SOLUBLE FRACTION OF SARDINE PROTEIN HYDROLYSATES IN THE FEEDING OF THE SOUTH AMERICAN CATFISH

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ABSTRACT

The objective of this study was to evaluate the effect of the inclusion of the soluble fraction of sardine protein hydrolysate on the stability of diets and the excretion of ammonia in South American catfish. Seven experimental diets were prepared with soluble fractions of sardine muscle and viscera hydrolysates. The stability of the pellets in the water was determined at different time intervals (5 min, 10 min, 20 min, 30 min, 1 h and 1.5 h). Total ammonia was determined every five hours. Significant difference was detected for diet stability only at 5 minutes, where the feed containing 20% muscle soluble hydrolysate demonstrated a higher leaching rate when compared to the control diet. Starting at 10 hours of observation, the ammonia concentration was higher in the control when compared to the other treatments. The sardine waste protein hydrolysate demonstrated to be a promising alternative protein source for South American catfish. The inclusion of up to 10% of the soluble fraction of the sardine muscle and viscera hydrolysates had no effect on the stability of diets; however, the ammonia excretion was reduced exhibiting eco-friendly diets with lower environmental impact.

Key words: water quality, jundiá, leaching, ammonia, *Rhamdia quelen*, *Sardinella brasiliensis*

FRAÇÃO SOLÚVEL DE HIDROLISADO PROTEICO DE SARDINHA NA ALIMENTAÇÃO DO JUNDIÁ

RESUMO

O objetivo deste trabalho foi avaliar o efeito da inclusão da fração solúvel do hidrolisado proteico de sardinha sobre a estabilidade das dietas e a excreção de amônia de juvenis de jundiá. Foram elaboradas sete dietas experimentais com as frações solúveis dos hidrolisados de músculo e vísceras de sardinha. A determinação da estabilidade do pellet na água foi medida em intervalos de tempo diferentes (5 min, 10 min, 20 min, 30 min, 1 h e 1,5 h). A determinação de amônia total foi realizada a cada cinco horas. Como resultado do teste de estabilidade foi detectado diferença significativa somente no período de 5 minutos, onde a dieta contendo 20% de hidrolisado solúvel de musculo teve maior taxa de lixiviação em comparação com a dieta controle. A partir de 10 horas de observação, a concentração de amônia foi maior no controle em relação aos outros tratamentos. O hidrolisado proteico de resíduo de sardinha tem grande potencial para ser utilizado nas rações do jundiá. A inclusão de até 10% da fração solúvel de hidrolisados de músculo e de vísceras de sardinha não afeta a estabilidade das dietas, porém reduz a excreção de amônia, minimizando o impacto ambiental.

Palavras-chave: Qualidade da água, jundiá, lixiviação, amônia, *Rhamdia quelen*, *Sardinella brasiliensis*

Artigo Científico: Recebido em 09/08/2016 – Aprovado em 27/11/2016

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INTRODUCTION

The Brazilian sardine (*Sardinella brasiliensis*) is a pelagic fish with a geographic distribution from the Gulf of Mexico to Brazil and northern Uruguay (FISHBASE, 2016). The global capture production was 98,315 tons in 2013 with Brazil representing the largest fraction of the production (FAO, 2015). The sardine industry is well established, but some waste is still discarded into the environment with virtually no treatment and ultimately contributes to pollution. Enzymatic hydrolysis is an alternative to transform the by-products from the processing of sardines into a product valuable to other industries.

Fish protein hydrolysate is produced from the by-products from industrial fishing processes that include the use of hydrolytic enzymes to break proteins, which results in a higher proportion of free amino acids and low molecular weight peptides (BERGE and STOREBAKKEN, 1996). These compounds are absorbed directly by the intestine and enter intact in the bloodstream, favoring their absorption and resulting in a greater metabolic use of the diets (RYAN *et al.,* 2011). Thus, protein hydrolysates can be used in feeds for aquatic organisms (OLIVA-TELES *et al*., 1999) and have been evaluated in recent studies (ZHENG *et al*., 2013; BUI *et al.,* 2014; KHALED and KTARI, 2014; COSTA-BONFIM *et al*., 2016).

