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# Effect of the Addition of High-Protein Hydrolyzed Flour from *Oncorhynchus mykiss* Byproducts on the Properties of an Extruded Feed

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**ABSTRACT:** This work aims to evaluate the effect of the addition of a high-protein hydrolyzed (HPH) flour from the chemical silage of trout (*Oncorhynchus mykiss*) residues on the parameters of the extrusion system physicochemical transformations and the microstructure of the extrudate. During the extrusion process, the materials used for the study were the HPH flour obtained from trout by chemical silage, fishmeal, and cassava starch. The extrudate's microstructural changes were evaluated by determining the porosity, scanning electron microscopy, the chemical changes, the amino acid profile, residual formic and lactic acid content, the molecular mass profile, the grade of hydrolysis, and in vitro digestibility. The results showed pellets with high durability due to the cohesiveness of the hydrolyzed protein flour but at the same time with low hardness due to the high porosity achieved. The monitoring carried out to the changes in the protein, such as the degree of hydrolysis, water-soluble protein, and molecular mass profile, verify the binding effect of the high-protein hydrolyzed flour during the extrusion process. Finally, the high-resolution optical microscopy methodology presented a high correlation with the phenomena presented in the experiment.
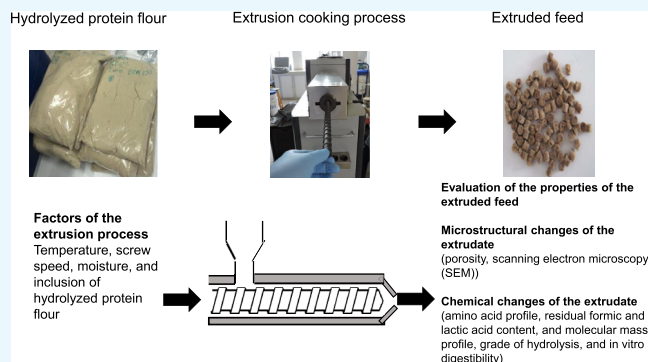
## INTRODUCTION

Aquaculture is one of the fastest-growing food industries, with a sustained increase of 3.2% in the past 5 decades[1] and an annual production in continental aquaculture of 47.1 million tons.[2] This sector demands quality food that ensures consumption, adequate conversion rate, and productive parameters.[3] Therefore, balanced animal feed must have nutritional and physical characteristics that meet the needs for development and satisfaction of the metabolic functions of the animal.

In evaluating the physical quality of granulated feed in fish farming, a subdivision is made into tests that evaluate the hardness and durability of a given feed and additional characteristics, such as porosity, buoyancy, expansion index (EI), density, water absorption, and solubility in water.[4,5] The physical quality check of experimental diets should be carried out routinely, mainly if new ingredients or processing conditions are investigated, requiring standardized methods for their evaluation.[6] In addition to nutritional aspects, fish food contains polymers such as starch and proteins of animal and vegetable origin, where their interaction has an impact on the physical characteristics,[7] such as having enough porosity to allow good oil absorption and a durable structure that remains during storage, transport, and pneumatic feeding of the product.[8−10]

Extrusion cooking of fish food is a complex process that requires several variables to obtain the attributes of the desired product. Independent process parameters such as temperature, screw speed, throughput, feed composition, and moisture content affect system variables such as specific mechanical energy (SME) and residence time. These system variables, also known as intermediate process variables, induce reactions that affect the final product's nutritional value and physical properties.[11,12] The composition of the feed is a dominant parameter that influences the variables of the intermediate process.

Therefore, the objective of the present work is to evaluate the effect of the addition of a high-protein hydrolyzed (HPH)
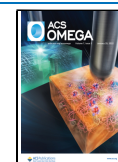
**Table 1. Regression Analysis of the Response Variables**[a]

| parameter | SME F | SME coef. | MRT F | MRT coef. | EI F | EI coef. | density F | density coef. | porosity F | porosity coef. | hardness F | hardness coef. | durability F | durability coef. | buoyancy F | buoyancy coef. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| constant | 27.02 | 57.941[b] | 32.18 | 91.560[b] | 53.97 | 1.518[b] | 8.97 | 0.9495[b] | 30.81 | 0.061 | 24.24 | 170.11[b] | 21.90 | 97.433[b] | 11.25 | 34.38[b] |
| temperature | 4.03 | −4.617 | 0.86 | 1.006 | 115.3 | −0.254[b] | 3.74 | −0.035 | 6.32 | 0.019 | 2.75 | −8.920 | 9.57 | −0.628[c] | 2.90 | 10.47 |
| moisture | 20.09 | −10.313[c] | 56.68 | −8.192[b] | 0.00 | 0.001 | 12.37 | 0.065[c] | 3.97 | −0.016 | 1.68 | −6.972 | 52.26 | 1.467[b] | 0.94 | 5.97 |
| HPH | 67.02 | −18.838[c] | 161.8 | −13.842[b] | 153.9 | 0.293 | 2.29 | −0.027 | 30.72 | 0.043[b] | 5.31 | −12.391 | 44.64 | 1.356[b] | 3.28 | 11.14 |
| rpm | 0.046 | 1.796 | 40.11 | −6.891 | 0.00 | −0.001 | 0.25 | 0.009 | 2.06 | 0.011 | 6.25 | −13.441 | 30.59 | −1.122[b] | 0.56 | −4.58 |
| temperature*temperature | 27.00 | −10.862 | 3.75 | −1.919 | 51.21 | −0.306[b] | 25.18 | −0.167[b] | 89.74 | 0.135 | 93.12 | −95.074[b] | | | 32.09 | 63.86[b] |
| moisture*moisture | | | 6.00 | −2.428[c] | | | 8.43 | −0.097[b] | 36.26 | 0.086 | 29.53 | −53.593[b] | 19.51 | −0.813[b] | 36.78 | 68.36[b] |
| HPH*HPH | 13.22 | 7.587 | | | | | 26.78 | −0.172[b] | 142.4 | 0.170 | 104.88 | −100.89[b] | 5.03 | −0.413 | 30.93 | 62.69[b] |
| rpm*rpm | | | 13.75 | −3.676 | | | 4.27 | −0.069[b] | 2.30 | 0.022 | 16.77 | −40.350[b] | | | 2.65 | 18.36 |
| temperature*moisture | | | | | | | | | | | | | | | | |
| temperature*HPH | | | 5.29 | 3.065 | 3.43 | −0.1075 | 10.10 | −0.1412 | 6.86 | −0.049 | 6.91 | 34.632 | | | | |
| temperature*rpm | | | | | | | | | | | | | | | | |
| moisture*HPH | | | | | | | | | | | | | 4.9 | −0.550 | | |
| moisture*rpm | | | | | | | | | | | | | 10.26 | 0.796 | | |
| HPH*rpm | | | 4.71 | −2.893 | | | | | | | | | | | | |
| adjusted $R^2$ | 81.26 | | 90.34 | | 92.78 | | 70.51 | | 89.94 | | 87.47 | | 84.79 | | 73.22 | |
| lack of fit (*P*-value) | 0.081 | | 0.616 | | 0.813 | | 0.759 | | 0.051 | | 0.346 | | 0.901 | | 0.069 | |

