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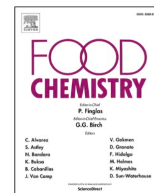

Evaluation of functional and nutritional potential of a protein concentrate from *Pleurotus ostreatus* mushroom

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ABSTRACT

Edible mushrooms used as a protein-rich food may be an attractive alternative to conventional protein sources, while promoting its valorization. This work aimed to obtain a protein concentrate from a *Pleurotus ostreatus* mushroom flour, its characterization, and nutritional and functional properties evaluation. Methodologies applied for extraction and precipitation of protein were optimized - pH 4 and 12, respectively; and flour-solvent ratio of 1:20 w/v. The protein density was increased by 78%. *P. ostreatus* flour and concentrate were characterized by proximal composition. The content of total phenolic compounds in the protein concentrate decreased, leading to a positive effect on protein digestibility, while the DPPH radical scavenging activity was not significantly affected. Peptides with molecular weights from 12 to 35 kDa, with possible bioactivity, were identified by electrophoresis. Protein digestibility assessed by *in vitro* gastrointestinal digestion showed a 4.2-fold higher hydrolysis degree in the protein concentrate than the flour.

1. Introduction

Protein is an essential macronutrient to human body growth and maintenance due to its important physiological functions, such as vital performance of hormones and enzymes action (Bhutta, Sadiq, & Aga, 2013). Proteins of animal origin have a high nutritional quality. However, its associated production cost is extremely high when compared to the production of vegetable proteins. For instance, the production of 1 kg of meat requires between 5 and 40 times more water than the production of 1 kg of cereal, which increases the meat production cost up to 10 times. Meat production also leads to high greenhouse gas emission, resulting in a relevant environmental impact (Kumar et al., 2017). In addition, some foods of animal origin (e.g., red and processed meats) have been associated with human health risks, namely cardiovascular diseases and colon cancer (Windey, Preter, & Verbeke, 2012; Wolfe, Baum, Starck, & Moughan, 2018). One possible way to overcome these issues is to promote the consumption of other protein sources (e.g., protein from vegetal and fungal sources).

The interest in proteins from plant sources as an alternative to animal proteins has been growing in the last decades. Mainly due to its reduced production cost, abundant supply, and content of bioactive and phytochemical substances (Sá, Moreno, & Carciofi, 2020). However, the use of plant protein is still limited, as it lacks one or more essential amino acids to classify it as a complete protein (Kurpad, 2013). For example, cereals contain low-values of lysine, and legumes are deficient in sulfur amino acids (e.g., methionine and cysteine) (Sá et al., 2020).

Recently, proteins of fungal origin have gained the attention from food industry players and scientific community, due to its high nutritional values associated to the rich level of essential amino acids when compared to vegetables (Bach et al., 2017). Generally, the cultivation of mushrooms is faster and cheaper as compared to vegetables, since they can be easily cultivated using agro-industrial residues (Lavelli, Proserpio, Gallotti, Laureati, & Pagliarini, 2018). Moreover, mushroom proteins have high thermal and pH stability (Erjavec, Kos, Ravnikar, Dreo, & Sabotič, 2012). The use of edible mushrooms for the development of protein rich food products could provide an attractive alternative to an animal protein source while promoting its valorization.

It has been reported that the protein digestibility of mushrooms

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ranges from 60 to 70% (Lavelli et al., 2018). However, the digestibility of proteins can be improved by removing food components that hamper its hydrolysis in the gastrointestinal tract. For instance, fiber content decreases protein density; reducing sugars are subject to Maillard reaction and decrease the assimilable lysine, methionine, and tryptophan; residual lipids can be oxidized leading to rancidity, or when associated with proteins can cause thickening problems during food processing and storage (Vioque, Sánchez-Vioque, Pedroche, Yust, & Millán, 2001). In addition, it has been reported that edible mushrooms possess bioactive peptides with beneficial effects for human health (e.g., antimicrobial, hypocholesterolemic), which are inactive within the protein, but can be released during enzymatic proteolysis (e.g., gastrointestinal digestion, *in vitro* hydrolysis with proteases) or by food processing (e.g., cooking, fermentation, ripening), providing the functional properties associated to such peptides (Xu, Yan, Chen, & Zhang, 2011). Moreover, phenolic compounds in mushrooms have been related with antioxidant activity, inhibition of lipid peroxidation, scavenge reactive oxygen species, and chelating activity on ferrous ions (Jayakumar, Thomas, Sheu, & Geraldine, 2011).

Despite all these studies on mushrooms, there is a lack of information regarding the processing of its protein concentrate and on elucidation of how this process affects the protein digestibility and its antioxidant activity. In this regard, this study aims to evaluate the edible mushroom *P. ostreatus* flour as a raw material for development of a protein concentrate.

2. Materials and methods

2.1. Reagents

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): sodium carbonate (≥99%), copper(II) sulfate pentahydrate (≥98%), potassium sodium tartrate tetrahydrate (≥99%), sodium hydroxide (≥97%), Folin-Ciocalteu's phenol reagent, Bovine Serum Albumin (BSA), sodium dodecyl sulfate (SDS), β-mercaptoethanol, bromophenol blue, Coomassie Brilliant Blue (R-250), tetramethylethylenediamine (TEMED), gallic acid (3,4,5 trihydroxybenzoic acid), 2,2 diphenyl-1picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), pepsin from porcine gastric mucosa, α- amylase and pancreatin from porcine pancreas, bile extract porcine and pefabloc® SC. Acrylamide/Bis solution (29:1) was purchased from Bio-Rad (California, USA), whereas calcium chloride was obtained from Panreac (Barcelona, Spain). Hydrochloric acid, *n*-hexane (≥95%), methanol, glacial acetic acid, and 2,4,6 trinitrobenzene sulfonic acid (TNBSA) were purchased from Thermo Fisher Scientific (Bremen, Germany) whereas serine was obtained from Acros Organics (Belgium).

2.2. Raw material preparation

P. ostreatus mushrooms were grown at the Antonio Narro Autonomous Agrarian University. The mushrooms were cultivated in dark conditions for 40 days, at temperature ranging between 25 and 28 °C with 60% moisture, using sorghum forage as substrate. *P. ostreatus* flour was obtained by drying the fruiting bodies in an electric oven, at a temperature of 60 °C for 72 h (Tolera & Abera, 2017) followed by grinding in a Retsch® SM 100 grinder and sifting through a 355 µm sieve.

