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Enhancement of fish protein hydrolysates for salad dressing through high shear and sterilization pre-treatments

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Abstract

Thermal and mechanical treatments may affect the structure of hydrolyzed proteins, thus influencing the obtaining of peptides with improved bioactivity. In this work, tilapia muscle was treated by thermal sterilization or homogenization with ultra-turrax (UT) and hydrolyzed with alcalase to obtain FPHs with antioxidant properties in salad dressing. To evaluate the bioactive potential of FPHs, the acetylcholinesterase inhibition assay was applied, resulting in up to 45.87% inhibition for the UT sample (60 mg/mL). Also, no cytotoxicity was detected by *Allium cepa* model for all FPHs. The emulsifying activity index and emulsifying stability index of FPHs indicated better emulsifying capacity in basic pH. As a proof of concept, FPHs were used as an emulsifying/antioxidant agent to prepare a salad dressing. FPHs increased the formulation's protein content, pseudoplastic behavior, color, and texture. In addition, FPHs aided the oxidative stability of salad dressing (evaluated by oil's extinction coefficient), demonstrating potential application in emulsified foods by acting on the elimination of radicals generated in lipid oxidation.

Practical applications

Fish protein hydrolysates (FPHs) offer diverse bioactive properties such as antioxidant, antimicrobial, anticancer, antihypertensive, and acetylcholinesterase (associated with Alzheimer's disease) inhibitory effects. However, optimizing their technological properties poses a challenge, affecting applicability and bioactivity. Industrial processes such as thermal and mechanical treatments can alter protein structures, influencing peptide bioactivity post enzymatic hydrolysis. This study investigates the impact of substrate pre-treatments, sterilization via thermal heating, and homogenization using a rotor-stator system (ultra-turrax) on FPHs' technological properties after hydrolysis with alcalase, including emulsifying capacity and acetylcholinesterase inhibitory capacity. In addition, it explores the application of pre-treated FPHs in a

real food system (French salad dressing), assessing rheological properties, texture, and oxidative stability. Such evaluations are crucial for ensuring the feasibility of industrial FPHs production and their application.

KEYWORDS
enzymatic hydrolysis, food application, homogenization, pre-treatment, sterilization

1 | INTRODUCTION

Fish protein hydrolysates (FPH) are products of the hydrolysis of abundant and high-quality native proteins, such as fish muscle, skin, and wastes. Since FPHs are rich in essential amino acids and peptides, making them a valuable source of high-quality protein for human nutrition. The health benefits of FPHs, including potential effects on chronic disease prevention and overall wellness, highlight their importance in dietary interventions and functional foods. They have already demonstrated various bioactive properties such as anti-inflammatory (Da Rocha et al., 2018), anticancer (Yaghoubzadeh et al., 2020), antimicrobial (Jemil et al., 2016), antioxidant (Bashir et al., 2020; Cheng et al., 2020), and acetylcholinesterase (AChE) inhibitory activity (Moreira et al., 2022). This broad bioactivity is directly linked to smaller peptides and free amino acids formed during hydrolysis, which are affected by pH, temperature, enzyme, time, and substrate properties (Halim et al., 2016). The health benefits of FPHs, including potential effects on chronic disease prevention and overall wellness, highlight their importance in dietary interventions and functional foods. By incorporating FPHs into diets, there is potential to improve nutritional intake and support better health outcomes. Furthermore, FPHs offer practical solutions for enhancing food products, reducing waste, and meeting consumer demands for nutritious, sustainable, and functional foods. Their ability to improve texture, stability, and sensory attributes makes them valuable in the development of diverse food products.

Fish muscle, which makes up 15%–25% of the total protein in fish, is an interesting protein source. Fish muscle proteins can be divided into three groups: structural protein (~70%–80% of total protein content), myofibrillar protein, and sarcoplasmic protein. All of them contain the essential amino acids (lysine, tryptophan, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, and valine) (Heffernan, Giblin, & O'Brien, 2021). The growing demand for advanced pharmaceutical and nutritional products has spurred interest in fish muscle protein hydrolysates' high-quality nutritional components and bioactivities. These hydrolysates, which are highly sensitive to proteolytic breakdown, offer significant potential. Fish muscle protein hydrolysates and their amino acids are attractive functional ingredients due to their natural availability, cost-effective extraction methods, and beneficial effects on human health (Ryu et al., 2021).

Tilapia production in 2023 rose to 579.080 t and reached 65% of Brazilian production, and its consumption has grown 93% in one decade in Brazil (Brazilian Fish Farming Association, 2024). Brazil is the fourth-largest producer of Nile tilapia (*Oreochromis niloticus*), and the country with the highest growth potential in the world (Castilho-Barros et al., 2020). However, it is important to note that key aspects of how pre-treatments affect the technological and bioactive properties of enzymatically hydrolyzed Nile tilapia muscle still require further investigation.

Recent studies have evaluated pre-treatments, before hydrolysis of the substrate, to modify the protein structure and improve enzyme access, facilitating the exposure of the N and C terminals of peptides (Noman, Qixing, et al., 2020). Among these technologies are the microwave (Ketnawa et al., 2018), ultrasound (Li et al., 2020), high-pressure processing (Hemker et al., 2020), heat treatments (Rivero-Pino et al., 2020a), and high shear (Silvestre-De-León et al., 2021). By altering the structure of proteins, pre-treatments can also aid in modifying functional properties while maintaining high nutritional quality (Moya Moreira et al., 2023; Rivero-Pino et al., 2020a), allowing FPH to be applied in the formulation of food products for better physical, functional, and nutraceutical properties (Noman et al., 2018). In the case of salad dressings, which are oil-in-water (Kangsanant et al., 2014) emulsions with high-fat levels (Tekin-Cakmak et al., 2021), FPH can provide better formation and stability of emulsions by favoring the adsorption of peptides at the oil/water interface (Ruiz-Álvarez et al., 2022). In addition, during storage, antioxidant peptides can be released to interact with other ingredients and retard lipid oxidation (García-Moreno et al., 2016; Ghorbani Gorji et al., 2016).

Sterilization has been previously utilized as a pre-treatment for various plant and animal proteins, including okara (a soybean by-product) and whey protein (Du et al., 2021), and porcine skin by-products (Min et al., 2017). However, its application to fish proteins has been explored less. Notable studies include Korczek et al. (2020), who investigated the effects of various pre-treatments (cooking, frying, baking, and sterilization) followed by hydrolysis with flavourzyme on the antioxidant and antihypertensive activities, and in vitro digestion stability of mackerel (*Scomber scombrus*) protein hydrolysates. Their findings indicated that the highest DPPH radical scavenging capacity (91.56%) was achieved with sterilization pre-treatment, compared to 13.88% for the control hydrolysate without pre-treatment. In another study by Korczek et al. (2021), similar pre-treatments and flavourzyme hydrolysis were applied to herring (*Clupea harengus*) fillets, with sterilization resulting in the highest radical scavenging ability, measured at 2.55 μmol TE/mg for the FRAP value.

Another pre-treatment applicable for enzymatic hydrolysis is high-shear processing, which can be achieved through methods such as extrusion and high-speed shearing homogenization with devices like ultra-turrax (Silvestre-De-León et al., 2021). Extrusion has been used to evaluate the effects of combined extrusion pre-treatment and

enzymatic hydrolysis on soy protein isolate's emulsifying properties (Chen et al., 2011) and to assess the antihypertensive effects of chickpea protein hydrolysate before and after protein extrusion (Chávez-Ontiveros et al., 2022). In addition, Shiao et al. (2021) evaluated the physicochemical and antioxidant properties of gelatin hydrolysates from tilapia scales pre-treated with single-screw extrusion. Hao et al. (2022) employed high-speed shear homogenization with ultra-turrax post-hydrolysis to reduce the particle size of soy protein isolate. However, there is a gap in research regarding the use of ultra-turrax as a pre-treatment for enzymatic hydrolysis of fish protein hydrolysates.

This study aimed to investigate whether substrate pre-treatments, sterilization (steam sterilization under high pressure), and high shear homogenization using an ultra-turrax could affect the cytotoxicity, bioactive (AChE inhibition), and functional properties of fish protein hydrolysates (FPH) after enzymatic hydrolysis. In addition, the study sought to analyze the technological properties of a real food system, specifically salad dressing, when incorporated with FPHs.