[a]SME: specific mechanical energy; MRT: mean residence time; EI: expansion index. [b]Significant at $P < 0.01$. [c]Significant at $P < 0.05$.
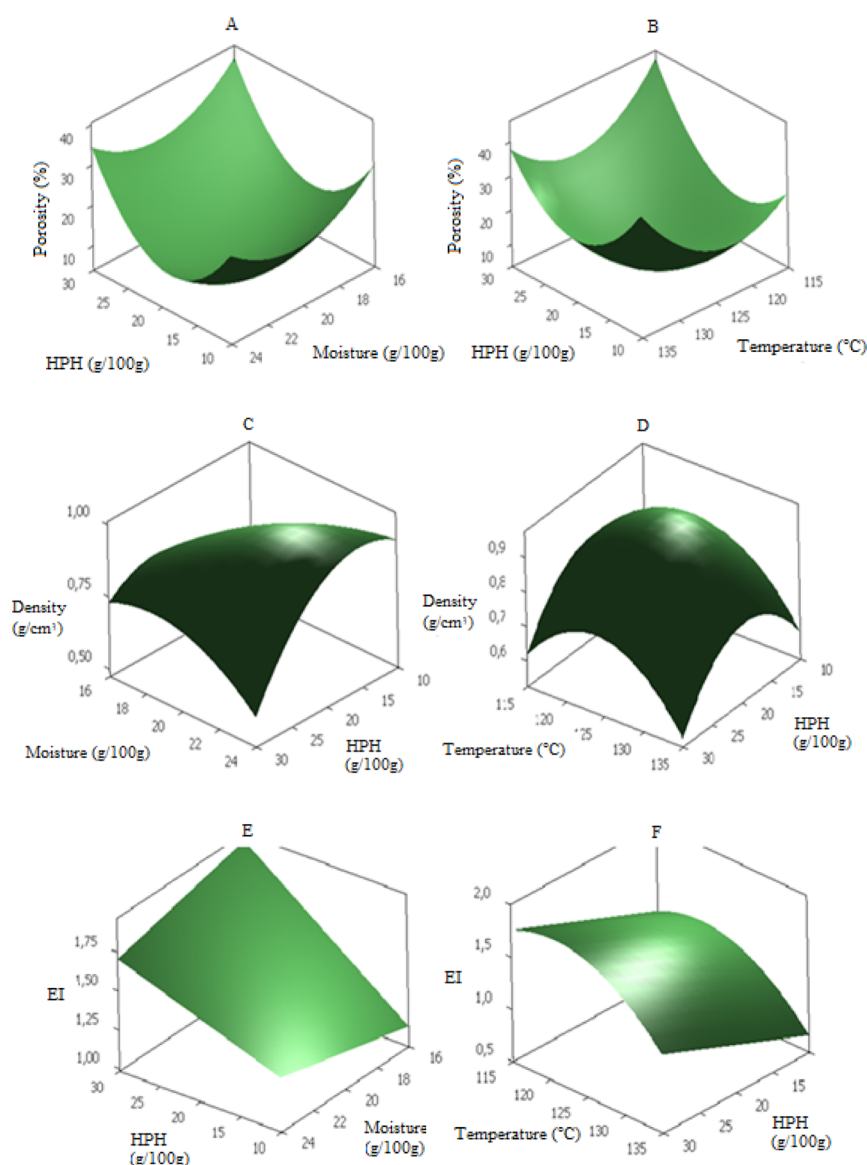
**Figure 1.** Effect of HPH, moisture content, and temperature on porosity, density, and EI. (A) Moisture vs HPH (temperature 125 °C and screw speed 220 rpm); (B) HPH vs temperature (screw speed 220 rpm and moisture content of 20 g/100 g); (C) moisture vs HPH (temperature 125 °C and screw speed 220 rpm); (D) HPH vs temperature (screw speed 220 rpm and moisture content of 20 g/100 g); (E) moisture vs HPH (temperature 125 °C and screw speed 220 rpm); and (F) HPH vs temperature (screw speed 220 rpm and moisture 20%).

flour from chemical silage of trout (*Oncorhynchus mykiss*) residues on the parameters of the extrusion system, the physicochemical transformations, and the microstructure of the extrudate.

## ■ RESULTS AND DISCUSSION

**Evaluation of the Microstructural Changes of the Extrudate.** *Porosity.* The values of *F* estimated in the ANOVA (Table 1) indicate that the quadratic terms of the HPH flour (142.4), temperature (89.74), and moisture (36.26) have a significant influence on the variation of the porosity data.

Porosity develops as a product of the expansion or blowing of the material as part of the extrusion cooking process, creating pockets of air or water vapor.[13,14] These bags or cells allow/do not allow gas exchange depending on their complete to incomplete structure.[15] In addition, the expansion of an extrudate is dependent on the development of the said cells. At

the same time, the steam expands when the mixture leaves the nozzle and experiences a decrease in pressure, thereby increasing the size of the extrudate.[16] During this process, it is likely that both available starches and low-molecular-weight proteins are converted into a melt, in which highly elastic bonds and networks are formed, capable of retaining the water vapor for a longer time at its exit from the extruder; finally, the empty spaces will be the cells that constitute the internal structure of the extrudate.[17]