2.3. Optimization of process conditions to obtain a protein concentrate

Initially, the *P. ostreatus* flour was defatted according to Cruz-Solorio, Garín-Aguilar, Leal-Lara, Ramírez Sotelo & Valencia-del Toro (2014) methodology. Mushroom flour was dispersed in *n*-hexane solution at a ratio of 1:5 (w/v), continuously stirred with magnetic agitation at a rotation speed of 80 rpm during 8 h at 4 °C. Subsequently, the hexane was decanted to remove fat, and the powder was allowed to dry completely. The methodology used to obtain the protein concentrate was studied in terms of the effect of pH value and flour-solvent ratio upon protein solubilization.

2.3.1. Protein solubility

The protein solubility was evaluated according to the method described by Cruz-Solorio et al. (2014) with some modifications. Briefly, 1% (w/v) *P. ostreatus* flour was dispersed in distilled water, and the pH value of the solution was adjusted by ranging between 2 and 12 using 1 mol L^{-1} HCl and/or 1 mol L^{-1} NaOH, as necessary. Subsequently, the resulting solutions were agitated in a vortex and centrifuged at 12 400 × *g* for 30 min Then, the soluble protein was evaluated in the supernatant following Lowry, Rosebrough, Farr & Randall (1951) methodology. For this purpose, the sample (100 µL) was mixed with 25 µL of a solution containing 2% Na_2CO_3, 1% $CuSO_4 \cdot 5\ H_2O$ and 2% sodium–potassium tartrate (at 1:1:8 ratio), and maintained in dark conditions, at room temperature for 10 min. Then, 10 µL of the diluted Folin-Ciocalteau reagent (1:2) was added to the sample, which remained for 30 min in the same conditions as previously described. The protein solubility was quantified by measurement of the absorbance at wavelength 630 nm in Synergy™ HT Multi-mode Microplate Reader (Biotek Instruments, Winooski, VT, USA). Standard solutions of BSA were used to establish an appropriate calibration curve ($y = 0.0008x + 0.0129$; $R^2 = 0.98$; where *y* and *x* were the absorbance and concentration of BSA, respectively).

2.3.2. Mushroom-solvent ratio

Mushroom flour was dispersed in distilled water at several ratios (1:5, 1:10, and 1:20 w/v). The pH of the solution was adjusted to the value whereby the maximum protein solubility was achieved in the previous assay (pH 12) using 2 mol L^{-1} NaOH. Afterward, the mushroom flour solution was centrifuged at 12 400 × *g* for 20 min at 4 °C. The supernatant was collected, and the solution was adjusted to the isoelectric pH value (pH 4, previously assessed) with 2 mol L^{-1} HCl. Then, the sample was centrifuged again using the same conditions established previously, and the precipitate was collected. An aliquot (1 mL) of each supernatant was used for soluble protein content determination, as described in Section 2.3.1. Protein concentrate samples were dehydrated by freeze-drying, in order to obtain a lyophilized powder. Protein yield was estimated by the following equation:

$$Yield(\%) = \left(\frac{EP}{TP}\right)x100 \quad (1)$$

where *EP* is the amount of extracted protein (g) and *TP* is the amount of total protein (g) in the mushroom flour.

2.4. Proximate analysis

The amount of moisture, ash, lipids, protein, crude fiber, and carbohydrates of *P. ostreatus* flour and the protein concentrate were determined by AOAC methods (A.O.A.C, 1995).

2.5. Electrophoresis

The electrophoretic profile of the protein concentrate was determined following the protocol used by Kimatu et al. (2017) with some modifications. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad®) at 400 mA and 115 V. The resolving and stacking gel contained 30% acrylamide/bis solution (29:1) at concentrations of 12% and 30%, respectively. Samples were prepared at 20 mg mL^{-1} in deionized sterile water and filtered through 0.45 µm nylon membrane filter (Corning®). The loading buffer containing 2% w/v SDS, 5% v/v β-mercaptoethanol, 1% v/v glycerol and 0.001% w/v

bromophenol blue, was mixed with protein concentrate samples (4:1 ratio). The resulting solution was heated at 95 °C during 5 min through a temperature-controlled water bath to promote protein denaturation. The gel was stained with a Coomassie Blue (R-250) solution, containing 40% and 10% of methanol and glacial acetic acid, respectively. Then, the gel was destained with acetic acid and methanol solution, both at concentration of 10%. Standard marker Bio-Basic® prestained protein ladder (molecular weight ranging from 10 to 250 kDa) was used to identify the sample by their molecular weight.

2.6. Total phenolic content and antioxidant capabilities

2.6.1. Determination of total phenols

Total phenolic content of mushroom flour and protein concentrate was determined by the Folin-Ciocalteu assay according to the method used by Goiris et al. (2012). Sample (0.2 mL) at concentration of 20 mg mL^{-1} was mixed with 1.5 mL of the diluted Folin-Ciocalteau reagent (1:2). The resulting solution was maintained during 5 min at room temperature (25 °C). Then, 60 g L^{-1} sodium bicarbonate solution (1.5 mL) was added to the mixture and incubated for 90 min at room temperature. The sample absorbance was evaluated spectrophotometrically at wavelength 750 nm. Methanolic solutions of gallic acid with concentration ranging from 25 to 150 mg L^{-1} were used as a standard in an appropriate calibration curve ($y = 0.0042x + 0.0713$; $R^2 = 0.97$; where y and $\times$ were the absorbance and concentration of gallic acid, respectively). The results were expressed as mg gallic acid equivalent (G.A.E.) g^{-1} of mushroom flour or protein concentrate.

2.6.2. DPPH radical scavenging activity

The DPPH assay was performed to evaluate the radical scavenging ability (*RSA*) of mushroom flour and protein concentrate, according to the procedure described by Coelho, Aquino, Latorres, & Salas-Mellado (2019). A sample (0.4 mL) at concentration of 20 mg mL^{-1} was added to 0.4 mL of 0.2 mmol L^{-1} $DPPH^{+}$ and mixed vigorously. The resulting solution was incubated at 25 °C for 45 min in the dark before the analysis. The absorbance of the samples was measured at wavelength 515 nm. The DPPH scavenging ability was calculated according to a Trolox calibration curve ($y = -0.0002x + 0.1543$; $R^2 = 0.95$; where y and $\times$ are the absorbance and concentration of Trolox, respectively).