2 | MATERIALS AND METHODS

2.1 | Material

Nile tilapia muscle was obtained in a local market in 2020 (Campo Mourão, Paraná, Brazil). The enzymatic hydrolysis was started using Alcalase 2.4 L enzyme (≥2.4 U/g, P4860, Sigma-Aldrich). For FPH characterizations, bovine albumin (A7030, Sigma-Aldrich), ninhydrin (2%, Sigma-Aldrich), and glycine amino acid (Sigma-Aldrich) were used. The acetylcholinesterase (AChE) activity assay reagents used were tris hydroxymethyl aminomethane (Tris–HCl, Dinâmica, Brazil), monobasic potassium phosphate (Dinâmica, Brazil) and dibasic potassium phosphate (Neon, Brazil) for the potassium phosphate buffer (TFK) preparation, acetylcholinesterase enzyme from *Electrophorus electricus* (electric eel, Sigma-Aldrich) (1.25 U mL^{-1} in Tris–HCl buffer (20 mM, pH 7.5)), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, 98%, Sigma-Aldrich), and acetylthiocholine iodide (ASCh) (Sigma-Aldrich, 99%). The emulsifying capacity reagents were soybean oil (Coamo, Brazil) and sodium dodecyl sulfate (SDS, Isofar, Brazil). Cytotoxicity and genotoxicity assay reagents used were methyl methanesulfonate (MMS, CAS 66-27-3, Sigma-Aldrich), ethanol (99%, Dinâmica, Brazil), and acetic acid (Dinâmica, Brazil). In the French salad dressing control formulation, the commercial antioxidant butyl hydroxy toluol (BHT) (Êxodo Cientifica, Brazil) was used, and potassium sorbate (Casa dos Químicos, Brazil) was applied in all formulations as an antimicrobial agent. For oxidative stability evaluation, isooctane was used as a solvent (Neon, Brazil).

2.2 | Proximate composition of Nile tilapia muscle (*Oreochromis niloticus*)

Fish muscles were ground, homogenized, and stored at −80°C. The proximate composition of the fish muscle was determined according to the method described by Lutz (2008) in wet basis (w.b.), where moisture (gravimetric method at 105°C), ash content (muffle at 550°C), lipids (Bligh and Dyer method), and the protein content (Micro-Kjeldahl method with a correction factor of 6.25) were applied.

2.3 | Substrate pre-treatment

To investigate the effects of ultrasound and sterilization applied previously to enzymatic hydrolysis, the following methodology was used. The frozen fish muscles were thawed and separated into three substrate groups: control (C, no treatment before hydrolysis), sterilization (EST), and ultra-turrax (UT). In the group EST, the substrate was directly sterilized in an autoclave (Phoenix, AV, 18 L) at 120°C under 1 kgf cm^{-2} for 15 min. In the group UT, the substrate was first solubilized in distilled water (1:2, wt:v) and then homogenized in an ultra-turrax (IKA, T25) at 20,000 rpm for 15 min.

2.4 | Production of fish protein hydrolysates (FPH)

The production of protein hydrolysates was performed according to Alvares et al. (2018) with minor adaptations. The substrate samples of C and EST treatments were solubilized in distilled water (1:2, wt:v). The sample UT was already solubilized for the pre-treatment process. Then, all treatments (C, EST, and UT) had the pH adjusted to 7.5 with NaOH 1 M or HCl 0.1 M, and after that, the alcalase enzyme was added (0.8%v/wt, [enzyme:substrate]). Samples were taken to a Dubnoff bath, where the temperature was adjusted to 55°C, and the mixture was kept under gentle agitation for 120 min. The enzymatic reaction was halted by raising the temperature to 90°C for 15 min. After that, the mixture was cooled at room temperature and centrifuged at 6000 rpm for 20 min. The supernatant was collected and filtered with a cellulose filter with a vacuum pump. The filtrate FPH was frozen in an ultra-freezer (−80°C) for 24 h and freeze-dried (L101, Liotop, Liobrás).

2.5 | FPH characterization

2.5.1 | Degree of hydrolysis (DH) and yield

The following methodologies were used to assess the degree of hydrolysis by measuring the proportion of cleaved peptide bonds and to evaluate the hydrolysis yield in Nile tilapia muscle samples. The degree of hydrolysis (DH) was determined using the methodologies described by Hoyle and Merrltt (1994) and Baek and Cadwallader (1995), with modifications already described by Moreira et al. (2022). It was calculated by Equation (1), where TCAsp represents the amount (mg) of soluble protein in FPH (Lowry's method using bovine serum albumin as the standard) and TP is the total protein in the sample (muscle proximate composition).

$$\mathrm{DH}(\%) = \left(\frac{\mathrm{TCAsp}}{\mathrm{TP}}\right) \times 100\%. \quad (1)$$

For yield determination (Equation 2), after the complete hydrolysis procedure, samples were weighed (HW, g) and then centrifuged (6000 rpm for 20 min). Afterward, the aqueous fraction (containing the protein hydrolysates) was transferred, weighed (AFW, g), and stored at −80°C.

$$\mathrm{Yield}(\%) = \left(\frac{\mathrm{AFW}}{\mathrm{HW}}\right) \times 100\%. \quad (2)$$

2.5.2 | Free amino acid

The free amino acid determination was used to evaluate how thoroughly FPHs have been broken down into their constituent amino acids. The determination was performed through the ninhydrin test, as indicated by Moore and Stein (1954), with adaptations. For this, 1 mL of 2% ninhydrin solution was added in 1 mL FPH samples (50 mg mL^{-1} or distilled water as blank) and kept in a bath at 100°C for 15 min. After this, 15 mL of ethanol (50%v/v) was added to all samples. The absorbance at 570 nm was determined using a UV–Vis spectrophotometer (Ocean Optics USB650UV, USA), and results were calculated from a glycine calibration curve ($y = 0.4543x - 1.6067$; $R^2 = 0.9925$), reported as μmol of glycine equivalents per gram of sample ($\mu mol_{Gly\ E}/g_{sample}$).

2.5.3 | FTIR

To determine the molecular characteristics of FPHs samples, spectra were collected with an Infrared Spectrophotometer with Fourier Transform (IR AFFINITY-1, Shimadzu), in the range of 4000–400 cm^{-1}, using 32 accumulations and a resolution of 4 cm^{-1}. The samples were previously conditioned in a desiccator containing anhydrous calcium chloride ($CaCl_2$) for 7 days before the analysis, and spectra bands were normalized for spectrum comparison.

2.5.4 | Protein solubility

Solubility influences the incorporation of protein hydrolysates into various formulations, making its determination essential for optimizing production and formulation processes. With modifications, the protein solubility of the FPHs was determined according to Chalamaiah et al. (2010). FPH (300 mg) was diluted in 30 mL of distilled water. The solutions had the pH adjusted to 3, 5, 7, 9, and 11 with HCl 0.5 M or NaOH 0.5 M. Each solution was magnetically stirred at room temperature (25 ± 2°C) for 30 min and centrifuged at 6000 rpm for 30 min at 4°C. The protein content in the supernatant was determined using the Biuret method, and the total protein was determined using the Micro-Kjeldahl method. The solubility was calculated according to Equation (3): Psup represents the protein in the supernatant fraction (g) and TP is the total protein before the fractionation (g):

$$\mathrm{Solubility}\,(\%) = \left(\frac{\mathrm{Psup}}{\mathrm{TP}}\right) \times 100. \quad (3)$$

2.5.5 | Bioactivity: AChE inhibitory activity

Acetylcholinesterase (AChE) is an enzyme that breaks down acetylcholine, a neurotransmitter involved in muscle movement and cognitive functions. Inhibition of AChE can lead to increased levels of acetylcholine, which may be beneficial in treating conditions such as Alzheimer's disease, where acetylcholine levels are reduced (Leimann et al., 2023). AChE inhibitory activity was performed according to the methodology described by Ellman et al. (1961), with some modifications previously described by Moreira et al. (2022).

The method is based on the hydrolysis of acetylthiocholine by AChE, followed by the reaction of thiocholine (hydrolysis product) with DTNB (5,5′-dithiobis(2-nitrobenzioc) acid), resulting in a yellow-colored compound (2-nitro-5-thiobenzoate anion, TNB). Initially were added to a 96-plate well, 90 μL (50 mM) of potassium phosphate buffer (pH 7.5), 45 μL of Milli-Q water, and 15 μL of the enzyme from electric eel (1.25 U mL^{-1} in Tris–HCl buffer (20 mM, pH 7.5)) and 10 μL of the FPH solution (final concentrations of 15, 45, or 60 mg mL^{-1}). For the blank sample, the FPH solution was substituted by 10 μL of Milli-Q water. This medium was incubated at 25°C for 10 min and then 20 μL of DTNB (2 mM) and 20 μL of acetylthiocholine iodide (ASCh, 0.8 mM) were added to the wells in the dark. Reaction was kept for 4 min and absorbance measurements were performed in a plate reader (Thermo-Plate Reader) at 405 nm. Results were expressed as percent of enzyme activity in comparison to the control (mixture without FPH solution).