According to Figure 1a, it is verified that the porosity percentage of the extrudate is affected by the content of the HPH flour and moisture. It is also observed that the lowest porosity is achieved in the intermediate proportions of the HPH flour (20%) and water (20 g/100 g) in the mixture. These results agree with the increase in density under similar conditions (Figure 1c). The explanation for this behavior is attributed to the fact that under these process conditions, proteins are coupled in partially modified starch structures as a
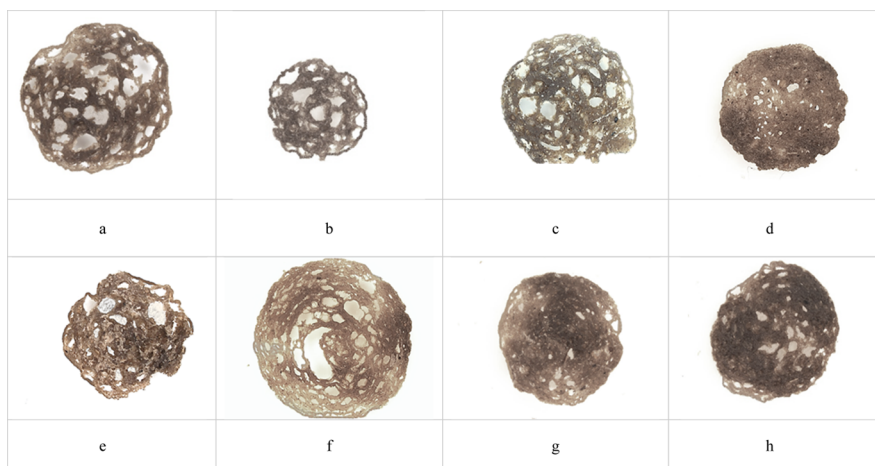
**Figure 2.** High-resolution optical microscopy micrographs of the axial section of some feeds obtained by the extrusion process. (a) Treatment 17, $T$ 115 °C, moisture 20 g/100 g, HPH 20%, and rpm 220 min$^{-1}$; (b) treatment 18, $T$ 135 °C, moisture 20 g/100 g, HPH 20%, and rpm 220 min$^{-1}$; (c) treatment 19, $T$ 125 °C, moisture 16 g/100 g, HPH 20%, and rpm 220 min$^{-1}$; (d) treatment 20, $T$ 125 °C, moisture 24 g/100 g, HPH 20%, and rpm 220 min$^{-1}$; (e) treatment 21, $T$ 125 °C, moisture 20 g/100 g, HPH 10%, and rpm 220 min$^{-1}$; (f) treatment 22, $T$ 125 °C, moisture 20 g/100 g, HPH 30%, and rpm 220 min$^{-1}$; (g) treatment 23, $T$ 125 °C, moisture 20 g/100 g, HPH 20%, and rpm 190 min$^{-1}$; (h) treatment 24, $T$ 125 °C, moisture 20 g/100 g, HPH 20%, and rpm 250 min$^{-1}$.

filler material.[15] However, when thermal conditions are extreme due to increased shear (low moisture content) or temperature, proteins contribute to the formation of networks and pores, allowing the extrudate to grow and reducing its density.[18,19]

In Figure 1b, it is confirmed that the percentage of porosity is affected by the content of the HPH flour in the mixture and the temperature. The changes in porosity due to temperature are more significant than those reported due to moisture, as verified by the $F$ values of their corresponding quadratic terms (Table 1). The lowest porosity is achieved at an intermediate level of the HPH flour concentration with 20% as at the medium temperature level (125 °C). Similarly, it is observed that the highest level of porosity is achieved when the temperature profile is at its lowest level. At the same time, the content of the HPH flour in the mixture is high.

Similarly, the highest porosity levels are recorded at a moisture content of 16 g/100 g and a high-protein hydrolyzed flour content of 30% behavior that could be influenced by the shear achieved inside the extruder. The low moisture content maximizes this effect in the mixture. The diffusion of humidity inside the matrix is possibly favored by the hydrolyzed protein content, facilitating starch's gelatinization.[17,20]

These results show that the hydrolyzed protein contents have an essential effect on the percentage of porosity developed. In addition, the structural changes due to the accumulation of proteins are characteristic, with the formation of tiny bubbles of cross-linked protein, like an expanded and porous sponge, which leaves the water vapor free at the exit of the extruder.[19] In addition, depending on the properties of the protein and its interaction with the rest of the components in the mixture, the bubbles may or may not coalesce.[12] Other studies reported similar results with the expanded ones based on corn and hydrolyzed soy protein, finding that the samples that contained hydrolyzed protein achieve higher porosity than protein without any modification.[21]

The highest EI was obtained with a moisture content of 16 g/100 g and a HPH flour of 30% (Figure 1e). Likewise, porosity is inversely correlated with density under the same operating conditions.[21−24] It is observed that the lowest

density achieved is reported with a HPH flour content of 30% and a moisture content of 24 g/100 g (Figure 1e). When there is a high proportion of moisture in the mixture, a greater proportion of water vapor is generated, forcing many cells to collapse and undergo coalescence, generating air spaces in a greater proportion and, therefore, a low density, even reducing the level of expansion.[19]

Regarding the speed of the screw and according to the regression coefficients, a positive quadratic effect is observed (Table 1), which means that increasing the speed of the screw advances the level of porosity of the extrudates, and this effect increases if a higher concentration of hydrolyzed fish protein is incorporated into the mixture.[17] The result of the screw speed could be attributed to a more significant shear effect inside the extruder and increased pressure.[25]

Figure 2 summarizes the results obtained from the high-resolution optical microscopy analysis. Figure 2a (T17) and Figure 2b (T18) present the axial temperature points. It is observed that at the higher temperature, the EI is reduced, a behavior related to an increase in the breaking of starch and protein, which decreases the possibility of maintaining a stable structure at the extruder outlet due to the change in pressure and loss of steam of water. However, the increase in temperature increases the interaction of the components in the mixture, improving the buoyancy for the T18 treatment. It is observed that the greater buoyancy is not related in this case to more significant expansion, indicating the possibility of less gelatinization of starch for the T17 treatment. Figure 2c (T19) and Figure 2d (T20) present the axial moisture points. According to Table 1, the moisture factor significantly impacts the density and durability, being observed in Figure 2c lower density according to the porosity, verifying larger pores and therefore a higher percentage of porosity, in contrast to the T20 treatment, which has higher density, low pore formation, and high durability; probably, the higher moisture content produces a lower cooking degree of starch, reducing expansion and increasing density.

Figure 2e,f (T21 and T22) presents the axial points of hydrolyzed protein flour. In this case, the influence of the addition of HPH is significant for all response variables,

