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Evaluation of *in vitro* whey protein digestibility in a protein-catechins model system mimicking milk chocolate: interaction with flavonoids does not hinder protein bioaccessibility

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Abstract

Flavonoids are largely present in plant food such as cocoa and derived products. These compounds can interact with proteins inherently contained in the food matrix and/or the proteolytic enzymes involved in gastrointestinal digestion. The flavonoid/protein interaction might hamper protein bioaccessibility and digestibility, affecting the nutritional quality. However, information on the digestion fate of proteins in food matrices containing both proteins and flavonoids is limited. The aim of this work was to evaluate the interaction between proteins and flavonoids and verify the potential effects of this interaction on protein digestibility. Taking milk chocolate as model, first a simple whey proteins/catechins mixed system was evaluated, and then the effects on digestibility were also verified in a real sample of commercial milk chocolate. The effects of the catechins/whey proteins interaction in the model system was evaluated by optical and chiro-optical spectroscopy, outlining a slight protein structure modification upon interaction with catechins. The digestibility of the protein fraction both in the model system, with and without catechins, and also in milk chocolate, was then determined by the application of INFOGEST *in vitro* digestion method: the bioaccessibility was evaluated in terms of protein hydrolysis and protein solubilisation, and major peptides generated by the digestion were also determined by LC/HR-MS. Despite the slight interaction with proteins, flavonoids were found to not hinder nor modify protein solubilization, protein hydrolysis and peptide profile by digestive enzymes. Also protein digestibility in milk chocolate, evaluated by SDS-PAGE, was found to be complete. The present data clearly indicate that the interaction of the proteins with the flavonoids present in the cocoa matrix does not to affect protein bioaccessibility during digestion.

KEYWORDS: flavonoids; catechin; epicatechin; *in vitro* digestion; protein solubilization; protein-flavonoid interaction; milk chocolate; whey proteins

Abbreviations C, catechin; CA, catechins; CD, Circular dichroism; DH, degree of protein hydrolysis; EC, epicatechin; GD, gastro-duodenal digestion; MC, milk chocolate; WP, whey proteins; WP/CA, whey proteins added with catechins; SSFe, electrolytic solution of simulated salivary fluid, SGFe, electrolytic solution of simulated gastric fluid, SIFe, electrolytic solution of simulated intestinal fluid.

1. Introduction

Flavonoids are defence-linked phytochemicals extensively found in the plant kingdom. Fruits, vegetables, and hence plant-based derived food (e.g., chocolate products) and beverages (e.g., tea, wine) are rich in these compounds (Dias et al., 2021; Panche et al., 2016). Among the commonly eaten foods, cocoa and chocolate products contain a high concentration of flavonoids such as, catechin and epicatechin, which belong to the flavan-3-ols subgroup (Dias et al., 2021).

During the last few years, some investigations underlined how flavonoids could interact with proteins leading to several structural changes (Cao & Xiong, 2017; de Morais et al., 2020; Han et al., 2019; Pal et al., 2012; Sęczyk et al., 2019; Shen et al., 2014). These modifications might involve reversible or not-reversible reactions which impact the structure and functional properties of proteins (Ozdal et al., 2013). Reversible protein/flavanols interactions occurring in food, include non-covalent binding such as hydrogen bonds, hydrophobic interactions and van der Waals interactions (Yildirim-Elikoglu & Erdem, 2018), whereas irreversible interactions include covalent bonds obtained under specific conditions (Dai et al. 2022). Among proteins, dairy proteins were explored for the interaction with flavonoids, mainly due to the high binding affinity for milk proteins of some dietary flavonoids, both in pure compounds and natural extracts (Han et al., 2019; Yildirim-Elikoglu & Erdem, 2018). The latter, was also demonstrated at molecular level, using a computational docking simulation. For instance, Li et al. (2018) observed that flavonoids can interact with specific amino acid residues of β-LG. Another example was given by Li et al. 2021 who evaluated the mode of interaction between flavonoids and whey proteins isolate (β-lactoglobulin and α-lactalbumin in mix): in that conditions all flavonoids interacted with the same hydrophobic cavity of β-lactoglobulin and α-lactalbumin in correspondence with specific amino acid residue.

Some studies also suggest that flavonoid/dairy protein interaction might lead to the modification of the secondary structure. However, information on the subject is contradictory, indicating both increase and decrease in β-sheet and α-helix (Yildirim-Elikoglu & Erdem, 2018; Li et al., 2018; Han et al. 2022). In other studies, the structural modification consisted of an increase in molecular size related to the formation of protein aggregates (Han et al., 2019; Yildirim-Elikoglu & Erdem, 2018).

As a consequence of the protein/flavonoid interaction also the digestibility of proteins could be affected. As reviewed by Cirkovic Velickovic & Stanic-Vucinic (2018), flavonoids can bind both the protein substrates in food matrices and the proteolytic enzymes involved in the digestive process. The alteration of the activity of the enzymes and the accessibility of food proteins can then hinder the protein fate during digestion. For instance, Świeca et al., 2013 reported that the main enzymes involved during gastrointestinal digestion of protein, i.e. trypsin, chymotrypsin, pepsin and pancreatin, could be affected by the presence of flavonoids by consequently reducing the protein digestibility. Anyway, the effect on protein digestibility depends on the different treatment conditions tested, such as type of protein substrate, type and concentration of the

flavonoid, protein/flavonoid ratio, enzyme/substrate concentration and digestion time (Cirkovic Velickovic & Stanic-Vucinic, 2018).

The other important aspect concerns the effect on digestibility of the direct interaction between the protein substrate and flavonoids. In products which contain proteins (bread, legumes, dairy products), the addition of flavonoids as supplements in fortified food demonstrated a decrease in the protein digestibility with an improved stability of the flavonoid/protein complex (Świeca et al.,2014). In contrast with previous results, de Morais et al. (2020) did not observe differences of digestibility between whey protein/flavonoid complex and whey protein control, except for some peptides resistant to digestion observed at pH 7. In addition, an improved solubility and protein hydrolysis, hence digestibility, was observed in a whey protein/gallic acid/epigallocatechin gallate system (Cao & Xiong, 2017) suggesting an increase in nutritional quality and biological properties.

Thus, information on the digestibility of proteins in presence of flavonoids are both contradictory and limited. In addition, the variability of the *in vitro* digestion protocols used, and the different substrates and protein/flavonoids concentrations also introduced a further element of complexityIn this context, and to fill the gaps existing in the current literature, the aim of this work was to evaluate protein digestibility in presence of flavonoids, using state-of-the art validated digestive models . Milk chocolate was chosen as a prototypical complex food system consisting of proteins and flavonoids. Using that food matrix as model, a simplified mixed system of whey proteins and catechins was prepared, taking into account the real protein/flavonoids ratio (and the actual flavonoids present). The interaction between whey proteins and the main flavonoids of milk chocolate [(+)-catechin and (-)-epicatechin)] was verified using optical and chiro-optical spectroscopyThen protein digestion was determined by the application of the harmonized INFOGEST protocol: the digestate was analysed in terms of protein solubilisation, degree of protein hydrolysis and peptide profile determined by LC/MS. Protein digestibility was also verified on a commercial sample of milk chocolate by analysing the protein fraction before and after digestion with SDS-PAGE.