The radical inhibition rate was assessed by Equation 2.

$$InhibitionRate(\%) = \langle 1 - \left(\frac{A_{sample}}{A_{control}}\right)\rangle x100 \quad (2)$$

where A_{sample} is the absorbance value of the sample and $A_{control}$ is the absorbance value of the control.

The results of radical scavenging ability were expressed as IC_{50}. Namely, the amount of antioxidant required to reduce the concentration of the free radical by 50% and was estimated as follows:

$$IC_{50} = \left(\frac{C_{sample}}{IR}\right)x50 \quad (3)$$

where C_{sample} is the concentration of antioxidants in the sample (determined by the Trolox calibration curve formula) and *IR* is the inhibition rate of the sample (determined by Equation 2).

Aditionally, the IC_{50} of Trolox (10.81 ± 0.1 for DPPH and 23.15 ± 4.0 for ABTS) was used to calculate the Trolox Equivalent Antioxidant Capacity (TEAC) of the samples by using Equation 4 (Ferreira-Santos et al., 2020):

$$TEAC = \left(\frac{IC_{50}Trolox}{IIC_{50}Sample}\right) \quad (4)$$

The higher *TEAC* value means the higher *RSA*.

2.6.3. ABTS radical scavenging assay

The $ABTS^{\cdot+}$ scavenging activity was determined according to the method used by Coelho et al. (2019). Briefly, 7 mmol L^{-1} ABTS solution was dispersed in 2.45 mmol L^{-1} potassium persulfate (at 1:1 ratio). Then, the resulting solution was stored for 16 h at room temperature (25 °C) in dark conditions. The ABTS solution was diluted in ethanol in order to achieve ABTS absorbance value of 0.70, at a wavelength of 734 nm. Afterward, ABTS radical solution (0.6 mL) was added to 0.2 mL of mushroom flour or protein concentrate solution, both in the concentration of 20 mg mL^{-1}. An accurate determination of absorbance value was performed in a spectrophotometer at wavelength 734 nm started after 5 min. An appropriate calibration curve was used with Trolox as a standard ($y = -0.0002x + 0.1543$ $R^2 = 0.95$; where y and $\times$ were the absorbance and concentration of Trolox, respectively). The rate of inhibition of the $ABTS^{\cdot+}$ radical was determined using Equation 2.

2.7. Gastrointestinal digestion

2.7.1. In vitro digestion of protein concentrate and mushroom flour

The *in vitro* digestion experiment was performed as previously described by Nobre et al. (2018). The procedure was based on the harmonized INFOGEST *in vitro* digestion protocol to simulate the oral, gastric, and intestinal conditions (Minekus et al., 2014). Samples (5 mL) were prepared by dispersing 20 mg mL^{-1} protein concentrate or mushroom flour in deionized water and stored at refrigeration temperature (5 °C) overnight to ensure the sample full rehydration.

Oral phase simulation consisted in the addition of simulated salivary fluid (SSF) (KCl 15.1 mmol L^{-1}, KH_2PO_4 3.7 mmol L^{-1}, $NaHCO_3$ 13.6 mmol L^{-1}, $MgCl_2{\cdot}(H_2O)_6$ 0.15 mmol L^{-1}, $(NH_4)_2{\cdot}CO_3$ 0.06 mmol L^{-1}, and HCl 1.1 mmol·L^{-1}), $CaCl_2{\cdot}(H_2O)_2$ (to reach the concentration of 1.5 mmol L^{-1}), and α-amylase enzyme solution (to obtain 75 U mL^{-1} activity). Samples (5 mL) were incubated for 2 min at 37 °C in a water bath under constant shaking (120 horizontal strokes per minute).

The stomach conditions were mimicked by the addition of simulated gastric fluid (SGF) (KCl 6.9 mmol L^{-1}, KH_2PO_4 0.9 mmol L^{-1}, $NaHCO_3$ 25 mmol L^{-1}, NaCl 47.2 mmol L^{-1}, $MgCl_2{\cdot}(H_2O)_6$ 0.1 mmol L^{-1}, $(NH_4)_2{\cdot}CO_3$ 0.5 mmol L^{-1} and HCl 15.6 mmol L^{-1}), $CaCl_2{\cdot}(H_2O)_2$ (to achieve the concentration of 0.15 mmol L^{-1} in the fluid), and porcine pepsin solution (with an activity of 2000 U mL^{-1} in the final mixture). The pH value was adjusted to 3.0 with 1 mol L^{-1} HCl, and the gastric phase volume was complete with milli-Q water. The samples were incubated in a shaking water bath at 37 °C during 2 h.

The intestinal phase conditions were mimicked by the addition of simulated intestinal fluid (SIF) (KCl 6.8 mmol L^{-1}, KH_2PO_4 0.8 mmol L^{-1}, $NaHCO_3$ 85 mmol L^{-1}, NaCl 38.4 mmol L^{-1}, $MgCl_2{\cdot}(H_2O)_6$ 0.33 mmol L^{-1}, and HCl 8.4 mmol L^{-1}), $CaCl_2{\cdot}(H_2O)_2$ (to obtain 0.6 mmol L^{-1} in the fluid), pancreatin suspension (with an activity of 100 U mL^{-1} in the final mixture) and bile solution (to obtain 10 mmol L^{-1} in the final mixture), both prepared in SIF solution. The pH value was adjusted to 7.0 with 1 mol L^{-1} NaOH, when necessary. Samples were incubated in the shaking water bath at 37 °C for 2 h. At the end of the intestinal phase, the reaction was stopped by adding 1 mmol L^{-1} of enzyme inhibitor pefabloc® SC.

Samples were collected after the oral phase, every 30 min during gastric and intestinal phases, and cooled in ice before measurements. All samples were tested at least in triplicate.

2.7.2. Protein hydrolysis degree during in vitro digestion

The protein hydrolysis degree (*HD*) of mushroom flour and protein concentrate during *in vitro* digestion was performed according to Simões, Martins, Pinheiro, Vicente, & Ramos (2020) procedure. Briefly, a sample (0.5 mL) of each *in vitro* digestion phase was diluted in 0.1 mol L^{-1} $NaHCO_3$ at pH 8.5 to achieve a final protein concentration range from 0.05 to 0.2 mg mL^{-1}. Afterward, 0.25 mL of 0.1% TNBSA was added to the resulting solution (0.5 mL) and incubated at 37 °C during 2 h with gentle agitation. Subsequently, 0.25 mL of 10% SDS and 0.125 mL of 1 mol L^{-1} HCl were added to each sample and homogenized. Taking into consideration the potential interference of the pepsin from

the gastric juice and pancreatin from intestinal phase on the measurements, an *in vitro* digestion without sample was performed and used as blank.

