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Digestion-resistant whey peptides promote antioxidant effect on Caco-2 cells

Juliana Santos de Espindola [a], Milena Ferreira Taccóla [a], Vera Sônia Nunes da Silva [a], Lucilene Delazari dos Santos [b], Bruno Cesar Rossini [b], Bruna Cavecci Mendonça [b], Maria Teresa Bertoldo Pacheco [a], Fabiana Galland [a,*]

[a] Quality and Science Center of Food, Institute of Food Technology (ITAL), Brasil Ave. 2880, P.O. Box 139, Campinas, SP 13070-178, Brazil
[b] Institute of Biotechnology, São Paulo State University (UNESP), Botucatu, SP 18607-440, Brazil

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ABSTRACT

Enteric endothelial cells are the first structure to come in contact with digested food and may suffer oxidative damage by innumerous exogenous factors. Although peptides derived from whey digestion have presented antioxidant potential, little is known regarding antioxidant pathways activation in Caco-2 cell line model. Hence, we evaluated the ability to form whey peptides resistant to simulated gastrointestinal digestive processes, with potential antioxidant activity on gastrointestinal cells and associated with sequence structure and activity. Using the INFOGEST method of simulated static digestion, we achieved 35.2% proteolysis, with formation of peptides of low molecular mass (<600 Da) evaluated by FPLC. The digestion-resistant peptides showed a high proportion of hydrophobic and acidic amino acids, but with average surface hydrophobicity. We identified 24 peptide sequences, mainly originated from β-lactoglobulin, that exhibit various bioactivities. Structurally, the sequenced peptides predominantly contained the amino acids lysine and valine in the N-terminal region, and tyrosine in the C-terminal region, which are known to exhibit antioxidant properties. The antioxidant activity of the peptide digests was on average twice as potent as that of the protein isolates for the same concentration, as evaluated by ABTS, DPPH and ORAC. Evaluation of biological activity in Caco-2 intestinal cells, stimulated with hydrogen peroxide, showed that they attenuated the production of reactive oxygen species and prevented GSH reduction and SOD activity increase. Caco-2 cells were not responsive to nitric oxide secretion. This study suggests that whey peptides formed during gastric digestion exhibit biological antioxidant activity, without the need for previously hydrolysis with exogenous enzymes for supplement application. The study's primary contribution was demonstrating the antioxidant activity of whey peptides in maintaining the gastrointestinal epithelial cells, potentially preventing oxidative stress that affects the digestive system.

1. Introduction

The maintenance of the gastrointestinal epithelial cells is of fundamental importance for human health. Due to the continuous exposure to exogenous factors, the intestine is very susceptible to oxidative stress that results from the imbalance between the production of reactive oxygen species and endogenous antioxidant systems (Qiao et al., 2022). These changes are common in gastrointestinal pathologies, such as colon cancer, Crohn's disease, as well as immune and metabolic changes (Xu et al., 2017). In a redox imbalance, only the endogenous antioxidant defense becomes inefficient, and the intake of antioxidants from food is a good strategy for preventing oxidative damage (Carocho & Ferreira, 2013; Power, Jakeman, & Fitzgerald, 2013; Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Hence, a demand for natural antioxidants has grown due to their potential beneficial effects on health allied to a lower risk of immunoreactivity (Sarmadi & Ismail, 2010) (see Table 1).

Food peptides have been explored for their health benefits and nutritional function. Bioactive peptides are protein fragments with

* Corresponding author at: Centro de Ciência e Qualidade de Alimentos (CCQA), Instituto de Tecnologia de Alimentos (ITAL), PO Box 139, 13070-178 Campinas, SP, Brazil.
E-mail addresses: juliana.s.espindola@gmail.com (J.S. de Espindola), milenaftaccola@gmail.com (M. Ferreira Taccóla), vera.silva@ital.sp.gov.br (V.S.N. da Silva), lucilene.delazari@unesp.br (L.D. dos Santos), bruno.rossini@unesp.br (B.C. Rossini), brucavecci@gmail.com (B.C. Mendonça), mtb@ital.sp.gov.br (M.T.B. Pacheco), fabiana.pe@ital.sp.gov.br (F. Galland).

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chains of 2–20 amino acids joined by covalent bonds, which differ in bioactivity according to their sequence, composition, charge, and molecular weight (Sánchez & Vázquez, 2017). They are inactive within the original structure of the protein, but may have activity when released from hydrolysis, fermentation, or digestion processes.

Peptides formed from whey protein have presented several bioactivities, including the ability to act as antioxidant molecules, even showing higher antioxidant activity than synthetic compounds such as BHA (Corrochano, Buckin, Kelly, & Giblin, 2018; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005). Several studies have explored processing methods (enzymatic, thermal, fermentative and pressure) that increase the antioxidant potential of whey peptides (Adjonu, Doran, Torley, & Agboola, 2013; Corrochano, Sariçay et al., 2019; Hernández-Ledesma et al., 2005; Iskandar et al., 2015; Zhidong et al., 2013; Virtanen, Pihlanto, Akkanen, & Korhonen, 2007). Peptide bioactivity improvement can be advantageous when considering the application of these compounds for industrial food preservation purposes; however, this property is not always kept for functional food production purposes, which may pass through digestion (Corrochano, Sariçay et al., 2019).

Peptides are more accessible to enzyme action compared to intact protein complexes and are easier to be degraded to amino acids (Corrochano, Sariçay et al., 2019; Koopman et al., 2009; Moughan, Cranwell, & Smith, 1991). Although antioxidant activity of whey hydrolysates has been extensively explored, most studies have used exogenous enzymes for hydrolysis or chemical *in vitro* assays, which are not always reproducible when extrapolated to complex biological systems (Amigo, Martínez-Maqueda, & Hernández-Ledesma, 2020; Corrochano, 2018; O'keeffe et al., 2017). Only peptides resistant to the digestive process are considered bioaccessible, being able to act on intestinal cells promoting antioxidant effects at the cellular level (Mann, Athira, Sharma, Kumar, & Sarkar, 2019).

After peptides reach the target tissue, they must be able to interact with the cellular environment, be internalized, and act as proton donors to free radicals, chelating metal ions, or inhibiting lipid peroxidation (Mann et al., 2019; Zou, He, Li, Tang, & Xia, 2016). At the same time, they can modulate important transcriptional regulatory pathways and stimulate the synthesis of antioxidant cell defense compounds (Corrochano, Buckin et al., 2018).

Although Caco-2 cells simulate the gastrointestinal epithelium and are the first cells to come into contact with food, little has been evaluated regarding the antioxidant effect of whey after digestion (Piccolomini et al., 2012). At this stage, whey hydrolysates were able to decrease the production of reactive oxygen species in myoblasts, hepatocytes, and neuronal cells (Corrochano, Ferraretto et al., 2019; Zhang & Shi, 2015). In enterocytes, there is no information regarding the possible intracellular defense mechanisms activated by whey digests, such as glutathione production and superoxide dismutase activity. Here, we associated antioxidant activity with peptide sequence to better explore structure–activity relationships.

For simulate digestion we used an internationally recognized static *in vitro* digestion system, Infogest, which establishes standardized hydrolysis conditions, such as enzyme concentration and origin, temperature, pH, and reaction time, in order to minimize variability between protocols (Brodkorb et al., 2019). Several studies have applied this method, which results in more homogeneous results(Egger et al., 2017; Mat, Le Feunteun, Michon, & Souchon, 2016; Sousa, Portmann, Dubois, Recio, & Egger, 2020,2023).

In this study, we characterized whey peptides resistant to the digestive process with respect to their molecular size, hydrophobicity, and amino acid content. Additionally, we evaluated their antioxidant capacity using chemical assays *in vitro* and in Caco-2 cells, which were stimulated with hydrogen peroxide to cause oxidative damage and evaluate the whey peptides antioxidant capacity in the cellular machinery of enterocytes. The sequence of peptides was identified using LC-MS/MS and compared to prior analyses using bioinformatics tools.