2. Materials and methods

2.1 Chemicals

20x XT reducing agent, 4x XT sample buffer, Coomassie brilliant blue protein stain powder R-250, Criterion XT Bis-Tris Precast Gel (10 % and 12 % Bis-Tris, 13.3 × 8.7 × 0.1 cm), and Precision Plus Protein Standards XT MES running buffer, were purchased from Bio-Rad (Hercules, CA, USA). Quant-iT™ Protein Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA). Kjeldahl defoamer, Acetonitrile (CH_3CN) were purchased from Merck (Darmstadt, Germany). Copper (II) oxide were purchased from VWR Chemicals (Radnor, PA, USA). Kjeldahl tablets catalyst (3.5 g/tablet), sulfuric acid (96 %, H_2SO_4) were purchased from Merck Millipore (Burlington, MA, USA). (-)-Epicatechin, (+)-Catechin hydrate ≥ 98 %, bile salts, hydrochloric acid (37 %, HCl),

pancreatin (containing enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease) from porcine pancreas 8 × USP, porcine pepsin from gastric mucosa 727 U/mg, porcine α-amylase 15 U/mg, salts for electrolytic solution of simulated salivary fluid (SSFe), electrolytic solution of simulated gastric fluid (SGFe), electrolytic solution of simulated intestinal fluid (SIFe), ultra-pure formic acid (HCOOH), SDS (sodium dodecyl sulphate), were purchased from Sigma Aldrich (St. Louis, MO, USA). Boric acid (H_3BO_3), Calcium chloride ($CaCl_2$), N-acetylcysteine (NAC), *o*-phthaldialdehyde (OPA), and sodium hydroxide (NaOH) were purchased from Carlo Erba (Milan, Italy).

2.2 Analysis on commercial milk chocolate

2.2.1 Proximate composition

The proximate analysis of a commercial milk chocolate, bought in a local shop, included the determination of moisture, proteins, lipids, and ashes according to the standard procedures (AOAC, 2002). Moisture content was determined after drying the sample in an oven for 24 h at 105 °C. Total protein content was quantified after measuring total nitrogen by using Kjeldahl system (VELP SCIENTIFICA, Usmate Velate, Italy). The general 6.25 conversion factor was used to convert the percentage of nitrogen to the protein content found in the sample (US Department of Agricolture (USDA)a) . To determine the lipid fraction, an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) was employed. The extraction was carried out by using diethyl ether as extraction solvent. For the determination of ashes, the sample was incinerated in a muffle furnace at a controlled temperature of 550 °C for 5 h. All the analyses were performed in triplicate, and the results were expressed as a percentage of dry matter (g/100 g of sample). The percentage of the other compounds present in the samples was calculated as a difference.

2.2.2 Quantification of catechins by UHPLC/ESI-MS/MS

The determination of (-)-epicatechin and (+)-catechin was performed on commercial milk chocolate. The sample and the standard curve were prepared as previously described by Shumow & Bodor (2011) with some modifications. Briefly, 2 g of ground milk chocolate was defatted twice by the addition of 40 mL of hexane, mixed and centrifuged at 4 °C, 3220*g* for 5 min (5810R Eppendorf, Hamburg, Germany). The supernatant was decanted while the defatted pellet was dried under nitrogen flux. For the extraction, the sample was added with 10 mL of 30 % methanol, 70 % water and 1 % formic acid solution, mixed, sonicated for 5 minutes and centrifuged for 5 min; the supernatant was then decanted in a 25 mL volumetric flask. The extraction was repeated, and the supernatant was decanted into the same 25 mL volumetric flask made up to volume with the extraction solvent. Before the analysis, the sample was filtered through a 0.45 µm nylon filter membrane into UHPLC vials. In addition, a stock standard solution (100 µg/mL) with (+)-catechin hydrate and (-)-epicatechin was prepared for the analysis. For the calibration curve, the stock standard solution was diluted and prepared in duplicate with concentrations ranging from 25 µg/mL to 1.56 µg/mL for (-)-epicatechin, and from 1.25 µg/mL to 0.078 µg/mL for (+)-catechin hydrate. The analysis was carried out using UHPLC (Dionex

Ultimate 3000, Sunnyvale, CA, USA) coupled to a triple quadrupole ESI-MS/MS (TSQ Vantage, Thermo Scientific, Waltham, MA, USA). A reversed phase column was used for the chromatographic separation (Aeris Peptide 1.7 µm XB-C18, 150 × 2.10 mm, Phenomenex, Torrance, CA). Eluent A was Milli-Q water, 0.2 % CH_3CN and 0.1 % HCOOH, eluent B was CH_3CN, 0.2 % Milli-Q water, 0.1% HCOOH. The injection volume was 2 µL, flow rate was maintained at 0.2 mL/min; the applied gradient was 0–1 min, 95 % A; 1–10 min, from 95 % A to 20 % A; 10–13 min, 20 % A; 13–14 min, from 20 % A to 95 % A; 14–22 min, 95 % A. The run time was 22 min; the column temperature was 35 °C; the autosampler temperature was set at 10 °C. For the detection, the analysis was performed in SRM scan type; negative ion mode (ESI-); MS acquisition time 22 min; $[M-H]^-$ m/z 289: ion fragment 245 (Collision E 16); 205 (Collision E 19), 203 (Collision E 21); S-Lens: 98; scan width (m/z): 0.200, scan time: 0.100 s, Q1 peak width: 1, Q3 peak width: 0.70. The data, including the peak integration, were processed using Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA). The analyses were performed in triplicate.

2.3 Preparation and analysis of whey proteins (WP) and whey proteins/catechins (WP/CA) model system

2.3.1 Preparation of whey proteins (WP) and whey proteins/catechins (WP/CA) samples

Commercial whey proteins were purchased in the market. Ingredients declared in the label: whey proteins, thickener: xanthan gum, aroma, sweeteners: acesulfame potassium and sucralose, emulsifiers: sunflower and soy lecithin. Composition declared in the label (g/100 g): fats 2.4 g (saturated fats 1.5 g), carbohydrates 3 g (sugars 2.2 g), proteins 83.0 g, fiber 0.8 g and salt 1.0 g. Whey proteins were used as such without any further purification for the preparation of the flavonoids/protein mixture.

5 g of the above commercial whey proteins mixed with (10:1 wt./wt.) (-)-epicatechin and (+)-catechin up to 1:35 wt./wt. between catechins and whey proteins. The mix was solubilized with 50 mL of ultrapure water (w/v 1:10) under stirring for 30 min, at room temperature. Then, the suspension was quick-frozen under liquid nitrogen, lyophilized (Lio 5P, 5Pascal, Milan, Italy) and stored at -20 ° C. Simultaneously, a negative control (WP) was prepared following the same procedure but without the addition of catechins.

