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Physicochemical and Microbiological Characterization of an Endogenous Enzymatic Hydrolysate Obtained by Adding Formic Acid to Trout Viscera (*Oncorynchus mykiss*)

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Abstract: By-products obtained in the development of fish farming such as trout entrails have a high protein and oil content in their composition with potential use in the food agribusiness. There are alternative methods such as chemical silage which facilitates fat extraction and obtaining of hydrolysed protein due to the action of endogenous proteases which are activated in an acidic medium during this process. The main objective of this work was to obtain and characterise physicochemical and microbiologically a hydrolysed protein meal from trout by-products (*Oncorhynchus mykiss*). Proximal analysis (humidity, ash, ethereal extract and crude protein) and microbiological analysis were performed to finally obtain an endogenous hydrolysate to which the degree of hydrolysis, pH, titratable acidity and oil yield was determined. It was found that the treatment carried out using chemical silage generates an acidic environment (pH 3.2) that prevents microbial decomposition and facilitates the extraction of the lipid fraction. The hydrolysis process obtained, close to 62%, allows obtaining a product called hydrolysed protein meal that according to its composition could be used to formulate farm animal's diet.

INTRODUCTION

In the department of Cauca, fish farming has been carried out for >25 years by producers from the La Salvajina dam area in the municipality of Suarez. In the town of Silvia the trout culture (*Oncorynchus mykiss*) has been strengthened in recent years, being currently the chief cultivated species in the department with 737.4 t/year, generating approximately 133.6 t of by-products per year. The Ministry of Agriculture and Rural Development in its National Agenda for Research in Fisheries and Aquaculture prioritized, since, 2012 the development of activities for the management of by-products and the need to consolidate alternatives other than fishmeal due to dependency and high cost in fish farming. Various methodologies have been proposed for the management of by-products generated by fish farming and silage is a widely accepted process[1]. In which a wide range of interactions and chemical reactions occur, improving nutritional properties, availability of nutrients and conferring higher stability and durability at a low cost.

The silage process is considered an interesting alternative to fishmeal, since, it constitutes a simple, safe, environmentally friendly and low-investment technology compared to obtaining flour. It also reduces unpleasant odours by preventing deterioration in the forage due to its low pH[2]. In this way, whole fish or parts of it are added with lactic acids, enzymes or bacteria, causing the hydrolysis of the protein[3]. The physical-chemical and microbiological changes produced can prevent processes undesirable as the fat oxidation, putrefaction processes and allow the recovery of essential fatty acids and other functional ingredients like protein hydrolysate, collagen, oil, among others[4]. It consists of the addition of inorganic and organic acids which favours endogenous enzymatic hydrolysis due to the decrease in pH.

The characterisation of new raw materials as by-products of the post-capture of fish farming activity, facilitate the optimization of processes for obtaining animal feed. The quality of these feeds is significantly determined by the composition of ingredients used the formulation of the diet and the processing methods used in its elaboration[5], achieving a positive economic and environmental impact. This work aimed to obtain and characterise physicochemical and microbiologically a hydrolysed protein meal from trout (*Oncorhynchus mykiss*) by-products.

MATERIALS AND METHODS

Next, the materials and methods used to obtain the hydrolysed protein meal from trout entrails are described.

Viscera simples: By-products of rainbow trout (*Oncorhynchus mykiss*) were obtained from the slaughter of animals in the fattening stage with an average size and weight of 18.5±1.0 cm and 400±10.0 g, respectively, subjected to 24 h of fasting and supplied by the Silvia Cauca Aquaculture and Agricultural Products Producer and Marketer Association-APROPESCA. The sample was obtained in an average production pond which has an average load of 1500 animals. A 50 kg sample of viscera was taken. Immediately, the viscera of the animal's body were separated, stored in portable refrigerators and transferred to the Biotechnology Laboratory of the University of Cauca on dry ice for proximal and microbiological analysis, carried out in triplicate.

Proximal analysis: Humidity was determined according to the standard. Therefore, 5.0 g of sample was subjected to dehydration at a temperature between 100 and 105°C in a forced convection oven Lab Companion OF-01E (USA), to constant weight. Ash content was determined according to standard. Subsequently, 1.0 g of dry sample was taken in a porcelain crucible into a muffle type oven Thermolyne Fumace 1400 (USA) at 550°C for 2 h. The ether extract was determined according to standard (AOAC 962.09, 2005). Accordingly, 1.0 g of sample was taken into Micro Soxhlet extraction cartridges and processed in a SOXHTEXT RAYPA extraction system (Spain) in ethyl ether with reflux for 4 h. Crude protein content was determined according to standard. A sample of 0.8 g was taken which was digested with 10 mL concentrated H_2SO_4 in a compact RAYPA digestion system MBC (Spain), neutralized with NaOH and distilled in 3% H_3BO_3 solution with Tashiro indicator in distiller equipment RAYPA DNP-2000 (Spain). For protein quantification (Factor 6.25) each sample was titrated with 0.1 N HCl. Crude fibre content was determined according to the method. In which 1.0 g of the residue resulting from fat determination was taken to the system fibre quantification of FIBERTEST (Spain). Acid digestion was carried out with H_2SO_4 0.255 N and basic digestion employing NaOH 0.313 N. The residue was transferred to a crucible, oven dried at a temperature of 100°C to constant weight and taken to a muffle type oven at 550°C for 20 min. The Non-Nitrogenous Extract (NNE) was calculated by difference between 100 and the content of crude fibre, ether extract, crude protein and ash.

