box in Fig. 1).

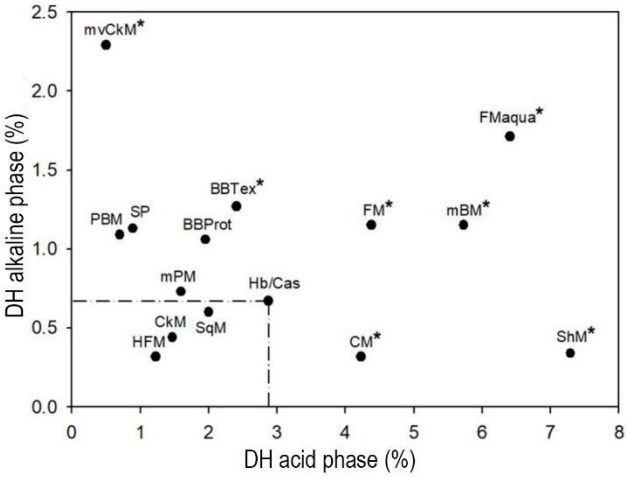

Figure 1. Degree of hydrolysis (DH, %) of proteinaceous ingredients in acid and alkaline conditions using gastrointestinal extracts of *A. tropicus* juveniles. Hb: hemoglobin; Cas: casein; ShM: shrimp meal; mBM: meat bovine meal; FM: fish meal; FMaqua: fish meal aqua; CM: crab meal; BBTex: beef bovine meal Texcoco; BBProt: beef bovine meal PROTMAGRO; mPM: meat porcine meal; PBM: poultry blood meal; mvCkM: meat and viscera of chicken meal; SP: soy past; mPM: meat porcine meal; SqM: squid meal; CkM: chicken meal; HFM: hydrolyzed fish meal. Values are presented as mean (n = 3). Means with asterisk are significantly different ($P < 0.05$).

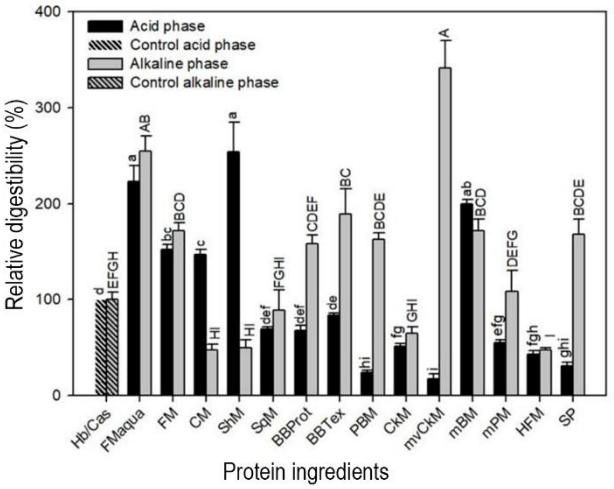

Figure 2. Relative digestibility (%) of proteinaceous ingredients in acid and alkaline conditions using gastrointestinal extracts of *A. tropicus* juveniles. The relative digestibility of each ingredient expressed as a percentage concerning the controls. Hb: hemoglobin; Cas: casein; ShM: shrimp meal; mBM: meat bovine meal; FM: fish meal; FMaqua: fish meal Aqua; CM: crab meal; BBTex: beef bovine meal Texcoco; BBProt: beef bovine meal PROTMAGRO; mPM: meat porcine meal; PBM: poultry blood meal; mvCkM: meat and viscera of chicken meal; SP: soy past; mPM: meat porcine meal; SqM: squid meal: CkM: chicken meal; HFM: hydrolyzed fish meal. Values are presented as mean ± standard error (SE), n = 3. Means with different letters are significantly different ($P < 0.05$).

The relative digestibility of each ingredient expressed as a percentage concerning the controls, Hb (acid phase) and Cas (alkaline phase) are shown (Fig. 2). During the acid phase, meals with a percentage significantly higher than the control ($P < 0.0001$) were ShM, FM, mBM, FMaqua, and CM. In the alkaline phase, the mvCkM, FMaqua, BBTex, FM, and mBM meals were significantly higher than the control ($P < 0.0001$) (Fig. 2).

For meals with DH higher than the control hemoglobin, only mBM and FMaqua had a TFA concentration like the control. While in the alkaline phase, all the meals with DH significantly higher than the control (Cas) had a TFA concentration like Cas (Fig. 3). The correlation was not significant for DH and TFA; for both, acid (r-value = 0.21, $P = 0.44$) and alkaline (r-value = 0.11, $P = 0.61$) phase.