Samples (0.2 mL) were transferred to a 96-well microplate and the absorbance measurements were carried out at wavelength 335 nm. All samples were analyzed in triplicate.

The free amino groups content was determined using serine standard curve (y = 0.226x + 0.2431; R^2 = 0.98; where *y* and *x* were the absorbance and serine concentration, respectively). The *HD* was estimated according to the percentage of peptide bonds cleaved in the total number of peptide bonds using the following equation:

$$HD(\%) = \left(\frac{h_{sample}}{h_{total}}\right)x100 \tag{4}$$

where h_{sample} is the number of peptide bonds cleaved in sample after each *in vitro* digestion phase and h_{total} is the total number of peptide bonds in the sample after the intestinal phase.

2.8. Statistical procedures

All experiments were carried out at least in triplicate and data were expressed as the average ± standard deviation (SD). One-way analysis of variance (ANOVA) was carried out coupled with Tukey mean comparison test with a significance level of 5% using Statistica® software version 7.0 (Statsoft, USA). Results were considered significantly different for *p*-values $\leq$ 0.05.

3. Results and discussion

3.1. Optimization of the protein concentration process

The protein extraction process from mushroom flour is influenced by several factors, for instance, pH value, temperature, solvent type, solvent ratio, extraction time and ionic strength (Arango, Patiño, García, Calcedo, & Guerrero, 2012). Several studies have demonstrated that the pH value and solvent ratio were the main factors that impacted the protein extraction yield (Firatligil-Durmus & Evranuz, 2010; Jarpa-Parra et al., 2014). Thus, its influence was herein studied.

3.1.1. Effect of pH on protein solubility

Results obtained regarding the influence on mushroom flour protein solubility by adjusting the supernatant to various pH values are shown in Fig. 1. The solubility of the protein varied between 0.035 ± 0.005 and 0.51 ± 0.02 mg mL^{-1}. The results showed that by increasing the pH value from 4.0 to 12.0, the protein solubility of mushroom flour also increased. The lowest protein solubility was achieved for samples at pH between 3.0 and 4.0, namely 0.065 ± 0.005 and 0.035 ± 0.005 mg mL^{-1}, respectively. This pH range is relatively close to the isoelectric point, at which the protein net load is close to zero and the repulsive electrostatic force is weakened. Consequently, the solubility of the protein tends to decrease and precipitate (Simões, Araújo, Vicente, & Ramos, 2020). These observations agree with those reported by Cruz-Solorio et al. (2014), which by alkaline extraction and isoelectric precipitation of the protein obtained from three strains of *Pleurotus* spp., identified an isoelectric point in a range between pH 3.96 and 4.15.

The maximum protein solubility of 0.51 ± 0.02 mg mL^{-1} ($p \leq 0.05$) was reached at pH 12 (Fig. 1). A high net load is achieved by the proteins at alkaline environments, which results in higher protein solubility. In addition, it has been reported that high alkaline concentrations contribute to the breakdown of hydrogen bonds and to the dissociation of hydrogen from sulfate and carbonyl groups (Hadidi, Khaksar, Pagan, & Ibarz, 2019; Jarpa-Parra et al., 2014). These results are also in agreement with those reported by Cruz-Solorio et al. (2014), who observed a maximum protein solubility of a *Pleurotus* flour by adjusting the pH value to 12. Thus, pH 12 was selected as the optimal for protein extraction as it promotes protein solubility, and pH 4 was chosen for the precipitation phase because at this pH the net load decreases and the proteins tend to aggregate and precipitate, allowing a better separation from the rest of the compounds present in the solution.

3.1.2. Effect of mushroom flour-solvent ratio on protein extraction yield

The effect of the mushroom flour-solvent ratio on the protein extraction yield is shown in Fig. 2. The solubility of the protein varied from 5.4 ± 0.2 to 9.7 ± 0.2 mg mL^{-1} ($p \leq 0.05$). The yield was estimated by Equation 1 (section 2.3.2). Considering the initial protein content of the mushroom flour of 32% d.w. (section 3.2), a protein extraction yield of 17%, 25% and 30% was determined for extractions using 1:5, 1:10 and 1:20 solvent ratios, respectively.

Results showed that the protein yield increases with the amount of solvent applied to the mushroom flour. Significant differences were found for assays where the mushroom flour-solvent ratio varied from 1:5 to 1:20 ($p \leq 0.05$). While when increasing the ratio from 1:5 to 1:10 and from 1:10 to 1:20 no significant differences were found on the protein solubility.

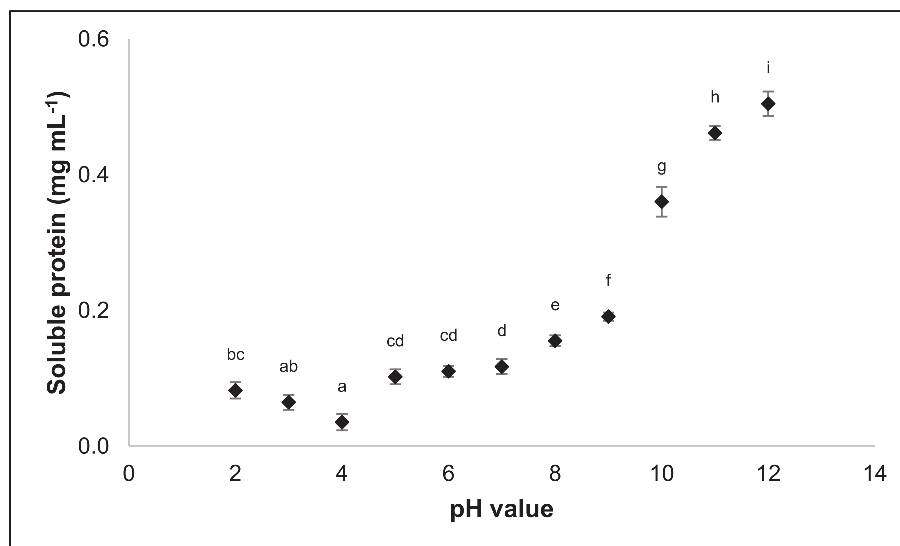

Fig. 1. Effect of the pH on the protein solubility. Results are given as mean ± standard deviation. Different superscript letters correspond to significantly different solubility ($p \leq 0.05$).