2.3.2 Optical and chiro-optical spectroscopy

Electronic absorption, fluorescence and circular dichroism were measured on i) whey proteins (WP), ii) whey proteins added with catechins (WP/CA), iii) catechins (CA) [as a sum of (-)-epicatechin and (+)-catechin]. Electronic absorption (near and middle UV regions) spectra were measured with an Agilent Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in transmission mode, in 1 cm × 1 cm quartz cuvettes. Fluorescence emission and excitation spectra were measured with an Edinburgh Instruments FLS1000 fluorometer, in 1 cm × 1 cm quartz cuvettes. For the latter analysis, the samples were solubilized as follows: WP and WP/CA (0.125 mg/mL) in milliQ water and catechins (3.57 µg/mL) in H_2O: MeOH solution. Then, each sample were diluted 1:5 in H_2O: MeOH solution to maintain linearity following the Beer–Lambert law. Circular dichroism spectra were recorded at 24 °C by using Jasco J-1500 CD Spectrometer (Jasco, Tokyo,

Japan) in a wavelength range from 200 to 350 nm. For circular dichroism, samples were solubilized as follows: WP and WP/CA (0.125 mg/mL) in milliQ water and catechins (8.57 µg/mL) in H_2O: MeOH solution (50:50). Moreover, using the open web server BestSel on data collected by CD, the secondary structure content (%) was estimated (Micsonai et al., 2018). For the determination of secondary structure, the following parameters were used: input units, measured ellipticity (mdeg); concentration (µM), 6.25 (assuming protein average MW 20000 g/mol); number of residues, 150; Pathlength (cm), 1.

2.4 *In vitro* digestion study

2.4.1 *In vitro* gastroduodenal (GD) digestion

Samples were digested using an *in vitro* static gastro-duodenal digestion model, previously described by Brodkorb et al. (2019). The following procedure was slightly adapted as reported below. In short, 0.250 g of lyophilised sample was added with 2.5 mL of Milli-Q water, while 1 g of commercial milk chocolate was added with 1 mL of Milli-Q water, to obtain a better bolus consistency. Simulated salivary fluid which included SSFe (salivary electrolytic salt solution), 75 U/mL α-amylase and 1.5 mM $CaCl_2$ was added in 1:1 ratio (v/v). The samples, at pH 7, were incubated under agitation for 2 min at 37 °C using an orbital shaker incubator (ES-20, Biosan, Buenos Aires, Argentina). Then, simulated gastric fluid which included SGFe (gastric electrolytic salt solution), 2000 U/mL pepsin and 0.15 mM $CaCl_2$ was added (1:1 v/v). The samples, adjusted at pH 3, were incubated under stirring at 37 °C for 2 h. During the duodenal phase, the simulated intestinal fluid was added (1:1 v/v). Intestinal fluid was prepared by combining SIFe (intestinal electrolytic salt solution), 10 mM bile salts, 100 U/mL pancreatin (based on trypsin activity) and 0.6 mM $CaCl_2$. The samples were moved from an acidic pH to a neutral environment by adding 1 M NaOH, and a final incubation was performed for 2 h at 37 °C under shaking. Then, the incubation of samples at 100 °C for 5 min was carried out to inactivate the enzymes added during the process. Finally, the supernatant was recovered after 45 min, 4 °C, 3220*g* centrifugation (5810R Eppendorf, Hamburg, Germany) and stored at -20 °C. Negative digestion controls i) without sample ii) without the addition of enzymes, were prepared following the same steps and conditions. The digestion procedure was executed in triplicate for each sample.

2.4.2 Determination of protein amount before digestion and of protein solubilization after digestion

The determination of protein amount in raw lyophilized WP, WP/CA, and of protein solubilization in digested samples was carried out using the official Kjeldahl method. 0.5 g of undigested sample, or 1 mL of the supernatant coming from gastrointestinal digestion of WP, WP/CA were mineralized for 30 min at 420 °C using a heating digester (DKL heating digester, VELP SCIENTIFICA, Usmate Velate, Italy). Then, a distillation was performed using a semiautomatic distillation unit (VELP SCIENTIFICA, Usmate Velate, Italy). Finally, a titration was carried out to quantify the nitrogen present in the sample. The percentage of protein was determined using a nitrogen-to-protein conversion factor of 6.38 for WP and WP/CA both digested and raw undigested materials (Accardo et al., 2022; US Department of Agricolture (USDA)b).

2.4.3 Determination of the degree of protein hydrolysis after digestion

The degree of protein hydrolysis (DH%) was used as a key parameter to evaluate the percentage of the cleaved peptide bonds. To estimate the DH %, an OPA-based method was used, following the previously described procedure (Accardo et al., 2022).

The reaction solution was prepared by combining 50 mM OPA in methanol, 50 mM NAC water solution, 2 % (w/v) SDS and 0.1 M borate buffer at pH 9.5. The solution was stirred for 1 h covered from the light. 3 µL of the supernatants, resulting from *in vitro* gastro-duodenal digestion, and 17 µL of Milli-Q water were added to 2.4 mL of OPA/NAC solution and incubated for 15 minutes protected from the light. The same procedure was also applied for negative digestion controls. For calibration L-isoleucine water solutions of 0, 0.125, 0.25, 0.5, 1, and 2 mg/mL were prepared. The absorbance with the spectrophotometer (B530 JASCO, Oklahoma City, OK, USA) was read at 340 nm. The concentration of the free amino groups for each unknown sample was estimated by using the standard curve. The DH % was calculated as a ratio between free nitrogen groups present in the soluble fraction of the digestate, measured by OPA, and the total (free and bound) nitrogen groups quantified in the digestive mixture by using the Kjeldahl method.

2.4.4 Peptide analysis by LC-HRMS

To identify peptides generated during *in vitro* digestion, supernatant from simulated gastrointestinal digestion of each sample (WP and WP/CA) was filtered through a 0.2 µm PTFE membrane filter (for LC-MS) and analysed directly by VIon IMS QTof Mass Spectrometer (Waters, Milford, MA, USA). For analysis, chromatographic separation was achieved using a reversed-phase column (ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7 µm, 2.1 mm × 150 mm, Waters, Milford, MA, USA). Eluent A was Ultrapure water and 0.1 % HCOOH (MS grade). Eluent B was CH_3CN and 0.1% HCOOH (MS grade). The injection volume was 1 µL and the flow rate was maintained at 0.25 mL/min; total run time was 36 min and applied gradient was 0–3.5 min, 100 % A; 3.5–25 min, from 100 % A to 50 % A; 25–26.3 min, 50 % A; 26.3–26.5 min, from 50 % A to 0 % A; 26.5–29.1 min, 0 % A; 29.1–29.5, from 0 % A to 100 %; 29.5–36 min, 100 %. The column temperature was fixed at 35 °C; the autosampler temperature was set to 10 °C. Acquisition parameters were set as previously described by Accardo et al. (2022) following a positive ion mode (ESI+); MS acquisition time 3.5–29.1 min; analyser mode, sensitivity; mode, standard transmission; capillary, 3.00 kV; sample cone voltage, 40 V; source offset voltage, 80 V; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas, 50 L/h; desolvation gas, 800 L/h. MSe mode, high definition MSe; scan range, 100–2000 m/z; scan time, 0.4 s; low collision energy, 6 V; high collision energy ramp, 20 to 45 V; automatic lock correction (leucine enkephalin). Data were processed using UNIFI software (Waters, Milford, MA, USA). The list of identified peptides includes the following Uniprot protein accession numbers: P02754 (β-lactoglobulin), P00711 (α-lactalbumin), P02666 (β-casein), P02662 (αS1-casein), P02663 (αS2-casein), P02668 (k-casein), P24627 (Lactotransferrin). The variable amino acid modifications allowed were deamidation (N, Q), N-terminal pyroglutamic acid (E, Q),