**Table 2. Experimental Design Results**

| treatment | temperature (°C) | moisture (g/100 g) | HPH (g/100 g) | rpm (min⁻¹) | SME (W·h/kg) | MRT (s) | EI | density (g/cm³) | porosity (%) | hardness (N) | durability (%) | buoyancy (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 120 | 18 | 15 | 205 | 76.67 | 109.78 | 1.37 | 0.832 | 13.52 | 111.17 | 96.13 | 72.3 |
| 2 | 130 | 18 | 15 | 205 | 73.54 | 116.48 | 1.15 | 0.761 | 17.35 | 98.76 | 93.70 | 100.0 |
| 3 | 120 | 22 | 15 | 205 | 61.06 | 88.94 | 1.4 | 0.869 | 13.11 | 109.93 | 96.87 | 93.7 |
| 4 | 130 | 22 | 15 | 205 | 46.96 | 90.61 | 1.17 | 0.849 | 15.8 | 87.72 | 97.90 | 98.3 |
| 5 | 120 | 18 | 25 | 205 | 57.72 | 82.44 | 1.68 | 0.830 | 20.49 | 92.28 | 99.87 | 100.0 |
| 6 | 130 | 18 | 25 | 205 | 27.95 | 83.22 | 1.48 | 0.789 | 17.36 | 116.58 | 97.83 | 99.0 |
| 7 | 120 | 22 | 25 | 205 | 26.17 | 71.42 | 1.65 | 0.760 | 16.33 | 78.77 | 100.87 | 100.0 |
| 8 | 130 | 22 | 25 | 205 | 26.87 | 77.85 | 1.4 | 0.814 | 17.98 | 103.64 | 97.20 | 98.3 |
| 9 | 120 | 18 | 15 | 235 | 110.64 | 97.25 | 1.35 | 0.820 | 11.69 | 110.77 | 92.03 | 85.7 |
| 10 | 130 | 18 | 15 | 235 | 80.30 | 108.40 | 1.06 | 0.703 | 18.59 | 70.76 | 89.97 | 99.3 |
| 11 | 120 | 22 | 15 | 235 | 67.00 | 86.96 | 1.48 | 0.953 | 15.6 | 97.45 | 97.57 | 90.0 |
| 12 | 130 | 22 | 15 | 235 | 49.63 | 86.46 | 1.1 | 0.918 | 17.25 | 72.17 | 95.57 | 98.7 |
| 13 | 120 | 18 | 25 | 235 | 32.47 | 66.34 | 1.69 | 0.802 | 20.99 | 83.03 | 95.10 | 100.0 |
| 14 | 130 | 18 | 25 | 235 | 30.56 | 61.37 | 1.5 | 0.815 | 17.72 | 80.83 | 94.50 | 100.0 |
| 15 | 120 | 22 | 25 | 235 | 25.06 | 64.00 | 1.65 | 0.774 | 17.4 | 84.04 | 97.70 | 98.0 |
| 16 | 130 | 22 | 25 | 235 | 24.16 | 50.21 | 1.43 | 0.795 | 17.24 | 75.68 | 98.80 | 99.7 |
| 17 | 115 | 20 | 20 | 220 | 25.23 | 79.08 | 1.54 | 0.851 | 15.72 | 97.58 | 98.30 | 64.0 |
| 18 | 135 | 20 | 20 | 220 | 18.23 | 87.41 | 1.01 | 0.738 | 22.39 | 74.71 | 96.10 | 100.0 |
| 19 | 125 | 16 | 20 | 220 | 97.76 | 103.15 | 1.54 | 0.765 | 17.03 | 134.91 | 90.93 | 73.7 |
| 20 | 125 | 24 | 20 | 220 | 55.47 | 59.27 | 1.55 | 0.964 | 11.23 | 120.45 | 96.87 | 99.3 |
| 21 | 125 | 20 | 10 | 220 | 129.84 | 120.94 | 1.28 | 0.790 | 15.27 | 106.52 | 92.90 | 61.7 |
| 22 | 125 | 20 | 30 | 220 | 61.21 | 68.85 | 1.85 | 0.789 | 29.87 | 54.11 | 98.10 | 100.0 |
| 23 | 125 | 20 | 20 | 190 | 50.60 | 92.63 | 1.54 | 0.884 | 5.48 | 150.16 | 98.80 | 52.7 |
| 24 | 125 | 20 | 20 | 250 | 60.72 | 59.81 | 1.55 | 0.901 | 9.92 | 131.57 | 94.90 | 20.3 |
| 25 | 125 | 20 | 20 | 220 | 69.00 | 88.59 | 1.44 | 0.979 | 6.59 | 170.17 | 97.53 | 41.7 |
| 26 | 125 | 20 | 20 | 220 | 62.42 | 90.48 | 1.52 | 1.011 | 5.94 | 186.6 | 99.53 | 20.0 |
| 27 | 125 | 20 | 20 | 220 | 51.85 | 91.98 | 1.49 | 1.003 | 4.95 | 168.54 | 98.40 | 26.3 |
| 28 | 125 | 20 | 20 | 220 | 52.36 | 98.25 | 1.62 | 0.956 | 6.16 | 184.94 | 97.90 | 47.0 |
| 29 | 125 | 20 | 20 | 220 | 65.17 | 90.36 | 1.54 | 0.918 | 4.65 | 158.64 | 95.80 | 32.7 |
| 30 | 125 | 20 | 20 | 220 | 55.15 | 79.87 | 1.6 | 0.891 | 7.69 | 162.15 | 96.20 | 36.0 |
| 31 | 125 | 20 | 20 | 220 | 45.80 | 93.44 | 1.59 | 0.888 | 7.03 | 159.76 | 97.17 | 37.0 |
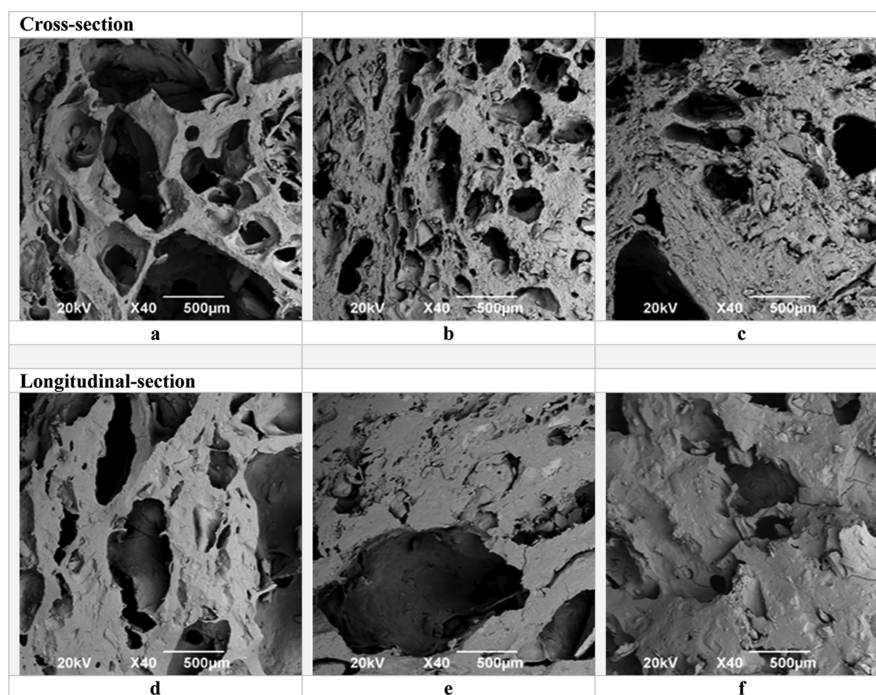


**Figure 3.** SEM micrographs of the cross section of some feeds obtained by the extrusion process. Effect of the addition of the hydrolyzed protein flour. (a,d) 30% of HPH. (b,e) 20% of HPH. (c,f) 10% HPH.

exhibiting greater expansion with its complement, uniform pore distribution, with homogeneous edges, a behavior related to the ability of the low-molecular-weight protein to distribute moisture homogeneously in addition to the formation of molecular interactions that give it stability, with a positive effect on the physical characteristics, as shown in Table 2. Its plasticizing effect allows the reduction of the average retention time and specific mechanics energy, a behavior related to low-molecular-weight peptides that facilitate the mobility of starch chains. Finally, Figure 2g,h (T23 and T24) shows the results of the axial points of the screw speed factor (rpm) with a negative correlation on hardness, where pellets with the lowest values of expansion are obtained at higher screw speeds, related to a higher percentage of porosity, as observed in Figure 2h.

