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Research Article

In vitro protein digestibility of animal, vegetal and microbial feed ingredients for *Macrobrachium tenellum*

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ABSTRACT. Due to the cost of raw materials, the need to formulate balanced feeds with highly digestible ingredients is indispensable for the aquaculture feed industry. For this reason, the protein *in vitro* digestibility, assessed by the pH-stat method, of ingredients with potential of using them on the balanced feed for *Macrobrachium tenellum*, were evaluated. The relative protein digestibility was assessed in twelve feed ingredients, including animal (pork meal, feather poultry meal, prime poultry meal, turkey meal, fish meal, shrimp meal), vegetal (coconut paste, chickpea meal, soybean meal, wheat gluten) and microbial (yeast and *Spirulina* meal); casein (Hammerstein grade) was used as the reference protein. The highest relative protein digestibility was found in: *Spirulina* meal (52.6%); following by pork meal (45.6%), and feather poultry meal (39.6%). The lowest digestibilities were found in soybean meal (15.9%), chickpea meal (12.1%), and fish meal (11.6%). The protein digestibility value should be considered for selecting potential ingredients for the formulation of balanced feeds for *M. tenellum.*

Keywords: *Macrobrachium tenellum*, prawns, nutrition, protein, enzyme activity, digestibility, aquaculture.

INTRODUCTION

The production of the aquaculture feed is one of the fastest growing sectors of animal feed worldwide (Rust *et al*., 2012). In aquaculture, feeding represents one of the highest production costs, according protein content and source, therefore the protein quality on the commercial feeds, is one of the most important nutritional parameters, as influence the prawns growth and the body composition (Chisty *et al*., 2009). The feed digestibility and assimilation are also very important to reduce its conversion into a source of water pollutants, with negative effects on the ecosystem (Terrazas *et al*., 2010a). An efficient feeding depends on the nutritional characteristics of the feed, its digestibility, and feeding strategy since they are essential elements in providing nutrients and energy needed for efficient growth of cultivated species (Carrillo-Farnés *et al*., 2007; Chisty *et al*., 2009).

The digestive capacity of the aquaculture species is sustained by the activity of digestive enzymes present in their digestive tract (Ali *et al*., 2009). The study of the digestive enzymes and nutrient digestibility is essential for understanding the mechanisms of digestion, and for being useful for selection of ingredients and feeds with higher nutritional value, for each species (Cruz-Suárez *et al*., 2002).

Since the *in vivo* digestibility methods are lengthy and expensive, it has been necessary to apply *in vitro* methods, that are fast and reliable, for assessing protein digestibility, using the digestive enzymes of the species of interest (Nolasco *et al*., 2006). The pH-stat *in vitro* method has been widely used in the evaluation of the nutritional quality of raw materials and processed feeds, because it is simple, fast, and with high reproducibility (Moyano *et al*., 2014). The use of pH-stat system has proved to be suitable for checking the quality of the protein sources, for marine shrimp feed

formulation (Ezquerra *et al*., 1998; Lemos *et al*., 2004, 2009).

Although *M. rosenbergii* production has been profitable in some countries, just few studies have been performed on ingredient and feed digestibility (*in vivo* methods: Taechanuruk & Stickney, 1982; Ashmore *et al*., 1985; Ellis *et al*., 1987; Chin, 1988; Gomes & Peña, 1997). In general, information about native American species is limited (*in vitro* method: Manríquez-Santos *et al*., 2011).

This research contributes to the knowledge of the protein *in vitro* digestibility, by using the pH-stat method, of protein from animal, vegetal and microbial origin, with actual or potential for inclusion in feeds for *M. tenellum*.

MATERIALS AND METHODS

Preparation of enzyme reagent

Twenty six pre-adults prawns (4-10 g) of *M. tenellum*, at intermolt stage, randomly collected from an artificial pond located at the Centro Universitario de la Costa (CUCOSTA), Universidad de Guadalajara, in Puerto Vallarta, Jalisco, Mexico, were used. The prawns were weighed (0.01 g precision, Ohaus, NJ, USA) and then were sacrificed by immersion in ice water, and subsequent freezing (-20ºC).