Microbiological analysis: The determination of mesophilic aerobes, moulds and yeasts, lactic acid bacteria and total coliforms was carried out based on the Colombian Technical Standards (NTC) with some modifications. Serial dilutions up to 10-6 were made for the analyses, starting from an initial dilution of 10.0 g of sample in 90 mL of sterile peptone water (10-1 dilution) and in triplicate in Binder BD 115 UL incubator (Germany).

Mesophilic aerobic counts were performed based on NTC 4519, 2009 by surface inoculation in sterile Plate Count Agar (PCA) medium at 30°C for 48 h. The mould and yeast count was determined based on NTC 4132, 1997 by surface inoculation in sterile Yeast Glucose Chloramphenicol (YGC) medium at 30°C. Later, counting was performed after 3-5 days of incubation. The lactic acid bacteria were determined based on NTC 5034, 2002 by surface inoculation in Agar Man, Rogosa and Sharpe (MRS) medium added with aniline blue at 35°C for 48 h. In the case of presence of total coliforms, the most probable Number Method (NMP) was used based on the NTC 4516, 2009 standard by inoculating in Lauril Sulfate Tryptosa Broth at 35°C for 24 h. The tubes with gas formation were inoculated in Brilliant Green Bile Broth (BRILA) and incubated at a temperature of 35°C for 24 to 48 h. Gas-forming tubes were counted as positive. The NMP is based on the combination of the positive tube or negative tube results and was determined according to NTC 4092, 2009.

Obtaining the endogenous enzymatic hydrolysate: For the preparation of the endogenous enzymatic hydrolysate (chemical silage), 85% formic acid was added in a ratio of 25 g kg^{-1} of viscera, according to the methodology proposed by Goosen *et al*.[6]. This procedure ensures to obtain a pH between 3.0 and 3.2. Butylhydroxytoluene in a concentration of 0.1% was used as an antioxidant.

Monitoring of endogenous enzymatic hydrolysis: Monitoring was performed for 10 days at a temperature of 15°C±0.5. The experimental units corresponded to plastic containers of 3000 capacity. Analyses such as degree of hydrolysis (GH) and microbiological monitoring were performed on day 0, 5 and 10, in triplicate.

Determination of the degree of hydrolysis: It was performed using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method. Firstly, 8.0 g of sample was weighed and 12 mL of 0.2 M phosphate buffer, pH 7.0 was added. The mix was centrifuged (Hermle Z326 K-Germany) for 10 min at 12000 rpm at a temperature of 4°C, the supernatant was filtered, and 128 µL were added to a screw cap test tube with 1 mL of 0.01% TNBS and 2 mL of 0.2 m phosphate buffer, pH 8.2 and vortexed vigorously for 15 sec. Then, it was taken in a water bath at 50°C for 30 min and the reaction was ended by adding 2 mL of 0.1 m sodium sulphite. The reading was carried out on a UV-VIS SHIMADZU UV 1800 spectrophotometer (Japan) at a 410 nm wavelength interpolated on standard L-Leucine curve. The degree of hydrolysis was calculated according to Eq. 1:

$$DH(\%) = \frac{\left[(NH_2)_{tx}\right]}{\left[(NH_2)_{T}\right]} \times 100 \quad (1)$$

Where:

$(NH_2)_{tx}$ = Concentration of groups αAmino-terminal, expressed as mMol of L-Leucine at the time tx

$(NH_2)_T$ = Concentration of groups α-amino-terminal, expressed as mMol of L-Leucine after total acid hydrolysis

For the determination of the total hydrolysis, 0.5 g of previously ground viscera were taken, 4.5 mL of 6N HCl was added and kept at 100°C for 24 h in a test tube with screw cap. Subsequently, it was neutralized with 4.5 mL of 6N NaOH, filtered and read in UV according to the previously described methodology.

Determination of pH and Total Titratable Acidity (TTA): The pH measurement was made directly on the silage matrix with a HORIDA D-51 (USA) brand solids pH meter. For the determination of titratable acidity, 2.0 g of sample was taken and mixed with 25 mL of distilled water. Finally, it was titrated with 0.1 N NaOH until reaching a pH of 8.3[7].

