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Assessment of the effects of soy varieties and processing on protein quality, digestibility and intestinal bioactivity (DPP-IV inhibition and total GLP-1 release)

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ABSTRACT

Soybean is widely used to produce food and feed livestock for its high nutritional value and technological properties. Traditionally, soy proteins are wet-extracted from meal using heat and basic pH to produce high yields extracts. However, when proteins were extracted under these conditions some aspects related to protein quality were still overlooked. In this study, the effect of processing of two varieties of soy, Energy and Namaste, on protein digestibility and bioactivity were evaluated. Soybean meal was characterised and subjected to two different protein extraction conditions to evaluate their impact on *in vitro* gastrointestinal digestion, estimated by characterising the protein solubilisation. Furthermore, the bioactivity of the digested mixtures was studied by evaluating the effects on the total GLP-1 secretion and on the inhibition of the DPP-IV enzyme. In all conditions, soy meals and extracts showed potential bioactivity on total GLP-1 secretion. Depending on the variety and extraction conditions, some changes were observed. Noteworthy, different results were observed when proteins were extracted under more rough basic conditions, presenting the highest protein extraction yield (60–70 % in rougher and 40 % in milder conditions), hydrolysis of the high-molecular-weight proteins and stronger potential inhibitory activity on DPP-IV enzyme (27 % and 17 % respectively from Energy and Namaste, at the lowest concentration tested). However, this condition produced an increasing % of D-amino acid formed and lower protein solubilisation after digestion. These findings highlighted the relevance of employing an integrated approach, combining the evaluation of chemical and biological properties when the nutritional value is a target.

1. Introduction

Soybean (*Glycine max*) is one of the most appreciated legumes due to its high nutritional value, cost-effectiveness, and technological properties [1]. Soybeans are rich in proteins and lipids, which are the constituents of the leading products of these seeds: oil and meal [2]. In the industry, soy meal is mainly employed for protein-rich livestock feeds. In contrast, a lower amount is employed for producing soy-based products such as tofu, tempeh, and meat analogues, or introduced as a supplement to fortify various foods intended for human consumption [3]. However, the increasing demand for alternative foods to those of animal origin from consumers is reflected in the growing application of plant proteins in the food industry. Soy represents the main food matrix consumed in the vegan diet due to its nutritional and health benefits [4,5]. Furthermore, several protein extraction techniques have been developed to promote the valorisation of plant proteins, including the

Abbreviations: **CCK**, cholecystokinin; **DPP-IV**, enzyme Dipeptidyl peptidase IV; **E**, Energy meal; $\mathbf{E}_{def}$, Energy defatted meal; **EM**, Energy meal extract in mild condition; **ER**, Energy meal extract in rough condition; **GI**, gastrointestinal; **GLP-1**, glucagon-like peptide-1; **N**, Namaste meal; $\mathbf{N}_{def}$, Namaste defatted meal; **NM**, Namaste meal extract in mild condition; **NR**, Namaste meal extract in rough condition; **PYY**, peptide YY; **SGF**, simulated gastric fluid; **SIF**, simulated intestinal fluid; **SSF**, simulated salivary fluid.

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[1] In memory of our mentor and friend Stefano.

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reuse of food wastes and by-products [6]. The most common and effective method for extracting plant proteins is wet extraction, involving water solubilisation under alkaline conditions (pH > 8) combined with the application of temperature at different treatment times [7]. However, plant protein extraction represents a complex procedure. For this reason, several steps are introduced to remove, as much as possible, the anti-technological factors and consequently improve the protein extraction yield [7]. For instance, pre-extraction of lipids from seeds is used to aid solubilisation of seeds by improving the characteristics of the extracts. Traditionally, soy extracts are prepared starting from defatted soy meal and dispersed in an aqueous solution adjusted to a pH between 7.5 and 11, under controlled temperature conditions from 25 °C to 60 °C [2,8]. The most employed condition to extract commercial soy proteins is pH 8.5 at 60 °C [8]. As for the detailed composition of these proteins, the main storage proteins of soybean are β-conglycinin (7S fraction) and glycinin (11S fraction), which account for approximately 80–90 % of the total seed protein. At the same time, a minor amount is composed of other proteins, including the main protease inhibitors, i.e., Kunitz and Bowman-Birk inhibitors [9].

However, the protein composition of soybean can be affected by extrinsic factors such as environmental conditions and intrinsic factors such as genetic variety [10], which may also change the peptide release after gastrointestinal digestion [9].

In addition, another important aspect concerns the growing interest in the potential health-promoting effect linked to plant protein consumption. For instance, it was proven that dietary proteins and hydrolysates could modulate the release of the main enterohormones involved during the gastrointestinal process, including glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY), which are implicated in different metabolic and physiological functions, e.g., glucose and energy homeostasis, food intake via satiety modulation, gastric and intestinal motility [11]. Among the most relevant functions, GLP-1 is involved in the stimulation of insulin secretion, in the inhibition of glucagon secretion by the pancreas, and in delaying gastric emptying, increasing the sense of satiety as a response to food intake. The enzyme Dipeptidyl Peptidase IV (DPP-IV) is also implicated in this mechanism by inactivating GLP-1 [12]. Moreover, several authors indicated that the nature of the protein source, as well as its composition, plays an essential role by producing a differential response on the secretion of the enterohormone and its natural inhibitors [13,14]. As for soy, the latter is considered a source of bioactive peptides with a wide range of biological functions [15–19]. Previous work has shown that soy hydrolysate with different enzymes could act as a source of DPP-IV inhibitory peptides [20,21]. In addition, other authors evaluated the effect of bioactivity when soybeans were subjected to natural processing processes, such as germination or fermentation. The results highlighted that several soy peptides could modulate glycaemic response by exhibiting DPP-IV inhibition activity [22]. Moreover, in a comparative study on the effect of different protein sources on the release of CCK and GLP-1, Geraedts et al. [23] demonstrated that all tested proteins, including soy proteins, can directly stimulate the hormone release in the STC-1 cell line. However, some aspects related to the bioactivity of soy and how processing can influence bioactivity still need to be fully explored. For this purpose, the impact of different processing and varieties on protein digestibility was investigated by evaluating both nutritional quality and potential bioactivity of the released products, by expanding the current knowledge on this topic. In this study, we have selected two varieties of soybeans, Energy and Namaste, used by the food and feed industry for different purposes. Energy is used for food preparations such as tofu, whereas Namaste is used for the production of feed for livestock. Furthermore, two different protein extraction protocols were performed based on the data present in the literature for legume-protein extraction [24,25]. First, the selected soy meals and their extracts were characterised and evaluated for their protein quality. Then, after *in vitro* digestion, the possible implications of the different varieties and extraction protocols were assessed from chemical and biochemical points of view. Thus, the solubilised proteins, the total secretion of GLP-1 and the inhibitory activity of the DPP-IV enzyme on the digested products were analysed.