2. Material and methods

2.1. Sample and chemicals

Whey protein isolate (WPI) was acquired commercially from Glanbia nutritionals. The following enzymes, standards, and chemicals for antioxidant activity techniques were obtained from Sigma Aldrich (St. Louis, USA): pepsin (P7000), pancreatin (p1750), bile extract (B8631), phthaldialdehyde or OPA (P1378), DL-dithiothreitol or DTT (S43819), α-Lactalbumin (L6385), Insulin (I2643), Vitamin B12 (V2876), 3,4-Dihydroxy-L-phenylalanine (D9628), DL-2-Aminobutyric acid or AAAB (162663), phenyl isothiocyanate or PITC (P1034), 2,2-Diphenyl-1-picrylhydrazyl or DPPH (D9132), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt or ABTS (A1888), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid or Trolox (238813), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride or APPH (440914), Fluorescein (46955). Amino acid standards (P/N20088, Standard H) and L-Serine (36323) were obtained from Pierce Biotechnology (Massachusetts, USA). In vitro analysis with cell culture used Follin-ciocalteu (Dinâmica Química, Indaiatuba, São Paulo, Brazil), Thiazolyl Blue Tetrazolium Bromide or MTT (M5655), L-Glutathione reduced, or GSH (G6529), 2′,7′-dichlorofluorescin diacetate or DCF-DA (D6883), and bovine serum albumin (A4503) obtaining from Sigma Aldrich (St. Louis, USA). Deionized water and other reagents were used in the analytic grade. The analyzes were performed in triplicate.

2.2. Simulated gastrointestinal digestion of whey protein isolates

Static *in vitro* digestion analysis was performed according to the procedure recently described by Brodkorb and coworkers, as amended from the first version (INFOGEST 2.0) (Brodkorb et al., 2019). This protocol is based on human gastrointestinal digestion, where the food samples are subjected to sequential oral, gastric, and intestinal digestion with standardized physiologic parameters, such as electrolytes, enzymes, bile, dilution, pH, and time of digestion. Some conditions were adapted considering the protein samples to obtain a hydrolysate compatible with incubation in cell culture, such as heat inactivated enzyme, centrifugation and filtration. No enzyme was used to digest starch and fat (amylase, bile, and pancreatic lipase) since our sample was protein isolate. We used all simulated salivary, gastric, and intestinal fluids, as well as times of incubation as indicated by the protocol.

The activity of pepsin and pancreatin enzymes were measured prior to digestion process to ensure a correct final activity value. The assay used for measuring pancreatin and pepsin were based on those suggested by the INFOGEST supplementary material protocol (Brodkorb et al., 2019). Pepsin presented 3743 U/mg and pancreatin 7,3 U/mg. These values were corrected to obtain final concentration of 2000U/mL of pepsin in gastric phase and 100U/mL of pancreatin in intestinal phase. A control sample contained all the reagents included in the digestion protocol, but the sample was replaced with protein free ingredient maltodextrin as indicated by the INFOGEST group (Sousa et al., 2020).

For the oral phase, the sample was mixed with salivary fluid in the proportion of (1:1 w/v) with 10 mL total volume. The 0.3 M $CaCl_2$ was added to salivary fluid immediately before assay and no α-amylase enzyme was added due to the low carbohydrate content. This mixture formed a swallowable bolus with saliva consistency. In a bath at 37 °C with agitation, we incubated the mixture for 2 min. In the gastric phase, a ratio of 1:1 w/v oral bolus was mixed with simulated gastric fluid with 0.3 M CaCl2 freshly added, pH adjusted to 3.0 and sequentially added the pepsin enzyme (2000 U/mL), with volume adjusted to 20 mL. This mixture was placed in a bath at 37 °C under agitation for 2 h. For the intestinal phase, gastric chyme was mixed in a ratio of 1:1 w/v of intestinal fluid with the addition of 0.3 M CaCl2, pH adjusted to 7.0 with 5 M NaOH and addition of 5 mL of pancreatin dilution to reach final concentration of 100 U/mL in the intestinal phase. The total volume was

adjusted to 40 mL in this stage. The intestinal mixture was placed in a bath at 37 °C for 2 h. After this time, the enzyme activity was inhibited by incubation of samples in a bath at 85 °C for 10 min. Then, it was placed in a cold bath and centrifuged at 7000 g for 20 min at 4 °C. The supernatant was collected and lyophilized and stored under freezing for further analysis.

2.3. Physical-Chemical characterization of WPI and WPH

Whey protein isolates (WPI) and whey protein hydrolysates (WPH) were characterized measuring total protein content (Nx6.38), moisture, degree of hydrolysis, molecular size, total amino acids, and degree of hydrophobicity (LATIMER, 2012). The degree of hydrolysis of the samples was determined by the o-phthaldehyde (OPA) method described by Adler-Nissen (1986). The degree of hydrolysis (DH) was calculated according to Eq.

$$DH(\%) = B \times Nb \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{htot} \times 100$$

where: B = base consumption (mL); Nb = normality of the base; α = average degree of dissociation of the α-NH groups; MP = mass of protein (g); and htot = total number of peptide bonds in the protein substrate (meqv/ g protein), whey protein htot = 8.8.

2.4. Molecular size by size exclusion chromatography (FPLC)

WPI and WPH samples were analyzed using Fast Protein Liquid Chromatograph (Akta Pure Chromatograph, GE Healthcare) by the size exclusion method (SE-FPLC) with gel columns (Superdex 200 and Superdex 30 models) and UV detector (280 nm). Samples (5 mg protein/ mL) and standards (1 mg/mL) were diluted in 25 mM sodium phosphate buffer (pH 7.4 with 150 mM NaCl) and run at a flow of 0.5 mL/min for 120 min. The molecular weight standard α-Lactalbumin (14178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.4 Da), L-β-4-Dihydroxyphenylanine (197.2 Da) were used at a 1 mg/mL concentration (Sisconeto Bisinotto et al., 2021; Vander Heyden, Popovici, & Schoenmakers, 2002).

2.5. Total amino acids by High-Performance liquid chromatography (HPLC)

Total amino acids were obtained through acid hydrolysis, using a 6 N HCl hydrochloric acid and phenol solution, for 22 h in a digester block at 120 °C. Next, a pre-column reaction was carried out with phenyl isothiocyanate (PITC). Mobile phases A and B consist of sodium acetate, acetonitrile, ultra-pure water, and disodium EDTA. Quantitation was performed using a Shimadzu liquid chromatograph and a diode array detector (DAD) (Shimadzu, Brazil), and a C18 Luna-Phenomenex reversed-phase column (4.6 mm × 250 mm; particle size 5 μm) (Phenomenex Inc., Torrance, USA). The quantification was performed by comparing it with the amino acid standard and the internal standard α-aminobutyric acid (AAAB) at a wavelength of 254 nm (Hagen, Frost, & Augustin, 1989; White, Hart, & Fry, 1986). Tryptophan was not analyzed.

2.6. Hydrophobicity profile by chromatography

Reverse-phase high-performance chromatography (RP-HPLC) using a C18 column (4,6mm × 250 mm; particle size 5 μm/ Phenomenex/ Torrance, California, USA) and diode array detector (DAD) was used (Shimadzu, Brazil). The technique was based on previous work from our group (Caetano-Silva et al., 2017). The samples were diluted in water (3 and 1 mg protein/mL for WPH and WPI respectively), and 50 μl was injected. The solvent A (0,04% de TFA in ultrapure water) and solvent B (0,03% TFA in acetonitrile) was eluted with following gradient: 0 a 70% solvent B until 40 min, 100% solvent B in 45 min, and return 0% solvent B in 50 min. The flow rate was 1 mL/min at 25 °C/ 60 min. Samples were diluted in solvent A, filtered in polytetrafluoroethylene hydrophilic membrane (PTFE; 0,45 μm), and taken in the ultrasonic bath for 10 min after injection. The chromatograms obtained to WPI and WPH were divided into three zones, classifying the peptides as (I) Low hydrophobicity zone, with retention time from 0 to 14 min and elution gradient of 25%; (II) medium hydrophobicity zone with B solution gradient the up to 50% (17 and 29 min) and, (III) high hydrophobicity zone with B solution gradient over 50% (29 and 40 min) (Legay, Popineau, Bérot, & Guéguen, 1997).