2.5.6 | FPHs functional properties

To evaluate the ability of FPHs to form stable emulsions (which is crucial in products such as dressings, sauces, and beverages), how well the produced emulsions retain their stability over time, and to identify aggregation or instability of the FPHs, emulsifying activity index (EAI), emulsifying stability index (ESI), as well as turbidity were measured. The EAI and ESI were determined according to the method described by Pearce and Kinsella (1978) with some modifications. The pH values of 90 mL of the FPH samples (1 mg mL^{-1}) were adjusted to 3, 5, 7, 9, and 11 after adding 30 mL of soybean oil, respectively. Each mixture was homogenized at 20,000 rpm for 1 min (ultra-turrax, T25, IKA). Next, 100 μL of the emulsion was pipetted from the bottom of the mixture and diluted in 10 mL of a 0.1%wt/v SDS solution. The resulting mixture was then

homogenized additionally for 10 min. The absorbance values were measured at 500 nm, just after the emulsion dilution (A_0) and after 10 min (A_{10}). The absorbance values were used to calculate the EAI (m^2/g) and ESI (min), as shown in Equations (4) and (6), where T is the turbidity (Equation 5), ϕ is the oil volume fraction (v/v), L is the optical path of the cuvette (m), C is the initial concentration of FPH (g mL^{-1}) in the first emulsion, and t is the time interval (10 min). Each sample was measured in triplicate.

$$EAI = 2.T\left(\frac{A_0.L}{C.\phi.10^4}\right), \quad (4)$$

$$T = \frac{2.03.A_0}{L}, \quad (5)$$

$$ESI = \left(\frac{A_0}{A_0 - A_{10}}\right) \times t. \quad (6)$$

2.5.7 | Cytotoxicity and genotoxicity tests in *A. cepa* root meristems

The cytotoxicity and genotoxicity of FPHs were assessed to determine their potential harm to cells and their capacity to induce genetic damage, including mutations or chromosomal alterations, which, for long-term exposure, could potentially lead to cancer or other genetic disorders (dos Santos et al., 2022). Both tests were accessed using five onion bulbs (beta crystal variety from organic vegetables) per sample. The bulbs were placed in containers with distilled water, constantly aerated, and germinated in a dark environment until roots of 2.0 cm in length were obtained. Before treatment, some roots were collected as a control (Co—0 h). The roots were then subjected to their respective treatments for 24 and 48 h, with samples collected every 24 h. Two controls were prepared: a negative control, treated only with distilled water, and a positive control, treated with methyl methanesulfonate (MMS), a substance known to be cytotoxic and genotoxic for the *A. cepa* test system (4×10^{-4} mol L^{-1}). The collected roots were fixed in Carnoy 3:1 solution (ethanol:acetic acid) for up to 24 h. Slides were analyzed in an optical microscope (Nikon Eclipse E200) with a 40× objective lens.

The mitotic index (MI%) was used to evaluate the cytotoxic potential. Cells in interphase, prophase, metaphase, aphase, and telophase were counted and calculated according to Equation (7), where TNDC is the total number of dividing cells and TNC is the total number of cells:

$$MI(\%) = \frac{TNDC}{TNC} \times 100. \quad (7)$$

The genotoxic potential was evaluated using micronucleus frequency, colchicine metaphases, anaphase and telophase bridges, gene amplifications, adhering cells, nuclear buttons, and multipolar anaphases, among other changes.

2.6 | French salad dressing added with FPHs

2.6.1 | Production of French salad dressing

To apply FPHs in a practical food system that demands emulsion stabilization, texture consistency, and oil oxidation stability, French salad dressing was selected. This widely consumed product offers an opportunity to substitute synthetic antioxidants, such as butylhydroxytoluene (BHT), and enhance its health benefits through the bioactivity and protein content of FPHs. Five French salad dressing oil-in-water emulsions were prepared as described by Gomes et al. (2008). FPH samples (C, EST, and UT) were used as antioxidants in 3 g FPH per 100 g of salad dressing, and the produced salad dressings were coded as C (control FPH, without pre-treatment), EST (FPH submitted to sterilization pre-treatment), and UT (FPH submitted to ultra-turrax pre-treatment). Also, one formulation was produced with 0.5 g BHT per 100 g (coded as BHT) and a control formulation without antioxidants (coded as FC).

The following ingredients were solubilized in water (32 g/100 g): tomato extract (9 g/100 g), skimmed milk powder (8 g/100 g), sugar (5.7 g/100 g), vinegar (3 g/100 g), salt (2 g/100 g), garlic powder (0.5 g/100 g), sweet paprika (0.5 g/100 g), mustard powder (0.2 g/100 g), monosodium glutamate (0.5 g/100 g), potassium sorbate (0.1 g/100 g), and FPHs or BHT. Next, sunflower oil (40 g/100 g) was added slowly under agitation, and then the salad dressing was homogenized in ultra-turrax at 15,000 rpm for 1 min in an ice bath. The salad dressings were stored at 4°C, and on the next day (day 1 of storage), the analyses of proximate composition (same methodology described in Section 2.2), optical microscopy, and rheology were performed. Color, texture, and oxidative stability analyses were performed at 0, 15, and 30 days of storage.

2.6.2 | French salad dressing's physical–chemical characterization

The morphology of the emulsions was evaluated to evaluate the emulsifier efficiency and to identify the presence of droplet aggregation or flocculation that could indicate potential instability issues. Samples were diluted in the proportion of 1:5 (v:v) with distilled water, and a drop of this dilution was placed on a glass slide and carefully covered with a coverslip. An optical microscope (Nikon Eclipse E200) was used and the images were captured using a 100× objective lens with a Moticam 2.0 MP digital camera.

Proper rheological properties ensure that the dressing achieves the desired consistency, whether creamy, thick, or pourable. In addition, understanding flow characteristics helps design stable salad dressings that do not separate or break down during storage or application. The rheological behavior of the formulations was evaluated in duplicate at 25°C using a rheometer (Brookfield DV-III Ultra) with spindle velocity from 0 to 100 rpm. The results obtained by the shear stress and viscosity curves were adjusted to the models: Power Law

(Equation 8), Herschel–Bulkley (Equation 9), Casson (Equation 10), and Bingham (Equation 11), where "τ" is the shear stress (N m^{-2}), "τ_0" is the yield stress (Pa), "η" is the apparent viscosity (mPa s), "γ" (s^{-1}) is the shear rate, "K" (Pa s^{-1}) is the consistency index, and "n" is the dimensionless flow behavior index. Rheological model parameters were determined using non-linear regression using Statistica 7.0 software (Statsoft, USA).

$$\tau = \eta \times \dot{\gamma}^{n}, \tag{8}$$

$$\tau = \tau_0 + K \times \dot{\gamma}^{n}, \tag{9}$$

$$\tau^{\frac{1}{2}} = K_0 + K \times \dot{\gamma}^{\frac{1}{2}}, \tag{10}$$

$$\tau = \tau_0 + \eta \times \dot{\gamma}. \tag{11}$$

During product development, texture and color evaluations are important since they provide insights into sensory properties, stability, and consumer experience. Texture measurements were carried out in a texture analyzer (TA.XT Express, Stable Micro Systems) with a 10 kg load cell with a back extrusion cell (compression probe of 35 mm diameter). The methodology was adapted from Rojas et al. (2019), in which the samples were subjected to 50% depth compression with a speed of 1 mm s^{-1} in cylindrical containers with 50 mm diameter and 75 mm height (50 mL of the sample). The firmness parameter was determined by measuring the maximum force. The consistency parameter was calculated by measuring the area under the curve until the maximum force was reached. The cohesion parameter was obtained by measuring the maximum negative force. Finally, the adhesion parameter was determined by measuring the area under the curve until the maximum negative force was reached (Liu et al., 2007). Samples of each treatment were analyzed in triplicate.

Color analysis was performed with a Delta Color colorimeter (Delta Vista 450G) in triplicate from each salad dressing formulation, determining L^*, a^*, b^*, and Chroma (C^*).

To monitor the effect of FPHs on salad dressing oil oxidative stability, the extinction coefficients (K_{270}, K_{232}, and ΔK) were determined to provide a detailed understanding of the oil's quality and shelf life. The Bligh and Dyer method was applied for oil extraction from the salad dressing samples. After that, oil samples were filtered in 0.45 µm syringe filters and diluted 1000× with isooctane solvent. The procedure was performed in duplicate, and the salad dressings' oxidation state was evaluated using a UV–Vis spectrophotometer, with the absorbance of the solutions at 232 (A_{232}), 266 (A_{266}), 270 (A_{270}), and 274 (A_{274}) nm (1 cm quartz cuvette). The extinction coefficients were determined according to Equations (12–14), respectively (Santos et al., 2020).

$$K_{270} = \frac{A_{270}}{c.l}. \tag{12}$$

$$K_{232} = \frac{A_{232}}{c.l}. \tag{13}$$

$$\Delta K = A_{270} - \left(\frac{A_{266} + A_{274}}{2}\right). \tag{14}$$

The extinction coefficients obtained were analyzed by hierarchical cluster analysis (HCA) (Santos et al., 2018) using the software MATLAB R2021a (Mathworks Inc., Natick, MA).