2. Materials and methods

2.1. Reagents and solvents

All the chemicals used to produce the simulated salivary fluid (SSF), the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF), α-amylase from porcine pancreas (13 U/mg), pepsin from porcine gastric mucosa (727 U/mg), pancreatin from porcine pancreas (100 U/mg), bile Salts, boric acid, TRIZMA base ($NH_2C(CH_2OH)_3$), DL-norleucine, hydroxyproline, cysteic acid, methionine sulfone, tryptophan, dichloromethane (DCM), sodium sulphate (Na_2SO_4) and sodium dodecyl sulphate (SDS) were from Sigma Aldrich (St. Louis, MO, USA). n-hexane, 96 % sulphuric acid (H_2SO_4), copper (II) oxide, and sodium hydroxide (NaOH) were from VWR Chemicals (Radnor, PA, USA). The ultrapure water was obtained with a Milli-Q® system (Merck Millipore, Darmstadt, Germany). Potassium chloride (KCl) was by Carlo Erba (Milan, Italy). The defoamers and catalyst tabs for the Kjeldahl and α-methyl-tryptophan came from Merck (Darmstadt, Germany). 0.1 M hydrochloric acid was from ITW reagents (Barcelona, Spain). The ELISA kit for total GLP-1 and DPP-IV enzyme from porcine kidney were from Millipore (Billerica, MA, USA). Gly-Pro-7- amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) was from Bachem AG (Bubendorf, Switzerland). Diprotin A (Ile-Pro-Ile) was purchased from Enzo Life Sciences International (New York, USA). The LDH kit was from QCA (Tarragona, Spain). The AccQ-Tag Ultra Derivatization Kit for Amino Acid Analysis was from Waters (Milford, MA, USA). The Amino Acid Standard Solution H, BCA assay and molecular marker Spectra™ Multicolor Broad Range Protein Ladder were from Thermo Fisher (Thermo Fisher Scientific, Waltham, MA, USA). Tris-Glycine from Bio-Rad (Hercules, CA, USA). All other chemicals were analytical grade and from Sigma Aldrich (St. Louis, MO, USA).

2.2. Samples preparation and protein extraction

In the present study, soybeans (*Glycine max*) of two varieties named Energy (E) and Namaste (N) were used. These latest, soybeans samples were donated by the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine, Italy. The soybeans were pre-treated by low-temperature drying at approximately 45 °C.

Soy meal was obtained by finely grinding soybeans. Protein extracts were prepared according to Bu et al. [24] with some modifications using two conditions: i) mild; ii) rough.

Under mild condition, Energy soy meal (EM) and Namaste soy meal (NM) were defatted overnight with stirring by using n-hexane (w/v 1:10). Then, the hexane was removed and evaporated completely. The defatted meal was dispersed in 30 mM Tris-HCl buffer at pH 7.5 (w/v 1:15) and mixed for 1 h at room temperature. Instead, under the rough condition, Energy soy meal (ER) and Namaste soy meal (NR) were defatted overnight under stirring by using n-hexane (w/v 1:10). Then, the defatted meal was dispersed in 30 mM borate buffer at pH 11 (w/v 1:15) and mixed for 3 h at a controlled temperature of 60 °C. Finally, the mixtures were centrifuged at 4 °C, 3220*g* for 45 min (5810R Eppendorf, Hamburg, Germany) and the supernatant was freeze-dried using Lio 5P freeze drier (5 Pascal, Milan, Italy) and stored at −20 °C until the analysis. A schematic view of protein extraction procedures is shown in Fig. 1.

2.3. Proximate analysis and protein extraction yield

Moisture, proteins, lipids, and ashes for the proximate analysis of soy meals and extracts were determined according to the following standard procedures. For moisture analysis, the samples were dried in an oven