Stomach and digestive gland of *M. tenellum* were dissected and weighed. One single pool of organs was homogenized, adding distilled water (1:4 w/v) in cold bath, with a homogenizer (Pro Scientific, Pro 200, Oxford CT, USA). The enzymatic extract (EE) was clarified by centrifugation (20,800 x *g*, 8 min, 10°C), the lipid fraction was removed and the supernatant was recovered. The EE was immediately stored at -20°C until use for protein content and enzymatic activity.

The enzymatic reactive (ER) for determining *in vitro* protein digestibility of feed ingredients, was prepared as follows: the enzymatic extract was homogenized (homogenizer Pro Scientific) pH was adjusted to pH 8.0, with sodium hydroxide (NaOH 1M). The ER was stored at -20ºC until use. All assays were performed by triplicate.

Determination of protein and enzyme activity in enzymatic extract (EE)

The EE concentration for soluble protein was determined by the Bradford method (1976), by using bovine serum albumin (A4503, Sigma-Aldrich, St. Louis, MO, USA) as a standard.

The activity of total proteases was determined according Vega-Villasante *et al.* (1995) using azocasein (2% in Tris-HCl 50 mM, pH 7.5) as substrate. The specific activity of general protease was expressed in U protease/mg protein (a unit of protease is defined as the amount of the enzyme required to increase 0.01 U absorbance by min, at 440 nm).

The chymotrypsin activity was determined according Delmar *et al*. (1979), and trypsin activity by the modified micromethod based on Erlanger *et al*. (1961), using SAPNA (No. S7388, SIGMA, St. Louis, MO, USA), and BAPNA (No. B3133, SIGMA, St. Louis, MO, USA) (9.6 mM in dimethyl sulfoxide (DMSO)), as a substrate, respectively. The specific activity of trypsin and chymotrypsin was expressed as U/mg protein (a unit of trypsin and chymotripsin is defined as the amount of enzyme required to release 1 µMol of p-nitroanilide/min).

The amylase activity was determined to Vega-Villasante *et al*. (1993), using a starch solution (1% in Tris-HCl, 50 mM pH 8.0) as substrate. The specific activity of amylase was expressed U amylase/mg protein (a unit of amylase is defined as the amount of enzyme required to release 1 µMol of maltose/min).

The lipase activity was determined according to Versaw *et al*. (1989), using ß-naphthylcaprilate as substrate. The specific activity of lipase expressed in U lipase/mg protein (a unit of lipase is defined as the amount of the enzyme required to release 1 µMol of β-naphthol/min).

In vitro protein digestibility

The twelve ingredients used were: pork meal (meat and bones, USA); poultry meal (poultry products prime, USA); hydrolyzed feather meal (USA); turkey meal (60% turkey and 40% poultry products, USA); fish meal (sardine, Mexico); shrimp meal (Mexico), all animal meal were supplied by Proteínas Marinas y Agropecuarias, S.A. de C.V.; coconut paste hydrolyzate by Copreros Unidos por Tabasco S. de P.R. de R.L. (Mexico); chickpea meal elaborated in the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR-IPN unidad Sinaloa, Mexico); soybean meal by Procesadora de Ingredientes, S.A. de C.V. (Mexico); wheat gluten by Arancia Ingredientes Especiales (Mexico); *Spirulina* meal by Producción y Comercialización de Microalgas y sus Derivados GENIX (Cuba); Bakers' yeast (*Saccharomyces cerevisiae*) by SAFMEX, S.A. de C.V./FERMEX, S.A. de C.V. (Mexico) and Hammerstein grade bovine casein (No. 101289, MP Biomedicals, Santa Ana, California, USA). The chemical composition is presented in Table 1. All the ingredients were ground, in a coffee grinder, and sieved to 250 µm.

The *in vitro* protein digestibility was performed by the pH-stat method (Nolasco, 2008). Adding the corresponding amount of ingredient to have 6.25 mg of protein mL^{-1}, the pH was adjusted 8.0, with sodium hydroxide (NaOH 1M). To start the digestion 10 U protease of the ER, of *M. tenellum*, were added, with an initial reaction volume of 5 mL, and incubated in the pH-stat (Metrohom 842 Titrando, Herisau, Switzerland) at pH 8, for 30 min at 25°C, under stirring (120 rpm). The consumption NaOH (0.02 M) required to maintain the pH at 8.0 was recorded, and the number of moles of NaOH /min was calculated, using casein as reference protein. All tests were performed in triplicate. For the controls, of each ingredient, the procedure was performed in the same way, but using denatured (heat treatment at boiling water bath, for 10 min) ER. The relative protein digestibility (RD) was expressed according to the following formula:

$$\text{RD (\%)} = \frac{\text{Consumption of NaOH/min by the hydrolysis of the problem sample}}{\text{Consumption of NaOH/min by the hydrolysis of the reference protein}} \times 100$$