2.7 | Statistical analysis

Results were evaluated using analysis of variance (ANOVA). Averages were compared using the Tukey test at a 5% significance level ($p < 0.05$) using the software MATLAB R2021a. *A. cepa* results were analyzed by analysis of variance (ANOVA), and the mean values were compared by the Scott–Knott test with a significance of 0.05, using the software BioEstat®.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of FPHs

The yield, degree of hydrolysis (DH), free amino acids, and antioxidant activity (DPPH, FRAP, and ABTS) of fish protein hydrolysates obtained with different pre-treatments are described in Table 1.

The hydrolysis yield values determined for the hydrolysates submitted to the pre-treatments (EST and UT) and the control sample demonstrated that enzymatic hydrolysis was conducted properly. Pre-treatments did not affect this result since the values were statistically equal ($p > 0.05$). DH represents the percentage of cleavage of peptide bonds (Kristinsson & Rasco, 2000), being the ultra-turrax pre-treatment responsible for allowing a significantly higher ($p < 0.05$) cleavage by the enzyme during hydrolysis. The high physical shear promoted by the homogenization in ultra-turrax can disrupt protein aggregates (Koh et al., 2014), resulting in a higher exposition to enzyme action.

Concerning the free amino acids, it can be verified in Table 1 that results were significantly influenced by the different pre-treatments ($p < 0.05$). The FPH in which the substrate was submitted to the pre-treatment of sterilization (EST) presented a higher value of free amino acids, possibly due to the modifications in protein structure and denaturation resulting from the thermal treatment (Rivero-Pino et al., 2020a). On the other hand, among all FPH samples, the substrate pre-treated by homogenization (UT) presented the lowest free amino acid result. Thus, it is possible to conclude that although the UT pre-treatment has led to better contact between protein and enzyme, the hydrolysate produced was not as rich in free amino acids as the EST pre-treated sample.

3.1.1 | AChE activity inhibition of FPHs

Since AChE is one of the main enzymes related to Alzheimer's disease by the cholinergic hypothesis (Ju & Tam, 2022), its inhibition leads to

TABLE 1 Yield, degree of hydrolysis (DH), free amino acids, AChE activity inhibition, and mitotic indices (%) observed in root meristems of *Allium cepa* exposed to FPH at 24 and 48 h exposure times of FPHs undergoing pre-treatment before hydrolysis.

	C	EST	UT
Yield (%)	$81.01^{a} \pm 3.07$	$81.68^{a} \pm 2.87$	$77.19^{a} \pm 3.30$
DH (%)	$37.90^{a} \pm 0.34$	$37.66^{a} \pm 0.05$	$40.55^{b} \pm 0.25$
Free amino acid ($\mu mol_{Gly\ E}/g_{sample}$)	$1.057^{a} \pm 0.008$	$1.073^{b} \pm 0.001$	$1.013^{c} \pm 0.004$
AChE activity inhibition (%)			
15 mg mL^{-1}	$7.46^{a} \pm 1.50$	$9.54^{a} \pm 1.70$	$12.14^{a} \pm 3.90$
45 mg mL^{-1}	$20.07^{a} \pm 1.68$	$20.99^{a} \pm 2.18$	$29.58^{b} \pm 1.12$
60 mg mL^{-1}	$30.09^{a} \pm 2.52$	$27.39^{a} \pm 1.23$	$45.87^{b} \pm 3.20$
MI[#] (%)			
Co (0 h)	$19.6^{A} \pm 1.23$	$23.7^{A} \pm 0.97$	$20.4^{A} \pm 1.34$
24 h	$17.4^{A} \pm 1.74$	$14.5^{A} \pm 1.82$	$10.8^{A} \pm 1.69$
48 h	$15.3^{A} \pm 1.99$	$16.9^{A} \pm 1.92$	$13.5^{A} \pm 1.52$

Note: [a,b]The same letters in a row for yield, DH, free amino acids, and AChE activity inhibition refer to similar average result ($p > 0.5$ by Tukey's test) between the treatments. [A,B]The same letters refer to similar result averages between the exposure times considered (Co: 0, 24, and 48 h) at the same treatment by the Scott–Knott test at 0.05. MI (%) determined for the positive control methyl methanesulfonate (4×10^{-4} mol L^{-1}) = 4.78 ± 0.94%.
Abbreviations: C, control without pre-treatment; Co, control; EST, sterilization; MI: mitotic index; UT, ultra-turrax.

increased communication between neurons in the cholinergic pathway and nerve endings, resulting in lesser symptoms of Alzheimer's disease (Yener et al., 2020). AChE inhibition by FPHs ranged between 7.46% and 45.87% (Table 1). The AChE inhibition increased for all samples according to the concentration analyzed (15, 45, and 60 mg mL^{-1}). The results are similar to those found in previous work, in which Moreira et al. (2022) found 10.51%–40.45% of inhibition at FPH (Nile tilapia residues) concentrations of 20, 30, and 50 mg mL^{-1} under different hydrolysis conditions (without pre-treatment). Still, in Table 1, it is possible to note that for the for a lower concentration of FPH (15 mg mL^{-1}) resulted in no significant difference ($p > 0.05$) among the treatments. On the other hand, for concentrations of FPH equal to 45 and 60 mg mL^{-1}, the UT sample resented significantly higher ($p < 0.05$) inhibitory activity than the control and EST samples. Thus, it is possible to conclude that to guarantee significant inhibitory activity, the FPH must be pre-treated by homogenization and be applied at least at 60 mg mL^{-1}.

3.1.2 | Cytotoxic and genotoxic analysis in *Allium cepa* L.

The results of cytotoxicity and genotoxicity in vivo analyses of *Allium cepa* L. bulbs exposed to FPHs are presented in Table 1. It can be verified that there were no differences between the mitotic indices obtained for each exposure time within the same treatment and that none of the FPH treatments evaluated caused cytotoxicity to the meristematic cells of *A. cepa* root meristems. The results obtained through the bioassay with *A. cepa* have a satisfactory correlation with the effects observed in genetic tests performed in other bioassays, such as those with mammals and in cell culture (Herrero et al., 2012). This result indicates that the FPH produced in this work can be safely applied as an additive or bioactive ingredient in food.

3.1.3 | Functional and chemical properties of FPHs

Figure 1 presents the results of the solubility (a), emulsifying activity index (EAI) (b), emulsifying stability index (ESI) (c), and FTIR (d) analysis. The solubility of the FPHs was evaluated in the pH range from 3 to 11, as can be observed in Figure 1a. Solubility is an indicator of protein functionality widely used to evaluate the denaturation or aggregation of proteins. This property is highly pH-dependent and is linked to the performance of proteins when applied to foods, mainly in emulsions, foams, and gels (Chalamaiah et al., 2010; Zhang et al., 2019). The solubility remained stable in the range of pH 5–7. The lowest solubility for all samples was observed at pHs 3 and 9, possibly due to the difference in isoelectric points of the peptides, which were influenced by a load of acid and basic lateral groups. At pH 11, an intermediate solubility was found for all samples. However, the UT pre-treated sample had the highest stability for solubility among the samples. This difference in solubility between the samples may have resulted from modifications to the protein structure of the substrate (Thoresen et al., 2020). The high solubility of the UT sample may be attributed to peptide hydrolysis, which was the highest among all samples, resulting in smaller peptide sizes and greater hydrophilic properties. These findings suggest that applying the UT sample in food formulations could enhance sensory attributes such as appearance and mouthfeel, as reported in previous studies (Thiansilakul et al., 2007).