oxidation (single or double, M or W) and phosphorylation (S, T, Y). Finally, the lists of identified peptides were filtered selecting peptides as follows: % matched ions, more than 20 %; first-generation fragment ions, at least 2. Then, the in source fragments were dropped from the final peptide lists. Analyses were performed in triplicate.

2.4.5 Protein integrity before and after digestion by SDS-PAGE

One-dimensional gel electrophoresis (SDS-PAGE) was performed to visualize the main proteins before and after digestion. In brief, for raw materials 0.5 g of lyophilized samples were solubilized in 5 mL of water under shaking for 30 min and centrifuged at 4 ◦C, 3220*g* for 10 min (5810R Eppendorf, Hamburg, Germany). Then, to calculate the amount to be loaded in each well, quantification of proteins in the raw sample, gastro-duodenal digestate and negative digestion controls was performed, following the manufacturer instructions, using Quant-iT™ Protein Assay Kit and a Qubit fluorimeter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The samples were mixed with 20× reducing agent and 4× sample buffer, incubated at 95 °C for 5 min and quick-frozen for 5 min at -20 °C. For the electrophoretic run of WP and WP/CA digested samples, 10 µg of protein and a molecular marker standard (Precision Plus Protein unstained standard, Bio-Rad, Hercules, CA, USA) were loaded into precast 10 % gel (Criterion XT Bis-Tris, Bio-Rad, Hercules, CA, USA). Instead, the electrophoretic run for milk chocolate was carried out loading 50 µg and a molecular marker standard (Precision Plus Protein unstained standard, Bio-Rad, Hercules, CA, USA) into precast 12 % gel. The run was performed using an XT MES running buffer for 1 h at 150 V into a Criterion™ cell (Bio-Rad, Hercules, CA, USA). The gel was stained for 2 h with distilled H_2O/MeOH/Coomassie blue solution mixture (50:40:10). Then, the gel was destained with distilled H_2O/MeOH/CH_3COOH solution (50:40:10) and incubated under a slow rotatory agitation for 20 min until the bands were displayed.

2.5 Statistical analysis

Statistical analysis was performed by using GraphPad Prism software 8.0.1 version (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± standard deviation, and all the experiments were performed in triplicate. For the analyses, ANOVA (with Tukey post-hoc test) or an unpaired t-test were used to evaluate the differences between samples ($p < 0.05$ was considered statistically significant).

3. Results and discussion

3.1 Preparation of the protein-flavonoids simplified model system

The digestibility of whey proteins in presence of the flavonoids was the aim of this study. Milk chocolate was used as a prototypical example of a food where this interaction occurs, thus the chosen flavonoids were the ones mostly present in chocolate, i.e., (-)-epicatechin and (+)-catechin. The study was performed following different phases, a flow chart is presented in Figure 1.

<<Insert Figure 1>>

Milk chocolate is a complex food matrix, and for this reason, in order to evaluate the protein digestibility when whey proteins and flavonoids are present together without interferences from other proteins, a mixture simulating a simplified model of the whey protein-flavonoid composition of milk chocolate was first used.

To determine the actual protein content and the ratio between proteins and catechins in milk chocolate, a commercial sample was first characterized for its proximate composition and catechins content. The gross composition of milk chocolate sample (details in the experimental section) is reported in Table 1. These data were in line with the data reported by the manufacturer

Table 1 Proximate composition of commerciaal milk chocolate (g/100g)

Composition g/100 g	Commercial milk chocolate
Moisture	0.96±0.14
Proteins	7.96±0.35
Lipids	32.57±0.19
Ashes	2.02±0.02
Other compounds	56.91±0.51

Catechin composition and amount were also determined by LC/MS (details in the experimental section). The amount of catechins found in the commercial milk chocolate are shown in Table 2. These results were in line with Shumow & Bodor (2011). As expected, the content of epicatechin in milk chocolate is higher than catechin, as observed by Shumow & Bodor (2011).

Table 2 Quantification of of (+)-catechin and (-)-epicatechin in commercial milk chocolate

Compound	Retention time (min)	µg compound /g sample
(+)-Catechin	9±0.1	33.53±3.5
(-)-Epicatechin	9.6±0.1	175.12±2.66

The quantification was carried out by UHPLC/ESI- MS/MS. [M-H]- m/z 289 → 245; 205, 203.

All the above data were used to calculate the whey protein/catechin composition most similar to what is actually found in milk chocolate. The 1:10 (wt./wt.) ratio between catechin and epicatechin was chosen, as

also reported by Jalil & Ismail (2008), while the relative amount of catechins and whey proteins was chosen at 1:35 (wt./wt.), based on an average of our results and the data available on milk, whey protein and catechins in commercial milk chocolate products (Beckett, 2008; Gottumukkala et al., 2014; Gu et al., 2006; Shumow & Bodor, 2011; Gutiérrez, 2017). In order to prepare the model sample, commercial whey proteins, (-)-epicatechin and (+)-catechin were dissolved in water solution. Then the solutions were lyophilized, in order to ensure a proper interaction among molecules (details in the experimental section 2.3.1).
This simplified model sample was used to first evaluate the interaction between proteins and flavonoids, and then to characterize the protein digestibility.