2.7. LC/MS/MS analysis (Nano-coupled liquid chromatography QExactive mass)

Samples were resuspended in LC/MS water with 0.1% formic acid and quantified with Qubit Protein Assay. Chromatography was performed on a PicoChip source (Model 1PCH-550; 75 μm ReproSil Pur C18 3 μm silica matrix; New Objective, USA) at a continuous flow rate of 0.300 μl/min. A 1ug of sample was injected into a 2 cm Acclaim PepMap 100 trap (75 μm ID, C18 3 μm; Thermo Fisher Scientific) pre-column. The sample was then separated using a 2–40% mobile phase B gradient for 120 min, followed by 10 min in 80% mobile phase B, and re-equilibration of the column for 10 min in 2% mobile phase B. The chromatography mobile phases included: mobile phase A water/0.1% formic acid, and phase B acetonitrile/0.1% formic acid. Mass spectra were acquired on a Q Exactive mass spectrometer (Thermo Fisher Scientific) by the DDA (data dependent acquisition, FullMS/MS) method with top 10 count selection. The precursor ion search was conducted with 300–1,750 *m/z* at 70,000 resolution. An isolation window of 2 *m/z* was selected, NCE collision energy 15 and 30, followed by MS/MS acquisition at 17,500 resolution. Automatic gain control (AGC) target of 1^e 4 and maximum injection time of 100 ms. Loads 1 and greater than 5 were excluded. Dynamic exclusion time of 30 s was used. Samples were run in duplicate. Spectra analysis was performed with PatternLab for Proteomics (Carvalho et al., 2015) using the *Bos taurus* database from the UNIPROT database (https://www.uniprot.org). The search for de novo peptides was performed with the NOVOR software (Ma, 2015) (available at https://novor.cloud/), using default settings and the *Bos taurus database* as a reference. The peptides found were cross-referenced with the Milk Bioactive Peptide Database (Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides with 100% similarity to the database were considered. The sequencing results were cross-checked with the MBPDB and BIOPEP-UWM databases. The hydrophobicity of the sequenced peptides was calculated based on amino acid side chain by peptide sequence length using the open access web-based bioinformatic tools (Acquah, Stefano, & Udenigwe, 2018). To show the frequency of each amino acid in each position, a heat map graph was constructed based on iceLogo, a free, open-source Java application for analyzing and visualizing consensus patterns in aligned peptide sequences (Colaert, Helsens, Martens, Vandekerckhove, & Gevaert, 2009). The online software (https://iomics.ugent.be/icelogoserver/) was used.

2.8. Antioxidant assay -ABTS radical scavenging method

The antioxidant assay measured the ability of whey peptides to scavenge the ABTS, as described by (Dryáková, Pihlanto, Marnila, Čurda, & Korhonen, 2010). Briefly, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical solution (ABTS +) was performed by mixing potassium persulfate in aqueous solution 2.45 mM and ABTS 7 mM. The mixture was kept in the dark for 16 h and its absorbance was adjusted to 0.7 ± 0.02 at 734 nm using a UV–Vis spectrophotometer prior to analysis. An aliquot of 10 μl of the sample (1, 5, 10, 20 mg/mL) was placed in a microplate well followed by the addition of 190 μl of ABTS working solution (Abs 0.7 ± 0.02) to react for 6 min. The absorbance was then measured at 730 nm on the microplate UV–Vis Reader

Synergy (Varioskan Lux, Thermofisher). A Trolox standard curve in 75 mM phosphate buffer and pH 7.4 was used to express the ABTS values. The volume of 240 µl of sodium phosphate buffer (75 mM, pH 7.4) was subtracted from the absorbances of the samples and standard by the analytical curve. The final results were expressed in µmol Trolox equivalent/g of sample, or by the percentage of antioxidant activity (AA), calculated and expressed according to the following equation: AA (%)=((Blank Abs-(Sample Abs)/(Blank Abs))x100).

2.9. Antioxidant assay - free radical scavenging activity of DPPH.

The free radical scavenging capacity of the test samples were estimated following (Pires, Torres, dos Santos, & Chow, 2017), with minor modifications. An 80 µM solution of 2,2-diphenyl-1-picrylhydrazyl was prepared in a dark bottle using methanol. It was then mixed with serially diluted concentrations of sample (WPI and WPH at 1, 5, 10, 20 mg/mL) or standard (3–100 uM). The mixture was shaken and incubated at room temperature for 30 min, centrifuge 5000 g for 5 min at −4°C. A volume of 200 µl was dispensed into a 96-well microplate and read at an absorbance of 517 nM. The scavenging activity of the tested samples was extrapolated into the percentage DPPH inhibitory using the following equation: AA(%) = ((Blank Abs-(Sample Abs)/(Blank Abs)) × 100).

2.10. Antioxidant assay - oxygen radical absorption capacity-ORAC

ORAC assay measures the ability of an antioxidant to reduce the peroxyl radical (ROO-) generated by the thermal degradation of (2,2′-azobis(2-amidinopropane) dihydrochloride) (AAPH) and preserve the fluorescein molecule against the action of the peroxyl radical (Corrochano, Sariçay et al., 2019). Samples (WPH and WPI at 0.01; 0.25 and 1 mg/ml) and Trolox standard curve (5 to 80 µM) were diluted in 75 mM potassium phosphate buffer (pH 7.4). In a microplate, 20 µl of sample or standard, 120 µl of 0.17 µM fluorescein solution, 60 µl 40 mM AAPH solution Fluorescence were added and monitored during 2 h, with 90-second intervals, at 485 nm wavelength excitation and emission of 520 nm, using a microplate reader (Varioskan Lux, Thermofisher, Singapore). The samples and protection standard (AUCnet) were calculated by the difference between the area under the fluorescence decay curve of samples/standard (AUC sample/standard) and the area under the fluorescence decay curve without sample or addition of Trolox (white AUC). The results were expressed in µmol equivalent of Trolox/g of sample, in triplicate.

2.11. Caco-2 cell culture

Human lineage of adenocarcinoma derived from the epithelium (Caco-2) was kindly donated from Dra Juliana Macedo, professor of the Faculty of Food Engineering at UNICAMP (Campinas, São Paulo, Brazil). The cells were cultivated in a bottle of 25 or 75 cm^2, using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 8.4 mM Hepes, 23.8 mM NaHCO3, 1% sodium pyruvate, L-glutamine, 1% non-essential amino acids, 1% penicillin and streptomycin. Cells were incubated at 37 °C and 5% CO2 in a water-saturated atmosphere and the culture medium was replaced every two days. After growth and reaching 90% confluency, cells were trypsinized with 0.25% (v/v) trypsin-EDTA and seeded in 24-well plates (with a cell density of 10×10^4 cells/well) or 96-well plates (2×10^4 cells/well), depending on the analysis.

2.12. Cells treatment design

Cells were pretreated for 1 h with WPH at concentrations (0; 0.01; 0.25 and 1 mg/mL) or with the Infogest control (C). Cells were then stimulated with hydrogen peroxide at a concentration of 1 mM for 3 more hours. Treatment time was defined at 4 h (1 h of pretreatment and 3 h of stimulus) based on the physiological time of *in vitro* digestion.

2.13. Evaluation of cell viability

Cell viability was based on the cellular uptake of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide or MTT and the effect of metabolic cell activity that results in the conversion of MTT to purple-stained insoluble crystalline formazan (Crespo et al., 2012). MTT 0.05 mg/ml was incubated 30 min before the end of treatment. Then, the medium was removed, and dimethylsulfoxide (DMSO) was added to dissolve the crystals under stirring. The absorbance reading at wavelengths of 560 nm and 650 nm in a microplate reader (Varioskan Lux, Thermofisher, Singapore). The reduction of MTT was calculated as (abs at 560 nm) - (abs at 650 nm) and expressed as a percentage of basal.