An increase in the emulsifying capacity and stability in basic pH can be observed in Figure 1b,c. In addition, EAI results corroborated the solubility values since EAI was higher in the pH range where the

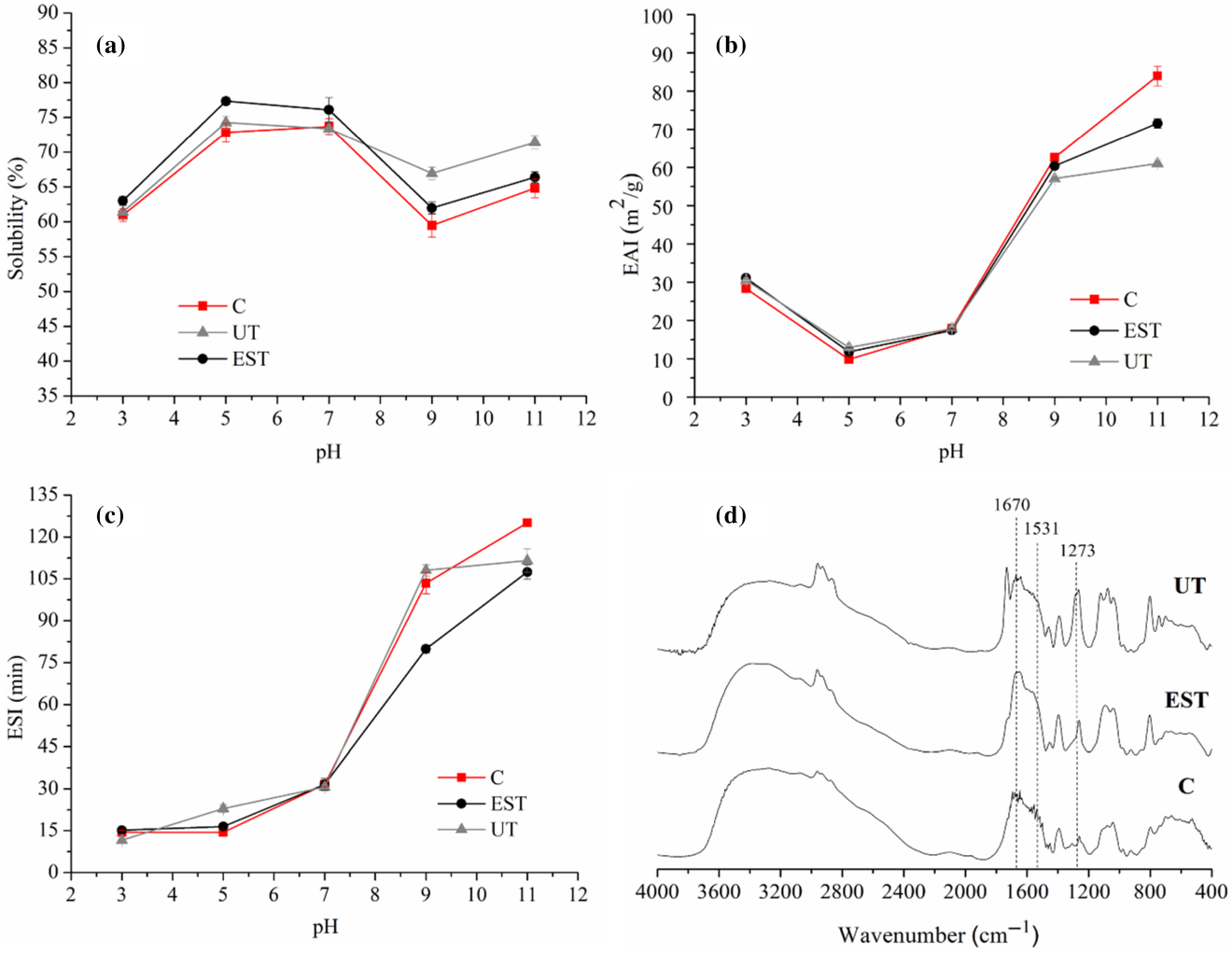

FIGURE 1 (a) Solubility, (b) emulsifying activity index (EAI), (c) emulsifying stability index (ESI), and (d) FTIR spectra of FPHs undergoing pre-treatment before hydrolysis. C, control without pre-treatment; EST, sterilization; UT, ultra-turrax.

solubility was lower. It is possibly related to the increased hydrophobicity of proteins in pH 9 and 11. Due to their negative charges, peptides can aggregate or undergo self-assembly interactions, forming a protective membrane around oil droplets during homogenization. This membrane prevents the coalescence of the droplets, thus stabilizing the emulsion (Gbogouri et al., 2004; Yesiltas et al., 2021). On the other hand, the higher solubility found from pH 5 to 7 may have resulted in an increased exposition of the peptides' hydrophilic sites, decreasing the hydrophobic–hydrophilic balance between the interaction sites at the oil–water interface and, consequently, reducing the emulsifying property. With this, undissolved peptides accumulate at the bottom of the oil drop and can cause deformation and decrease the surface tension of the emulsion (Yesiltas et al., 2021).

In the FTIR spectra (Figure 1d), the chemical structure of FPHs peptide-binding groups was evaluated by the characteristic bands: amide I (1670 cm^{-1}), amide II (1531 cm^{-1}), and amide III (1261 cm^{-1}) (Andrade et al., 2019). Usually, the amide I band at 1700–1600 cm^{-1} results from C=O stretching vibration, C–N stretching, and N–H bending vibrations (Stani et al., 2020). Amide I bands are widely associated with the secondary structure of proteins, the 1670 cm^{-1} band observed in FPH may be related to β-turn or β-sheet structures, which occur in 1675–1662 cm^{-1} (Vaskoska et al., 2021). Amide II (1600–1500 cm^{-1}), observed at 1531 cm^{-1}, may have been caused by the combination of the C–N elongation vibrations of the peptide with vibrations of curvature N–H. Amide III (1310–1175 cm^{-1}) band identified at 1261 cm^{-1} may be related to the elongation C–N, bending vibrations N–H, elongation C–C, and flexion C–H (Stani et al., 2020).

3.2 | Application of FPHs in French salad dressing

Figure 2 shows the emulsion microstructure and the appearance of the prepared salad dressings. The emulsion characteristics for the sample added with the FPH without pre-treatment (C) and the one with the synthetic antioxidant (BHT) are comparable. FC sample (control, without antioxidant or FPH) presented smaller droplets when compared to these samples. In contrast, the salad dressing produced

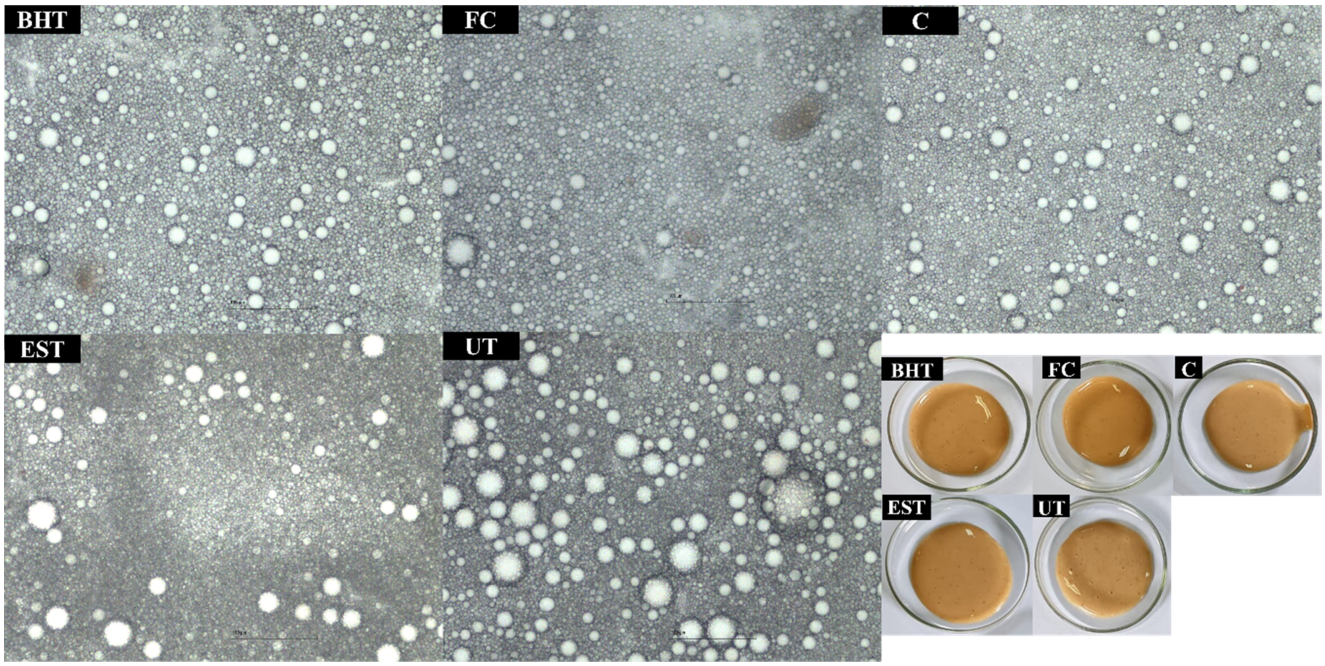

FIGURE 2 Optical microscopy (10×) and images of salad dressing emulsions added with BHT antioxidant (BHT), control formulation (FC, without antioxidants or FPHs), and FPHs samples (C, EST, and UT).

with sterilization pre-treated FPH (EST) shows fewer large droplets, while the UT pre-treated sample contains more large droplets. Since, in both cases, small and big droplets are present simultaneously, the emulsion may go through coalescence (when droplets merge together) or Ostwald ripening (the tendency of the molecules from the smaller droplets to diffuse and migrate to larger droplets due to differences in surface energy) (Hu et al., 2017). Coalescence and Ostwald ripening can affect the stability of the emulsion, leading to changes in droplet size distribution and potentially compromising the quality of the salad dressing.