Determination of oil extraction yield: Oil separation was performed by centrifuging (Hermle Z326 K-Germany) of the silage mixture from each experimental unit at 12,000 rpm for 10 min at 5°C and the recovered oil was weighed on a scale (KERN AES 220-4-Germany). The yield was expressed as the amount of oil extracted concerning the content of the ether extract of the by-products[4].

Microbiological analysis: The determination of mesophilic aerobes, moulds and yeasts, lactic acid bacteria and total coliforms was made for each type of silage based on Colombian Technical Standards, according to the methodology described above.

Statistical analysis: Oil extraction percentage and the changes in the degree of hydrolysis were evaluated using a simple repeated measures design in which the intra-subject factor corresponded to time. Statistical analysis of the data obtained was performed using the IBM SPSS Statistics 20 Software in which the normality of the data was verified and the Analysis of Variance (ANOVA) was performed for the intra-subject factors as well as the pairwise comparison to determine differences in time and between treatments. All effects are reported with a significance of $p<0.05$.

RESULTS AND DISCUSSION

Chemical and microbiological characterization of trout gut by-products (*O. mykiss*): The results of the proximal composition of the rainbow trout viscera show a majority component corresponding to lipids with 69.3%, followed by protein with 27.2%, macromolecules that represent about 90% in dry matter. Table 1 shows the results from the counts of aerobic mesophyll bacteria, mould and yeast, Lactic acid bacteria and total coliform. Regarding the proximal analysis, researcher report similar values for the same species[8] with fat content close to 70% and protein content of 23%. In contrast, the muscle has an inverse relationship with a higher protein content (65%) than fat (20%). Fat in the viscera has a function of energy reserve, of metabolic temperature regulation, mainly evidenced in cold water animals such as rainbow trout and as mechanisms of organ cushioning, therefore its higher proportion in these tissues. The minority fraction of carbohydrates and fibre is related to the glycogen accumulated in the liver and the striated and pentose muscles present in nucleic acids, released as a consequence of post-mortem autolytic changes.

The gastrointestinal tract of fish is an ecosystem where microorganisms colonize the gastrointestinal tract, with aerobic, facultative anaerobic, strict anaerobic and yeast bacteria being the main colonizers. The mesophilic aerobic content for the present study of 7.03 Log (CFU g^{-1}) is similar to the intestinal microbiota reported in fish with counts of 7.0 Log (CFU g^{-1})[9].

The viscera harbour great bacterial diversity[10], part of which can be considered transitory while another part exists as endogenous flora and associated with intestinal mucosa in the prevention, control of pathogens and beneficial function intermediaries. Induction of the host's innate immune response and nutrient exchange[11]. Changes in the profile of the intestinal microbiota can be related to negative impacts on the growth and health of fish[12].

Yeasts can constitute a significant fraction of the fish's gastrointestinal microflora and have a significant influence on their metabolism. Gatesoupe[13] reported counts in a range of 2 and 7 Log (CFU g^{-1}) in the intestine of salmonids, a value similar to that obtained in the present study with 4.6 Log (CFU g^{-1}). Lactic Acid Bacteria (LAB) in particular the Lactobacillus species, have received considerable attention regarding their beneficial effects as probiotics in humans and animals. The genera Lactobacillus, Aerococcus, Leuconostoc, Pediococcus, Streptococcus, Vagococcus and Weissella are part of the normal microflora of healthy fish[14] with counts between 2 and 5 Log (UFC g^{-1})[15], similar to the count reported in the present study of 4.38 Log (CFU g^{-1}).

Table 1: Microbiologic profile of the by-products of gutted trouts

Microbiological analysis	Values
Total coliform count (NMP g^{-1})	>1100
Mesophilic aerobic count (log (UFC g^{-1}))	7.03±0.05
Mold and yeast count (log (UFC g^{-1}))	4.60±0.01 yeast type
Lactic acid bacteria count (log (UFC g^{-1}))	4.38±0.04

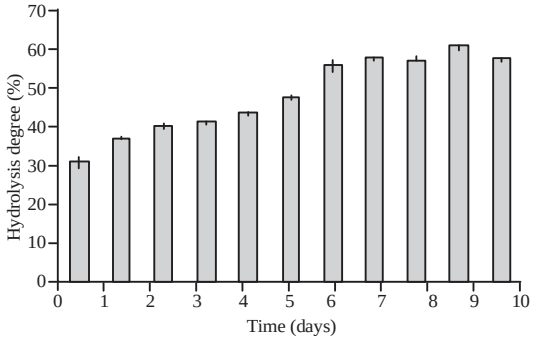

Fig. 1: Degree of hydrolysis

Monitoring of endogenous enzymatic hydrolysis: Once the by-products were characterised, the endogenous enzymatic hydrolysis was monitored for ten days. Below are the results obtained.