The correlation coefficient between the percentage of crude protein (CP) of the ingredients and RD was evaluated, with the purpose to determine if these data could be used to predict the digestibility of ingredients in prawn.

A test for normality Kolmogorov-Smirnov (α = 0.05) and homogeneity of variance Bartlett (α = 0.05) before the variance analysis (ANOVA) of a track, was applied. Statistically significant differences ($P < 0.05$) between treatment means were determined by the method of multiple comparisons of Tukey. All tests were performed using the statistical software version 11.0 SigmaPlot (Systat Software, Inc. Chicago, IL, USA).

The values of the concentration of CP ingredients were correlated with the results of RD and statistically analyzed to determine their correlation coefficient.

RESULTS

The enzyme activity in the enzymatic extract (EE) is shown in Table 2. The finding significant differences ($P < 0.05$) of RD, of ingredients used in this study is shown in Fig. 1. *Spirulina* meal showed the highest digestibility (52.6%), followed by the pork meal (45.6%); and the lowest digestibility was found in chickpea meal (12.1%), and fish meal (11.6%). The correlation between the CP concentration of the ingredients and the RD, was not significant, with a correlation coefficient of $r^2 = 0.25$, with $P > 0.05$.

Table 2. Digestive enzyme activity (average ± SE) in *M. tenellum*.

Enzyme	U/mg protein
General proteases	1.254 ± 0.31
Trypsin	0.018 ± 0.01
Chymotrypsin	0.004 ± 0.0005
Amylases	0.634 ± 0.47
Lipases	0.746 ± 0.23

DISCUSSION

The search for new sources of available, nutritious and cheaper protein has been a constant need in the development of aquaculture feed (García-Galano *et al*., 2007). The digestibility of an ingredient depends not only on its protein content, as show the results of correlation between the CP concentration and RD, obtained in this study. This depends on many other factors such as the physical properties (*i.e.*, particle size, solubility, etc.), and chemical properties (*i.e.*, amino acid content); but also biological characteristics of the animal; the architecture of the digestive tract, and physiology will affect protein digestibility, and also environmental conditions are important (pH, temperature, salinity, ions); all these aspect should be considered for the determination of *in vitro* protein digestibility of ingredients or feed (Cruz-Suárez, 1996; Ezquerra *et al*., 1998; Carrillo-Farnés *et al*., 2007). However, most of the new protein ingredients have not been assessed from the digestibility point of view (Álvarez-González, 2003).

The digestibility results of the different protein sources, as potential ingredients for *M. tenellum* in this research, confirms that *M. tenellum* is omnivorous; and that the species is able to efficiently digest a variety of ingredients of microbial, animal and plant origin. Bhavan *et al*. (2010) in *M. rosenbergii* postlarvae fed enriched *Artemia* with *Spirulina* or yeast, found that both produced favorable growth, but *Spirulina* produced higher growing effect than yeast. This is probably due not only to the quality of its nutrients but also to its digestibility, the highest recorded in the present study.

In this context, Zhao *et al*. (2015) proved that shrimp feed in which fish meal was replaced by yeast extract, showed a higher apparent protein digestibility (APD) than control treatment in *Penaeus vannamei*, stating that approximately 45% of fish meal can be replaced by the yeast extract, in the presence of fish oil, phosphorus and calcium. For *M. rosenbergii* postlarvae, Prasad *et al*. (2013) suggest that feeds containing 0.5% *S. cerevisiae* yeast, are suitable to promote growth, although Seenivasan *et al*. (2014) reported that yeast in-