2.14. Generation of reactive oxygen species (ROS)

Reactive oxygen species (ROS) production was evaluated by DCF-Da assay after cell treatment (Wan, Liu, Yu, Sun, & Li, 2015). DCF-DA is absorbed by passive diffusion by cells and cleaved by the action of intracellular esterases forming DCFH. DCFH is oxidized to dichlorofluorescein (DCF) by radicals. Cells were seeded and treated in a 96-well black plate with a density of $2x10^4$ cells/well. DCF-DA at 20 µM was incubated 30 min before the end of treatment with WPH at different concentrations (0; 0.01; 0.25 and 1 mg/mL). Then, cells were washed with PBS twice and a volume of 100 µl of 1 mM hydrogen peroxide medium or basal medium was incubated; the microplate was immediately placed on microplate reader (Varioskan Lux, Thermofisher, Singapore) for fluorescence reading at a wavelength of 485 nm excitation and 520 nm emission, in kinetic time, with readings every 5 min for a total time of 2 h at 37 °C. The result was expressed as arbitrary fluorescence unit per microgram of protein (UF/mg protein).

2.15. Glutathione reduced content (GSH)

This assay detects reduced glutathione levels according to the method of (Browne & Armstrong, 1998). After treatment, cells were scraped from wells, resuspended, and lysed in sodium-phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA. Proteins were precipitated in MPA-PR solution (Metaphosphoric acid and NaCl) and centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was pipetted in triplicate into a 96-well black plate. The standard curve was performed with a reduced glutathione standard (G-6529, Sigma Aldrich, St. Louis, USA) in the concentration range of 0–500 µM. The 1 mg/mL o-phthaldehyde (P-1378, Sigma Aldrich, St. Louis, USA) is the fluorescent compound of the reaction after its addition to the plate was protected from light for 15 min before reading. The fluorescence reading in the excitation parameters at 350 nm and emission at 420 nm was performed in a microplate reader (Varioskan Lux, Thermofisher, Singapore). The readings were discounted from the blank, and the result was expressed in umolGSH/mg protein.

2.16. SOD activity

The SOD activity of the previously treated Caco-2 cells was evaluated using the Cayman kit and the instructions according to the manufacturer were followed. The intracellular content of SOD was obtained by lysis each well (24 well plates) in 300 µl of kit indicated solution.

2.17. Nitric oxide production (NO)

Oxide nitric production was evaluated after treatment, following (Soliman & Mazzio, 1998). Griess reagent contains 1 part sulfanilamide (0.75%) and 0.5 N HCl to 1 part N-(1-naphthyl) ethylenediamine dihydrochloride (0.75%) in water. After treatment in cell culture, the supernatant was collected and mixed with the Griess reagent pipetted triplicate into the 96-well plate. Then, plates were incubated for 10 min in the dark at 25 °C, and the absorption was read at 550 nm on a

microplate reader (Varioskan Lux, Thermofisher, Singapore). The standard curve used was sodium nitrite (Synth, São Paulo, Brazil). Data were expressed as nM nitrite/mg protein/h.

2.18. Soluble proteins

Most of the techniques used in cell culture were corrected for soluble protein (μg/μL) by the Lowry method using bovine albumin (Peterson, 1979). The standard used was bovine albumin. The amount of protein was expressed in μg/μL.

2.19. Statistical analysis

Statistical analysis was conducted using SPSS 16.0 software (SPSS Inc., Chicago IL). ANOVA followed by Duncan's post-test was performed to evaluate differences between the groups. The mean ± standard deviation was used to represent data from each *in vitro* experiment, which was repeated at least five times. Statistical significance was determined as $p < 0.05$.

3. Results and discussion

3.1. Degree of hydrolysis and molecular weight of digested peptides

The effectiveness of the digestion using the Infogest method was evaluated by the degree of hydrolysis, and the molecular size of the peptides formed. The whey hydrolysate (WPH) obtained a degree of hydrolysis of 35.2 ± 2.4 g/100 g, similar to other results from the literature (values of 31, 49 and 52%) for with similar matrix (Ariëns et al., 2021; Mat et al., 2016).

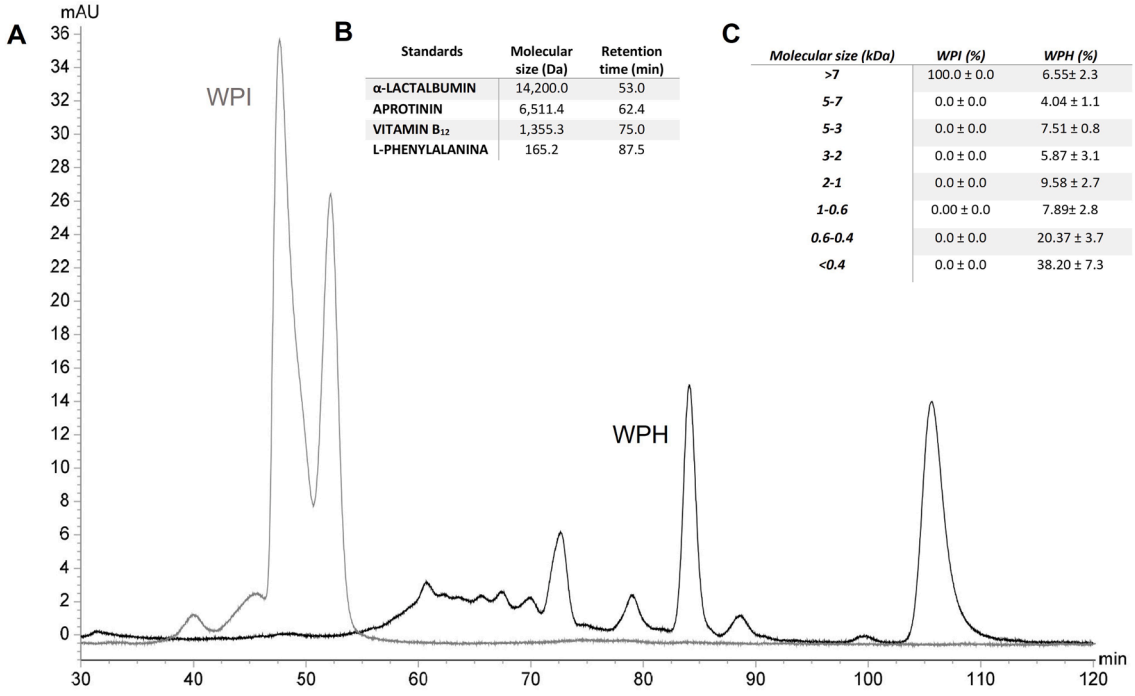
The molecular size of WPH was compared with whey isolate (WPI) by FPLC analysis (Fig. 1). Regarding WPI, we could observe that 100% of the sample corresponds to fractions larger than 7KDa, probably attributed to the proteins α-lactalbumin (14 KDa) and β-lactoglobulin (18.4 KDa), major constituents of whey. While the molecular mass distribution of WPH shows a contrasting profile, where most of the molecular weight fractions are below 0.6 KDa (Fig. 1 A and B). These data confirm the high proteolysis of proteins by enzymes present in the digestive process, resulting in low molecular size peptides. Studies in computer-controlled dynamic *in vitro* digestion system show that some whey proteins were resistant to gastric compartments; after intestinal phase, however, all the major proteins were hydrolysates into molecular sizes smaller than 2kDA, suggesting a high susceptibility to pancreatic enzymes (Nabil, Gauthier, Drouin, Poubelle, & Pouliot, 2011). Peptides of lower molecular mass are more resistant to gastric digestion, thus increasing their bioaccessibility for absorption (Sarmadi & Ismail, 2010).

3.2. Amino acid composition and hydrophobicity profile

The function and bioactivity of peptides are related to their amino acid composition and hydrophobicity. As expected, the amino acid composition did not change after the hydrolysis process (Table 1). The most abundant amino acid group in our sample was Aliphatics/Nonpolar (30.1%), followed by Acids (28.9%), Basics (13.4%), Hydroxylated (11.8%), Iminoacids (5.8%), Aromatics(5.7%), and Sulphurized (4.3%).