The pH of all the salad dressings produced was ~4. While the data presented in Figure 1a–c provide some insights into the behavior of the produced fish protein hydrolysates as emulsion stabilizers across different pH levels, it is important to note that the salad dressing formulation includes numerous other ingredients. Consequently, FPHs may have interacted with other components of the formulation (Rivero-Pino et al., 2020b), such as the proteins from skimmed milk powder or been affected by changes in ionic strength due to the presence of salt and potassium sorbate. Among all the samples, the control (without any antioxidants or FPH (FC)) exhibited the most stable emulsion microstructure. This stability is likely due to the presence of skimmed milk in the salad dressing formulation, which acts as an effective emulsion stabilizer. In contrast, the interactions between BHT and other components in the BHT salad dressing formulation may have compromised its emulsifying effectiveness (Saga et al., 2013).

Table 2 shows that the protein content of Nile tilapia muscle in this study is 35.25%. This is notably higher than the protein content reported in other studies, which ranges from 13% to 19% (Dawit Moges et al., 2022; Dulal et al., 2023; Uehara et al., 2022). The discrepancy may be attributed to water loss during thawing (during sample preparation), which could have concentrated the sample and artificially elevated the protein content.

An analysis of the proximate composition of the salad dressings (Table 2) revealed a significant increase in protein content in the formulations containing FPH, up to twofold higher than FC and BHT formulations. The protein content of samples UT and C was similar. The same trend was determined between samples C and EST. The EST formulation exhibited the highest protein content of all treatments at 9.19%. Unnikrishnan et al. (2020) demonstrated that substituting egg yolk with yellowfin tuna red meat hydrolysate in mayonnaise led to a statistically significant increase in protein content, rising from 3.58% in the control formulation with egg yolk to 6.16%. Additionally, Lima et al. (2021) developed a functional yogurt by incorporating a protein hydrolysate from stripped weakfish (*Cynoscion guatucupa*) in both free and microencapsulated forms (the latter achieved via spray drying with maltodextrin as a wall material). The resulting protein contents for the control yogurt (without hydrolysate), yogurt with free hydrolysate, and yogurt with microencapsulated hydrolysate were 3.1%, 4.4%, and 4.35%, respectively.

The ash content was also significantly higher ($p < 0.05$) in the EST salad dressing. For moisture content, the highest values were found in the EST and BHT samples, which were statistically similar. No significant differences ($p > 0.05$) were observed in lipid content among the treatments.

TABLE 2 Proximate composition (w.b.) of Nile tilapia muscle (*Oreochromis niloticus*) and French salad dressing formulations.

	Fish muscle	FC	BHT	C	EST	UT
Proximate composition (%)						
Moisture	62.15 ± 2.35	32.44^{ab} ± 0.09	34.27^{ab} ± 0.31	33.12^{ab} ± 0.35	31.48^{a} ± 2.12	34.78^{b} ± 1.26
Ash	0.87 ± 0.09	2.99^{a} ± 0.10	3.044^{ab} ± 0.14	3.12^{ab} ± 0.02	4.22^{c} ± 0.02	3.41^{b} ± 0.29
Protein	35.25 ± 2.29	4.21^{a} ± 0.15	4.19^{a} ± 0.62	8.65^{bc} ± 0.09	9.19^{c} ± 0.09	7.78^{b} ± 0.15
Lipids	1.19 ± 0.74	44.39^{a} ± 0.92	45.30^{a} ± 3.37	46.19^{a} ± 1.36	45.38^{a} ± 2.10	45.85^{a} ± 3.14
Rheological characterization						
Power Law						
K^{c} (mPa s)	-	22.8	7166	3533	5675	3967
n	-	0.57	0.53	0.53	0.61	0.52
R^2	-	91.6	95.3	92.6	94.6	93.4
Herschel–Bulkley						
K (mPa s)	-	60.3	8916	2633	6708	2870
τ_0 (N/m^2)	-	0.05	1.23	1.95	0.53	1.60
n^{b}	-	0.40	0.47	0.58	0.56	0.59
R^2	-	99.8	99.2	97.4	99.1	98.1
Casson						
η (mPa s)	-	0.90	513.2	247.7	639	291.4
$\tau_0{}^{d}$ (N/m^2)	-	0.06	6.38	3.32	4.88	3.24
$R^{2\ e}$	-	93.9	95.4	95.1	96.0	95.9
Bingham						
η^{a} (mPa s)	-	1.44	842.5	422.3	962	481.2
τ_0 (N/m^2)	-	0.15	15.2	7.53	13.5	7.64
R^2	-	71.2	81.2	84.1	82.8	88.1

Note: ^{a,b}The same letters in a row (for proximate composition analysis) refer to similar average results ($p > 0.5$ by Tukey's test) between the treatments. Abbreviations: $^{a}\eta$, apparent viscosity; ^{b}n, behavior index; ^{c}K, consistency index; $^{d}\tau_0$, yield stress; $^{e}R^2$, coefficient of determination.

3.2.1 | Rheological behavior

Understanding the rheological key parameters (viscosity at different shear rates, flow behavior (pseudoplasticity or Newtonian behavior), and yield stress) helps in assessing how the addition of FPH influences the dressing's thickness and pourability. A stable and desirable viscosity contributes to a consistent product texture and ease of use.

Shear stress and viscosity curves (Supplementary Material) for salad dressing formulations were adjusted to the mathematical models (Power Law, Herschel–Bulkley, Casson, and Binghan) and are presented in Table 2.

All the mathematical models evaluated demonstrated the ability to adjust the experimental data. The Herschel–Bulkley model provided the best fit for all samples, with R^2 values exceeding 97%. All samples exhibited pseudoplastic behavior, as indicated by behavior index values (*n*) consistently below 1. In addition, all samples containing antioxidants (BHT and FPH) had a higher consistency index (*K*) and shear stress (τ_0) when compared to the control sample for all the mathematical models evaluated. It demonstrates the samples' viscous nature, and the shear stress is an important factor for salad dressing because it relates to a greater retention capacity of the sauce on the surface of the salad (Bortnowska et al., 2014; Liu et al., 2007).

Concerning the Herschel–Bulkley model parameters, the behavior index (*n*) showed an increase in the function of FPH addition to the salad dressing formulations. The highest value was found for UT, followed by C and EST. The samples presented shear thinning behavior ($0 < n < 1$). In emulsions, this behavior represents that the structure was irreversibly broken due to the shear rate, resulting in the redistribution of the oil drops that form the emulsion. The parameter $K > 0$ represents consistency and indicates that the FPH and BHT provided the natural viscosity of the fluid (Kumar et al., 2021; Saramito, 2009). On the other hand, the values of the τ_0 parameters observed in the samples indicate a high suspension capacity, an important property for FPH to be used as stabilizers in food, especially in sauces and mayonnaises (Hosseini-Parvar et al., 2010).

Among the samples containing FPHs, the EST formulation exhibited the lowest yield stress (τ_0), indicating a weaker emulsion structure and resulting in a more fluid or less cohesive system. Additionally, the EST sample displayed the highest consistency index among the FPH-enriched samples. This index reflects the viscosity of the salad dressing at low shear rates, meaning the EST dressing is thicker and more viscous when not in motion or under low-shear conditions. Considering both parameters, it can be concluded that the EST salad dressing will maintain a more stable texture and resist thinning under low-

TABLE 3 Color, texture parameters of salad dressings, and extinction coefficients (K_{270}, K_{232}, and ΔK) of the oil extracted from samples.