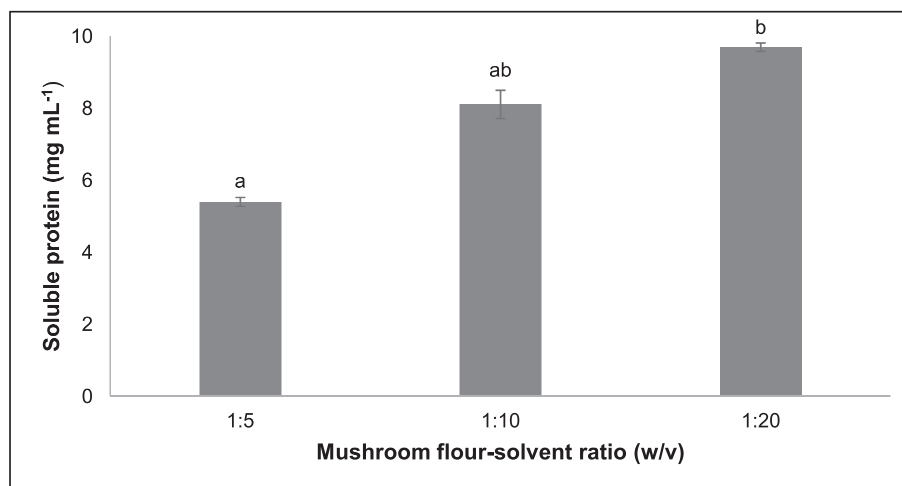

Fig. 2. Effect of mushroom flour-solvent ratio on the protein extraction yield. Results are given as mean ± standard deviation. Different superscript letters correspond to significantly different solubility ($p \leq 0.05$).

According to literature, by increasing the solvent ratio, the mass transfer coefficient is enhanced. Results showed that the diffusivity of the solvent in the solid and the protein desorption improve, resulting in a better extraction of protein (Arango et al., 2012; Hadidi et al., 2019). Results achieved in this study were in the same line of those observed in the literature. For example, in a study where a protein concentrate from *Erythrina edulis* flour was obtained, the protein yield raised by increasing the flour-solvent ratio from 1:20 to 1:40 (Arango et al., 2012). In another study, aiming at extract alfalfa protein, it was observed that by increasing the flour-solvent ratio from 1:20 to 1:45 (w/v) an enhanced extraction yield and protein content was obtained (Hadidi et al., 2019).

Based on the best results obtained, considering the effect of pH in the protein solubility and the mushroom flour-solvent ratio on the protein yield, a pH value of 12 and 4 were selected for the extraction and precipitation phases, respectively, and a 1:20 (w/v) mushroom flour-solvent ratio was selected for the extractions. These conditions were applied in the further experiments.

3.2. Proximate composition of mushroom flour and protein concentrate

The nutritional value of mushrooms, as well as its bioactive compounds content, change according to the species, strain, the stage of maturation, the substrate and the growing conditions used (Bach et al., 2017; Valverde, Hernández-Pérez, & Paredes-López, 2015). Table 1 shows the proximate composition of *P. ostreatus* mushroom flour and protein concentrate.

The lipid content obtained for the mushroom flour (1.9 ± 0.1% d.w.) was similar to those reported for *P. ostreatus* fruiting bodies cultivated on printed paper (1.68 ± 0.49% d.w.) or olive press cake (1.64 ± 0.35% d.w.) (Fernandes, Barros, Martins, Herbert & Ferreira, 2015; Koutrotsios, Mountzouris, Chatzipavlidis, & Zervakis, 2014). The lipid content of *P. ostreatus* mushrooms is generally low, ranging from 0.5 to 7.6% d.w. (Carrasco-González, Serna-Saldivar, & Gutierrez Uribe, 2017). Nevertheless, it contains essential fatty acids, such as oleic, linoleic and linolenic acids, which take part in a wide range of physiological functions, such as the prevention of cardiovascular diseases and arthritis, the decrease of triglyceride levels and blood pressure, and the promotion of brain, eye and heart health (Majesty, Ijeoma, Winner, & Prince, 2019; Valverde et al., 2015). Edible mushrooms mostly contain polyunsaturated fatty acids, thus, its regular intake may contribute to the reduction of serum cholesterol. The major sterol produced by edible mushrooms is ergosterol, a precursor of vitamin D_2, which has antioxidant properties and prevents cardiovascular diseases (Valverde et al., 2015). Foods with low-fat content, such as *Pleurotus* mushrooms, are suitable for healthy and calorie restriction diets.

Table 1
Proximate composition of mushroom flour and protein concentrate in % dry weight.

	Mushroom flour	Protein concentrate
Lipids	1.9 ± 0.1[a]	7.1 ± 0.5[b]
Protein	32 ± 1[a]	57 ± 2[b]
Carbohydrates	50.5 ± 0.2[a]	20.0 ± 0.4[b]
Crude fiber	5.6 ± 0.5[a]	0.5 ± 0.1[b]
Moisture	5.6 ± 0.3[a]	6.5 ± 0.9[a]
Ash	9.9 ± 0.2[a]	9.4 ± 0.3[a]

NOTE: Results are given as mean ± standard deviation. Different superscript letters correspond to significantly different values ($p \leq 0.05$).

The lipid density of the mushroom flour significantly increased by 274% after concentrating the protein ($p \leq 0.05$). The high lipid content found in the protein concentrate can be explained by the binding mechanism between protein and lipids, which results in the emulsification of the lipids by the protein (Cruz-Solorio et al., 2014).

The protein content of the mushroom flour grown on sorghum forage (32 ± 1%) was close to the values reported for *P. ostreatus* mushroom grown on spent beer grain with wheat bran (32.4 ± 0.1%) or in almond and walnut shells (31.36 ± 0.57%) (Lavelli et al., 2018). These three substrates seem to result in similar protein content of the harvested mushrooms. Nevertheless, the protein values reported for *P. ostreatus* varies between 7.3% and 53.3% (Carrasco-González et al., 2017). The protein content of the substrate affects the final concentration in the mushroom fruiting bodies. Substrates such as spent beer grains, wheat bran and wheat stalk are reported to result in high-protein mushrooms (Lavelli et al., 2018). Also, post-harvest treatments, such as the irradiation of a low dosage of gamma rays (1 kGy or less) on the fruiting bodies have shown to be an effective strategy to increase protein content by 37% (Carrasco-González et al., 2017).