Degree of hydrolysis: Changes in the degree of hydrolysis during monitoring are observed in Fig. 1. These are related to endogenous enzymatic hydrolysis in which pepsin which is the most important aspartate protease in fish viscera can act. Vannabun *et al*.[16] report high protease activity in fish by-products at pH 3.0 with stability >95% under these conditions.

Concerning the behaviour of the degree of hydrolysis, two marked stages are observed. The first stage with incremental values from day 0 until day 6 where the increase in the degree of hydrolysis is directly related to the endogenous content of gastric enzymes, mainly pepsins in the rainbow trout viscera. Pepsins, classified as aspartic end opeptidases (EC 3.4.23) has an affinity for aromatic amino acids and are responsible for the digestion of proteins in the stomach of animals in pH ranges between 2.0 and 3.0[17]. Wald *et al*.[18] report the isolation of three pepsinogens in rainbow trout with superior enzymatic activity in pH ranges between 1.5 and 4.5 and temperature between 30 and 40°C. During the elaboration of chemical silage, the addition of formic acid generates a pH of 3.0. In this condition the pepsinogen precursor, synthesised in the gastric mucosa and secreted in the lumen, auto-catalytically becomes pepsins by removal of an N-terminal segment[19], a condition in which it achieves its highest activity, initiating the process of protein hydrolysis. The second stage, after the sixth day, shows stability in the degree of hydrolysis, statistically corroborated, since, no significant difference was reported

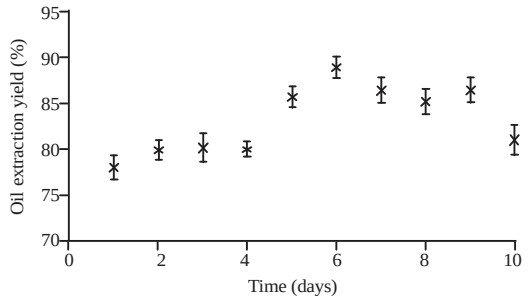

Fig. 2: Oil extraction yield

in the pairwise comparison (p>0.05). This behaviour is possibly related to the depletion of catalysing amino acids which reduces the available substrate as the degree of hydrolysis increases[21] as well as enzymatic inhibition due to the increase in low molecular weight peptides.

pH and titratable acidity: Total titratable acidity and pH did not show significant differences in the pairwise comparison (p>0.05). As the behaviour was expected, given that unlike processes such as biological silage, there is no continuous production and accumulation of lactic acid by the action of lactic bacteria[21]. Likewise, the proteolytic action produces amino acids which according to the degree of ionization can generate buffering capacity. The presented acidity and the pH stability at values close to 3.0, prevents the proliferation of spoilage-causing microorganisms that can lead to the production of nitrogenous compounds. These affect the buffering capacity and physicochemical characteristics of the product.

Microbiological monitoring: The microbiological analysis did not show the presence of microorganisms during the monitoring period, according to the applied methodology. The addition of formic acid in the evaluated matrix generated a strong antimicrobial effect. This acid has high hydrophilicity, a short-chain and easily ionisable, spreading within microorganisms cell's, acidifying the cytoplasm whose pH usually is close to neutrality[23]. In similar investigations[1], a decrease in aerobic mesophilic microorganisms and total coliforms was obtained for silage of rainbow trout, Epinephelus malabaricus and mackerel by-products, respectively also attributed to the low pH maintained during the process.

Oil extraction yield: Figure 2 shows the evaluation made for this parameter. On the sixth day, it showed a maximum value of 88.43%. Subsequent stability was observed, statistically corroborated, since, no significant difference was reported in the pairwise comparison (p> 0.05).

Autolysis of fish tissue forms an aqueous solution rich in peptides and amino acids that initially promote oil release[23]. Subsequently, the formation of emulsions due to the breakdown of the triacylglycerides and their interaction with low molecular weight peptides has an essential effect on obtaining oil, reducing the extraction yield. Besides, the content of phospholipids and other polar lipids generate an increase in emulsions[24], formed in high lipid contents, a condition that decreases the quality of oil[23].

CONCLUSION

The treatment carried out using chemical silage generates an acidic environment (pH 3.2) that prevents microbial decomposition and facilitates the extraction of the lipid fraction. On the other hand, the monitoring carried out on chemical silage showed that on the sixth day under the test conditions, the highest percentage of oil extraction is achieved as well as a constant degree of hydrolysis close to 62%. This fact allows it to obtain a product termed hydrolysed protein meal which leads to obtaining a product with stable conditions. The by-products resulting from the gutting of rainbow trout, have a content close to 90% of dry matter in protein and fat, macromolecules of great importance in the animal feed industry and according to the importance of looking for substitutes for conventional fishmeal, viscera become a viable alternative for harvesting.

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