Due to increased environmental monitoring and a greater pressure to use water resources more efficiently, new methods are needed to maximize the use of raw materials to limit waste released into the environment. The improvement of nutritional efficiency by formulating artificial fish diets with digestible nutrients is a way to reduce such waste. The assessment of digestibility is effective to evaluate the ingredients (LOVELL, 1989), however, it produces limited results in an environmental perspective since it considers only the loss of nutrients in the feces and disregards urine excretion. Furthermore, the metabolic losses of nitrogen in fish reach more than 40% of the total nitrogen ingested (BEVERIDGE and PHILLIPS, 1993) and should be evaluated in nutrition studies, as metabolic losses depend directly on the composition of the diets and the conditions in which the feeding is carried out (BUREAU *et al.,* 2002).

Many difficulties exist in the methods to determine the metabolizable energy of diets for fish, however, ammonia may serve as an excellent indicator for the loss of metabolic energy (BUREAU *et al.,* 2002). Ammonia corresponds to more than 80% of the nitrogen excreted from fish (WOOD, 1958) and has been accepted as a viable indicator for metabolism (CHAKRABORTY and CHAKRABORTY, 1998; MCGOOGAN and GATLIN III., 1999; CHENG *et al*, 2003). The evaluation of ammonia excreted from fish in laboratory conditions does not provide precise qualitative results for the quantity of excretion produced, but it does permit a basis of comparison between the use of different diets (CHENG *et al.,* 2003).

The South American catfish (*Rhamdia quelen*) demonstrates potential for fish farming in the southern region of Brazil. It is an omnivorous species that adapts well to captive conditions and has been the focus of several studies (MEYER and FRACALOSSI, 2004; SAHI *et al.,* 2004; MELO *et al.,* 2006). As the sardine waste protein hydrolysate is an innovative protein source that still needs further evaluation, the South American catfish could serve as a model for successive applications in similar species of higher economic value. The objective of the present study is to evaluate the effect of the inclusion of protein hydrolysate from sardine residue on the stability of diets and the ammonia excretion for juvenile South American catfish.

MATERIAL AND METHODS

The hydrolysates were produced in the Biochemistry Laboratory at the Universidade do Vale do Itajaí. The experiment was carried out at the Fish Culture Laboratory of CAV/UDESC, Universidade do Estado de Santa Catarina – Lages, SC. The experimental procedures were approved by the Ethics Committee of the Santa Catarina State University (protocol CEUA nº 8390300316). Two types of soluble fraction protein hydrolysates were evaluated from sardine waste (muscle and viscera) at three different concentrations (5%, 10% and 20%). The hydrolysates were included in diets for South

American catfish juveniles and compared to a control diet based on fishmeal. The stability test was carried out in a completely randomized design with seven treatments and three repetitions per treatment. The evaluation of the ammonia excreted was carried out using seven treatments with eight repetitions per treatment.

The fish waste used as the raw material to produce the hydrolysates was derived from the production line of GDC Alimentos S.A., a processing industry located in Itajaí, SC. The muscle protein hydrolysate was produced from clean sardine carcasses (*Sardinella sp.*; devoid of head, tail and viscera). The hydrolysates of the viscera were produced from viscera collected by the industry directly from the production lines, through suction, and designated as "Industrial Viscera". Whole specimens found were kept intact in a freezer at -20°C until processing. The samples were gathered by the industry and were collected in plastic bottles of 1 liter, labeled, and maintained on ice until arrival at the laboratory. Upon arrival, the samples were fractionated to avoid cycles of freezing and thawing, and kept in a freezer at -20°C until processing.

Aliquots with about 300 g of the sample were homogenized in a blender with 3 volumes of water and incubated with the bacterial protease enzyme from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (Novozymes A/S, Bagsvaerd, Denmark) 1:500 E:F (Enzyme:Fish) at 50°C for 90 minutes. The enzyme was then made inactive and the hydrolysates were pasteurized at 75-90°C for 15 minutes. The suspensions were mixed and subjected to Büchner filtration with 80 g filter paper (Niederlenz, Switzerland) and a vacuum in a kitassato flask. The withheld material was considered as the insoluble fraction and the filtrate as the soluble fraction. Only the soluble fractions were used in the present study. The soluble fraction was dried at 60°C in a forced-air convection oven. The drying time (4-16 hours) varied depending on the biomass placed in the oven and mainly on the distribution of the liquid layer (height/volume). The dried soluble fractions of the industrial sardine muscle and viscera were generated, with about 8% moisture (Table 1).