		Color parameters				Texture parameters				Extinction coefficients		
	Day	L^*	a^*	b^*	C^*	Firmness (N)	Cohesiveness (N)	Adhesion work (N s)	Consistency (N s)	K_{270}	K_{232}	ΔK
FC	0	$71.43^{ab,A} \pm 0.70$	$9.79^{ab,A} \pm 0.57$	$31.17^{a,A} \pm 0.39$	$32.68^{a,A} \pm 0.45$	$0.25^{a,A} \pm 0.02$	$-0.10^{a,A} \pm 0.00$	$-1.58^{a,A} \pm 0.10$	$3.64^{a,A} \pm 0.08$	$4.39^{ac,A} \pm 0.39$	$6.50^{a,A} \pm 0.41$	$4.04^{ac,A} \pm 0.35$
	15	$69.95^{a,B} \pm 0.49$	$10.88^{a,B} \pm 0.16$	$33.67^{a,B} \pm 0.50$	$35.38^{a,B} \pm 0.39$	$0.19^{a,B} \pm 0.00$	$-0.09^{a,A} \pm 0.00$	$-0.98^{a,B} \pm 0.12$	$2.98^{a,B} \pm 0.01$	$6.11^{a,AB} \pm 0.42$	$8.14^{ab,AB} \pm 0.03$	$5.61^{a,AB} \pm 0.40$
	30	$71.20^{a,A} \pm 0.64$	$10.20^{a,AB} \pm 0.81$	$33.84^{a,B} \pm 0.47$	$35.36^{a,B} \pm 0.36$	$0.25^{a,A} \pm 0.01$	$-0.09^{a,A} \pm 0.00$	$-1.12^{a,B} \pm 0.05$	$2.98^{a,B} \pm 0.13$	$6.65^{a,B} \pm 0.70$	$10.12^{b,B} \pm 0.93$	$6.11^{a,B} \pm 0.64$
BHT	0	$71.58^{b,A} \pm 0.47$	$10.23^{ab,A} \pm 0.38$	$31.23^{a,A} \pm 0.49$	$32.86^{a,A} \pm 0.49$	$0.18^{a,A} \pm 0.00$	$-0.10^{a,A} \pm 0.00$	$-1.39^{a,A} \pm 0.06$	$3.14^{a,A} \pm 0.07$	$5.37^{b,A} \pm 0.09$	$8.30^{c,A} \pm 0.09$	$4.93^{b,A} \pm 0.08$
	15	$70.22^{a,AB} \pm 0.60$	$10.73^{a,A} \pm 0.28$	$32.56^{b,B} \pm 0.33$	$34.29^{b,B} \pm 0.35$	$0.22^{a,B} \pm 0.00$	$-0.11^{a,A} \pm 0.00$	$-1.49^{a,A} \pm 0.20$	$3.52^{a,B} \pm 0.05$	$6.43^{a,B} \pm 0.11$	$9.46^{c,B} \pm 0.46$	$5.90^{a,B} \pm 0.10$
	30	$69.11^{a,B} \pm 1.50$	$10.64^{a,A} \pm 0.47$	$34.41^{a,C} \pm 0.64$	$36.02^{a,C} \pm 0.66$	$0.23^{a,C} \pm 0.00$	$-0.10^{a,A} \pm 0.00$	$-1.30^{a,A} \pm 0.08$	$3.45^{a,B} \pm 0.04$	$6.80^{a,B} \pm 0.04$	$9.76^{ab,B} \pm 0.10$	$6.23^{a,B} \pm 0.04$
C	0	$65.22^{c,AB} \pm 3.47$	$9.29^{b,A} \pm 1.023$	$33.15^{b,A} \pm 1.15$	$34.44^{b,A} \pm 1.13$	$1.33^{b,A} \pm 0.05$	$-0.78^{b,A} \pm 0.05$	$-15.25^{bc,A} \pm 1.11$	$19.85^{bc,A} \pm 0.94$	$5.14^{ab,A} \pm 0.05$	$6.95^{a,A} \pm 0.12$	$4.73^{ab,A} \pm 0.05$
	15	$67.11^{b,A} \pm 0.54$	$10.84^{a,A} \pm 0.33$	$34.07^{a,A} \pm 0.20$	$35.77^{a,A} \pm 0.15$	$1.31^{b,A} \pm 0.08$	$-0.92^{b,A} \pm 0.11$	$-15.30^{b,A} \pm 0.23$	$19.32^{b,A} \pm 1.28$	$6.24^{a,B} \pm 0.16$	$9.06^{bc,B} \pm 0.17$	$5.73^{a,B} \pm 0.14$
	30	$62.85^{b,B} \pm 2.53$	$10.03^{a,A} \pm 1.45$	$32.80^{a,A} \pm 1.45$	$34.30^{a,A} \pm 2.10$	$1.41^{b,A} \pm 0.10$	$-0.85^{b,A} \pm 0.10$	$-14.70^{b,A} \pm 0.42$	$19.74^{b,A} \pm 1.37$	$6.751^{a,C} \pm 0.007$	$9.23^{ab,B} \pm 0.18$	$6.197^{a,C} \pm 0.008$
EST	0	$68.74^{abc,A} \pm 3.02$	$10.83^{a,A} \pm 0.86$	$33.31^{b,A} \pm 0.71$	$35.05^{b,A} \pm 0.74$	$1.50^{c,A} \pm 0.09$	$-0.80^{b,A} \pm 0.12$	$-16.72^{b,A} \pm 0.97$	$21.80^{b,A} \pm 0.84$	$5.26^{ab,A} \pm 0.23$	$6.55^{a,A} \pm 0.05$	$4.83^{ab,A} \pm 0.20$
	15	$65.64^{c,AB} \pm 1.45$	$10.93^{a,A} \pm 0.19$	$33.87^{a,A} \pm 0.21$	$35.59^{a,A} \pm 0.20$	$1.39^{b,AB} \pm 0.09$	$-0.82^{b,A} \pm 0.05$	$-14.66^{b,AB} \pm 0.35$	$19.55^{b,A} \pm 2.05$	$5.83^{a,A} \pm 0.16$	$6.97^{a,AB} \pm 0.04$	$5.356^{a,A} \pm 0.008$
	30	$62.28^{bc,B} \pm 1.99$	$10.33^{a,A} \pm 1.02$	$33.25^{a,A} \pm 2.25$	$34.82^{a,A} \pm 2.40$	$1.26^{b,B} \pm 0.09$	$-0.83^{b,A} \pm 0.05$	$-14.37^{bc,B} \pm 1.03$	$18.30^{b,A} \pm 1.22$	$6.31^{a,A} \pm 0.54$	$7.79^{ac,B} \pm 0.50$	$5.80^{a,A} \pm 0.50$
UT	0	$67.75^{ac,A} \pm 1.75$	$10.53^{a,A} \pm 0.55$	$33.05^{b,AB} \pm 0.38$	$34.72^{b,AB} \pm 0.49$	$1.23^{b,A} \pm 0.05$	$-0.68^{b,A} \pm 0.02$	$-13.51^{c,A} \pm 0.44$	$18.54^{c,A} \pm 1.40$	$4.02^{c,A} \pm 0.27$	$5.20^{b,A} \pm 0.14$	$3.70^{c,A} \pm 0.25$
	15	$67.06^{b,A} \pm 0.67$	$10.74^{a,A} \pm 0.74$	$33.78^{a,A} \pm 0.63$	$35.46^{a,A} \pm 0.75$	$1.42^{b,B} \pm 0.09$	$-0.86^{b,B} \pm 0.07$	$-15.26^{b,B} \pm 0.40$	$19.42^{b,A} \pm 1.81$	$5.91^{a,B} \pm 0.39$	$7.11^{a,B} \pm 0.49$	$5.43^{a,B} \pm 0.36$
	30	$60.01^{c,B} \pm 0.62$	$9.75^{a,A} \pm 1.88$	$32.19^{a,B} \pm 1.11$	$33.67^{a,B} \pm 1.60$	$1.26^{b,AB} \pm 0.04$	$-0.78^{b,AB} \pm 0.03$	$-13.16^{c,A} \pm 0.06$	$18.29^{b,A} \pm 1.11$	$5.57^{a,AB} \pm 0.47$	$6.98^{c,B} \pm 0.48$	$5.12^{a,AB} \pm 0.43$

Note: ^{a,b}The same letters refer to similar average results ($p > 0.5$ by Tukey's test) between the treatments at the same storage time. ^{A,B}The same letters refer to similar average results ($p > 0.5$ by Tukey's test) for the same treatment across different storage times.

shear conditions. However, it will flow more easily and become less viscous when subjected to higher shear rates, making it easier to pour or mix (Martínez-Padilla, 2024).

The salad dressing with the control FPH (C) displayed the opposite behavior compared to the EST sample. Specifically, the higher yield stress and lower consistency index suggest that it will be more stable and cohesive at rest but may become less fluid and more resistant to mixing when disturbed, compared to the EST sample. These changes in the rheological behavior of the samples can be attributed to interactions between the FPHs and other components in the salad dressing formulation, as discussed in the results shown in Figure 2 (emulsion microstructure).

3.2.2 | Color and texture parameters

The back extrusion texture method measures the textural properties of the salad dressing, such as its firmness, consistency, and gel-like qualities. Color is a crucial aspect of food presentation and consumer appeal, so consistent and desirable color helps in creating a positive first impression and can affect salad dressing's perceived quality. It is important to ensure that salad dressings formulated with FPHs meet the expected standards for texture, color, and overall consumer satisfaction.