*Scanning Electron Microscopy.* Figure 3 presents the micrographs of the 10, 20, and 30% HPH flour treatments, processed under constant conditions of temperature (125 °C), moisture (0.2 g/g), and screw speed (220 min⁻¹).

In general, melted structures are observed with no residual starch granules, presenting excellent pore formation in the sample with 30% of HPH (29.87%), followed by 10% of HPH (15.27%), and finally 20% of HPH (<7.69%). In the pellet with 0.3 g/g of HPH, a porous pellet with a cohesive structure is physically evident due to its high content of low-molecular-weight protein[26−28] (Figure 3a,d).

Likewise, the physical characteristics such as the buoyancy, porosity, EI, density, hardness, and durability were positively affected by the treatment with 30%, compared with the treatments that contain 10 and 20% of HPH, this is associated with the content and type of protein, directly affecting the MRT (mean residence time). The pellet with 30% HPH presented the lowest MRT, a result related to the plasticizing effect of the low-molecular-weight protein, which facilitates the moisture distribution process, obtaining a product with superior physical characteristics. In contrast, pellets with 10 and 20% of HPH, under the experimental conditions evaluated, presented high MRT values due to the high protein content of fishmeal, which competes for available water, a condition that possibly makes interaction difficult between the water and the rest of the components in the mixture, achieving undesirable physical characteristics, such as low buoyancy, high hardness, low porosity, and high density[20,29] (Table 2), results consistent with the compact structure observed in the micrographs (Figure 3c,f,b,e),[30] which reported observations by scanning electron microscopy (SEM) of compact structures in pellets for fish feed, associating it with the low content of the plasticizer and high content of fishmeal.[29]

**Evaluation of the Chemical Changes of the Extrudate.** Table 3 shows the results of treatments 21, 26, and 22, corresponding to the treatments with 10, 20, and 30% of the HPH flour processed under constant conditions of temperature (125 °C), moisture content (20 g/100 g), and screw speed (220 min⁻¹). A decrease in total protein is observed as the content of inclusion of HPH increases, a behavior related to the high content of low-molecular-weight protein that can be lost in the form of volatile nitrogen during the extrusion process.

Other studies observed a considerable increase in deamidation during the extrusion process associated with high processing temperatures. Deamidation is described as the loss of amide from the glutamine or asparagine residue, resulting in ammonia release and transforming these amino acids into glutamic acid and aspartic acid.[31] Deamidation is a

**Table 3. Chemical Characterization Treatments Including 10, 20, and 30% HPH**

| analysis | 10% HPH (g/100 g) | 20% HPH (g/100 g) | 30% HPH (g/100 g) |
|---|---|---|---|
| total protein (g/100 g) | 25.1038 ± 0.13 | 23.9 ± 0.2 | 22.79 ± 0.04 |
| DH (%) | 22.12 ± 0.06 | 24.08 ± 0.09 | 37.64 ± 0.04 |
| water-soluble protein (g/100 g) | 16.32 ± 0.13 | 18.28 ± 0.07 | 19.14 ± 0.07 |
| total volatile nitrogen (mg/100 g) | 103.7 ± 0.2 | 104.24 ± 0.11 | 106.7 ± 0.2 |
| in vitro digestibility (g/100 g) | 89.94 ± 0.03 | 89.62 ± 0.08 | 94.1 ± 0.4 |
| formic acid (g/100 g) | 0.021 | 0.081 | 0.118 |
| lactic acid (g/100 g) | 0.076 | 0 | 0.094 |

hydrolytic reaction and therefore requires water to form products, being less favorable when the tertiary and quaternary structures of the proteins are stable, limiting water access to potential deamidation sites within the protein. Unfolding the protein by extrusion temperatures exposes more areas, producing negatively charged acids that accelerate cleavage and expose more internal amides to the aqueous medium, thereby obtaining additional deamidation.[32] This behavior is related to the use of HPH, which would have a greater possibility of deamidation, observing that, with greater inclusion, the percentage of the total protein decreased, possibly due to the greater chance of access to water and the consequent loss of ammonia at the outlet extruder. Table 4 complements this result since glutamic acid corresponds to the most abundant amino acid in the evaluated protein raw materials. Therefore, during the extrusion process, the deamidation process would have a greater possibility of success. Likewise, a high content of total volatile nitrogen is observed in the extruded treatments, with records more significant than 100 mg/100 g of the sample, constituting high values, considering that the content of protein in the extruded product does not exceed 25 g/100 g, compared to HPH and fishmeal, which contain a similar value of NVT with a protein content close to 65 g/100 g.

An increase is also observed in the degree of hydrolysis (DH), water-soluble protein, total volatile nitrogen, and in vitro digestibility as the inclusion of HPH increases. This behavior is due to the characteristics of the raw materials used as a protein source, where HPH presented a DH of 62.95% compared to the DH of fishmeal of 5.61%, which is why an increase in the inclusion of HPH, will represent an increase in the values related to this parameter in the final product. However, according to HPH and fishmeal proportions, higher DH values were expected in the final product, obtaining values between 22 and 37%. The water-soluble protein shows the exact behavior of 68.94 g/100 g for HPH and 17.33 g/100 g for fishmeal. However, values between 16 g/100 g and 19 g/100 g are obtained in the final product. This behavior indicates the occurrence of interactions between low-molecular-weight protein and carbohydrates, reducing the solubility and availability of soluble low-molecular-weight protein in the DH and water-soluble nitrogen tests.