Leucine (10 mg/100 g), valine (6.3 mg/100 g), and isoleucine (6.4 mg/100 g) stood out among the aliphatic amino acids. These amino acids have the highest hydrophobicity score within a hydrophobicity scale of 20 proteinogenic amino acids (Acquah et al., 2018; Kyte & Doolittle, 1982). Peptides with high levels of hydrophobic amino acids, particularly leucine, have been reported to exhibit enhanced antioxidant activity (Zou et al., 2016). The high hydrophobicity of peptides likely contributes to their permeability across the cell plasma membrane, facilitating their uptake. Indeed, the high hydrophobicity of peptide fraction of casein positively affected its bioavailability (Xie, Wang, Jiang, Liu, & Li, 2015).

According to the literature, casein peptides have shown that acidic amino acids in peptide sequences (highly present in our sample) contribute positively to digestive stability in simulated gastric and intestinal digestion models (Jing Ao, 2013). Aspartic acid and glutamic acid, along with glutamine, are considered the major energy substrates in the diet for enterocytes. These amino acids assist in maintaining the integrity of the intestinal barrier, indirectly preventing the entry of



Standards	Molecular size (Da)	Retention time (min)
α-LACTALBUMIN	14,200.0	53.0
APROTININ	6,511.4	62.4
VITAMIN B_{12}	1,355.3	75.0
L-PHENYLALANINA	165.2	87.5

Molecular size (kDa)	*WPI (%)*	*WPH (%)*
>7	100.0 ± 0.0	6.55± 2.3
5-7	0.0 ± 0.0	4.04 ± 1.1
5-3	0.0 ± 0.0	7.51 ± 0.8
3-2	0.0 ± 0.0	5.87 ± 3.1
2-1	0.0 ± 0.0	9.58 ± 2.7
1-0.6	0.00 ± 0.0	7.89± 2.8
0.6-0.4	0.0 ± 0.0	20.37 ± 3.7
<0.4	0.0 ± 0.0	38.20 ± 7.3

Fig. 1. Molecular size of whey isolates (WPI) and whey hydrolysate (WPH). (A) Chromatographic profile of molecular mass distribution of WPI and WPH; (B) Retention times of internal standards (C) Molecular mass ranges in percentage.

Table 1
Amino acid profile and concentration (g/100 g protein) of WPI and WPH.

Amino acids (AA)	WPI	WPH
Aliphatic/Nonpolar		
Ala	5.0 ± 0.01	5.0 ± 0.01
Gly	1.6 ± 0.01	2.6 ± 0.01
Leu	10.6 ± 0.02	10.0 ± 0.02
Val	6.2 ± 0.01	6.3 ± 0.01
Ile	6.5 ± 0.01	6.4 ± 0.01
Aromatics		
Phe	3.0 ± 0.01	2.9 ± 0.01
Tyr	2.9 ± 0.01	2.7 ± 0.01
Hydroxylated		
Ser	4.9 ± 0.01	5.0 ± 0.01
Thr	7.2 ± 0.01	6.5 ± 0.06
Sulphurized		
Cys	1.6 ± 0.06	1.4 ± 0.01
Met	2.9 ± 0.01	2.7 ± 0.01
Imino acids		
Pro	5.8 ± 0.02	5.8 ± 0.01
Acids		
Glu	17.9 ± 0.01	17.6 ± 0.03
Asp	10.9 ± 0.01	11.4 ± 0.02
Basics		
Arg	2.1 ± 0.01	2.7 ± 0.01
His	1.6 ± 0.01	1.7 ± 0.01
Lys	9.4 ± 0.01	9.2 ± 0.01

Note: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly;H = His; I = Ile; K = Lys; L = Leu;M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val;W = Thr; Y = Tyr.

microorganisms into the systemic circulation (P. Li, Yin, Li, Kim, & Wu, 2007; G. Wu & Morris, 1998). These negatively charged amino acids have demonstrated the ability to chelate free radicals due to the presence of excess electrons in peptides derived from colza (He et al., 2013). In addition, glutamic acid is an immediate precursor in the synthesis of glutathione, which plays an important role in eliminating oxidative compounds and regulating the immune response (Wu, Bazer, Cudd, Meininger, & Spencer, 2004).

Fig. 2 shows the hydrophobicity analyses of WPI and WPH at two wavelengths, with the 214 nm absorbance typically used to estimate the amount of peptides present in the hydrolysates, while the 280 nm absorbance is more related to the presence of aromatic amino acids (Asher, Ludwig, & Johnson, 1986; Kuipers & Gruppen, 2007). It is observed that the intensity of the peaks at 280 are lower than 214 nm, indicating a lower exposure of aromatic amino acids, corroborating with our amino acid composition analysis.

Fig. 2a, which refers to WPI, shows two peaks of higher intensity in zone II that correspond to α-lactalbumin and β-lactoglobulin(Caetano-Silva et al., 2017; Corrochano, Sariçay et al., 2019). Regarding the chromatograms of WPH (Fig. 2B), several peaks resulting from the hydrolysis process are distributed in the low and medium hydrophobicity zones.

Although both samples had a good proportion of amino acids classified within the hydrophobic group (Table 2), we observed a reduction in overall hydrophobicity after the hydrolysis process. This analysis evaluates the surface hydrophobicity of peptides, and not all amino acids are exposed in solution. Previous works observed a reduction of hydrophobicity after the hydrolysis process of whey (Melnikova, Bogdanova, & Koshevarova, 2022; Schröder, Berton-Carabin, Venema, & Cornacchia, 2017). The reduction in hydrophobicity could be attributed to a structural change of the peptides, leading to an increase in charges in the C- and N-terminal regions, after cleavage of the peptides between adjacent amino acids (Schröder et al., 2017).

3.3. Digestion-resistant peptide sequences identification

Peptides released after the digestion process were identified by HPLC-ESI-MS/MS, with a detection window of 300 to 1750 *m/z*. A number of 126 sequences were identified, however, after bioinformatics analysis, only 24 peptides exhibited 100% similarity to the databases (Table 2). We identified 12 peptides derived from β-LG protein; 11 peptides from β-casein and 1 from α-LA. Prior studies on whey hydrolysis have identified sequences derived from casein, even though this protein is mainly present in milk (Corrochano, Buckin et al., 2018). Interestingly, four peptide sequences (LIVTQTMK, IDALNENK, RELKDLK, ALPMHIR) were previously detected after hydrolysis with digestive enzymes originating from β-Lg and α-La and related to proliferative effect (Jacquot, Gauthier, Drouin, & Boutin, 2010).

Most of the identified peptides had between 6 and 13 amino acid

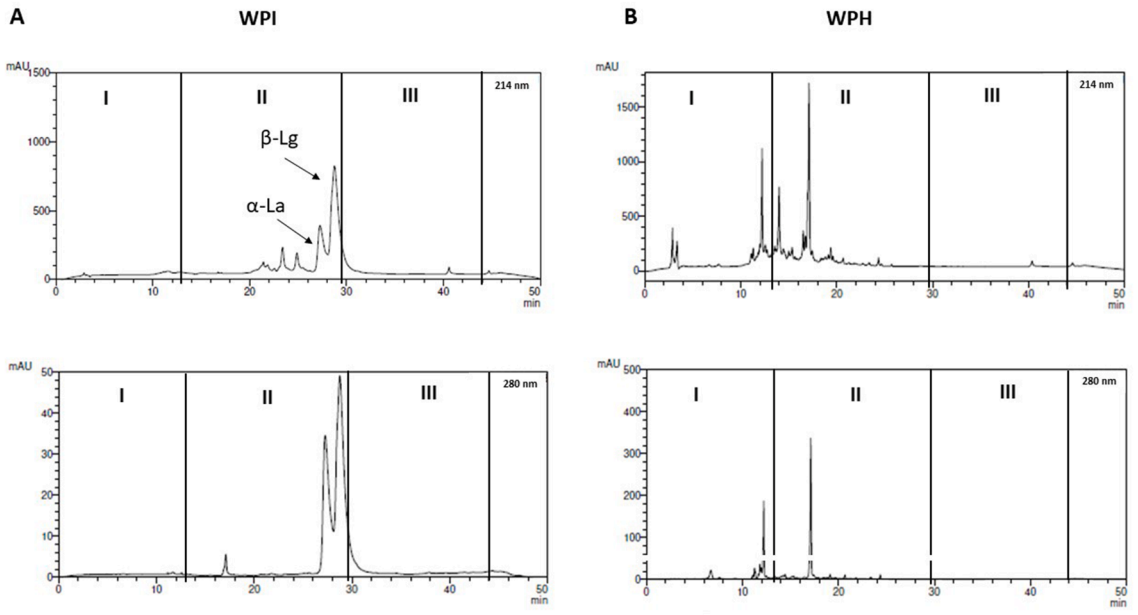


Fig. 2. WPH presented peptides with medium hydrophobicity. Chromatograms relating hydrophobicity zones of peptides of WPI (A) and WPH (B) obtained at 214 and 280 nm, respectively. The hydrophobicity zones are related to retention time and mobile phase concentration where: 0–25% Mobile Phase B corresponds to low hydrophobicity (I), 25–50% medium hydrophobicity (II), 50–100% high hydrophobicity (III).