The protein density of the protein concentrate increased by 78% as compared with the unprocessed mushroom flour. In other foodstuffs, such as flaxseed meal and sour cherry kernel, an increase in protein density of 49.6% and 91.3%, respectively, of the resulting protein concentrates as compared with their defatted flours was observed. These protein concentrates were also obtained by alkaline extraction and isoelectric precipitation (Tirgar, Silcock, Carne & Birch, 2017; Çelik, Güzel & Yildirim, 2019). This selective method separates the protein from insoluble residues resulting in significantly higher protein density in the protein concentrate ($p \leq 0.05$) (Simões, Araújo et al., 2020).

The carbohydrate content of *P. ostreatus* have been reported in a

range between 13.1 and 85.8% d.w. (Carrasco-González et al., 2017). This large variability may be related to genetic factors involved in the determination of the amount and type of saccharides present in the fungal cell wall. In this work, for the mushroom flour, a carbohydrate content of 50.5 ± 0.2% was determined, which is also in agreement with results found for a white oyster type of *P. ostreatus* (46.62 ± 0.92% dry matter) (Bach et al., 2017).

Most of the carbohydrates of *P. ostreatus* are polysaccharides that comprise the cell wall, such as α- and β-glucans, chitin and hemicelluloses (mannans, xylans and galactans). These polysaccharides are not digestible and are considered as a source of dietary fiber (Bach et al., 2017). The main fiber source in the edible mushrooms are the β-glucans, which have been associated with anticarcinogenic properties and immunoregulatory functions (Zhu, Du, Bian & Xu, 2015). A crude fiber content of 5.6 ± 0.5% was determined in the mushroom flour.

The carbohydrates and crude fiber content significantly decreased on the protein concentrate, as compared with mushroom flour ($p \leq 0.05$). While total carbohydrates decreased by 60%, the crude fiber was almost residual in the protein concentrate. The harsh conditions used during the concentration of the protein, namely the extreme alkaline and acidic conditions, may disrupt the cell wall of the mushroom, which is mainly composed of polysaccharides, such as chitin and glucans. Acid pH below 4.1 can cause hydrolysis of cell wall macromolecules. (Zivanovic, Buescher & Kim, 2003), while alkaline treatment can deacetylate and degrade chitin in the cell wall, improving its solubility and making it easier to remove (Pillai, Paul & Sharma 2009). On the other hand, adjusting the pH to the isoelectric point allows the selective precipitation of the protein, separating it from the rest of the soluble components in the solution, including carbohydrates and fiber.

The amount of minerals available in the growth substrates affects the final mineral level present in the mushroom (Lavelli et al., 2018). Ash content ranges from 4.1 to 15.9% d.w. in *P. ostreatus* (Carrasco-González et al., 2017) and its related to the presence of nutritionally important minerals. The main fraction of minerals available in mushrooms is constituted by potassium, phosphorus, and magnesium (Bach et al., 2017). The protein concentrate maintained the same moisture and ash content initially present in the mushroom flour (around 6 to 9%), without significant changes ($p > 0.05$).

3.3. Soluble protein composition

Mushrooms produce a large number of proteins with biological and pharmacological activities (Al-Obaidi, 2016). The determination of the molecular weight of the peptides that comprise the protein concentrate may provide an overview regarding those properties. The SDS-PAGE profile of the soluble protein from the concentrated sample is shown in Fig. 3.

Electrophoresis profile of the protein concentrate sample (analyzed in duplicate) revealed molecular weight (M_W) bands of 12, 13, 28, 32 and 35 kDa (Fig. 3).

The *Mw* bands found on the protein concentrate sample may correspond to several valuable proteins from *P. ostreatus*, which have been reported in the literature. For example, hydrophobins, which have a *Mw* ranging from 10 to 20 kDa, these proteins are useful for reversing surface hydrophobicity, stabilizing emulsions and as a coating for biomaterials (Cox & Hooley, 2009; Erjavec et al., 2012). The *Mw* bands ranging from 9 to 15 kDa in the protein concentrate samples may be also attributed to pleurostrin, a unique peptide from *P. ostreatus* mushrooms, with antifungal properties against several plant pathogenic fungi (e.g. *Michospaerella arachidicola, Fusarium oxysporum* and *Physalospora piricola*) (Alves et al., 2013; Erjavec et al., 2012). Additionally, it has also been reported the presence of lectins (*Mw* bands ranging from 12 to 190 kDa) and ribonucleases (*Mw* bands ranging from 8 to 18 kDa and from 28 to 45 kDa) in several mushrooms, including *P. ostreatus*, these peptides have been mainly recognized by their antitumor and antiproliferative activities (Erjavec et al., 2012; Xu et al., 2011).

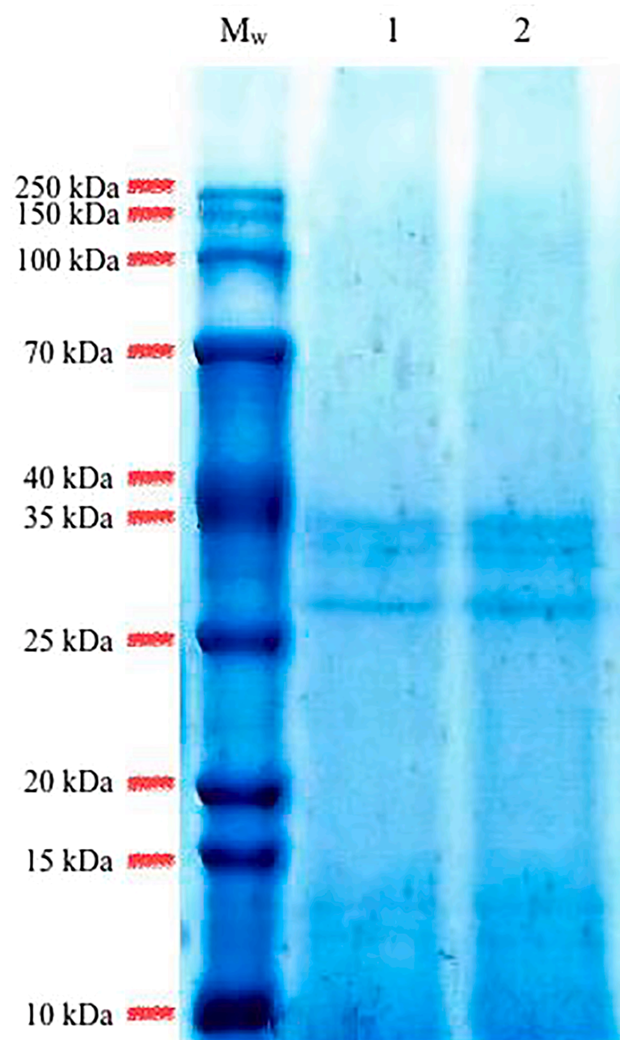

Fig. 3. SDS-PAGE pattern of the protein concentrate sample (1, 2). M_w: Standard marker Bio-Basic® .