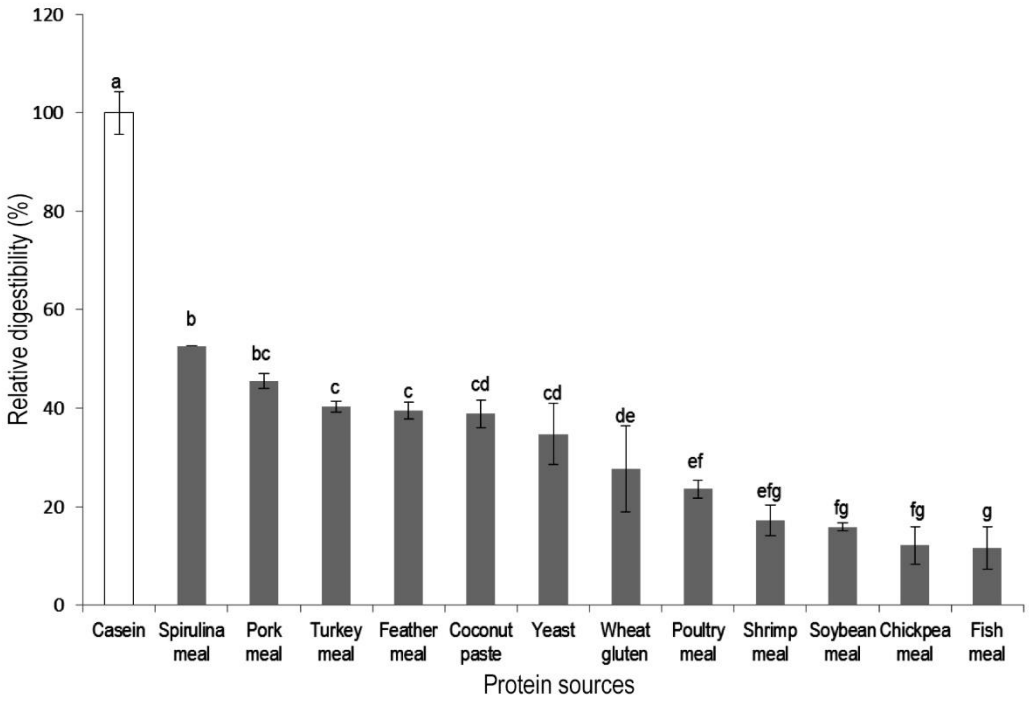

Figure 1. Relative protein digestibility (RD) of ingredients for *M. tenellum*. The digestibility of Casein Hammerstein type was considered as 100% (0.0068 µmol of peptide bonds hydrolyzed per min). Bars represent RD ± SE (standard error). Columns with differing letters are significantly different ($P < 0.05$).

clusion levels at 4%, improved growth, enzyme activity, and amino acid production. Similarly, Parmar *et al*. (2012) demonstrated that 1% yeast inclusion in the diet improve the immune response, and control of white muscle disease. Therefore, *Spirulina* meal and yeast could be used as feed supplements for *Macrobrachium* species.

Related to the *in vitro* protein digestibility evaluations on different protein sources, Lemos *et al*. (2004) by pH-stat method and using digestive proteases of *Farfantepenaeus paulensis* juveniles, reported that Brazilian fish meal, meat meal and wheat meal presented the highest values of protein hydrolysis; in contrast the less digestible ingredients were soybean meal, Mexican fish meal, and blood meal; particularly, soybean meal showed high inhibitory effects (38%) on shrimp proteases. Manríquez-Santos *et al*. (2011) evaluated the protein *in vitro* digestibility of nine ingredients by the pH-stat method, using a multienzyme extract of *M. carcinus*, and using casein as reference protein. They found the highest values on yeast, beef blood meal, and pork meal; in contrast, the lowest digestibility values were found on soybean paste, fish meal, and fish hydrolyzate. In general, relative digestibility and protein hydrolysis values were higher (88.3-189.0% and 27.8-59.5%, respectively), than those reported in other studies (Ezquerra *et al*., 1998; Lemos *et al*., 2004; Nieto *et al*., 2005).