Table 1. Bromatological composition of the hydrolysates

	Moisture (%)	CP (%)	GE (%)	EE (%)	A (%)	Lys (%)	Met (%)
Muscle sol. fraction	93.28	85.5	5108.8	2.00	8.0	4.61	1.19
Industrial visc. sol. fraction	90.40	61.4	4607.8	1.69	11.3	5.31	2.08

CP – Crude Protein, GE – Gross Energy (kcal kg^{-1}), EE – Ether Extract, A – Ash, Lys – Lysine (% dry material), Met – Methionine (% dry material).

The chemical analyses of the hydrolysates and their respective raw materials were carried out according to the methods described in HORWITZ (2000). The moisture content was determined by infrared radiation and the lipid content by the Soxhlet method. The ash content was determined by gravimetry, with incineration at 650°C for 2 hours and the total protein content was determined by the Kjeldahl method. The content of soluble proteins was determined using the method described in LOWRY *et al.* (1951) with bovine serum albumin as the standard to create a calibration curve. To determine the degree of hydrolysis (DH), an adaptation of the method described in NIELSEN *et al.* (2001) was used to exploit the reactivity of the o-phthaldialdehyde (OPA) with amino groups (Table 2). The tests were carried out in clear bottom microplates with the addition of 40 µL of sample and 260 µL of the OPA reagent. The absorbance readings were taken at 340 nm in a microplate reader model Genius (Tecan), and the results were expressed as DH (%) according to the equation:

DH (%) = [(Serine-NH2 – β) / α meqv / g protein] / htot * 100

Table 2. Degree of hydrolysis of the protein hydrolysates from the sardine residue

	DH (%)
Muscle soluble fraction	20.1
Industrial viscera soluble fraction	54.0

DH – Degree of hydrolysis

The α, β and htot values used were previously determined by ADLER-NISSEN (1986) for fish as 1.0, 0.4 and 8.6, respectively. The analyses of the amino acids were carried out in the laboratory CBO, which analyzed the amino acids through chromatography in High Performance Liquid Chromatography (HPLC). The individualization of the monomers through hydrolysis by HCl 6N at 110°C was initially carried out for 24 hours. Then, the HPLC with spectrophotometric detection was used, with a precision superior to ± 0.5%.

Seven experimental diets were formulated with 39% of CP and GE 4400 kcal kg^{-1}, and with complete mineral and vitamin supplementation (Table 3). The diets were prepared based on the requirements of the species (RADÜNZ-NETO and BORBA, 2013). The hydrolysates of the sardine muscle and viscera were included through the partial substitution of (5%, 10%, and 20%) fishmeal in the diets. The diets were prepared in the fish production laboratory of CAV/UDESC, using a hammer mill to grind the ingredients until passing through a 0.8 mm sieve and mixed according to the formulation. The diets were then pelleted, dried in a forced-air convection oven at 45 °C for 12 hours, stored in plastic containers and conserved in a refrigerator throughout the entire experiment.

Table 3. Composition of the experimental diets.

	Control	M 5%	M 10%	M 20%	V 5%	V 10%	V 20%
Soybean Meal	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Wheat Bran	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Corn grain	8.0	12.7	15.3	24.4	9.5	11.0	14.0
Fish meal	48.0	39.0	32.0	14.0	42.0	36.0	24.0
Muscle Sol. Hyd.	0.0	5.0	10.0	20.0	0.0	0.0	0.0
Viscera Sol. Hyd.	0.0	0.0	0.0	0.0	5.0	10.0	20.0
Soybean Oil	5.0	5.3	5.0	4.6	5.0	5.0	5.0
Fish oil	3.0	2.0	1.7	1.0	2.5	2.0	1.0
Premix*	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Estimated Composition							
DM (%)	91.20	91.31	91.53	91.78	91.48	91.76	92.32
CP (%)	39.32	39.07	39.74	39.22	39.24	39.16	39.01
GE (kcal kg^{-1})	4422	4420	4423	4429	4423	4423	4425
EE (%)	9.56	9.40	8.76	7.51	9.23	8.89	8.21
CF (%)	1.51	1.59	1.64	1.79	1.54	1.56	1.61
A (%)	13.52	11.93	10.77	7.59	12.74	11.96	10.40
Lys (%)	2.71	2.59	2.54	2.30	2.74	2.77	2.82
Met (%)	0.83	0.77	0.73	0.61	0.85	0.87	0.91