3.4. Antioxidant activity

Results obtained on the antioxidant activity of the mushroom flour and the protein concentrate, given by its total phenolic content and, DPPH and ABTS assays, are shown in Table 2.

The DPPH methodology is based on the principle of reducing the DPPH radical to DPPH-H, after link with a substance acting as hydrogen atom donor. Results showed that the mushroom flour and the protein concentrate had a similar effect inhibiting the formation of DPPH-H ($p > 0.05$).

Table 2
Total phenolic content (TPC), DPPH and ABTS radical scavenging activity of mushroom flour and protein concentrate.

	TPC	DPPH		ABTS	
	(mg GAE g^{-1})	IC_{50} (mg mL^{-1})	TEAC	IC_{50} (mg mL^{-1})	TEAC
Mushroom flour	36 ± 1^b	1.1 ± 0.2^a	10.03 ± 0.3^a	13.2 ± 0.2^b	1.75 ± 0.1^b
Protein concentrate	24.2 ± 0.8^a	1.4 ± 0.2^a	7.92 ± 0.6^a	15.3 ± 0.3^a	1.52 ± 0.1^a

Results are given as mean ± standard deviation. Different superscript letters correspond to significantly different values ($p \leq 0.05$).

A spectrophotometric method using ABTS radical is commonly used to estimate the total antioxidant activity of compounds. The ABTS scavenging ability of mushroom flour was significantly higher as compared to the protein concentrate ($p \leq 0.05$) which is consistent with the results obtained for the content of phenolic compounds. Although both the DPPH and ABTS radical scavenging assays are useful to determine the antioxidant capacity of several compounds, it has been reported that the antioxidant capacity detected by the ABTS assay is mainly associated with the content of phenolic compound and flavonoids present in the sample, while the DPPH mainly reflects the presence of high-pigmented and hydrophilic antioxidants (Floegel et al., 2011). High amounts of flavonoids such as chrysin (40 mg 100 g^{-1}) and rutin (31.2 mg 100 g^{-1}) have been detected in ethanolic extracts of *P. ostreatus,* as well as other compounds with antioxidant activity, including β-carotene (3.1 mg 100 g-1), ascorbic acid (25 mg 100 g^{-1}) and α-tocopherol (30.3 mg 100 g^{-1}) (Jayakumar et al., 2011). This suggests that the antioxidant compounds that decrease most in the protein concentrate are flavonoids and phenolic compounds, while hydrophilic (e.g. ascorbic acid) and highly pigmented antioxidants (e.g. carotenoids) remain almost unaffected.

It is important to find out whether the conditions used for obtaining the protein concentrate may alter its antioxidant activity. For example, when processing proteins, amino acid sequences which were embedded in the native protein may be released. These peptides, known as bioactive peptides, have positive health related effects, such as antioxidant activity and also others, e.g. they have been characterized as antimicrobial, hypocholesterolemic, antihypertensive and immunomodulatory (González et al., 2020; Li-Chan, 2015). On the other hand, as many of these compounds are sensitive to extreme temperatures, light or oxygen exposure, processing conditions can affect their antioxidant capacity (Ioannou, Chekir & Ghoul, 2020). Low temperatures used during freezing can affect the antioxidant capacity. For example, in a study where the antioxidant capacity of *P. ostreatus* stored at different temperatures was assessed by DPPH radical scavenging method, as the temperature dropped (from 20 to −40 °C), the DPPH IC_{50} increased (from 0.32 to 2.26 mg mL^{-1}), showing a decrease in antioxidant activity (Bakir, Karadeniz, & Unal, 2018). Also, the drying process might have an important effect on the phenolic content and the *RSA*. The freeze-drying process was reported to decrease the phenolic content and the DPPH IC_{50} of *P. ostreatus* (Ucar & Kardag, 2019). Considering that during its processing the protein concentrate was subjected to freezing and subsequent freeze-drying, this may somewhat explain its decrease in phenolic content and *RSA* as compared to the mushroom flour. However, phenolic compounds have been associated with a negative impact on protein digestion, since many of them may remain in the gastrointestinal tract inhibiting digestive enzymes (Cirkovic-Velickovic, & Stanic-Vucinic, 2018). Thus, the removal of these compounds, which are beneficial to health but can also act as anti-nutrients, could mean better digestibility of the protein present in the concentrate.

3.5. *In vitro protein digestibility test*

The protein hydrolysis kinetic of the mushroom flour and the protein concentrate during *in vitro* gastrointestinal digestion, comprising oral, gastric and intestinal phase simulation, is shown in Fig. 4.

The behavior of protein hydrolysis during the digestion process depends strongly on the nature of the proteins present in the food, since each digestive enzyme has its specific site to hydrolyze. Pepsin is the enzyme responsible for the hydrolysis of proteins during the food passage through the stomach and has an affinity for breaking bonds between aromatic amino acids (Bhutta et al., 2013). The mushroom flour and the protein concentrate achieved a *HD* of 16.5 ± 2.5% and 20.3 ± 3.1%, respectively, after gastric digestion. The most remarkable increase in the protein *HD* was observed for the protein concentrate sample, when passing from the gastric to the intestinal phase, reaching a *HD* of 76.2 ± 1.3%. During intestinal digestion several proteolytic enzymes are secreted, such as trypsin, which cuts the internal bonds of lysine or arginine, the chymotrypsin breaks aromatic or neutral amino acid bonds, the elastase hydrolyzes aromatic amino acid bonds, while carboxypeptidases A and B cut aromatic amino acids and arginine or lysine from the C-ends of proteins and peptides, respectively (Bhutta et al., 2013). Subsequently, at the end of the intestinal phase, it was observed that *HD* reached 100% for the protein concentrate ($p \leq 0.05$). Most probably, the proteolytic enzymes present in the pancreatin completely digested the proteins from the concentrate into smaller peptides (Simões, Martins, et al., 2020). The full digestion of proteins facilitates its absorption into the bloodstream. On the other hand, the mushroom flour only reached a *HD* of 23.5 ± 4.6% at the end of the digestion. These results indicated that the protein digestibility of the protein concentrate was 4.2-fold higher as compared with the mushroom flour ($p \leq 0.05$). Limited digestibility of unprocessed flours compared to their respective protein concentrates or isolates may be due to the high content of non-protein compounds, mainly polysaccharides, which hamper the accessibility to hydrolysis sites (Gbadamosi, Abiose & Aluko, 2012). The