Our results are in agreement with Manríquez-Santos *et al*. (2011) with *M. carcinus*, and Lemos *et al*. (2004) with *F. paulensis*, suggesting that pork meal, and poultry meal are a good alternative to be used in the feed formulation for prawns. In this regard Cruz-Suárez *et al*. (2007), reported that poultry meals are a good amino acids and cholesterol source for *P. vannamei*; moreover, according digestibility coefficients, fish meal protein can be replaced up to 80% with poultry meal. Yu (2006) considers that 60% fish meal replacement as the maximum, in order to do not affect the growth, due to a low essential amino acids (EAA) ingestion by reducing high digestible (84%) fish meal. In the case of *Macrobrachium*, Yang *et al*. (2004) evaluated the potential use of poultry by product meal and meat and bones meal as alternative dietary protein sources, finding that both could replace up to 50% protein fish meal in diets for *M. nipponense*. Hossain & Islam (2007) considers that the meat and bone meal could be replacing 14% the fish meal in diet of *M. rosenbergii* postlarvae.

The low digestibility of soybean meal, obtained in this investigation, coincide with values reported for *F. paulensis* (Lemos *et al*., 2004) and *M. carcinus* (Manríquez-Santos *et al*., 2011). Campaña-Torres *et al*. (2005) reported that APD of plant origin ingredients was higher than those of animal origin; the highest digestibility was found on soybean meal, in *Cherax*

quadricarinatus juveniles. Also, Chin (1988), evaluated APD in juveniles, adults and females of *M. rosenbergii*, reporting that coconut paste, wheat meal, and soybean meal were better digested than fish, and shrimp meal (by juveniles and adults); although soybean meal digestibility results do not coincide with our study, but match about the low digestibility of shrimp meal, and fish meal. Hasanuzzaman *et al*. (2009), in feeding trials with soybean meal, obtained a higher weight, protein efficiency ratio and feed conversion, in *M. rosenbergii* juveniles with diets with 80% of fish meal replacement by soybean meal. García-Ulloa *et al*. (2008), in *M. tenellum* juveniles, compared the growth of prawns fed isoproteic diets (40% CP), replacing fish meal by soybean meal (20, 40, 60, 80 and 100% (w/w) of substitution), finding no significant differences in any of the evaluated growth parameters. This could be due to that reported in present study, where no significant differences on protein digestibility between fish meal and soybean meal was found. Besides Montoya *et al.* (2016) when comparing the essential amino acids (EAA) profile, of different ingredients, with *M. tenellum* muscle, found that fish meal (CP > 60%), and soybean meal (47% CP), both only have three limiting amino acids (threonine, lysine, leucine, and methionine lysine, threonine, respectively).

The relative low digestibility of fish meal, obtained in the present study, is according with Campaña-Torres *et al*. (2005) who found low digestibility of fish meal by *C. quadricarinatus* juveniles. Lemos *et al*. (2004) concluded that the differences in protein digestibility of fish meals may be related to the freshness of the raw material used in its production, exhibiting higher digestibility less fresh fish meals, possibly due to hydrolysis during decomposition of the raw material. In this respect, García-Galano *et al*. (2007) indicate that fish meal used in aquaculture feeds, may vary its quality according fish species used as raw materials, methods of manufacture, cooking and drying temperature, used during processing; also fish meal quality affects the availability of nutrients (Terrazas-Fierro *et al*., 2010b). Our study is according with the results obtained by Nieto *et al*. (2005), who found significant differences between degree of hydrolysis (DH) (21.6 to 39.6%) of 15 different fish meals, by *in vitro* digestibility in pH-stat system using *P. vannamei* enzymes, these authors consider that the pH-stat method works better on fish meal with high level of protein and low level of ash. Therefore, the low digestibility, found for fish meal in this study may due to low quality (low protein content, <60% CP and high ash content, >18%), according to the classification used by Nieto *et al*. (2005). In addition, when comparing the EAA profile, according *M. tenellum* muscle profile (Montoya *et al*., 2016), the amino acid composition of sardine fish meal (used in our experiment) was adjusted to a lesser extent if compared with to tuna fish meal (with the highest protein content), also according to its amino acid content according to Terrazas *et al*. (2010a), who proposed that amino acid profile of the tissue of aquaculture animal, must be considered for selecting ingredient for feed formulation, for any species.

In conclusion, the best digestible ingredient for *M. tenellum* was the *Spirulina* meal, followed by pork meal, and poultry meal, with high potential be used to formulate diets for prawns, particularly to *Macrobrachium* species. However, future research work include *in vivo* digestibility test and feeding trials with diets with the best ingredients, selected by *in vitro* protein digestibility test, to determine the optimal inclusion level of these ingredients.

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