Table 2
Peptide identification of WPH based on de novo and database comparison strategy.

Protein Source	Peptide	MW average (Da)	Sequence length	Hydrophobicity (%)	Function related	Manuscript
Beta lactoglobulin	ALPMHIR	837.06	7	57.14	Proliferative effect	(Jacquot et al., 2010)
					Release of endothelin-1 by endothelial cells	(Maes et al., 2004)
					ACE-inhibition	(Yamada et al., 2015)
	DAQSAPLRV	956.07	9	44.44	DPP-IV Inhibition	(Lacroix & Li-Chan, 2012)
	GLDIQK	672.78	6	33.33	Hypocholesterolemic	(Nagaoka et al., 2001)
					DPP-IV Inhibition	(Pihlanto-Leppälä et al., 1998)
	IDALNENK	916.01	8	37.5	Proliferative effect	(Jacquot et al., 2010)
	LIVTQTMK	933.18	8	50	Proliferative effect	(Jacquot et al., 2010)
	LKPTPEGDL	969.11	9	22.2	DPP-IV Inhibition	(Lacroix et al., 2017)
	LKPTPEGDLE	1098.22	10	20.0	DPP-IV Inhibition	(Lacroix & Li-Chan, 2014)
	TPEVDDEALEK	1245.31	11	27.27	Antibacterial	(Power et al., 2014; Silveira et al., 2013)
					DPP-IV Inhibition	
	VLDTDYK	852.47	7	42.86	DPP-IV Inhibition	(Pihlanto-Leppälä et al., 2000)
	VLVLDTDYK	1065.24	9	55.56	DPP-IV Inhibition	(Silveira et al., 2013)
	VYVEELKPTPEGDLEILLQK	2313.69	20	40.0	Hypocholesterolemic	(Suwal et al., 2017)
	YVEELKPTPEGDL	1489.65	13	30.77	Antioxidant	(Basilicata et al., 2018)
Beta-casein	EMPFPK	747.91	6	33.33	Bradykinin-Potentiating	(Perpetuo et al., 2003)
					ACE-inhibition	(Plaisancié et al., 2015)
	HQPHQPLPPT	1151.30	10	10.0	ACE-inhibition	(Adams et al., 2020)
	LHLPLP	688.87	6	50.0	ACE-inhibition	(Quirós et al., 2008)
	LNVPGEIVE	969.11	9	44.44	ACE-inhibition	(Gobbetti et al., 2000)
	LVYPFPGPI	1002.23	9	55.56	ACE-inhibition	(Otte et al., 2007)
	NIPPLTQTPV	1079.27	10	30.0	ACE-inhibition	(Gobbetti et al., 2000)
	PFPGPIPN	837.98	8	25.0	ACE-inhibition	(Lin et al., 2017)
	SLPQNIPPL	978.16	9	33.33	DPP-IV Inhibition	(Rendón-Rosales et al., 2022)
	VYPFPGPI	889.07	8	50.0	Prolyl endopeptidase-inhibitory/ PEP-inhibitory	(Asano et al., 1991)
	VYPFPGPIPN	1100.29	10	40.0	Antioxidant	(Tonolo et al., 2020)
					ACE-inhibition	(Eisele et al., 2013)
	YPFPGPIPN	1001.16	9	33.33	DPP-IV Inhibition	(Uenishi et al., 2012)
					ACE-inhibition	(Saito et al., 2000)
					Antioxidant	(Amigo et al., 2020)
Alpha-lactalbumin	RELKDLK	901.08	7	28.57	Proliferative effect	(Jacquot et al., 2010)

Note: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly;H = His; I = Ile; K = Lys; L = Leu;M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val;W = Thr; Y = Tyr.

residues and a size between 0.6 and 1.5 kDa, which is consistent with the data found in the molecular size analysis using FPLC (refer to Fig. 1). Several studies have linked low molecular size peptides with improved antioxidant activity (Zou et al., 2016). A study using different sizes of whey hydrolysates (5 kDa, 1 kDa, and 0.6 kDa) showed that smaller peptides had greater antioxidant activity, evaluated by ORAC (Estévez et al., 2020; O'keeffe et al., 2017; O'Loughlin, Murray, FitzGerald, Brodkorb, & Kelly, 2014).

The majority of peptides had intermediate hydrophobicity, ranging from 30 to 50%, which is consistent with the hydrophobicity analysis using HPLC. The most hydrophobic peptides found in our sample (>42% hydrophobicity) had a higher proportion of leucine and valine, mainly in the N-terminal region, as show in the heating map (Fig. 3A), which is considered significant for antioxidant potential (Bougatef et al., 2010; Chen, Muramoto, & Yamauchi, 1995; Zou et al., 2016). QSAR modeling showed that the steric properties of amino acids in the N- and C-terminal region played a vital role in antioxidant activity (Tian et al., 2015). Furthermore, hydrophobic amino acid residues (such as leucine and valine) in the N-terminal region increased the interaction between peptides and fatty acids, which could lead to greater penetrability in the cellular lipid bilayer (Li, Li, He, & Qian, 2011).

A high proportion of the amino acid lysine and isoleucine was found in the C-terminal region of hydrophobic peptides (Fig. 3C). Isoleucine has an important antioxidant role as branched chain amino acids, such as leucine and valine. Isoleucine has presented an important protection against oxidative damage in mammalian epithelial cells, improving the deficit in peroxisome transport (Wu et al., 2022). Dietary lysine levels have improved antioxidant capacity in intestinal barrier function by upregulating Nrf2, and the expression of antioxidant enzyme genes (Zhao et al., 2023). Proline residue was presented in most sequenced peptides (Fig. 3). In fact, high content of Pro is characteristic of casein derived peptides, contributing to their open structure, which may increase the availability of the amino acid residues to act as antioxidants (Sabeena Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010; Zou et al., 2016).

As shown in Table 2 the sequences identified in our sample were previously identified in other studies for other biofunctionalities, mainly related to ACE and DPP-IV inhibitory action, as well as hypercholesterolemic function. Three sequences from our sample (YPFPGPIPN, VYPFPGPIPN, YVEELKPTPEGDL) were associated with antioxidant activity in previous chemical *in vitro* analyses (Basilicata et al., 2018; FitzGerald, Cermeño, Khalesi, Kleekayai, & Amigo-Benavent, 2020; Tonolo et al., 2020). Interestingly, the β-LB-derived sequence YVEELKPTPEGDL inhibited ROS production in intestinal crypt cells (IEC) and stimulated the translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) cytroprotectant, indicating a potential of intracellular redox mechanisms (Basilicata et al., 2018).