Hemoglobin was the ingredient that released the largest quantity of amino acids during the acid phase of digestion ($P < 0.0001$), with statistically similar values to those for HFM, SqM, mPM, mBM, mvCkM, and FMaqua. On the other hand, during alkaline digestion, most of the ingredients released an amount of TFA, like Cas meal ($P > 0.0001$), with only the exception of mPM, which had a lower concentration of TFA (Fig. 4).

In vitro digestibility of lipid ingredients

Most of the oils had an SH, like fish oil, except for beef tallow and sardine oil, which had significantly lower SH ($P = 0.0002$) (Fig. 5). The oils with the best relative digestibility were soybean oil (Soy), soybean lecithin (SoyLec), canola (Can), cod liver (Cod), olive (Oliv), and maize (Maiz) ($P = 0.0002$) (Fig. 6).

DISCUSSION

In the present study, the DH in acid and alkaline conditions showed that fishmeal (FM and FMaqua) and beef meal (mBM) had significantly higher values than the control ingredients (hemoglobin and casein). Likewise, the crustacean meals (ShM and CM) had DH significantly higher than hemoglobin in the acid phase.

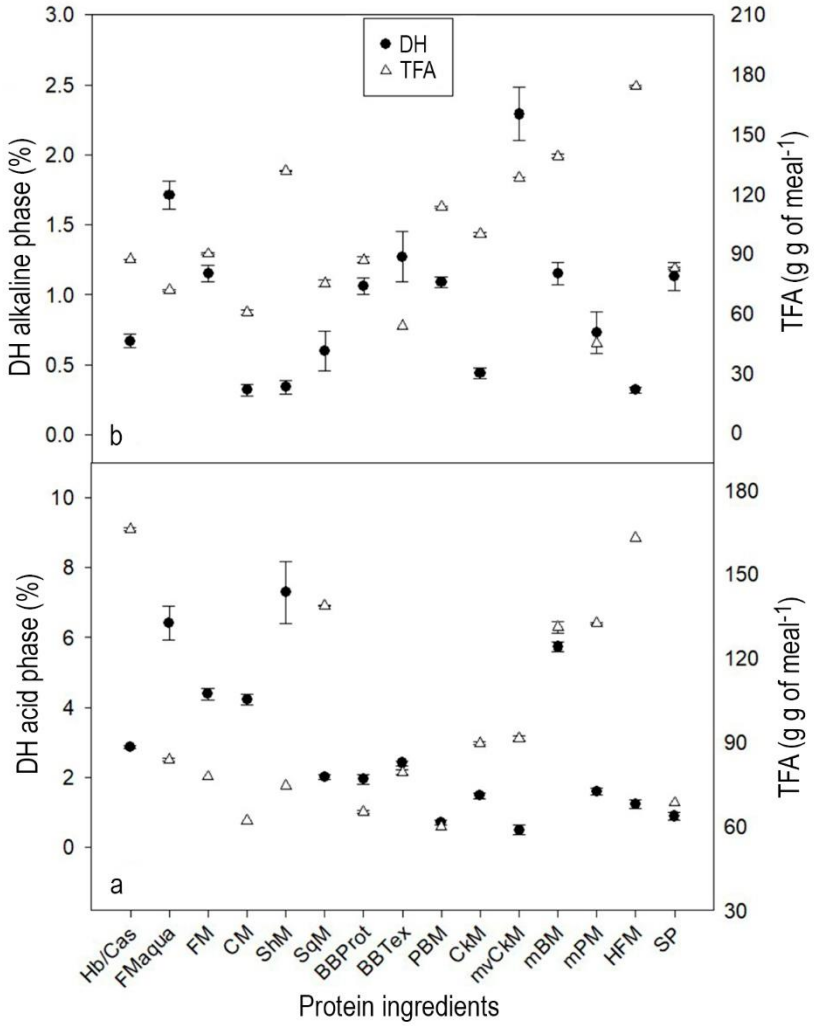

Figure 3. Degree of hydrolysis (DH) and the total amount of free amino acids (TFA, mg g meal^{-1}) from proteinaceous ingredients in a) acid and b) alkaline conditions using gastro-intestinal extracts of *A. tropicus* juveniles. Hb: hemoglobin; Cas: casein; ShM: shrimp meal; mBM: meat bovine meal; FM: fish meal; FMaqua: fish meal aqua; CM: crab meal; BBTex: beef bovine meal Texcoco; BBProt: beef bovine meal PROTMAGRO; mPM: meat porcine meal; PBM: poultry blood meal; mvCkM: meat and viscera of chicken meal; SP: soy past; mPM: meat porcine meal; SqM: squid meal; CkM: chicken meal; HFM: hydrolyzed fish meal. Values are presented as mean ± standard error (SE), n = 3. Means with asterisk are significantly different ($P < 0.05$).