3.2 Evaluation of the protein-flavonoids interaction by (chiro-)optical spectroscopy

Optical spectroscopy (UV absorption and fluorescence) and circular dichroism spectroscopy are common techniques used to acquire information on the structure and enantiomers of biomolecules (Ranjbar & Gill, 2009). Through these techniques, it is possible to obtain information on the secondary and tertiary structure (far and near-UV, respectively) of proteins as well as unveil details on the interactions with other chiral molecules (Kelly & Price, 2005; Khan et al., 2019; Ranjbar & Gill, 2009).
The UV absorption spectra measured for whey proteins (WP), catechins (CA) and the mixed whey proteins/catechins (WP/CA) samples are shown in Figure 2, panel a. The absorption spectrum of WP/CA is well superimposable to the sum of the absorption spectra of WP and CA. Unfortunately, WP and CA absorb in the same spectral range, with a weak absorption band around 280 nm (that has been enlarged in the inset of panel a), and a more intense band around 200 nm, with a shoulder at $\sim$ 230 nm. This makes difficult to evaluate the possible effect of WP/CA interactions on the shape and position of the absorption bands of the protein. To better evaluate the interaction effect, we also measured the fluorescence spectra (both emission and excitation) on the same samples (Figure 2, panel c). The fluorescence of the protein (picked at 330 nm) is due to the tryptophan residues, whose absorption falls at 280 nm. Fluorescence spectra were obtained upon excitation at that wavelength, for all the samples (WP, WP/CA and CA). The fact that CA also absorbs at 280 nm complicates the interpretation of the emission spectra, since CA is fluorescent (with a maximum at 310 nm) and its fluorescence is partially overlapped with that of tryptophan. Results show that the fluorescence spectrum of WP/CA is roughly the sum of the fluorescence spectra of WP and CA. A further confirmation is given by excitation spectra upon detection at different wavelengths (Figure 2, panels d and e): when detecting light emitted at 300 nm (where mainly CA emits), we retrieve an excitation spectrum that is superimposable to that of CA alone (panel d); when detecting light emitted at 360 nm (where mainly WP emits), we retrieve an excitation spectrum that is superimposable to that of WP alone (panel e); when detecting at an intermediate wavelength (such as 330 nm, panel c), we obtain an excitation spectrum where the absorption of both WP and CA contribute.

Electronic absorption and emission spectra, being roughly additive, thus suggest a negligible interaction between the fluorophores in WP and CA. It is worth underlying that tryptophan, responsible for the emission of the protein, is scarcely abundant in the specific protein that we used, so that its weak or negligible interaction with CA is not representative of the protein behaviour.

To better characterise the possible interaction between whey proteins and catechins and to verify possible protein structural changes in presence of (-)-epicatechin and (+)-catechin, circular dichroism (CD) spectroscopy was also used. In fact, the bands of CD spectrum characterizes the relative distribution of a secondary structure of a protein. Indeed, when a protein is rich in α-helix the CD spectrum shows two minimum at 208 and 222 nm while β-sheet shows a minimum at about 218 nm (Ranjbar & Gill, 2009). In Figure 2 (panel b) the CD spectra of whey proteins and of whey proteins after the addition of catechins are shown. The contribution to the spectrum of the catechins alone was measured and found to be negligible (data not shown).

For WP and WP/CA, a similar shape of the spectra was observed, with a minimum in the same wavelength region. An interaction between proteins and flavonoids, albeit minimal, was clearly detectable. Indeed, the slight shift of the minimum from 208 nm to 211 nm and a slight decrease of CD signal suggest an interaction of the secondary structure in presence of flavonoids, in agreement with results observed by Al-Shabib et al. (2020) on β-LG in presence of (+)-catechin at different concentrations. Also, these results are consistent with the data collected by Pal et al. (2012), who evaluated the variation of secondary structure of BSA in presence of different concentration of epicatechin (EC) and epicatechin gallate (ECG) displaying a slight decrease in ellipticity indicating an interaction between flavonoids and proteins resulting in a change in secondary structure.

<<Insert Figure 2>>

In order to better understand changes in the secondary structure when whey proteins and catechins are present together, the obtained CD data were used to estimate the content (%) of the secondary structure distinguishing helix, antiparallel, parallel and turn (Table 3). In WP/CA a change in the secondary structure was confirmed, with a slight increase both in the percentage of α-helix and β-sheets (~3 %). These data are consistent with the study on the interaction between β-LG and tea flavonoids ((+)-catechin, (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate) reported by Kanakis et al. (2011) who observed an increase of the percentages of α-helix and β-sheets whereas a reduction of random coil which suggest a enhancement of protein stability in the presence of flavonoids. On the other side, these results partially agree with Han et al. (2022) and Pal et al. (2012) which observed an increase in β-sheets and a decrease in α-helix in presence of flavonoids.

Overall, these data confirm previous observed results which demonstrated the high propensity of flavonoids to interact with proteins even at low concentrations (Cao & Xiong, 2017; Pal et al., 2012;).

The overall data from (chiro-) optical experiments generally outline a slight interaction between flavonoids and whey proteins, most likely taking place on the surface of the proteins.

Table 3 Secondary structure content (%) estimated by using the open web server BestSel on data collected from CD spectra of WP and WP/CA

Secondary Structure	Estimated secondary structure content (%)	
	WP	WP/CA
Helix	12.3	15.1
Antiparallel	25.5	28.1
Parallel	4.1	8.8
Turn	15.1	12.0
Others	43.0	36.0

3.3 Evaluation of the *in vitro* digestibility

3.3.1 Choice of digestion protocol

For the digestion of the protein/flavonoid mixture (and then for the digestion of milk chocolate), the *in vitro* static gastrointestinal digestion was applied following the validated consensus method named "INFOGEST" (Brodkorb et al., 2019).

In this model, the food sample is sequentially solubilized and degraded in different simulated compartments, i.e., oral, gastric, and duodenal (details in the experimental section 2.4.1). Considering the initial amount of the sample, the latter was added by specific simulated digestive fluids (simulated salivary fluid, simulated gastric fluid, simulated intestinal fluid) which consist of salt solutions, digestive enzymes (amylase, pepsin, pancreatin), bile extract and other components (i.e., HCl or NaOH solutions for pH adjustment). Each stage of the digestion was performed considering all key parameters such as controlled temperature, pH, and digestive enzymes concentration.

In addition, to evaluate the behaviour of the sample after exposure to the simulated digestive fluids, a negative control made by dissolving the samples in the fluids above without the enzymes were also tested. These controls were prepared in the same conditions as the digested sample but without the addition of the digestive enzymes and bile extract. Besides, also an enzymatic blank digestion was performed, following the same procedure as the digested sample but without the food sample. This protocol was chosen to understand the possible effect of the interaction between flavonoids and proteins on digestibility because of the advantages of reproducibility and simplicity.

3.3.2 Determination of protein solubilisation and degree of hydrolysis after simulated digestion of the protein/flavonoids mixture

After the digestion of the protein/flavonoid mixture, the soluble fraction of the digestate supernatant was first analysed in order to determine the percentage of protein solubilization, calculated as the percentage ratio between the protein found in solution after the digestion and the total content of protein in the starting material to be digested. In order to calculate that percentage, the Kjeldahl method for protein determination was first applied to the raw starting materials using 6.38 as conversion factor for WP and WP/CA (Accardo et al., 2022; US Department of Agricolture (USDA),b). Then the same method was applied to the soluble fraction after digestion in order to quantify the total solubilized proteins in the supernatant.