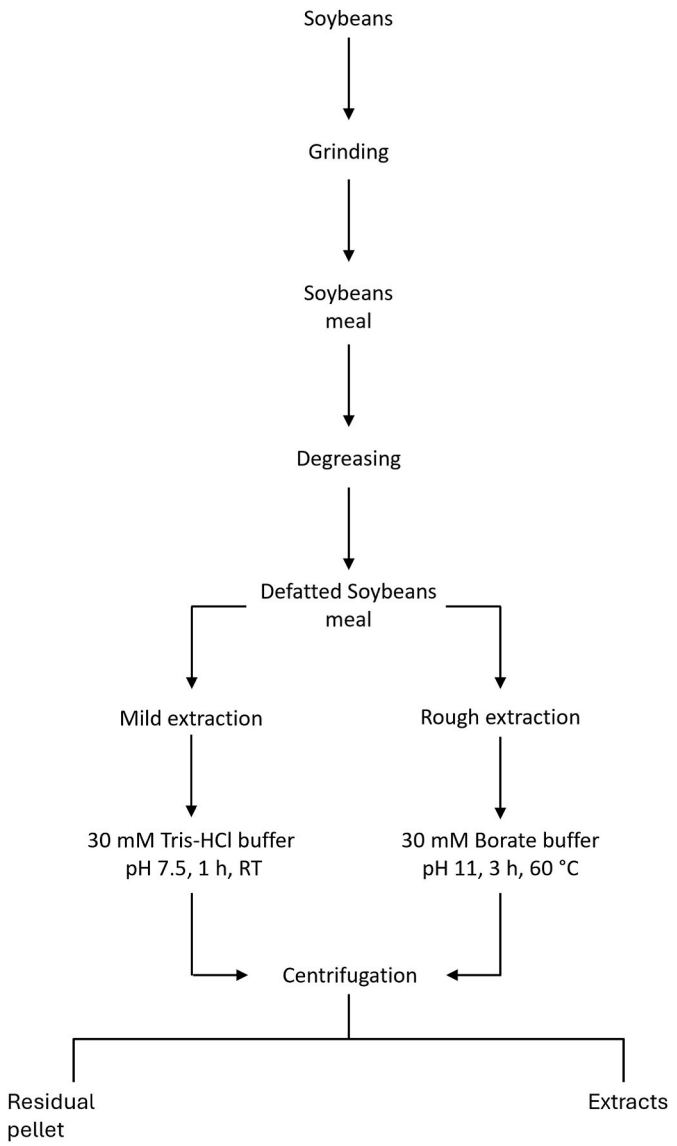

Fig. 1. Schematic view of the process of mild and rough extraction of soy protein.

(30–1060, Memmert GmbH, Schwabach, Germany) at 105 °C for 24 h until reaching a constant weight. Total nitrogen was determined following the official Kjeldahl method using DKL heating digester and semiautomatic distillation unit (VELP SCIENTIFICA, Usmate Velate, Italy) [26]. The percentage of proteins was calculated from total nitrogen by applying a 5.71 conversion factor for soy [27].

After the quantification of the protein percentage of soy meals and extracts, the protein extraction yield of mild and rough extracts was calculated in percentage as the ratio between the percentage of protein extract on the mass in g obtained after extraction and the initial protein percentage on the mass in g of the defatted meal used for the extraction × 100.

Extractable lipids were quantified after acidic hydrolysis using a Soxhlet apparatus (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy). Ashes were quantified after incineration of the sample at controlled temperature of 550 °C for 5 h in a muffle furnace (Ionos 201, Mazzali Fratelli, Monza, Italy). Carbohydrate content was estimated as the difference. All chemical analyses were performed in triplicate.

2.4. Determination of total amino acids

Three different procedures were followed to determine the total amino acids composition. All amino acids, except sulphur containing amino acids and tryptophan, were quantified by subjecting the samples to acid hydrolysis, followed by the derivatization step and analysed using UPLC-ESI-MS (Waters, Milford, MA, USA) [13]. Sulphur containing amino acids were determined by subjecting the samples to oxidation, hydrolysis, derivatization, and UPLC/ESI-MS analysis [13]. For the determination of all amino acids (except Trp) an external calibration curve was used. The calibration curve was prepared by mixing in a 1:1 ratio the Amino Acid Standard H (each amino acid at a concentration of 2.5 mM in 0.1 M HCl) and a mixture of the remaining amino acids (each amino acid, nor-leucine, hydroxyproline, cysteic acid, and methionine sulfone at a concentration of 2.5 mM in 0.1 N HCl). Thus, concentrations ranging from 1.25 mM to 0.078 mM were used. Lastly, tryptophan was determined after basic hydrolysis and UPLC/ESI-MS analysis as described by Prandi et al. [28]. For the determination of tryptophan, a response factor was prepared by mixing 150 μL of tryptophan (0.5 mg/mL) and 150 μL of α-methyl-tryptophan (0.5 mg/mL) into 10 mL volumetric flasks and bringing to volume with Milli-Q water.

2.5. Determination of enantiomeric purity of amino acids

The enantiomeric purity of amino acids was determined following the procedure previously described by Cutroneo et al. [29]. The analysis was performed using a GC/MS system (Agilent Technologies 7820° gas-chromatograph coupled with an Agilent Technologies 5977B mass spectrometer, Palo Alto, CA, USA). For each sample, the areas of the D- and L-amino acids were integrated (when both forms were detected). For each amino acid the percentage of D-amino acid was calculated as the ratio between the area of the D-amino acid and the sum of the areas of the D-form and the L-form x 100.

2.6. In vitro gastrointestinal digestion

Soy meals and soy protein extracts were subjected to *in vitro* simulated gastrointestinal (GI) digestion following the consensus INFOGEST static method [30]. The protocol was slightly adapted, as previously described by Accardo et al. [13]. The same digestion protocol was performed for the negative control, which did not include enzymes, and for the enzymatic blank, which did not include the sample.

2.7. Analysis after in vitro gastrointestinal digestion

2.7.1. Determination of solubilised protein after digestion

Nitrogen was quantified on 1 mL of the digested sample with the official Kjeldahl method, following the procedure described by Accardo et al. [31]. The sample was mineralized and distilled using the DKL heating digester and semiautomatic distillation unit (VELP SCIENTIFICA, Usmate Velate, Italy). A final titration was performed using 0.01 M HCl. Total nitrogen was converted to protein by 5.71 conversion factor [27].

2.7.2. Electrophoretic profile of digested materials

The protein profile of the digested sample, negative control and enzyme blank was visualised using an SDS-PAGE according to Luparelli et al. [32] with minor changes. The amount of protein present in the supernatant of the digested samples and digestion controls was quantified using the BCA assay following the instructions provided by the manufacturer. To perform the electrophoretic run, samples which contains 30 μg of proteins, and a molecular marker were loaded into a polyacrylamide gel. The run was performed using a 1x Tris-Glycine running buffer for 2 h at 120 V.

2.7.3. Determination of DPP-IV inhibition activity

The DPP-IV inhibition activity of digested soy meals and soy protein extracts was determined according to the procedure previously described by Casanova-Martí et al. [33]. The activity of the enzyme was checked using 35 μL of 100 mM Tris-HCl buffer at pH 8 and 15 μL of DPP-IV enzyme from porcine kidney. For the fluorimetric assay the following concentrations were tested: 0.5 mg/mL of protein and 6 mg/mL of protein of digested sample mixture. The same procedure was performed using Diprotin A (Ile-Pro-Ile) as positive control of DPP-IV inhibition. A blank, without enzyme and sample, was also prepared. The assay was performed in triplicate for each sample. The read was carried out at excitation and emission wavelengths of 380 nm and 460 nm respectively, using a microplate reader (FLx800™, BioTek Instruments, Winooski, Vermont, U.S). The functionality of DPP-IV was calculated in percentage, as the ratio of the enzymatic activity in the test samples to the enzymatic activity, and the inhibition was estimated as follows: *inhibition = 100 - functionality*.