The HPH content positively affects the digestibility in vitro when its inclusion percentage increases in the mixture, a behavior related to the ease of access to protein and carbohydrate fragmentation by hydrolytic enzymes during

**Table 4. Amino Acid Composition of the Treatments Including 10, 20, and 30% HPH**

| amino acids | fishmeal (g/100 g) | hydrolyzed protein flour (g/100 g) | 10% HPH (g/100 g) | 20% HPH (g/100 g) | 30% HPH (g/100 g) |
|---|---|---|---|---|---|
| | | Hydrophobic Amino Acids | | | |
| isoleucine (Ile; I) | 3.83 | 3.62 | 3.082 | 3.682 | 3.348 |
| leucine (Leu; L) | 6.72 | 6.80 | 6.674 | 6.715 | 7.468 |
| methionine (Met; M) | 2.47 | 2.38 | 1.581 | 1.834 | 1.546 |
| phenylalanine (Phe; F) | 3.44 | 3.25 | 3.482 | 3.698 | 3.626 |
| valine (Val; V) | 4.51 | 4.30 | 2.878 | 3.787 | 3.334 |
| alanine (Ala; A) | 5.24 | 3.64 | 1.439 | 0.978 | 0.820 |
| proline (Pro; P) | 4.16 | 3.80 | 4.305 | 4.374 | 4.445 |
| total | 30.37 | 27.78 | 23.440 | 25.067 | 24.587 |
| | | Basic Amino Acids | | | |
| lysine (Lys; K) | 10.34 | 9.31 | 9.131 | 9.644 | 8.470 |
| arginine (Arg; R) | 8.30 | 9.23 | 7.316 | 7.957 | 7.908 |
| histidine (His; H) | 3.11 | 2.93 | 2.670 | 2.926 | 2.744 |
| total | 21.76 | 21.47 | 19.116 | 20.527 | 19.122 |
| | | Polar Amino Acids | | | |
| tyrosine (Tyr; W) | 3.10 | 3.58 | 4.016 | 4.100 | 4.278 |
| threonine (Thr; T) | 4.57 | 5.70 | 6.118 | 2.645 | 5.268 |
| glycine (Gly; G) | 7.00 | 7.40 | 7.979 | 8.380 | 8.399 |
| serine (Ser; S) | 14.44 | 15.68 | 18.020 | 18.140 | 17.836 |
| total | 29.10 | 32.37 | 36.134 | 33.265 | 35.781 |
| aspartic acid (Asp; D) | 3.12 | 3.85 | 2.626 | 3.002 | 3.213 |
| glutamic acid (Glu; E) | 15.65 | 14.53 | 18.684 | 18.138 | 17.296 |

the test, a result correlated with the increased nutritional value,[33,34] corroborating that HPH positively stimulates nutritional aspects in fish.[35]

Table 4 presents the compositional summary of amino acids, showing a stable behavior of most amino acids during the extrusion process, except for changes in lysine, alanine, and glutamic acid. Lysine is the limiting amino acid during the extrusion process.[36] During thermal processing, reducing sugars or other carbonyl compounds can react with the amino group of lysine, thus reducing its biological availability.[37]

In extrusion processes, the temperature conditions ($T > 180$ °C), shear forces (rpm > 100), moisture content (<15 g/100 g), and especially the presence of reducing sugars (glucose, fructose, maltose, and lactose) affect the availability of lysine, related to the condensation or Maillard reaction between the $-NH_2$ groups of lysine residues and the $C=O$ groups of reducing sugars.[38] The loss of lysine observed is related to the reducing sugars generated from starch during the thermomechanical process, added to other factors such as moisture, screw speed, temperature, nozzle diameter, mean retention time, and SME.[1] A reduction in alanine is also observed, which can undergo cross-linking processes during food processing, forming lysin alanine, reporting formation in low amounts in extruded foods.[37]

Table 5 presents the frequency of masses in a range up to 1.2 kDa, and the reference data corresponding to HPH and fishmeal to the extruded treatments with 10, 20, and 30% of HPH presented a majority peptide composition in a mass range of 0.2−0.4 kDa, a behavior that changes in the extruded treatments, showing a decrease in the same mass range. As has been discussed, water-soluble peptides, due to their low molecular weight, positively affect the physical quality of the extruded feed, contributing to the formation of intermolecular networks through hydrogen bonds, ionic bonding, and hydrophobic interactions, increasing the efficiency of cooking due to its plasticizing effect.[39] These interactions decrease the

**Table 5. Frequency of Molecular Masses of Protein Less Than 1.2 kDa for Treatments with Inclusion of 10, 20, and 30% HPH**

| mass range (kDa) | hydrolyzed protein flour (g/100 g) | fish flour (g/100 g) | 10% HPH (g/100 g) | 20% HPH (g/100 g) | 30% HPH (g/100 g) |
|---|---|---|---|---|---|
| 0.0−0.2 | | | 14.3 | 12.5 | 6.7 |
| 0.2−0.4 | 82.4 | 20.0 | 28.6 | 25.0 | 13.3 |
| 0.4−0.6 | 17.6 | 6.7 | 14.3 | 25.0 | |
| 0.6−0.8 | | 33.3 | 42.9 | 37.5 | 40.0 |
| 0.8−1.0 | | 33.3 | | | 40.0 |
| 1.0−1.2 | | 6.7 | | | |

availability of these peptides and corroborate the formation of structures that give them desired physical characteristics in terms of porosity, expansion, buoyancy, and durability.

## ■ CONCLUSIONS

The HPH flour presented technofunctional properties related to its cohesiveness and plasticizer capacity. Its cohesiveness capacity (cross-link) was verified positively on the physical properties of the pellets, such as buoyancy, EI, porosity, hardness, and durability. The plasticizing capacity reduced the mean retention time in the extruder barrel and a decrease in the SME. Although HPH exhibits a cohesive action, the increase in the mass flow due to the plasticizing effect impacts SME, reducing its value. During the evaluation of the addition of the HPH flour in the extrusion process, a deamidation phenomenon is observed, which can generate disadvantages in thermal processing due to the loss of nitrogen in the form of ammonium, which was proportional to the inclusion of hydrolyzed protein flour. This characteristic can become limiting if remarkably high inclusions of HPH flour are made in the diet. However, the addition of HPH during the extrusion process supports increasing the in vitro digestibility of the food, improving the nutritional characteristics of the extrudate. The monitoring carried out for the changes in the protein, such

as the DH, water-soluble protein, and molecular mass profile, verifies the binding effect of the HPH flour during the extrusion process. Durability and hardness are response variables that generally present a positive correlation. Similarly, high-resolution optical microscopy (MOAR) demonstrated high correlation with the phenomena presented in the experiment. Finally, for the present study, pellets with high durability were achieved due to the cohesiveness of the HPH and low hardness due to the high porosity achieved.