These results agree with other carnivorous fish species, such as *A. tropicus*, mainly consuming fish and crustaceans (Reséndez & Salvadores 1983, Mora-Jamett et al. 1997).

The above has been confirmed through digestive physiology studies since *A. tropicus* can hydrolyze proteins from 30 days post-hatching (Frías-Quintana et al. 2010), and its protease activity increases during the juvenile stage, develop a functional stomach that secretes pepsin and hydrochloric acid (Guerrero-Zárate et al. 2014). Unlike the results found in *L. guttatus* (Peña et al. 2017) and *A. trimaculatus* (Toledo-Solís et al. 2020), in those that do not report ingredients with DH higher than those of hemoglobin during the acid hydrolysis phase, our study the fish, crustaceans and beef meals have higher DH than the control, which may be related to the presence of two pepsin isoforms in the stomach of *A. tropicus* (Frías-Quintanta et al. 2015).

The results obtained with the crustacean meals suggest that *A. tropicus* juveniles have the enzymatic digestive capacity to hydrolyze proteins of these by-products due to its carnivorous feeding habits (Márquez-Couturier & Vázquez-Navarrete 2015), which has been studied in other lepisosteid species were the

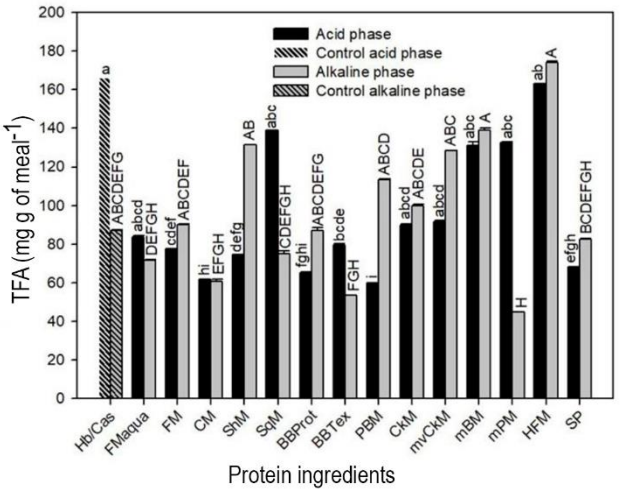

Figure 4. Total amino acids released (TFA, mg g meal^{-1}) from proteinic ingredients in acid and alkaline phases using multienzyme extracts of *A. tropicus* juveniles. Hb: hemoglobin; Cas: casein; FMaqua: fish meal aqua; FM: fish meal; CM: crab meal; ShM: shrimp meal; SqM: squid meal; BBProt: beef bovine meal PROTMAGRO; BBTex: beef bovine meal Texcoco; PBM: poultry blood meal; CkM: chicken meal; mvCkM: meat and viscera of chicken meal; mBM: meat bovine meal; mPM: meat porcine meal; HFM: hydrolyzed fish meal; SP: soy past; mPM: meat porcine meal. Values are presented as mean ± standard error (SE), n = 3. Means with asterisk are significantly different ($P < 0.05$).

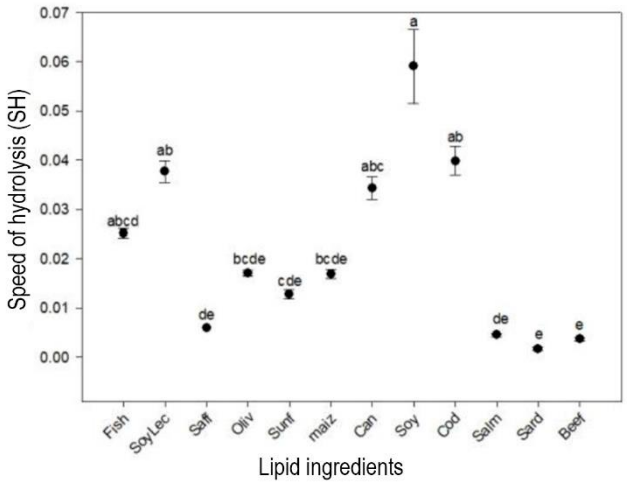

Figure 5. Speed of hydrolysis (SH) of lipid ingredients using intestinal extracts of *A. tropicus* juveniles. Fish: fish oil; SoyLec: soy lecithin; Saff: safflower oil; Oliv: olive oil; Sun: sunflower oil; maize: maize oil; Can: Canola oil; Soy: soy oil; Cod: cod liver oil; Salm: salmon oil; Sard: sardine oil; Beef: beef tallow oil. Means with different letters are significantly different ($P < 0.05$).