*PREMIX COMPOSITION: Folic acid – 2,400mg, nicotinic acid – 48 g, pantothenic acid – 24 g, biotine – 96 mg, vit. A – 2,400,000 IU, vit. D3 – 400,000 IU, vit. E – 24,000 IU, vit. B1 – 9,600 mg, vit. B2 – 9,600 mg, vit. B6 – 9,600 mg, vit. B12 – 9,600mg, vit K3 – 4,800 mg, vit. C – 96 g, iron – 100 g, manganese – 40 g, zinc – 6000 mg, cobalt – 20 mg, iodine – 200 mg, selenium – 200 mg. Antioxidants – 19.6 g. M5% = 5% of the soluble fraction of the sardine muscle hydrolysate; M10% = 10% of the soluble fraction of the sardine muscle hydrolysate; M20% = 20% of the soluble fraction of the sardine muscle hydrolysate; V5% = 5% of the soluble fraction of the sardine viscera hydrolysate; V10% = 10% of the soluble fraction of the sardine viscera hydrolysate; V20% = 20% of the soluble fraction of the sardine viscera hydrolysate. DM – Dry Matter; CP – Crude Protein; GE – Gross Energy; EE – Ether Extract; A – Ash; CF – crude fiber; Lys – Lysine; Met - Methionine

The stability of the pellets in water was determined using methods adapted from HASHIM and SAAT (1992). The stability of the pellets in the water was measured at different

time intervals (5, 10, 20, 30, 60 and 90 minutes), where three samples of each diet with approximately 4 g per sample were placed in small recipients (20 mL), and then immersed in aquariums (15 L) with aeration and controlled temperature. The parameters of the water quality were equal to those established throughout the experiment. A screen was fitted to the bottom of the recipients in which the pellets were collected, so that the material would have adequate contact with the aquarium water. After the immersion periods of the pellets, the samples were dried in a forced-air convection oven at 45°C until constant weight. The leaching of the diets was calculated by the difference between the weight of the pellets before immersion and the dry material after immersion.

To evaluate the excretion of the ammonia, 112 juveniles of South American catfish with a mean weight of 5.90 ± 1.90 grams were used. During the acclimatization period of ten days, the fish were divided into two polyethylene containers of 500 L each and the feeding was carried out with a 5 mm commercial feed with 32% CP and 5% EE. The containers were each equipped with a biological filter, constant aeration and a thermostat to stabilize the temperature between 26 and 28°C. The water was partially renovated daily (25%). The ammonia excretion was evaluated using 16 glass aquariums of 15 liters each, equipped with constant aeration and thermostat. Two experimental diets were evaluated per trial. Before the moment of evaluation, the animals from each container were adapted to an experimental diet for seven days, receiving the feed at 10:00h and 16:00h until apparent satiation.

On the day of the evaluations, the aquariums were properly sterilized with alcohol (70%) and filled with clean water. Two fishes were randomly sampled and placed in experimental aquariums immediately after the first feeding of the day, constituting eight aquariums for each treatment. Water samples were taken every five hours, totaling six sample collections at 0, 5, 10, 15, 20 and 25 hours. Approximately 50 mL of water were taken from each aquarium at each period, which was then filtered with filter paper and frozen. The water in each aquarium was also measured using a multi probe YSI Pro Plus (Ohio, USA) before each sampling for temperature (27.70 ± 1.06°C), dissolved oxygen (4.80±0.91 mg L^{-1}), and pH (7.76±0.56). Upon finishing the cycle of evaluations, the fish were returned to the 500 L container and another experimental diet was randomly chosen and provided for seven days before a new evaluation.

The analyses of the water were carried out at the Water and Waste Treatment Laboratory of the Department of Environmental Engineering, UDESC/Lages. The total ammonia was determined using the colorimetric method based on the generation of indophenol blue (Method 4500 NH3 F). This compound occurs through the reaction of ammonia with hypochlorite ions to form chloramines. These react with phenol in the presence of sodium nitroprusside as a catalyst to generate the aforementioned colorant. The reading was carried out in a spectrophotometer Pharo 300 (Merck) at 620 nm. To carry out the analysis, the sample was initially filtered through a cellulose acetate membrane with opening equal to 0.42 mm to eliminate the effect of turbidity of the sample in the reading (APHA, 2005).