## ■ EXPERIMENTAL SECTION

**Materials and Preparation of the Extruded Product.** The raw materials used for the evaluation during the extrusion process were HPH obtained from trout by chemical silage, fishmeal (Siquality SA, Guayaquil-Ecuador), and cassava starch (Sucre starch). Its proximal composition is described in Table 6. Additives such as the vitamin core formulated for fish farming (Premex), bentonite used as a binder (Premex), calcium carbonate ($CaCO_3$) (analytical grade 99%, Carlo Erba), and sodium chloride (NaCl) (analytical grade 99%, Carlo Erba) were incorporated into the diet. The formulation verified the influence of the HPH flour by incorporating it in a range between 10 and 30 g/100 g (HPH 10, 20, and 30%) as a substitute for fishmeal (Table 7). The starch source, vitamin nucleus, and minerals remained stable and were supplemented according to the requirements reported by the National Research Council.[40] The standard proximal composition of the diet obtained was isoprotein and isoenergetic and formulated for omnivorous fish in the fattening stage. The flours were sieved with a Tyler series no. 40 sieves and mixed in a kitchen aid mixer for 30 min. Later, they were packed in polyethylene bags and refrigerated for 24 h to be extruded afterward.

**Extrusion Process.** The extrusion process was developed using a compact single-screw extruder, Haake Polylab OS Rheomex 19/25 OS (Germany), which consists of a 475 mm long (L) and 19 mm diameter (D) worm with a 25/1 L/D ratio, a maximum working temperature of 450 °C, a total speed of 250 min$^{-1}$, and a maximum torque of 160 N m, coupled to a Haake RheoDrive 4 system, with a motor power of 4 kW. The extruder barrel has three independent heating or cooling zones; heating utilizes electrical resistances and cooling through channels using compressed air circulation. The compression ratio of the screw is 5:1, and the diameter of the hole of the nozzle used is 5 mm. The equipment is equipped with three thermocouples for monitoring the temperature of the barrel, an extra thermocouple placed in the die to measure the temperature of the product, and a device to measure pressure. The feeding was carried out using the HAAKE Metering Feeder OS. The extruded samples were cut into pellets (length: 5 ± 0.1 mm) and dried in a BINDER brand dehumidifier (Germany) at 50 °C until they reached a moisture content of 10 ± 0.05 g/100 g. The dried product was stored in polyethylene bags for subsequent property analysis. The MRT study was carried out using the methodology reported in the literature,[41] in which a marker (erythrosine dye) was added to carry out the follow-up.

**Evaluation of the Microstructural Changes of the Extrudate.** *Porosity.* The samples were obtained from cross sections made with Ultramicrotome, LKB, Bromma (Sweden), adjusted to a cut of 30 μm, from the extruded pellets. The internal diameters of the chambers formed in the pellet were captured by a Nikon DS-2Mv 2MP digital camera (Japan), coupled to a Nikon eclipse 80i optical microscope (Japan), and

**Table 6. Characterization of Raw Materials on a Dry Basis**

| | total protein (g/100 g) | ethereal extract (g/100 g) | ashes (g/100 g) | fiber (g/100 g) | free extract of nitrogen (ELN) (g/100 g) | total volatile nitrogen (mg/100 g sample) | pepsin digestibility (g/100 g) | DH (%) | soluble protein (g/100 g) | formic acid (g/100 g) | lactic acid (g/100 g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| hydrolyzed protein flour | 62.92 ± 0.05 | 6.45 ± 0.02 | 17.86 ± 0.12 | 0.6 ± 0.2 | 12.2 | 109.4 ± 0.2 | 91.2 ± 0.2 | 62.948 ± 0.011 | 68.94 ± 0.09 | 0.41 | 0.18 |
| fish flour | 63.20 ± 0.06 | 6.81 ± 0.02 | 18.67 ± 0.14 | 1.0 ± 0.2 | 10.3 | 109.70 ± 0.09 | 80.17 ± 0.006 | 5.61 ± 0.05 | 17.33 ± 0.06 | 0.19 | 0.18 |
| cassava starch | 1.60 ± 0.05 | 0 | 0 | 2.16 ± 0.04 | 96.24 | | | | | | |

**Table 7. Raw Materials Required for the Preparation of the Diet**

| raw materials | cassava starch | hydrolyzed protein flour | fish flour | vitamin | calcium carbonate | bentonite | sodium chloride |
|---|---|---|---|---|---|---|---|
| incorporation (g/100 g) | 55 | 10.0−30.0 | 30.0−10.0 | 2 | 0.8 | 1.7 | 0.5 |

processed in the Image Pro-Plus Analyzer Software (version 6.3, 2008). The percentage of porosity obtained from the different treatments was determined (eq 1).

$$\text{Porosity index (\%)} = \frac{\sum A_p}{A_t} \times 100 \tag{1}$$

where $\Sigma(A_p)$ is the cross-sectional pore area $(mm^2)$ and $A_t$ is the cross-sectional area of the pellet $(mm^2)$.

*Scanning Electron Microscopy.* The images of the cross sections of the pellets were captured with a JEOL JSM-6490LV (USA) scanning electron microscope from samples covered with conductive adhesive tape and subsequently with a gold bath. An acceleration voltage of 20 kV and magnifications of 50, 100, and 200 increases were used. The samples were processed in a specialized laboratory.

**Evaluation of the Chemical Changes of the Extrudate.** *Volatile and Soluble Nitrogen.* In the study, 1.0 g of the sample was weighed, and 50 mL of boiling distilled water was added with constant stirring for 10 min. Subsequently, the supernatant liquid was filtered through a Whatman no 1 filter paper, taking 15 mL of the filtrate for subsequent protein determination.[42] The decision of the total volatile essential nitrogen content (NBVT) was based on the reference method described in the official journal of the European Union EU, 2005. For this, the deproteinization of 10.0 g of the sample was carried out in 90 mL of 6% $HClO_4$. The homogenate was centrifuged (Hermle Z326 K, Germany) at 3000 rpm for 5 min, and the supernatant was filtered through a filter paper, thus obtaining the sample extract. Once alkalized, the 20% NaOH extract was subjected to steam distillation, and the volatile building blocks were absorbed in a 3% boric acid aqueous solution. The concentration of NBVT was determined by titration of the absorbed bases with HCl 0.01 N using Tashiro's reagent as an indicator.

*Crude Protein.* The crude protein content was determined according to the standard AOAC 968.06,[42] taking 0.8 g of the sample, which were digested with 10 mL of $H_2SO_4$ concentrated in a compact RAYPA MBC digestion system, neutralized with NaOH, and distillated in a 3% $H_3BO_3$ solution with the Tashiro indicator in RAYPA DNP-2000 still equipment. For protein quantification (factor 6.25), each sample was titrated with 0.1 N HCL.