2.7.4. GLUTag cell culture and total GLP-1 secretion test

GLUTag cells were donated by Prof. Staels of the Institut Pasteur de Lille (Lille, France) with the permission of Prof. Drucker of the Lunenfeld-Tanenbaum Research Institute (Toronto, Canada). GLUTag cells were cultured and pre-treated as described by Luparelli et al. [32]. To carry out the test, a density of 200.000 cells/mL (with ~80 % of confluency) was achieved. Before the secretion test, the protein present in the supernatant of the digested samples was quantified using the BCA assay. For the treatment, the cells were incubated for 2 h at 37 °C. Then, the medium was collected and stored at −80 °C until studying the GLP-1 secretion and LDH assay. The cell treatment was carried out at least in duplicate in three different biological passages. Total GLP-1 release was determined by using a sandwich ELISA assay according to the manufacturer's procedure. To determine total protein content, cells -added in RIPA lysis buffer-were sequentially lysed, collected and mixed in Eppendorf at 4 °C for 20 min. After, the samples were centrifuged at 13000*g* for 20 min at 4 °C and the supernatant was collected for the determination of total protein by using BCA assay. In addition, the LDH assay was carried out both in medium and cells to evaluate the potential cytotoxicity of the tested treatment, according to the manufacturer's instructions.

2.8. Statistical analysis

GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The ANOVA-Tukey was used to determine the statistical differences between samples whereas Dunnett's multiple comparisons test was used to compare the mean of each column with the mean of a control column ($p < 0.05$).

3. Results and discussion

3.1. Proximate analysis and evaluation of protein extraction yield after processing

Two different varieties were used for this study, Namaste, a low anti-nutritional variety (sowed on 2020) used as feed, and Energy (sowed on 2018), a variety used for food preparations. To assess the digestibility of the soy meals and their extracts, we first characterised the meals and extracts for their composition in terms of moisture, proteins, lipids, and ashes.

The proximate composition was performed on meal of the Energy and Namaste soy varieties (E, N), defatted meal (E*def*, N*def*), freeze-dried protein extract in mild conditions (EM, NM) and freeze-dried extract in rough conditions (ER, NR). The results are presented in Table 1.

The nutritional value obtained for both soy meals varieties was in line with the previous data published by the US Department of Agricolture [34].

The protein percentage, as expected, increased already after the degreasing pre-treatment but also after the extraction process in mild and rough conditions for both varieties, with significant differences compared to the initial value of the meal. For the Energy variety, the increase occurs equally in both mild and rough extracts while, for Namaste, the protein content is higher in rough extracts.

After the degreasing treatment, the lipid percentage undergoes a reduction of almost 50 % due to the solvent extraction, conducted before the extraction process. This percentage decreases with significant differences even after mild and rough extraction of both varieties.

The percentage of ash in the meal (E, N) and defatted meal (E*def*, N*def*) was around 5–6 % for both varieties, this percentage clearly increases for extracts under mild and rough conditions which were extracted using salt buffered solutions. However, after the extraction process, whether mild or rough, other molecules are still present.

Then, to further evaluate the effect of protein extraction under different conditions (mild and rough), the protein extraction yield was measured. The protein extraction yield was calculated as described in the experimental section (2.3); the results are shown in Fig. 2. The data shows that under mild conditions, the protein extraction yield is approximately 40 % with no significant differences between the Energy and Namaste varieties. Furthermore, protein extraction yield is higher under rough than mild conditions.

Our results confirm that extraction conditions represent a key parameter for increasing the extraction yield, demonstrating that the combination pH 11, 60 °C for 3 h (rough) is more effective than pH 7.5, RT, 1 h (mild).

Different pHs (final pH value from 8.73 to 10.85), temperatures (from 25 °C to 70 °C) and treatment times (from 25 to 100 min) were tested on soybean by-products [3]. Their results with those obtained here, showing the maximum extraction yield at pH 10.85 and 100 min at 60 °C and, confirming a better extractability of proteins in alkaline environment. However, they found a lower extraction yield (around 50 %). Other authors carried out aqueous extractions to evaluate the effect

Table 1
Proximate composition of soy meal and protein extract under mild and rough condition from Energy and Namaste variety. Values are reported based on the dry matter of three replicate analyses.

Parameter (%)	Energy (E)	Energy defatted (E *def*)	Energy mild (EM)	Energy rough (ER)	Namaste (N)	Namaste defatted (N *def*)	Namaste mild (EM)	Namaste rough (ER)
Protein	39 ± 0.2^{a}	43 ± 0.5^{b}	48 ± 0.2^{c}	46 ± 0.1^{c}	34 ± 0.3^{d}	40 ± 0.1^{a}	44 ± 1^{b}	47 ± 1^{c}
Lipid	22 ± 0.4^{a}	9.2 ± 0.7^{b}	$2.7 \pm 0.2^{c,d}$	4.9 ± 0.5^{e}	22 ± 1^{a}	8.2 ± 0.7^{b}	1.9 ± 0.2^{c}	$6.3 \pm 1.5^{d,e}$
Ash	$5.2 \pm 0.3^{a,c}$	5.0 ± 0.1^{c}	8.5 ± 0.2^{d}	11 ± 0.3^{e}	$5.4 \pm 0.1^{a,c}$	5.9 ± 0.5^{a}	11 ± 0.2^{f}	14 ± 0.1^{b}
Other compounds	34 ± 0.5^{a}	$43 \pm 1^{b,f}$	$41 \pm 0.4^{b,c,d}$	37 ± 0.1^{e}	$39 \pm 1^{d,e}$	45 ± 1^{f}	$43 \pm 1.5^{c,f}$	34 ± 1^{a}

[a-f] Different letters indicate statistically different samples ($p < 0.05$) (one-way ANOVA, Tukey test).