The normality (Kolmogorov-Smirnov) and homoscedasticity (Levene's test) were verified before all of the statistical analyses. The analysis of variance (ANOVA) was carried out and the means were compared by the Dunnett test (5%) using the program SAS, version 9.0 (SAS Institute Inc., North Carolina, USA).

RESULTS

For the diet stability test, a difference in the leaching was detected only in the first five minutes (Table 4). The feed containing 20% muscle soluble hydrolysate demonstrated a higher leaching rate ($P<0.05$) when compared to the control diet. No difference was detected in the other periods.

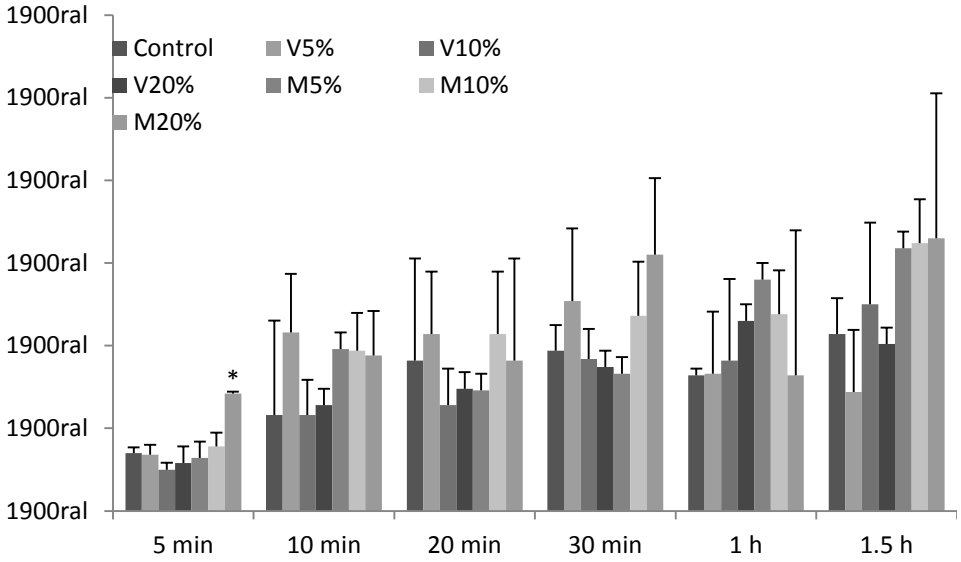

Figure 1. Variation of the stability of the diets in the water throughout the experiment. Results expressed as percentages of leaching.* Means differed from the control by the Dunnet test (P<0.05). Average of three replicates ± standard deviation. M5% = 5% of the soluble fraction of the sardine muscle hydrolysate; M10% = 10% of the soluble fraction of the sardine muscle hydrolysate; M20% = 20% of the soluble fraction of the sardine muscle hydrolysate; V5% = 5% of the soluble fraction of the sardine viscera hydrolysate; V10% = 10% of the soluble fraction of the sardine viscera hydrolysate; V20% = 20% of the soluble fraction of the sardine viscera hydrolysate. (5 min, 10 min, 20 min, 30 min, 1 h e 1.5 h)