3.4. Chemical antioxidant capacity in vitro (DPPH, ABTS and ORAC)

The antioxidant activity of the gastric digestion resistant peptides was assessed using chemical *in vitro* assays (ABTS, DPPH and ORAC) and compared with the whey protein isolate (see Table 3). Very low or undetectable levels of antioxidant activity were observed in the Infogest control sample, confirming that the fluids used in the *in vitro* digestion process did not affect the antioxidant capacity of the sample. Both samples showed concentration-dependent antioxidant activity in the assays. Nevertheless, the antioxidant activity of the digested peptides

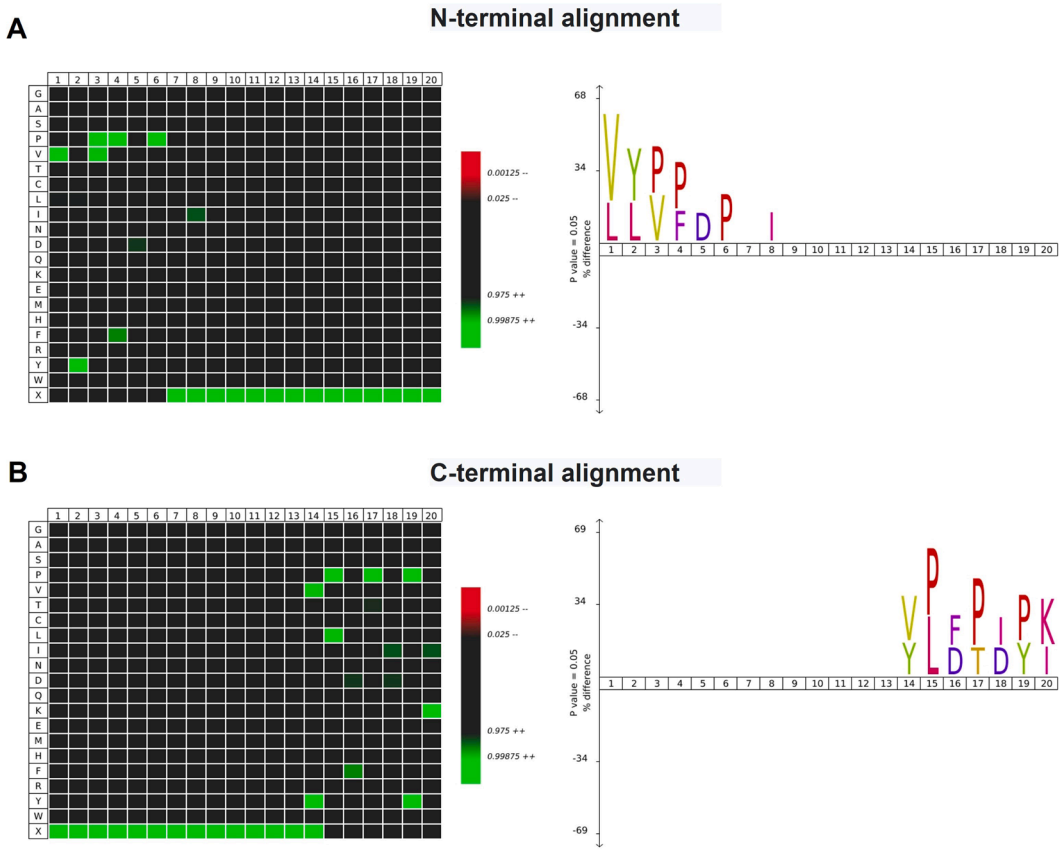


Fig. 3. Heat map graph of most hydrophobic sequences (greater than40%) using N-terminal alignment (A) and C-terminal alignment (B). Only significant amino acids ($P < 0.05$) are shown or colored in the heatmap. The difference in the frequency of an amino acid is expressed as the size of letters or color intensity. The P value of each amino acid at every position was calculated by testing the experimental frequency against the frequency of each amino acid in the reference set.

was approximately two times as that of the whey protein isolate in the three assays tested, confirming that protein breakdown and peptide formation enhance the antioxidant capacity of the sample by exposing hydrogen-donor amino acids previously hidden in protein structure.

A study observed an increase in the antioxidant activity of whey peptide isolate after digestion by ABTS, FRAP and ORAC method (Corrochano, Sariçay et al., 2019), further supporting our results. This study determined the antioxidant bioactivity of peptides formed by exogenous enzymes was not maintained after the digestive process, indicating some peptides must be degraded. In opposition, other studies have shown higher antioxidant activity of hydrolysates after gastrointestinal digestion (Wang, Han, Tan, Hong, & Luo, 2023). From a physiological perspective, the fortification of foods with antioxidant peptides may be dependent on peptide resistance during digestion process.

3.5. Treatment with WPH on Caco-2 cells

Our study evaluated the antioxidant capacity of the digestion-resistant peptides in Caco-2 cell cultures under oxidative stimulation by hydrogen peroxide (H_2O_2). Due to the higher antioxidant activity of hydrolysates in previous results, only these samples were evaluated in cell culture. To determine safe concentrations of WPH and H_2O_2 that would not compromise cell viability, a curve with different concentrations was performed. Safe concentrations for WPH were in the range of 1–0.01 mg/mL, while for H_2O_2 they were below 1 mM (Fig. 4A).

Stimulation with H_2O_2 1 mM induced an increase in reactive oxygen species (ROS) production which was partially prevented by WPH in a dose dependent manner (Fig. 4B). Moreover, the oxidation induced by H_2O_2 reduced glutathione content, which was reversed by pretreatment with WPH (Fig. 5A). Oxidation stimulus induced by H_2O_2 increased superoxide dismutase (SOD) activity, which was prevented by WPH (Fig. 5B). Nitric oxide secretion levels were not affected in the stimulated Caco-2 cells (Fig. 5C).

Like our results, digested fractions of β-LG and α-LA in HT-22 neuronal cells and in whey-based beverage and WPI pressurized in Caco-2 intestinal cells reduced ROS (Corrochano, Arranz et al., 2018; García-Casas et al., 2022). The hydrolysates prevented the increase of ROS and increased the content of glutathione, a major cellular antioxidant. This suggests two possible mechanisms of action of whey peptides: (I) peptides serving as hydrogen or electron donors for radicals, saving glutathione itself for its antioxidant function and/or (II) peptides acting on oxidative pathways, promoting increased cellular glutathione synthesis (Corrochano, Buckin et al., 2018). A previous study observed that whey digests increased the expression of glutathione peroxidase at the mRNA level in Caco-2 cells, suggesting that these peptides may play an important role at the transcriptional level (Corrochano, Buckin et al., 2018). Amino acid analysis revealed that WPH is rich in glutamate, a major cofactor for glutathione synthesis. An increase in glutathione levels induced by hydrolyzed whey concentrates (with exogenous enzymes) was observed in studies with human umbilical vein endothelial cells (O'Keeffe & FitzGerald, 2014), with hepatocytes (Pyo, Yang, Chun, Oh, & Lee, 2016), and with whole protein in myoblasts (Kerasioti et al., 2014).

In contrast to our result, previous studies have shown that WPH protected myoblast and fibroblast cells from oxidative stimulus by increasing SOD activity (Kong, Peng, Xiong, & Zhao, 2012; Xu, Liu, Xu, & Kong, 2011). Since these assessments were performed over a longer incubation period of 24 h, different levels of SOD activity may also be related to an increase in enzyme expression. In our acute treatment (4 h), an increase in SOD activity after oxidative stimulus could represent a

Table 3
WPH present antioxidant activity by ABTS, DPPH and ORAC assay.

ABTS % of antioxidant activity		
Concentration mg/mL	WPI	WPH
1	4.8 ± 5.9^{a}	$60.0 \pm 25^{c,d}$
5	19.6 ± 7.3^{b}	89.6 ± 8.9^{e}
10	45.3 ± 11.6^{c}	97.5 ± 3.2^{e}
20	$56.9 \pm 7.8^{c,d}$	99.5 ± 0.3^{e}
Infogest control	1.3 ± 0.5^{a}	
DPPH % of antioxidant activity		
Concentration mg/mL	**WPI**	**WPH**
1	22.0 ± 8.6^{b}	20.9 ± 9.8^{b}
5	$30.6 \pm 7.2^{b,c}$	$27.9 \pm 9.5^{b,c}$
10	$20.8 \pm 13.5^{b,c}$	51.6 ± 13.4^{d}
20	$40.0 \pm 2.3^{c,d}$	75.1 ± 8.1^{e}
Infogest control	3.7 ± 11.2^{a}	
ORAC % of antioxidant activity		
Concentration mg/mL	**WPI**	**WPH**
0.01	$11.6 \pm 4.2^{a,b,c}$	$8.9 \pm 8.2^{a,b}$
0.25	$11.8 \pm 1.5^{a,b,c}$	$44.9 \pm 9.4^{b,c}$
1	$29.2 \pm 7.7^{a,b,c}$	48.3 ± 14.3^{c}
10	49.0 ± 13^{c}	48.6 ± 14.3^{c}
Infogest control	4.7 ± 1.2^{a}	

Different concentrations of WPI and WPH tested for ABTS, DPPH, and ORAC assay. Concentrations are shown as percentage of antioxidant activity ± standard deviation. WPI: whey protein isolate WPH: whey protein hydrolysate. Different letters indicate they showed a significant difference between samples by the Duncan test ($p < 0.05$). N = 5.