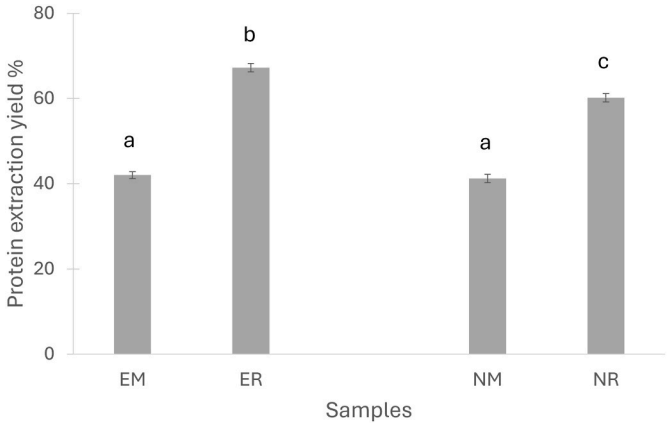

Fig. 2. Protein extraction yield evaluated after extraction in mild and rough conditions for both Energy and Namaste defatted meals. Samples from left to right: protein extract in mild condition from Energy defatted meal (EM), protein extract in rough condition from Energy defatted meal (ER), protein extract in mild condition from Namaste defatted meal (NM), protein extract in rough condition from Namaste defatted meal (NR). Different letters indicate statistically different samples ($p < 0.05$) (one-way ANOVA, Tukey's test).

of different pH on protein recovery from okara (soybean by-product) [35]. pH values 9, 10, 11, and 12 were tested at 60 °C for 1 h in 0.1 M phosphate buffer solution. Different pH values showed significant differences in protein extraction yield indicating increases protein recovery in a pH-dependent manner, with the highest protein extraction yield at pH 12 [35].

Furthermore, Perović et al. [36] evaluated the effect of basic extraction (pH 8) for three different treatment times (1 h, 2 h, 3 h) on the yield of protein extraction from defatted soy grit and observed a significant increase in the yield of protein extraction in a time-dependent manner, with the highest value in alkaline conditions after 3 h of treatment.

On the other hand, regarding the different varieties, Energy showed the highest extraction yield indicating that, depending on the treatment applied, also the variety can also influence the extraction process.

3.2. Evaluation of protein quality: amino acids profile and effect of processing on D-amino acids formation

To evaluate the possible change of the amino acid profile or depletion of certain amino acids in different varieties (Energy and Namaste) and conditions (mild and rough) of soy meal, total amino acids were determined (Table 2). The true protein content was estimated for each sample (E, EM, ER, N, NM) by subtracting the water molecules that are lost in the formation of peptide bonds in the proteins. Thus, a more realistic data is provided by excluding possible errors arising from the conversion factor, applied for the Kjeldahl method, or from the presence of nitrogen-containing salts in the buffer (i.e., EM, NM) used during protein extraction. No significant differences were observed for any sample, except for EM and NM due to the presence of nitrogen-containing salts, showing good agreement between the protein content assessed via the Kjeldahl method and the true protein content assessed via total amino acid. Consisting with what was found by the United States of Department of Agricolture [34], a high percentage of Glu/Gln and Asp/Asn were detected in all soy samples while among the essential amino acids these samples are rich in Leu and Lys. On the other side, among the essential amino acids the lowest are Met and Trp [34]. Overall, the amino acid profile does not show marked differences in the depletion of some amino acids after protein extraction. Some differences regarding concentrations were observed after rough extraction, which appears to be more effective than the mild process. As regards, the variety, Namaste meal exhibits the lower concentration of total amino acids compared to Energy meal, indicating some differences between the two varieties studied.

The percentage of D-amino acids formed in the meals and extracts was evaluated for the different varieties. To investigate the effect of processing among the samples, the mean of each treated sample (EM, ER and NM, NR) was compared to the mean of the control sample (E and N respectively). The percentage of D-amino acids found, expressed as the sum of Ala, Asp, Phe, Glu and Lys (Fig. 3) showed a significant increase in the D-form after rough processing. D-amino acids in Energy extract under rough condition (ER) showed the highest value, followed by mild extract (EM) and meal (E). In contrast, Namaste meal and the mild extract (N, NM) showed a similar percentage of D-amino acids formed while the latter, only under rough conditions showed the highest value. Differences were also observed between the two varieties, showing

Table 2
Amino acid profile in raw soy meal and their protein extract under mild and rough condition from Energy and Namaste varieties.