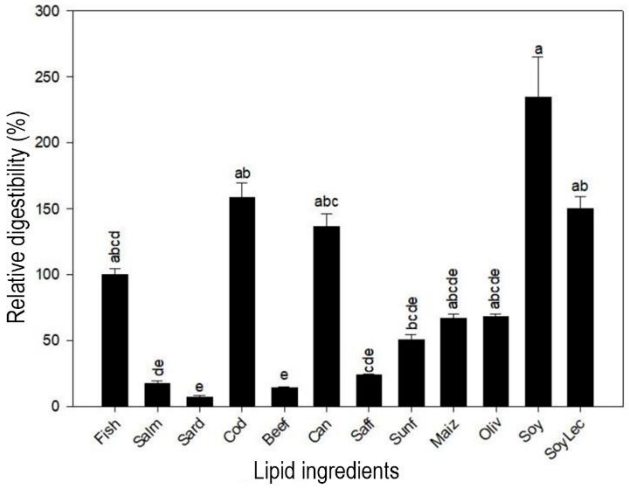

Figure 6. Relative digestibility (%) of lipid ingredients using gastro-intestinal extracts of *A. tropicus* juveniles. Fish: fish oil; Salm: salmon oil; Sard: sardine oil; Cod: cod liver oil; Beef: beef tallow oil; Can: Canola oil; Saff: safflower oil; Sun: sunflower oil; maiz: maize oil; Oliv: olive oil; Soy: soy oil; SoyLec: soy lecithin. Values are presented as mean ± standard error (SE), n = 3. Means with different letters are significantly different ($P < 0.05$).

presence of crustaceans is an essential item in the natural diet (Goodyear 1967). In this sense, it is probable that chitinases are present in the stomach and allow it to hydrolyze chitin; however, this aspect needs to be clarified. It has been described that fish are capable of synthesizing chitinases, and there are at least two groups: acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) (Ikeda et al. 2017). The presence and characterization of chitinases in *A. tropicus* should be studied in subsequent studies.

Another group of meals with high relative digestibility were terrestrial animal by-products, including meat and viscera of chicken meal, meat porcine, and beef blood meals. These ingredients have been used for several decades in the manufacture of feed for aquaculture; however, their use has been limited and even avoided for various reasons, such as low digestibility and aspects of biosafety and traceability of products that may endanger human health (Sapkota et al. 2007, Vidyarthi et al. 2021). Nonetheless, the results of the present study show that these ingredients are a viable alternative for substituting fishmeal in diets for *A. tropicus*. In this sense, studies in different fish species where high-quality animal-rendered meals (mainly poultry-by-product meals) were used showed that at least 30% of these meals could be included in their diet without affecting

digestibility, which adds to other benefits such as low cost, good palatability, absence of anti-nutritional factors and good profiles of essential amino acids and highly polyunsaturated fatty acids (Galkanda-Arachchige et al. 2020).

In the present study, beef blood meals (BBTex and BBProt) had DH values close to the reference ingredients. Studies such as those carried out on *P. maculatofasciatus*, *C. undecimalis*, and *A. tropicus* larvae have also indicated high DH values for the blood meal (Álvarez-González 2003, Álvarez-González et al. 2010, Frías-Quintana et al. 2010). Although they could constitute a good source of protein for the elaboration of diets for these species, given their high protein content (72 to 82%), care must be taken about using them only as a partial resource for the substitution of fishmeal since that the high iron content included in the prosthetic group may have anti-nutritional effects, in addition to its high lysine content, it could present an antagonism with arginine, affecting its use for growth (Ofori & Hsieh 2014). In addition, specify the quality in the process for its manufacture, biogenic amines, and protein denaturation can occur, causing amino acid imbalances and antagonism, which causes adverse effects on the growth and survival of organisms (Doeun et al. 2017, Wójcik et al. 2021).