*Amino Acid Profile.* The analysis was performed by high-performance liquid chromatography on a Prominence equipment with a Shimadzu brand UV visible detector (Japan), ACE C18-AR column.[43] The samples constituted of 200 pg of protein, and a volume of 10 $\mu$L of a 3 mM D,L-$\alpha$-aminobutyric acid solution was added. The hydrolysis was carried out with 600 $\mu$L of 12 N HCl at 110 °C for 20 h in vials and subsequent drying in a vacuum oven at 60 °C for 4 h. Then, 1 mL of 4.0 M NaOH was added to 3 mg of the sample to determine tryptophan amount. The hydrolysis was carried out at 110 °C for 4 h in vials and under a nitrogen atmosphere with subsequent adjustment of the pH to 7.0 with 12.0 N HCl. The contents were transferred to a 10 mL volumetric flask and capped with sodium borate buffer (pH 9.0). Finally, 100 $\mu$L of

the solution was filtered using 0.45 $\mu$m membranes. A volume of 10 $\mu$L was injected to determine amount.

The derivatization was carried out in the vials with the hydrolyzed and dry sample; a volume of 0.8 $\mu$L of diethyl ethoxymethylenemalonate (EMMDE) and 1 M sodium borate buffer (pH 9.0) was added until it reached a final volume of 1 mL. The reaction was carried out at a temperature of 50 °C for 50 min. After the reaction time, the vials were allowed to cool to room temperature, and a volume of 10 $\mu$L was taken to be injected into the chromatograph.

For the chromatographic analysis, gradient changes were made linearly. For the determination of tryptophan, the isocratic gradient was used in a 91:9 ratio. The analysis was carried out at 34 °C with sodium acetate 25.0 mM-0.02% sodium aside (pH 6.0) (A) and HPLC-grade acetonitrile (B). The flow was 0.9 mL/min, and the injection volume was 10 $\mu$L. Mobile phases and samples were filtered through Millipore membrane filters with a pore size of 0.45 $\mu$m before use.

*Residual Formic and Lactic Acid.* The analysis was performed by high-performance liquid chromatography on an LC-2010 equipment with a Shimadzu (Japan) brand visible−UV detector, Aminex HPX-87C Bio-Rad column.[44]

*Molecular Mass Profile.* The analysis was performed by ultrahigh performance liquid chromatography in an Ultimate 3000 equipment coupled with a Thermo Fisher Scientific (Germany) Exactive Plus Orbitrap mass detector. As a stationary phase, a reversed-phase C18 Titan column was used, with a diameter of 2.09 mm, a length of 100 mm, and a particle size of 1.9 mm. The mobile phase was a water:-acetonitrile (20:80) mixture adjusted to pH 4.0 with 0.1% formic acid with a gradient change to 100% acetonitrile and a constant flow of 0.300 mL/min. The column compartment temperature was 40 °C. The evaluated mass range was 100−1200 $m/z$. The mass analysis parameters adjusted the ionization source at 6 kW (spray voltage) and with nitrogen as the auxiliary gas at 190 °C. The information was processed using Thermo Xcalibur Version 3.1 software.

*Degree of Hydrolysis.* The study was performed using the 2,4,6-trinitrobenzene-sulfonic acid (TNBS) method;[45] 8.0 g of the sample was weighed, and 12 mL of 0.2 M phosphate buffer, pH 7.0, was added. It was centrifuged (Hermle Z326 K, Germany) for 10 min at 12,000 rpm at a temperature of 4 °C; the supernatant was filtered, and 128 $\mu$L was added in a screw-capped test tube with 1 mL of 0.01% TNBS and 2 mL of 0.2 M phosphate buffer, pH 8.2, and vigorously vortexed for 15 s. It was placed in a water bath at 50 °C for 30 min, ending the reaction by adding 2 mL of 0.1 M sodium sulfate. The reading was carried out using a UV-vis Shimadzu UV 1800 spectrophotometer (Japan) at a 410 nm wavelength interpolated on a L-leucine standard curve. The DH was determined (eq 2).

$$\% \text{ DH} = \frac{[(NH_2)_{tx}]}{[(NH_2)_T]} \times 100 \tag{2}$$

where $(NH_2)_{tx}$ is the concentration of terminal $\alpha$-amino groups, expressed as millimoles of L-leucine at time tx; $(NH_2)_T$ is the concentration of total terminal $\alpha$-amino groups

expressed as millimoles of L-leucine in the sample after total acid hydrolysis.

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

**In Vitro Digestibility.** In vitro, dry-matter digestibility was performed according to the methodology reported by Dierick and Vervaeke;[46] 10 g of the sample was taken, and grinding was carried out with an analytical mill A11 basic IKA (Germany) and sieved by sieve no. 100 Tyler series. 0.5 g of the sample was weighed, and 25 mL of 0.1 M phosphate buffer solution at pH 6.0 and 10 mL of 0.2 M HCl were added, adjusting the pH to 2.0 with 1 M HCl solution or 1 M NaOH. 1 mL of a 2.5% pepsin enzyme solution in 0.2 M HCl and 0.5 mL of a 0.5% chloramphenicol solution in ethanol were added. It was placed in a thermostatic bath at 39 °C for 2 h with constant stirring. Subsequently, 10 mL of a 0.2 M phosphate buffer solution at pH 6.8 and 5 mL of 0.6 M sodium hydroxide were added, adjusting the pH to 6.8 with 1 M HCl or 1 M NaOH solutions. 1 mL of pancreatin enzyme 10% in 0.2 M phosphate buffer at pH 6.8 was added, and the temperature was maintained at 39 °C for 4 h. Once the digestion was finished, the sample was filtered through a Whatman no. 2 filter paper previously dried and weighed and washed with distilled water. The filtered sample was dried at 105 °C to a constant weight. In vitro dry-matter digestibility (DivMS) was determined (eq 3). where $P_m$ is the weight of the sample, $M_s$ is the dry matter, $P_{filter}$ is the weight of the filter, and $P_{waste}$ is the weight of the waste.

**Statistical Analysis.** A $2^k$ factorial design was used where the factors temperature, screw speed, moisture, and inclusion of the HPH flour were evaluated with two levels each, as described in Table 2. The factorial experiment was expanded by adding axial and central points to obtain a response surface design, which for the case study was a central composite design, achieving a total of 31 treatments distributed as follows: 16 treatments[1−16] corresponding to the factorial portion, 8 treatments[17−24] corresponding to the axial points, and 7 treatments[25−31] corresponding to the central points, as described in Table 2. The axial points were obtained by applying the term $\alpha = (F)^{1/4}$, where $F$ is the number of points used in the factorial portion of the design, where $\alpha = 2$. This fact implies that the values of the axial points were extrapolated by that magnitude according to the coding presented in Table 2. For the surface analysis of response, Minitab 16 software was used.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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