g of amino acid/100 g of meal						
Amino Acid	Samples					
	E	EM	ER	N	NM	NR
Ala	1.45 ± 0.03^{a}	1.26 ± 0.04^{a}	1.8 ± 0.1^{b}	1.3 ± 0.2^{a}	1.32 ± 0.01^{a}	1.86 ± 0.03^{b}
Arg	3.2 ± 0.1^{ab}	4.2 ± 0.2^{a}	4.3 ± 0.3^{ac}	2.6 ± 0.6^{b}	2.8 ± 0.5^{b}	3.7 ± 0.2^{bc}
Asp/Asn	5.00 ± 0.05^{a}	4.3 ± 0.2^{a}	5.1 ± 0.4^{a}	4.1 ± 0.5^{a}	4.80 ± 0.03^{a}	6.3 ± 0.2^{b}
Cys	0.47 ± 0.01^{a}	0.49 ± 0.05^{a}	0.59 ± 0.07^{a}	0.50 ± 0.01^{a}	0.6 ± 0.1^{a}	0.56 ± 0.01^{a}
Glu/Gln	7.57 ± 0.03^{a}	6.3 ± 0.2^{ab}	9 ± 1^{abc}	6 ± 1^{ab}	7.0 ± 0.4^{abc}	9.3 ± 0.3^{ac}
Gly	0.97 ± 0.02^{ab}	1.7 ± 0.1^{ae}	1.35 ± 0.07^{ac}	0.92 ± 0.05^{bd}	0.94 ± 0.03^{b}	1.35 ± 0.01^{ce}
His	1.4 ± 0.2^{a}	2.20 ± 0.07^{a}	1.7 ± 0.2^{a}	1.3 ± 0.5^{a}	1.3 ± 0.4^{a}	1.54 ± 0.04^{a}
Ile	1.5 ± 0.1^{a}	1.5 ± 0.1^{a}	1.93 ± 0.01^{b}	1.31 ± 0.06^{a}	1.34 ± 0.02^{a}	1.86 ± 0.01^{b}
Leu	2.6 ± 0.1^{a}	2.58 ± 0.04^{a}	3.48 ± 0.01^{b}	2.4 ± 0.1^{a}	2.42 ± 0.01^{a}	3.40 ± 0.04^{b}
Lys	4.9 ± 0.1^{a}	3.2 ± 0.2^{b}	4.7 ± 0.7^{a}	3.2 ± 0.5^{ab}	3.65 ± 0.05^{ab}	4.5 ± 0.4^{a}
Met	0.49 ± 0.01^{a}	0.41 ± 0.04^{a}	0.63 ± 0.06^{b}	0.52 ± 0.01^{a}	0.43 ± 0.07^{a}	0.65 ± 0.02^{b}
Phe	1.56 ± 0.05^{a}	2.1 ± 0.2^{bc}	2.3 ± 0.2^{c}	1.6 ± 0.2^{a}	1.6 ± 0.1^{ab}	2.04 ± 0.08^{bc}
Pro	1.92 ± 0.04^{a}	1.9 ± 0.1^{a}	2.5 ± 0.1^{b}	1.67 ± 0.03^{a}	1.71 ± 0.06^{a}	2.33 ± 0.07^{b}
Ser	1.73 ± 0.06^{ab}	1.86 ± 0.06^{a}	2.38 ± 0.04^{c}	1.60 ± 0.01^{b}	1.7 ± 0.1^{b}	2.22 ± 0.01^{c}
Thr	1.35 ± 0.03^{a}	1.44 ± 0.07^{a}	1.77 ± 0.04^{b}	1.27 ± 0.01^{a}	1.31 ± 0.02^{a}	1.74 ± 0.03^{b}
Tyr	0.94 ± 0.02^{a}	1.3 ± 0.2^{c}	1.5 ± 0.2^{c}	1.0 ± 0.1^{ab}	1.0 ± 0.2^{ab}	1.33 ± 0.03^{c}
Trp	0.24 ± 0.02^{a}	0.27 ± 0.01^{a}	0.31 ± 0.01^{ac}	0.22 ± 0.02^{b}	0.25 ± 0.04^{ac}	0.31 ± 0.01^{ac}
Val	1.5 ± 0.1^{a}	1.41 ± 0.03^{a}	1.90 ± 0.04^{b}	1.34 ± 0.07^{a}	1.34 ± 0.04^{a}	1.86 ± 0.08^{b}
True protein content*	39 ± 1^{a}	38 ± 2^{a}	47 ± 4^{c}	33.1 ± 4.01^{b}	36 ± 2.2^{ab}	47 ± 1.6^{c}

[a-e] Significant differences were assessed with one-way ANOVA, Tukey's-b test ($p < 0.05$). Different letters indicate statistically different between each amino acid of the different samples.
*The true protein content represents the sum of total amino acids minus the water molecules present.

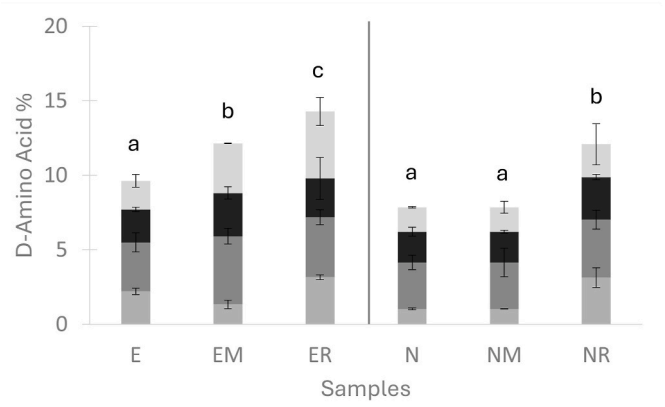

Fig. 3. Percentage of D-amino acids, expressed as the sum of the AA tested: Ala, Asp, Glu, Phe (from bottom to top). Significant differences were determined with one-way ANOVA, Dunnett's multiple comparisons test was used to compare the mean of each column with the mean of a control column (E and N) ($p < 0.05$). Different letters indicate statistically different samples.

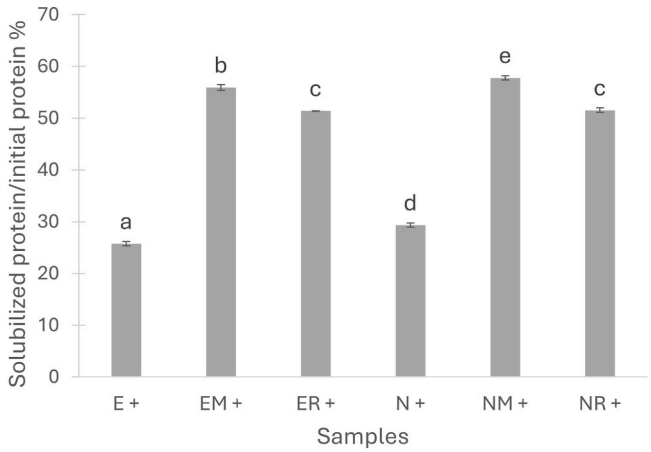

Fig. 4. Percentage of solubilised protein/initial protein. Samples from left to right: Energy soy meal (E), protein extract in mild condition from Energy defatted meal (EM), protein extract in rough condition from Energy defatted meal (ER), Namaste soy meal (N), protein extract in mild condition from Namaste defatted meal (NM), protein extract in rough condition from Namaste defatted meal (NR). One-way ANOVA and Tukey's test were used for multiple comparisons to evaluate significant differences ($p < 0.05$). Different letters mean statistically different samples.

different percentages of D-amino acids formed. These data are quite consistent with previous works which indicated that the formation of D-amino acid is influenced by the treatment involving the use of basic pH and heating [37,38]. The formation of D-amino acids (mostly observed after processing) within proteins represents an important parameter for evaluating the protein quality. Indeed, D-amino acids are not available for human protein synthesis, which lead to a decrease in the nutritional value of a protein food matrix, as well as hindering the digestibility of proteins in the gastrointestinal tract [37,39].