Table 3 presents the samples' color parameters (L^*, a^*, b^*, and C^*) and texture characteristics (firmness, cohesiveness, adhesion work, and consistency). Overall, there was minimal variation in color during storage. The samples maintained high luminosity (L^*), with a tendency toward red ($+a^*$), yellow ($+b^*$), and relatively low color intensity (C^*), which is consistent with the typical orange hue of French salad dressing.

In terms of texture, the formulations exhibited only slight variations over time. The BHT and FC formulations showed statistically similar texture parameters ($p > 0.05$) and differed significantly ($p < 0.05$) from those containing fish protein hydrolysates (FPHs). The FPH-containing formulations were similar to each other across all texture parameters. Their firmness, cohesiveness, adhesion work, and consistency values were comparable to those reported by Rojas et al. (2019) for mayonnaise samples.

3.2.3 | Oxidative stability

This test assesses the stability of the oil within the salad dressing, focusing on its resistance to oxidation over time. High oxidative stability indicates that the dressing has a longer shelf life and maintains its quality over time, so the presence of FPHs may impact this stability. The oxidative stability of salad dressings was evaluated using the extinction coefficients (K_{270}, K_{232}, and ΔK) as shown in Table 3 and Figure 3 by hierarchical cluster analysis (HCA).

The oxidation of polyunsaturated fatty acids can be verified at 232 and 270 nm by increased absorption in the ultraviolet region due

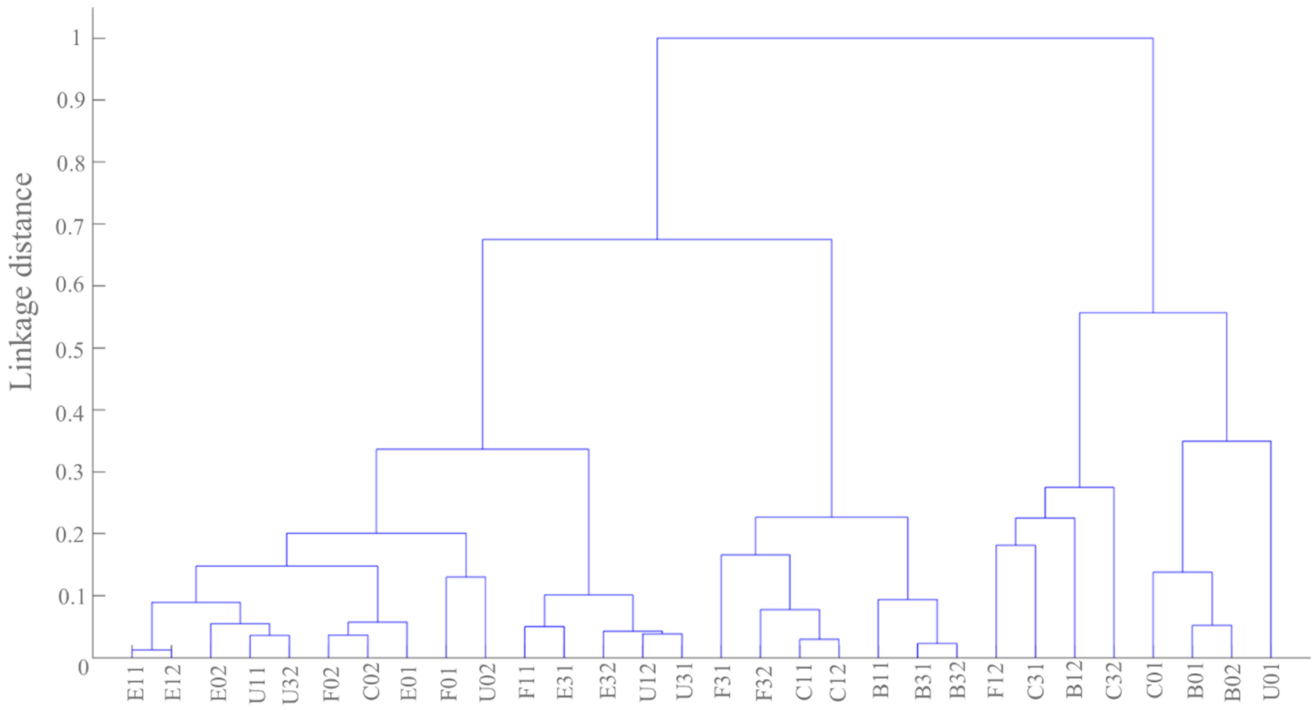

FIGURE 3 Hierarchical cluster analysis (HCA) of the extinction coefficients (K_{270}, K_{232}, and ΔK) data of salad dressing added with BHT (B), control formulation (F), and FPH samples: control (C), EST (E), and UT (U). *Samples codification: the letters represent the treatment followed by the storage time 0, 15, and 30 days (0, 1, and 3, respectively) and the replica of the experiment (1 or 2). For instance, B01 = BHT added salad dressing at 0 days of storage with its first replica.

to the formation of conjugated dienes and trienes (Santos et al., 2020). There was an increase in the extinction coefficient according to the storage time for all formulations, demonstrating the lipid oxidation in the salad dressing. It can be noted in Figure 3 that after 30 days of storage, the salad dressings without antioxidant added (F31 and F32) and added with FPH produced without pre-treatment (C31 and C32) are grouped, showing similarity. On the other hand, samples containing FPH that was pre-treated by sterilization and that were stored for 30 days (E31 and E32) were grouped with the control formulation (salad dressing without BHT or FPH), analyzed after 15 days (F11), as well as with the samples containing ultra-turrax pre-treated FPH, analyzed at 15 days (U12) and 30 days (U31). This indicates that the oxidation state of the oil in the salad dressing prepared without antioxidants (FC) was matched by the samples containing ultra-turrax pre-treated FPH after 15 days of storage, and this oxidation state remained similar after 30 days. Moreover, the salad dressing made with FPH pre-treated by sterilization took 30 days to reach the same oxidation level as the other FPHs tested, indicating superior preservation capacity. This result may be attributed to larger agglomerated oil droplets resulting from the higher solubility of UT, which reduced the proportion of amphiphilic proteins and protective membranes in the emulsions. Consequently, the oil in the UT-containing formulation had reduced exposure to water molecules, leading to less oxidation. Conversely, BHT, FC, and C samples were more susceptible to lipid oxidation.

4 | CONCLUSIONS

The FPHs pre-treatments by sterilization and homogenization were evaluated to characterize their effect on cytotoxicity (*Allium cepa* model), bioactive (AChE inhibition), and functional properties (solubility, emulsifying activity index, and emulsifying stability index) after enzymatic hydrolysis. Additionally, the study sought to analyze the technological properties of a real food system, specifically salad dressing, when incorporated with FPHs. These FPHs were considered concerning modifications in functional properties and their behavior when applied to salad dressing. The FPHs (C, EST, and UT samples) presented satisfactory yield values (up to 81.01%) and hydrolysis degree. In addition, FPHs showed AChE inhibition properties (significantly higher for FPH pre-treated by sterilization) and no cytotoxicity. Regarding functional properties, the highest solubility of FPHs occurred at pH 11. Emulsifying and emulsion stability capacity was also observed at basic pH. In salad dressings, the incorporation of FPHs resulted in pseudoplastic behavior, increased protein content, and preservation of the color and texture characteristics. Rheological analysis using the Herschel–Bulkley model revealed that the flow behaviors varied depending on the pre-treatment applied to the FPHs. Specifically, salad dressings with sterilized FPHs showed higher viscosity at low shear rates, while those containing ultra-turrax pre-treated FPHs, as well as those with non-pre-treated FPHs, exhibited lower viscosity under similar conditions. Also, FPHs demonstrated efficacy in assisting the oxidative stability of the salad dressings, thus indicating a possible action as radical scavengers. In the broader context of food science and nutrition, these findings highlight the potential of FPHs to improve both the functional and nutritional qualities of food products, making them valuable ingredients in enhancing food texture, stability, and health benefits. To advance the understanding of FPHs and their applications in food technology, the following steps should be considered: (i) Investigate novel uses of FPHs in food technology, such as developing functional foods, dietary supplements, or alternatives for specialized diets (e.g., hypoallergenic options). This exploration can open new market opportunities and applications for FPHs. (ii) Examine the digestibility of foods incorporating FPHs. Understanding how FPHs affect nutrient absorption and gastrointestinal health is important for ensuring that these hydrolysates enhance not only the functionality but also the nutritional value of the food. (iii) Assess the impact of FPHs on the sensory qualities of food, including taste, aroma, and mouthfeel, to ensure they enhance or do not adversely affect the overall consumer experience. (iv) Evaluate the economic feasibility of using FPHs in various food products.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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