After digestion, the percentage of the solubilized proteins in the two model samples constituted by whey proteins alone, or by whey proteins mixed with flavonoids, showed no differences, yielding a 100 % protein solubilization in both cases (Figure 3, panel a). The control experiment, i.e., the digestion procedure without the addition of the enzymes, did also not show any difference with or without the addition of flavonoids, but of course it yielded a much lower protein solubilization. These results strongly suggest that the flavonoids, albeit interacting with the proteins, do not affect the bioaccessibility of whey proteins by enzymes during the digestive process. Also, the above results also imply that flavonoids are not able to act as digestive enzyme inhibitors. Overall, the data strongly hint that the flavonoid presence does not reduce protein bioaccessibility by digestive enzymes.

The above results quite agree with the data on soluble nitrogen showed by de Morais et al. (2020) which observed no changes in solubility in the presence of flavonoids at all pH tested, except at pH 7. Cao & Xiong (2017) even suggested a significant increase of protein solubilization in presence of several flavonoids.

In order to better describe the protein digestibility and its extent, the degree of protein hydrolysis (DH) on the solubilized proteins was also measured for whey proteins (WP) and for whey proteins added in catechins (WP/CA) after digestion. The results, expressed as a ratio between hydrolysed amide bonds and total amide bonds (as a percentage, calculation details in the experimental section 2.4.3) are reported in Figure 3, panel b. The degree of hydrolysis found for both whey proteins (WP) and whey proteins added in catechins (WP/CA) was around 45 %, with no significant differences, again indicating that flavonoids, albeit interacting with the proteins, did not hinder the accessibility of the proteolytic enzymes involved during GD hydrolytic process. The above data partially agree with the observations made by Jiang et al. (2018) on interaction of chlorogenic acid with whey protein and casein, which even indicated an increase of the degree of hydrolysis of proteins during digestion by increasing the concentration of catechins, albeit with an overall lower DH%.

<<Insert Figure 3>>

3.3.3 Determination of peptide profile after simulated digestion of the protein/flavonoids mixture

To determine at more molecular level the effect of *in vitro* gastrointestinal digestion on protein/flavonoids mixture (WP/CA) and their control (WP), a peptidomic analysis was performed. The complete lists of the most abundant peptides identified after *in vitro* gastrointestinal digestion are reported in Supplementary materials (S1). The number of the identified peptides generating after gastrointestinal digestion was 77 and 73, respectively, for WP and WP/CA, suggesting that proteins also, when flavonoids are present, exhibit the same propensity to be hydrolysed. From a qualitative point of view, almost all of the identified peptides in WP were matched in WP/CA, indicating that no major changes, at the molecular level, happened during digestion of whey proteins, either in presence of flavonoids or not. These data on peptide profile totally agree with the results presented above on the degree of protein hydrolysis and the percentage of protein solubilisation, which strongly indicate that catechins do not change the high bioaccessibility of whey proteins of enzymes during the digestive process.

3.3.4 SDS-PAGE of proteins in the simplified mixture system and in milk chocolate after simulated digestion in absence and in presence of flavonoids

To further evaluate the effect of *in vitro* digestion, a protein separation based on molecular weight (MW) was carried out by using SDS-PAGE electrophoresis before and after the digestion, both in presence and in absence of flavonoids. In Figure 4 the protein pattern of whey proteins (WP) and whey proteins added in catechins (WP/CA) is shown before and after gastro-duodenal (GD) digestion, including negative digestion controls. The undigested samples (marked as raw lyophilized WP) and the digestion reference control (simulated digestion carried out without digestive enzymes) presented the typical protein profile of whey proteins, according to previous works (Accardo et al., 2022; Agyare & Damodaran, 2010). The bands' intensities of α-lactalbumin (14 kDa) and β-lactoglobulin (18 kDa) did not show any difference in presence of (+)-catechin and (-)-epicatechin.

After GD digestion, no bands were present, suggesting hydrolysis of the proteins by digestive enzymes, both in presence and absence of catechins. Thus, also from this further evaluation, protein digestibility was not particularly affected by the presence of flavonoids.

This also agrees with the data obtained by Cao & Xiong (2017) on α-lactalbumin and β-lactoglobulin with different amounts of gallic acid and epigallocatechin gallate at different pH after *in vitro* digestion.

<<Insert Figure 4>>

In addition, in order to also assess the effect of the simulated digestion on a real food sample, the effect of *in vitro* digestion was also evaluated for a commercial milk chocolate sample. In Figure 5 the protein profile of digested milk chocolate (MC), the negative sample control (without the addition of digestive enzymes) and the enzymatic blank control (without the addition of the sample) is reported. After digestion, the proteins of commercial milk chocolate also completely disappeared, while in the control sample, intact proteins were clearly visible between 20-25 kDa (which can be reliably assigned as milk proteins, even if the chocolate

matrix slightly altered the SDS profile) and 25-37 kDa (according to the MW, probably belonging to the globulin class such as cocoa vicilin-like storage protein). This result also indicated that, even in a complex matrix such as milk chocolate, the proteins were completely solubilised and hydrolysed through the action of digestive enzymes.

<<Insert Figure 5>>

4. Conclusions

In the present paper, in order to evaluate the impact on protein digestibility of the presence of flavonoids, a simplified protein-flavonoid model for milk chocolate was studied. The simplified mixed model was prepared by mixing whey proteins with catechins, in a ratio similar to what found in milk chocolate. UV absorption spectra show an additive behaviour, suggesting weak interactions between WP and CA. Similarly, fluorescence spectra point to a nearly additive behaviour of the protein/catechins system, as far as the fluorophores are concerned. Experiments with circular dichroism confirmed a weak but sizable protein-flavonoid interaction, as expected, with slight alteration of the protein secondary structure. This interaction clearly did not hamper protein digestibility. Indeed, after simulated gastrointestinal digestion, high levels of protein solubilisation were observed, both in whey proteins alone and in whey proteins added of catechins, with no significant differences. In addition, the presence of catechins also did not hinder the hydrolysis of the amide bond as measured by the degree of hydrolysis. Finally, the major peptides generated during digestion were mostly found not to differ between the samples with and without catechins. These data were also consisted with SDS-PAGE experiment which confirmed high levels of protein digestibility in both model samples, with and without catechins, and also in milk chocolate.

Thus, the data contained in the present study indicated that the presence of catechins does not seem to modify the digestibility of whey proteins and that the digestive process is not hampered by their presence, strongly suggesting that in a food rich in flavonoids, protein nutritional quality is not impaired.

More experiments, both *in vitro* and *in vivo*, will be needed in order to confirm the generality of these conclusions also in different food matrices and with different polyphenol classes.

Author contributions

Francesca Accardo: Investigation, Formal analysis, Writing – original draft. Barbara Prandi: Investigation, Writing – review & editing. Francesca Terenziani: Investigation, Writing – review & editing. Tullia Tedeschi: Investigation, Writing – review & editing. Stefano Sforza: Supervision, Conceptualization, Writing – review & editing.

Conflict of interest

Nothing declared.