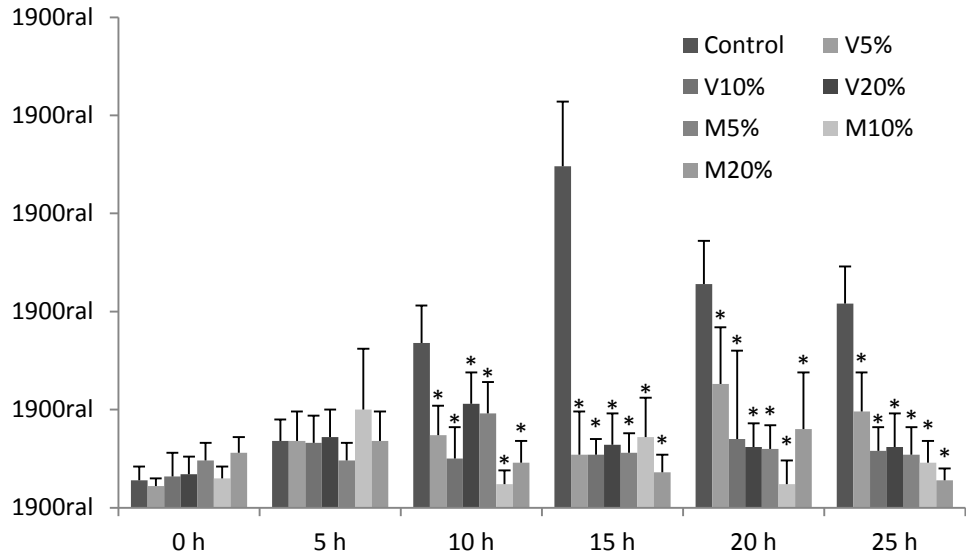

Figure 2. Concentration of the total ammonia (mg L^{-1}) in the water throughout the experiment.* Average of eight replicates ± standard deviation. Means differed from the control by the Dunnet test (P<0.05). M5% = 5% of the soluble fraction of the sardine muscle hydrolysate; M10% = 10% of the soluble fraction of the sardine muscle hydrolysate; M20% = 20% of the soluble fraction of the sardine muscle hydrolysate; V5% = 5% of the soluble fraction of the sardine viscera hydrolysate; V10% = 10% of the soluble fraction of the sardine viscera hydrolysate; V20% = 20% of the soluble fraction of the sardine viscera hydrolysate.

The results of the ammonia concentrations observed in the water are presented in Table 5. Starting at 10 hours of observation, the concentration of ammonia was higher in the control group (P<0.05) than in the other groups.

The results of the ammonia/biomass relationship also demonstrated a similar pattern (Table 6) to the total ammonia results.

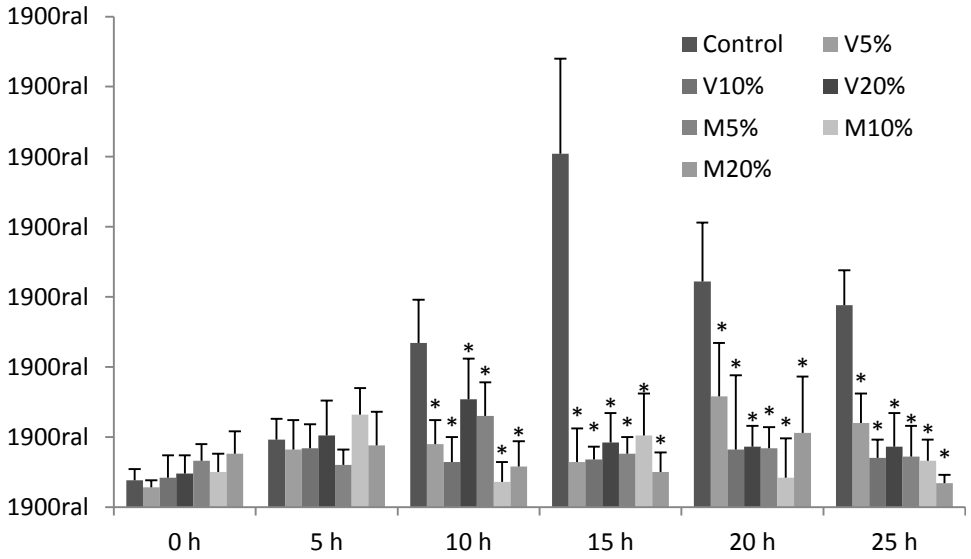

Figure 3. Ammonia/biomass relationship of the juveniles of South American catfish.* Means differed from the control by the Dunnet test (P<0.05). Average of eight replicates ± standard deviation. M5% = 5% of the soluble fraction of the sardine muscle hydrolysate; M10% = 10% of the soluble fraction of the sardine muscle hydrolysate; M20% = 20% of the soluble fraction of the sardine muscle hydrolysate; V5% = 5% of the soluble fraction of the sardine viscera hydrolysate; V10% = 10% of the soluble fraction of the sardine viscera hydrolysate; V20% = 20% of the soluble fraction of the sardine viscera hydrolysate.