Unlike the study by Frías-Quintana et al. (2010) with *A. tropicus* larvae, soybean paste showed a high hydrolysis capacity during alkaline digestion, even greater than casein. In this regard, there is extensive information regarding substituting fishmeal for vegetable ingredients, especially soybean paste, due to its high protein concentrations (Teves & Regaza 2016). One of the main problems is the processing to obtain it since if it is not done correctly, it can be raw or overcooked, which will influence the digestibility of the protein (Gasco et al. 2018). Likewise, the presence of some anti-nutritional factors that affect its nutritional value and reduce the palatability of feed when prepared with high levels of this ingredient, as well as its deficiency in sulfuric amino acids (methionine and cysteine), is well documented (Samtiya et al. 2020).

On the other hand, the analysis of the concentration of total free amino acids in the samples taken during the acid and alkaline phases of the digestion showed that hemoglobin had the highest TFA and that the HFM, SqM, mBM, mvCkM, CkM, mPM, and FMaqua, had amino acid concentrations like those of the reference ingredient during acid digestion. Likewise, in alkaline digestion, most meals, except for mPM, had amino acid concentrations (Li et al. 2021), like casein. Although no significant correlation was found between DH and TFA concentration, the nature of the ingredients and the industrial process could determine the existence of variable quantities of free amino acids, thereby determining the amounts of TFA. However, ultimately, these will be bioavailable to be assimilated by the fish's digestive system (Toledo-Solís et al. 2020).

We also determined the speed of hydrolysis and relative digestibility of 12 animal and plant lipid ingredients, using fish oil as the reference ingredient. Thus, it was found that in the alkaline phase, Soy, SoyLec, Can, olive, Maiz, and Cod oil showed similar hydrolysis rates to fish oil. Remarkably, most ingredients with the best relative digestibility were vegetable oils, probably due to their fatty acid profile. Vegetable oils are rich in monounsaturated and polyunsaturated fatty acids, while terrestrial animal fats contain more saturated fatty acids (Sutili et al. 2018). The action of lipases is affected by their selectivity, such that pancreatic lipases preferentially act upon ester linkages at positions 1 and 3 of the triacyl glycerides (TG) (Nolasco-Soria et al. 2018). Therefore, the type of TG and the structure of the fatty acids that compose it affect the digestibility of the ingredients. TG esterified in external positions with saturated fatty acids hinders the action of lipases due to their physicochemical characteristics (Song et al. 2022). In this regard, studies on the replacement of fish oil with vegetable oils have been carried out on warm-water and tropical freshwater fish such as the Mandarin fish (*Siniperca scherzeri*), large yellow croaker (*Larimichthys crocea*), and the Roho labeo (*Labeo rohita*), showing that fish oil could replace in considerable quantities with vegetable oils without affecting growth and feeding efficiency (Nasopoulou & Zabetakis 2012, Sankian et al. 2019, Mu et al. 2020, Siddiqua & Khan 2022, Xu et al. 2022); however, the selection of these oils is not based on *in vitro* digestibility studies.

Unlike the results described by Jiménez-Martínez et al. (2020) when using vegetable oils to feed *A. tropicus* larvae, our results are promising regarding the possibility of replacing fish oil with vegetable oils in *A. tropicus* juvenile stage because apparently, there is the ability to digest vegetable oils better. Furthermore, it was reported that an increased expression of *fads1*, *fads2*, *elovl2*, and *elovl5* genes during the early development of *A. tropicus* suggests that species possess the machinery to biosynthesize lipid C18 to LC-PUFA C20 and C22 (De la Cruz-Alvarado et al. 2021). This capacity is expected since freshwater fish species' physiological ability to elongate and desaturate n-6 and n-9 saturated and monosaturated fatty acids. Therefore, vegetable oils should be advantageous over

other animal oils, such as sardine, menhaden, salmon, and cod. In this regard, some vegetable oils have already been evaluated and substituted for sardine oil in experiments with larvae and juveniles of *A. tropicus* with excellent results (Huerta-Ortiz et al. 2018).

CONCLUSIONS

Based on the degree of hydrolysis and total free amino acids analyses, marine origin meals such as fish, shrimp, and crab meals, and terrestrial animal by-product ingredients such as beef, pork, and chicken are appropriate inputs as the protein base diet for *A. tropicus* juveniles. Likewise, based on the speed of hydrolysis, fish oil, lecithin and soybean oil, canola, olive, and corn oils could be included as the lipid source in diets for *A. tropicus* juveniles; however, the *in vitro* studies must be validated with the *in vivo* bioassays to know the appropriate mixes of protein and lipid sources and obtain the best growth of this species.

ACKNOWLEDGMENTS

The author thanks the National Council of Science and Technology of Mexico for the grant N°10098 and the project SAGARPA-2011-08-164673 for the financial support.

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Received: January 17, 2023; Accepted: August 30, 2023