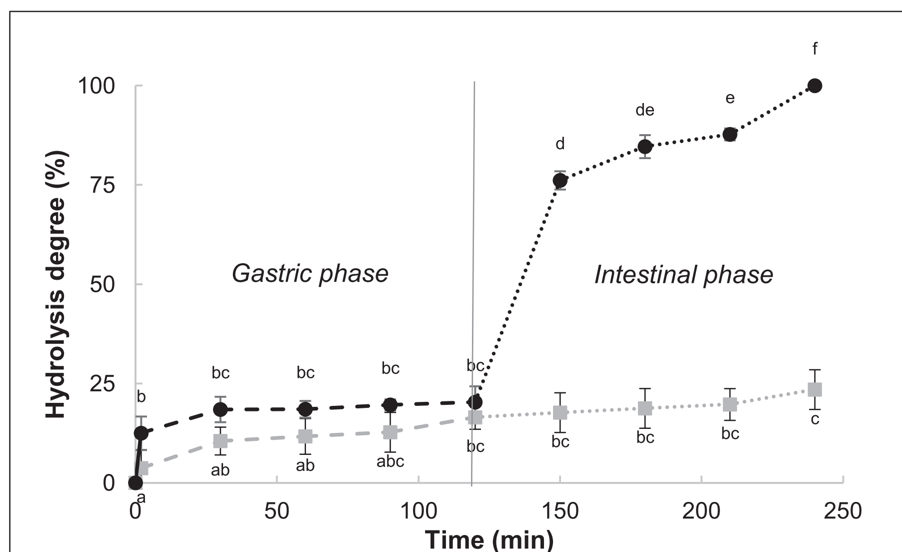

Fig. 4. Protein hydrolysis kinetic of mushroom flour (■) and the protein concentrate (●) during oral phase (Solid line), gastric phase (dashed line) and intestinal phase (dotted line). Results are given as mean ± standard deviation. Different superscript letters correspond to significantly different values ($p \leq 0.05$).

partial denaturation of proteins during processing for obtaining the concentrate may have contributed to improved digestibility, since the alkaline treatment used for protein extraction can cause a partial denaturation of protein and promote its hydrolysis into smaller peptides, reducing its molecular size and increasing its solubility and diffusivity (Hadidi et al., 2019). The increase in protein digestibility is an indicator of the higher number of hydrolyzed peptide bonds and the availability of a higher number of hydrolysis sites (Aryee & Boye, 2016). In accordance, a better *HD* was also obtained for oil/whey protein emulsions with denatured proteins (80%) than in its native form (52%). The authors suggested that denatured proteins were more sensitive to hydrolysis by intestinal proteases (Mat, Le Feunteun, Michon & Souchon, 2016).

Although few studies report the *in vitro* digestibility of food proteins, to our knowledge, data on mushroom protein concentrates is limited, if not inexistent. Studies addressing the digestibility of protein concentrates or isolates from other food flours have systematically observed a significant increase in the digestibility rate of the protein, as compared to the digestion in the raw material. For example, *HD* achieved at the end of the simulated digestion process for bean flour and its protein concentrate and isolate was 9.30, 13.60 and 28.29%, respectively (Sathe, Iyer & Salunkhe, 1982). Conophor nut flour enhanced the *HD* from 52.28% to 73.47% in its protein isolate (Gbadamosi et al., 2012). And the *HD* of a lentil protein isolate was 26% higher as compared to its raw flour (Aryee & Boye, 2016). This shows that the purest forms of protein are likely to have higher digestibility than when attached to a large amount of non-protein compounds that hinder their hydrolysis by digestive enzymes. A higher hydrolysis degree allows a better digestion and bioavailability in the body, because shorter peptides (mainly di- and tri-peptides) are better absorbed by the enterocytes (Bhutta et al., 2013).

Complementary studies on the amino acids profile of the extracted protein mushroom will help to determine the quality of this protein source. Nevertheless, the great results obtained during *in vitro* gastrointestinal digestion supports its high potential for application in a wide variety of food products, such as protein fortified foods, vegan foods and specialized nutrition products for people with limited digestive function.

4. Conclusions

The protein concentrate from *P. ostreatus* mushroom flour was successfully obtained, eliminating most of the soluble non-protein compounds by extraction at pH 12, followed by isoelectric precipitation at pH 4, applying a 1:20 (w/v) mushroom flour-solvent ratio.

Regarding to antioxidant activity, the ABTS radical scavenging ability was significantly diminished, possibly due to the important decrease in the content of phenolic compounds. Nonetheless, the removal of phenolics is reported to have positive impact on protein digestibility.

In vitro digestion process demonstrated that the protein concentrate was completely hydrolyzed in the intestinal phase. The protein concentrate digestibility was enhanced 4.2-fold compared to mushroom flour, since the non-protein components that possibly hinder its hydrolysis by the digestive enzymes were removed.

The findings of this work suggest that the mushroom *P. ostreatus* in its form of protein concentrate has a promising potential to be incorporated into foods, improving their nutritional and functional value.

CRediT authorship contribution statement

Conceptualization: AG, RB, MC. Methodology: AC, LSS, CN. Validation: LSS, CN. Formal analysis: AG, AL, RMRJ, CN. Investigation: CN, MC, RB. Resources: RB, MC, JT. Writing - original draft: AG. Writing - review and editing: AG, CN, RB, MC. Visualization: JC, AL, RMRJ. Supervision: RB, CN, MC, JT. Project administration: RB. Funding acquisition: RB, JT, MC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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