3.3. Evaluation of the protein fraction after in vitro digestion: solubilisation and protein integrity

The digestibility of proteins is affected by internal (e.g., the nature of the food matrix) and external (e.g., pH alteration and heating) factors. These could change the fate of the proteins upon digestion. For this reason, we decided to continue with the analysis of the effects of variety and processing by submitting E and N as well as extracts from rough and mild conditions to a simulated gastrointestinal (GI) digestion.

The supernatant coming from GI digestion represents the soluble, hence more available fraction for absorption after GI [40]. Thus, first the solubilised fraction was evaluated from a quantitative point of view. To do it so, the percentage of solubilised proteins (Fig. 4) was measured by Kjeldahl method and calculated as ratio between the protein found in the supernatant after the GI digestion and the total protein originally present in raw sample x100. After gastrointestinal digestion, statistically significant differences were observed for both varieties of soy meal and extracts under mild conditions. Thus, these results suggest that the variety represents a determining factor. However, no significant differences were observed between the two varieties extracted under rough conditions. This data could reasonably be due to the slight level of harshness of the treatment which seems to have more influence than the variety. In detail, comparing the solubilised protein present in the two varieties, soy meal showed the lowest solubility. Energy 26 % and Namaste 29 % showed lower solubility of protein than that found in extracts in rough conditions (around 51.5 %). In contrast, proteins solubilised under mild conditions showed the highest value of solubilised proteins (Energy 56 % and Namaste 58 %). Overall, the results suggest that both variety and extraction procedures can impact protein solubility [9,37]. In particular basic conditions lead to a major decrease in the digestibility of protein. This finding is consistent with previous research, which suggested that basic pH can change the structure of proteins by hindering their digestibility [37].

Then, the protein pattern after *in vitro* gastrointestinal digestion was qualitatively evaluated through a protein separation based on their molecular weight by an SDS-PAGE electrophoresis. Fig. 5 displayed the protein profile of digested soy meals and extracts.

The undigested samples show resolved protein bands at the different molecular weights. The overall protein patterns of both varieties in all conditions did not show marked qualitative differences. The most intense bands presumably belong to the most abundant soy proteins, namely glycinin and β-conglycinin and to the inhibitors of soybean, namely Kunitz and Bowman-Birk [9,41,42].

Considering the protein profile of the digested samples, it is noteworthy that the protein profiles of the two soy varieties are very similar in all conditions, suggesting that protein hydrolysis is not strongly variety dependent. Besides, it seems likely that it depends mostly on the treatment undergone.

Meal-derived proteins show significant hydrolysis after digestion, followed by protein extracts under rough conditions. In contrast, several high-molecular-weight proteins are still present under mild conditions even after digestion, suggesting increased resistance to hydrolysis by digestive enzymes. In a previous work, Rebollo-Hernanz et al. [9] investigated the protein profile in both raw and digested samples of seven different soyabean varieties. From a qualitative point of view, the profiles of the undigested samples of defatted soy meal are in line with what was observed by Rebollo-Hernanz et al. [9]. However, these authors observed a more resistant profile after the duodenal phase and a high variability of digestibility dependent on soybean varieties. These differences were strongly influenced by the specific composition of the evaluated varieties (e.g., distribution and abundance of certain proteins) [9].

3.4. Evaluation of potential bioactivity: DPP-IV inhibition and total GLP-1 release

We next aimed to analyse whether the different varieties and processing had differential effects on soy bioactivity after simulated gastrointestinal digestion. We focused on two functions that have previously been described as targets for bioactive peptides functions, that is DPP-IV inhibition and GLP-1 release [20–22].

3.4.1. DPP-IV inhibition

The effect of digested soy meals and extracts on DPP-IV inhibition

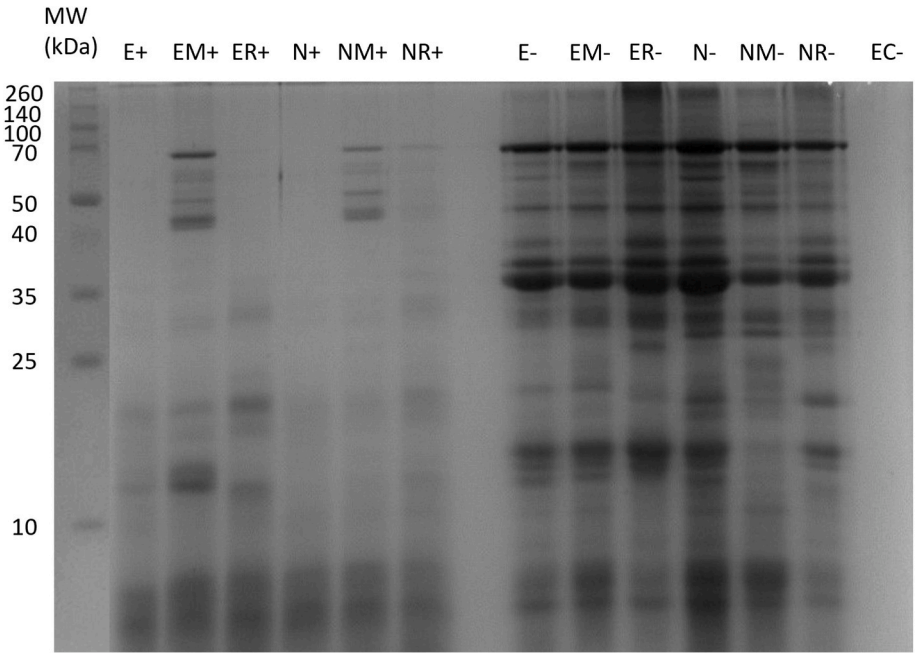

Fig. 5. SDS-PAGE electrophoretic profile of soy samples after digestion. Samples from left to right: Energy soy meal (E), protein extract in mild condition from Energy defatted meal (EM), protein extract in rough condition from Energy defatted meal (ER), Namaste soy meal (N), protein extract in mild condition from Namaste defatted meal (NM), protein extract in rough condition from Namaste defatted meal (NR). Samples marked "+" are digested; samples marked "−"are undigested samples (as negative control: sample with digestive fluids but without the addition of enzymes); EC- represents the negative enzymatic blank.

was evaluated. Fig. 6 shows that digested soy samples possess DPP-IV-inhibitory activity. Initially, a concentration of 6 mg/mL was tested, as a previously described strongly effective concentration for other protein sources [32]. At this concentration, a pronounced effect on the DPP-IV inhibitory activity was observed for soy samples in all conditions with no significant differences among the samples (Fig. 6A).