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Figure captions

Figure 1 Flow chart depicting the strategy of the present study

Figure 2 Panel (a): UV absorption spectra. Inset of panel (a): zoom of the absorption band located at 280 nm. Panel (b): CD spectra. Panel (c): Fluorescence emission (dashed lines) and normalized excitation spectra (full lines). Panel (d): comparison of the fluorescence excitation spectrum of WP/CA detected at 300 nm with the excitation spectrum of CA. Panel (e): comparison of the fluorescence excitation spectrum of WP/CA detected at 360 nm with the excitation spectrum of WP. Color code for all the panels: WP black lines, CA red lines, WP/CA green lines.

Figure 3 Panel (a): Percentage of total solubilized proteins (solubilized proteins/initial proteins x100), for whey proteins (WP), whey proteins added in catechins (WP/CA). The controls are percentage of solubilized proteins during the digestion procedure but without the addition of the digestive enzymes. ANOVA-Tukey test was used to assess significant differences between samples ($p<0.05$). Panel (b): Degree of protein hydrolysis for whey proteins (WP) and whey proteins added in catechins (WP/CA). T-test was used to assess significant differences between samples ($p<0.05$).

Figure 4 SDS-PAGE of whey proteins (WP) and whey proteins added in catechins (WP/CA) before and after digestion. Samples from left to right: protein profile of whey proteins before digestion (raw), whey proteins after gastro-duodenal digestion, negative digestion control of whey proteins (without enzymes); protein profile of whey proteins added in catechins before digestion, whey proteins added in catechins after gastro-duodenal digestion, negative digestion control of whey proteins added in catechins (without enzymes); enzymatic digestion control (enzymatic blank without sample). Marked bands: 1) β-lactoglobulin (18 kDa); 2) α-lactalbumin (14 kDa).

Figure 5 SDS-PAGE of milk chocolate (MC). Samples from left to right: milk chocolate (MC) after gastro-duodenal digestion, negative digestion control of milk chocolate (without enzymes), enzymatic digestion control (enzymatic blank without sample). Marked bands: 1) milk proteins (20-25 kDa); 2) cocoa vicilin-like storage protein (25-37 kDa)

Fig. 1.

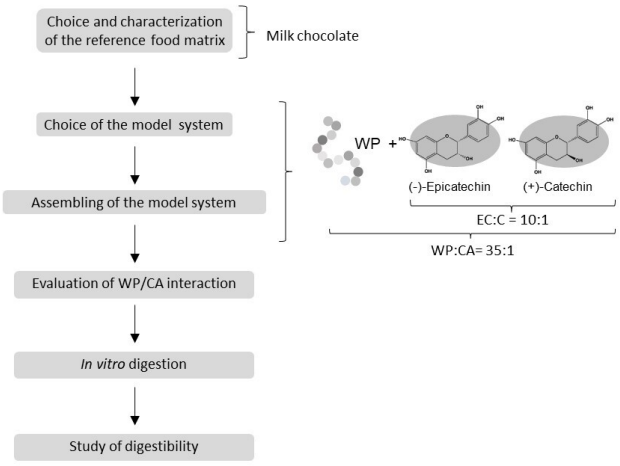


Fig. 2.

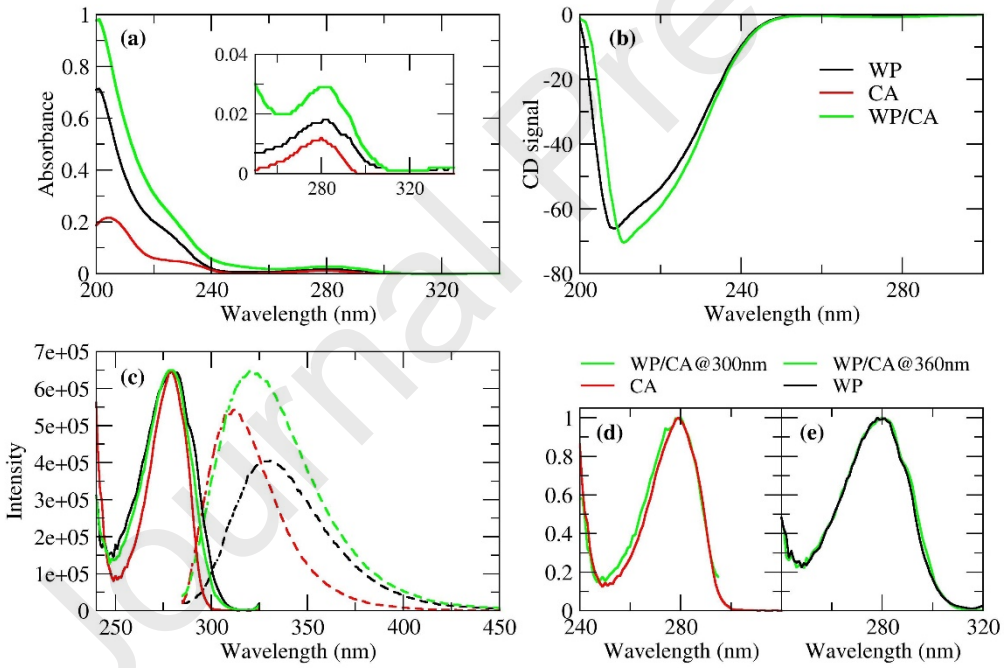


Fig. 3.

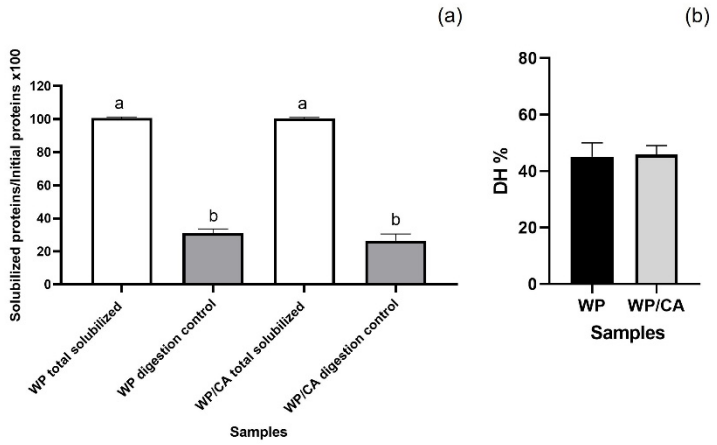


Fig. 4.