DISCUSSION

The positive effects of the soluble fraction of hydrolysates are described in the literature (ESPE *et al*., 1999; HEVROY *et al*., 2005; AKSNES *et al*., 2006). In elevated levels of inclusion above 15%, however, the soluble protein hydrolysates can reduce the stability of diets and compromise the feed conversion (GOOSEN *et al*., 2014). In the present study, inclusions of up to 10% of soluble fractions of the hydrolysates did not affect the stability of the diet. On the other hand, the 20% inclusion increased leaching. The change in the leaching was observed only with the soluble fraction of the muscle hydrolysate. The soluble fraction of the viscera hydrolysate had a higher degree of hydrolysis, but did not show greater leaching. The soluble fraction of the muscle hydrolysate was more rich in protein, however, which may explain the observed result of increased leaching.

The higher leaching rate was detected for the feed containing 20% muscle soluble hydrolysate five minutes after the feed was placed in the water. This time period is insufficient considering that the time to reach apparent satiation for some species can be greater than 40 minutes (BRETT, 1971; SINGH and SRIVASTAVA, 1985). The use of hydrolysates in elevated concentrations remains as a possibility, however, because many factors influence the leaching rate (PALANISWAMY and ALI, 1991; ALI *et al*., 2010). In addition, the development of production technology for more stable feed, with the use of binders (KNAUER *et al*., 1993) for example, may further reduce leaching. Furthermore, no difference was detected in

the leaching between the treatments in the periods above 10 minutes. This result indicates that the leaching was faster in the diet containing 20% muscle soluble hydrolysate, but after 10 minutes all of the diets were leached equally, which was expected because the pellets do not remain stable in the water for much time.

The inclusion of the hydrolysates in diets, even the soluble fraction, reduced the ammonia excreted from the South American catfish juveniles, which indicates metabolic loss in fish (MCGOOGAN and GATLIN III., 1999; CHENG *et al,* 2003). The decrease in the excreted ammonia can indicate a higher metabolic efficiency of the diets. The protein hydrolysates are rich in small peptides, which can be absorbed more efficiently than intact proteins or free amino acids (ZIEGLER *et al*., 1990). Other authors described that the inclusion of hydrolysates in diets can improve the productive performance of fish, even when the hydrolysates replace key ingredients such as fish meal (REFSTIE *et al.,* 2004; HEVROY *et al.,* 2005; ZHENG *et al*., 2013; BUI *et al*., 2014; COSTA-BONFIM *et al*., 2016).

Differences in the ammonia excreted were detected 10 hours after feeding. Ammonia is excreted through the gills by diffusion as NH_3 via the transcellular pathway, following the blood-water gradient (EVANS *et al*., 2013). Previous studies have described the peak of ammonia excretion to be between 4 and 8 hours after feeding for rainbow trout (*Oncorhynchus mykiss*) (GÉLINEAU *et al*., 1998) and the carp *Labeo rohita* (CHAKRABORTY and CHAKRABORTY, 1998). For other species, however, the response may be different. The tambaqui (*Colossoma macropomum*) exhibits rhythmic variation in excretion with different peaks, the first being after 4 hours and the last being up to 22 hours after feeding (ISMIÑO-ORBE *et al*., 2003).

The results observed in this study support the higher metabolic efficiency of diets containing the soluble fraction of sardine hydrolysate as compared to the diets containing fish meal. The improved metabolic efficiency is important to advance fish production, but the findings are even more relevant as related to the environment. Ammonia is the principal form of nitrogen excreted from fish (WOOD, 1958; ELLIOTT, 1976). The reduction of excretion would result in a reduced environmental impact, since nitrogen is considered one of the main pollutants in aquaculture. The development of strategies to control the ammonia released into the environment is vital to increase the sustainability of fish farming systems (FANG *et al*., 2015).

CONCLUSIONS

The sardine waste protein hydrolysate was effectively used in South American catfish feeds and decreased the impact of the diets on the environment.

The inclusion of up to 10% of the muscle and viscera soluble hydrolysates of sardines does not affect the stability of the diets of the South American catfish juveniles, however, higher levels can increase the leaching.

The soluble fraction of the protein hydrolysates, attained from the wastes from the processing of sardines and included in the diets, decreases the ammonia excreted by juvenile South American catfish.

ACKNOWLEDGEMENTS

The authors would like to thank the National Council for Scientific and Technological Development (CNPq) and the Foundation of Research and Innovation of the State of Santa Catarina (FAPESC) for financial assistance in this research.

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