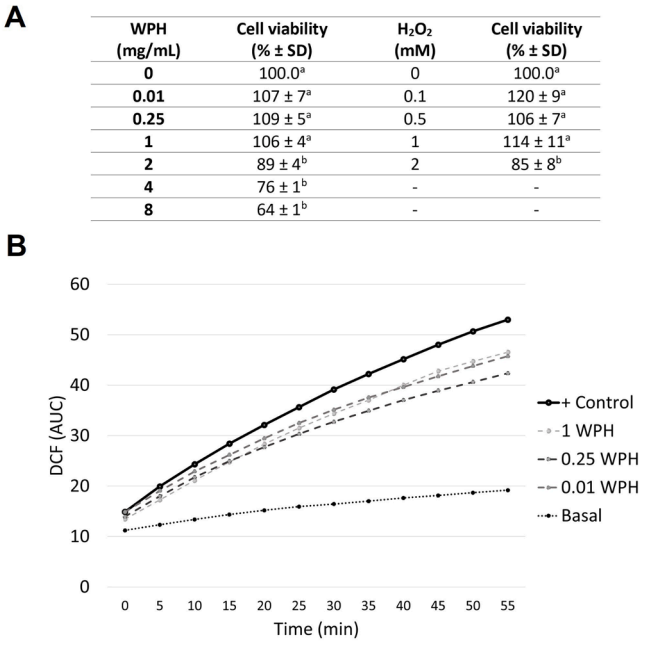


Fig. 4. WPH attenuates ROS production in oxidative stimulated Caco-2 cells. (A) To define concentrations that were not toxic to cells, Caco-2 were treated with different concentrations of WPH or H_2O_2 for 4 h for. Cell viability (MTT) was expressed by % of control (B) Cells were pre-treated with different concentrations of WPH (1 h) and stimulated with 1 mM H_2O_2 for 3 h. Stimulation with hydrogen peroxide increased the production of reactive oxygen species (ROS) by fluorescence increase of DCF, which was attenuated with WPH treatment.

cell response to attempt for a reduction of oxidative stress, while

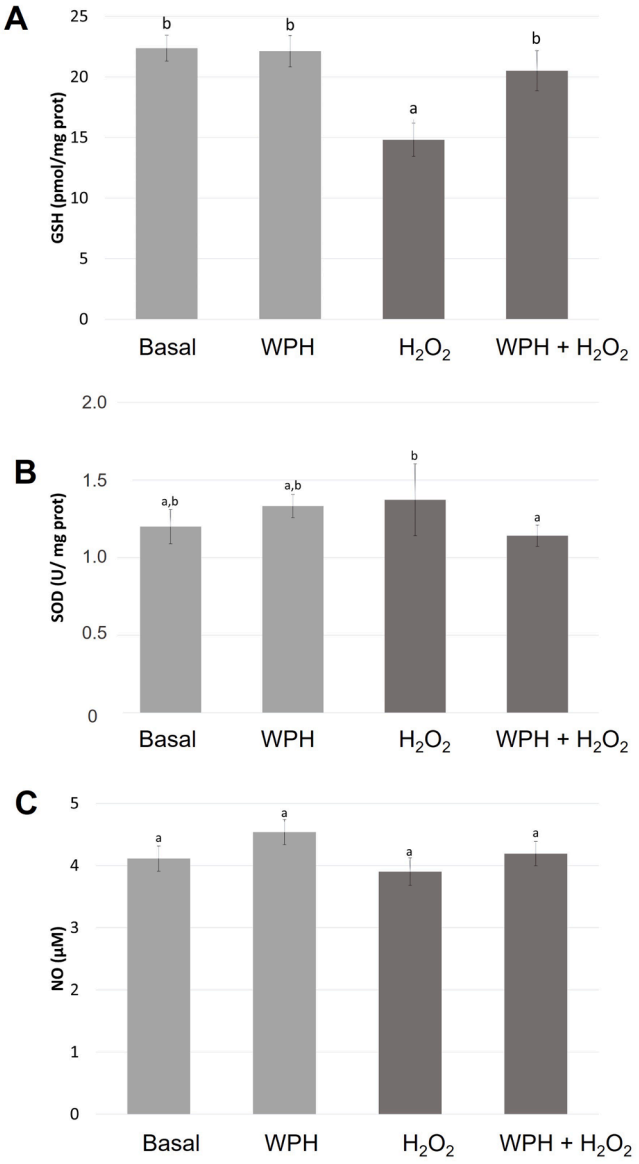


Fig. 5. WPH prevents GSH reduction and SOD activity in oxidative stimulated Caco-2 cells. Cells were pre-treated with WPH 1 mg/mL (1 h) and stimulated with 1 mM H_2O_2 or not for 3 h. (A) oxidative stimulus reduced GSH content, which was prevented by WPH treatment (B) Oxidative stimulus increased SOD activity, which was prevented by WPH. (C) Nitric oxide (NO) secretion levels were not affected in stimulated Caco-2 cells. Treatments were compared by ANOVA followed by the Duncan test. Different lowercase letters indicate statistical difference ($p < 0.05$). WPI: whey protein isolate WPH: whey protein hydrolysate.

coincubation with WPH may serve as an external antioxidant source, preventing the increase in SOD activity.

4. Conclusions

Hydrolysis of whey proteins during gastrointestinal digestion *in vitro* was able to produce peptides activating antioxidant pathways in human enterocytes and colonocytes model. The peptide digests were able to attenuate reactive oxygen species production, prevent GSH reduction and SOD consumption in Caco-2 cells stimulated with hydrogen peroxide. Although the peptides showed mostly hydrophobic and acidic amino acids in their chain, surface hydrophobicity was average. The identified peptides must be resistant to digestion, although some of them may be the result of incomplete digestion due to time constraints of the

digestive process. Structurally, the sequenced peptides showed predominantly the amino acids lysine and valine in the N-terminal region and tyrosine in the C-terminal region, with recognized antioxidant properties. These results reinforce the antioxidant properties of whey proteins, with the ability to form peptides that can promote health benefits even to the gastrointestinal epithelial cells.

Enteric endothelial cells are the first to come into contact with digested food and may suffer oxidative damage that compromises their functionality in inflammatory situations, such as in Crohn's disease (Iborra et al., 2011). Consuming foods with the potential to protect against this damage may offer a preventative approach.

Contribution

Juliana Santos de Espindola: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Milena Ferreira Taccóla: Investigation; Methodology; Vera Sônia Nunes da Silva: Methodology; Lucilene Delazari dos Santos: Investigation; Methodology, Software; Bruno Cesar Rossinib: Investigation; Methodology, Software; Bruna Cavecci Mendonçab: Investigation; Methodology; Maria Teresa Bertoldo Pacheco: Funding acquisition; Resources; Supervision; Writing – review & editing; Fabiana Gallanda: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; project administration; Resources; Supervision; Validation; Visualization; Roles/Writing, revising & editing.

CRediT authorship contribution statement

Juliana Santos de Espindola: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Milena Ferreira Taccóla:** Investigation, Methodology. **Vera Sônia Nunes da Silva:** Methodology. **Lucilene Delazari dos Santos:** Investigation, Methodology, Software. **Bruno Cesar Rossini:** Investigation, Methodology, Software. **Bruna Cavecci Mendonça:** Investigation, Methodology, Software. **Maria Teresa Bertoldo Pacheco:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Fabiana Galland:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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