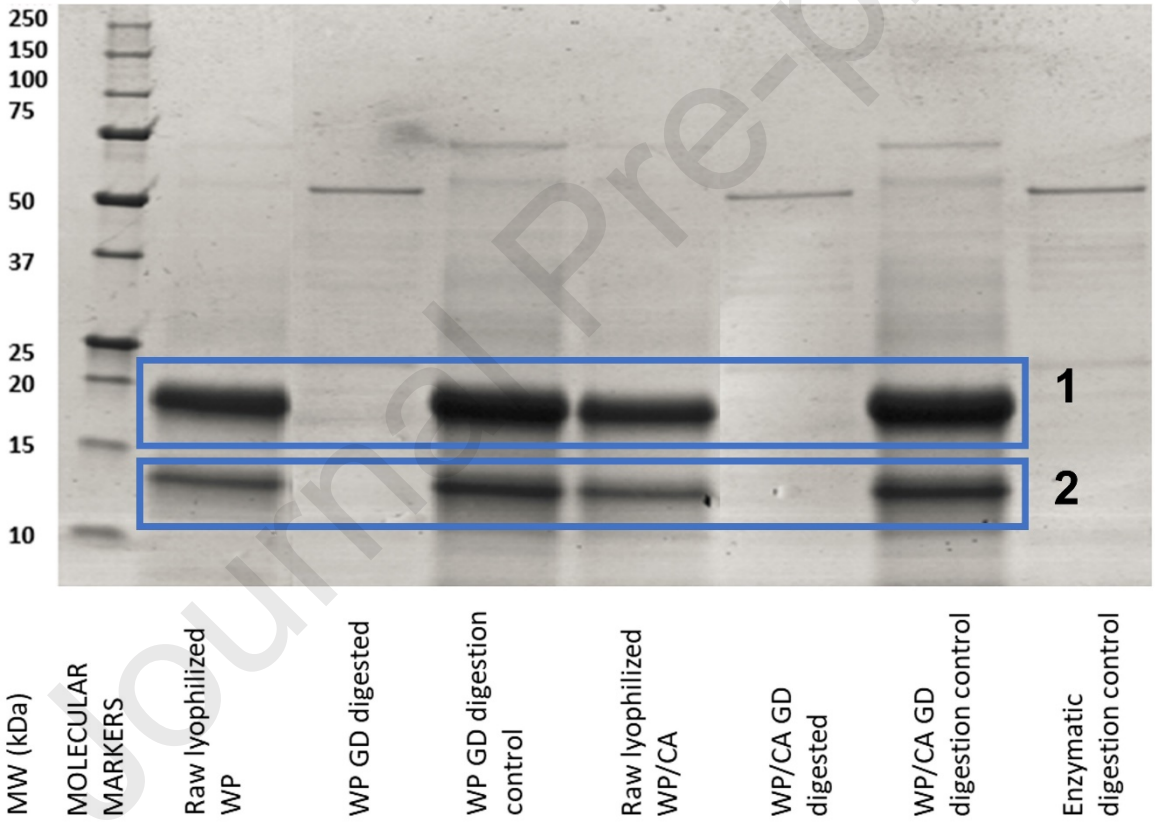
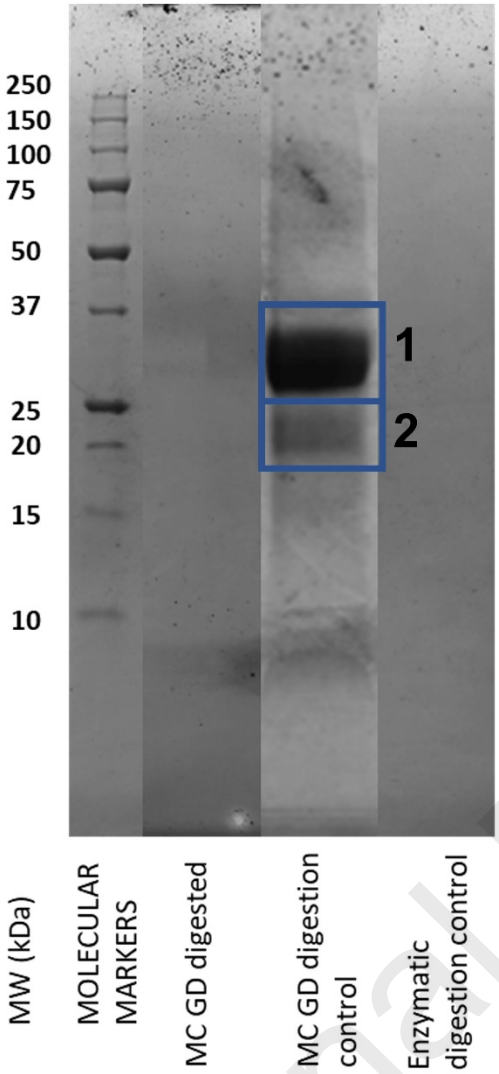
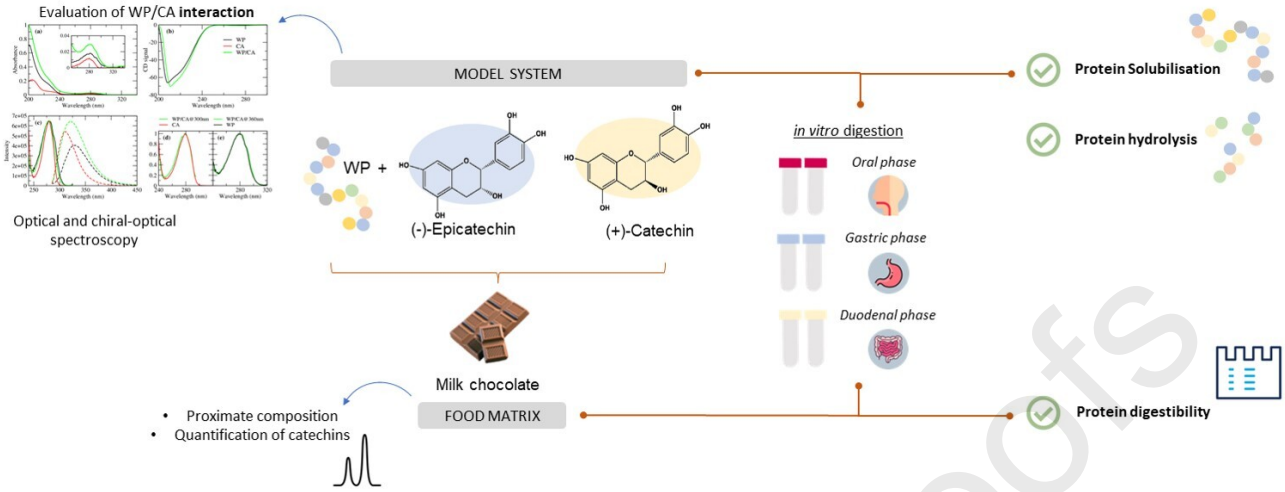


Fig. 5.



Graphical abstract



Highlights

- A simplified whey protein-catechins system was prepared in the same ratio as in milk chocolate
- Interaction between whey proteins and catechins determined by (chiro-)optical spectroscopy
- Protein hydrolysis/solubilisation of whey proteins during digestion unaffected by catechins
- Milk chocolate proteins completely digested
- Interactions of catechins with milk proteins does not impair *in vitro* protein digestibility

Credit Author Statement

Francesca Accardo: Investigation, Formal analysis, Writing – original draft. Barbara Prandi: Investigation, Writing – review & editing. Francesca Terenziani: Investigation, Writing – review & editing. Tullia Tedeschi: Investigation, Writing – review & editing. Stefano Sforza: Supervision, Conceptualization, Writing – review & editing.

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

X All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

X This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

X The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

o The following authors have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript:

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