In order to evaluate possible more subtle differences among samples, a 10 times lowest concentration (0.5 mg/mL) was tested. In this case, statistically significant differences among samples were observed (Fig. 6B). Notably, the soy protein extracts in mild conditions (EM, NM) showed the lowest and at comparable level to the enzymatic control, which showed no activity. On the other hand, Energy meal extract in rough condition (ER) and Namaste meal (N) showed the highest activity of DPP-IV inhibition. It is worth noting a possible correlation between the protein profile of the digested samples and the inhibitory activity of DPP-IV. Indeed, only some proteins of the initial protein pattern seem to be completely hydrolysed after the digestion process of the extracts in mild conditions. This could result in a lower presence of peptides with inhibitory activity on DPP-IV. In contrast, the rough conditions seem to promote the hydrolysis of the whole protein pattern, which could reasonably result in a higher concentration of bioactive peptides, confirming that the molecular size distribution of the digested proteins can influence the activity of DPP-IV enzyme [43]. This phenomenon was also confirmed in unprocessed meals by the electrophoretic profile and DPP-IV inhibitor activity.

Overall, previous work highlights that plant protein hydrolysates (e.g., soy, chickpea, pea, lupin, lentil) showed potential DPP-IV inhibitory activity after several enzymatic hydrolysis with commercial protease [44]. Furthermore, our data confirmed that protein-released peptides, derived from gastrointestinal digestion, produced great inhibition activity on DPP-IV [14,45]. In particular, the protein soybean-derived peptides showed a high DPP-IV inhibition activity, as suggested by different authors who found bioactive peptides after gastrointestinal digestion of soybean meal from different varieties and germinated soybean [9,22]. Provided the key role of DPP-IV inhibitors on type 2 diabetes [46], these data suggested that the soy could be considered a functional ingredient to potentially prevent the type 2 diabetes, as mentioned before [18,47].

3.4.2. *Total GLP-1 release*

Finally, the digested soy meals and extracts were tested for total GLP-1 release using *in vitro* cultured GLUTag cells. The enteroendocrine cells were treated with gastrointestinal digestate at a concentration of 5 mg protein/mL. Furthermore, the potential cytotoxicity of the tested treatment on the GLP-1 cells were excluded through the LDH method (LDH release to the medium was lower than 5 %).

The results obtained on the total release of GLP-1 after cell treatment with gastrointestinal digested samples are shown in Fig. 7. The results observed indicated that soy, in all condition and variety, can positively stimulate total GLP-1. In contrast, no significant differences were found between soy meals and extracts, probably due to complex mechanism involved the GLP-1 release and its receptors. GLP-1 secretion in cells can be activated by proteins [48] and appears to be closely related to the composition of the nutrient; so since digesta is a complex mixture derived from the same starting matrix with a very similar amino acid profile in the different conditions (as reported in section 3.2), thus can be driving to a similar GLP-1 release [49].

These results latter agree with the data observed by Nishi et al. [50,51] which suggested the remarkable ability of soy hydrolysate to stimulate enterhormone release. The study demonstrated that casein, soybean protein isolate, egg white, and gluten hydrolysed stimulated the CCK release. In particular, soy hydrolysate gave the most effective results on the stimulation among the others. In addition, another comparative study different protein matrices were evaluated on CCK and GLP-1 release, the latter showed that protein from different matrices can be effective on the stimulation of both enterhormones in STC-1 cell line, including soybean proteins [23]. This characteristic of soy to simulate the main hormones involved in appetite regulation is particularly interesting for human health.

4. Conclusion

In this study, different varieties of soybeans used for human and animal nutrition were chosen, and two commonly applied procedures

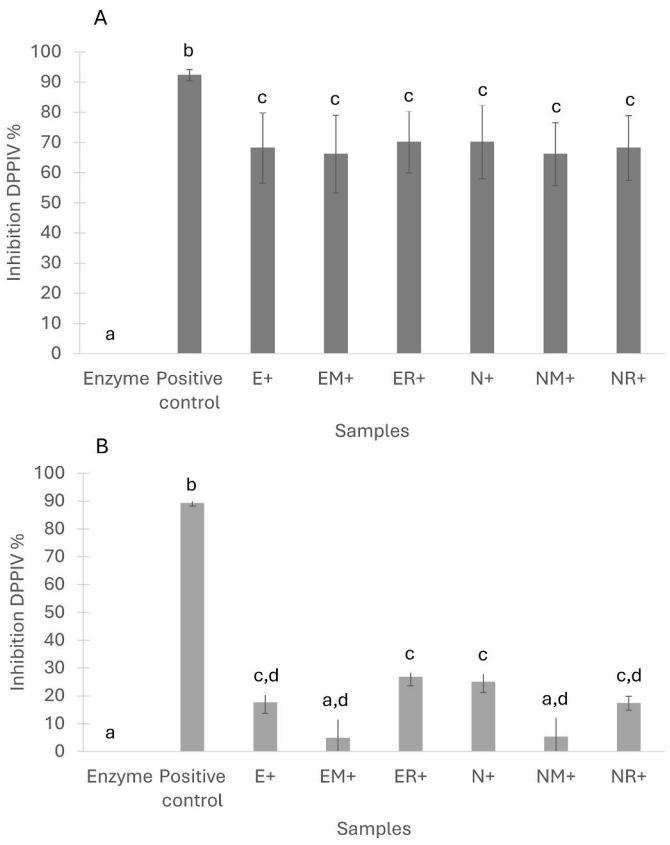

Fig. 6. Percentage of DPP-IV inhibition evaluated with the following concentrations 6 mg/mL of protein in the digestion supernatant (A) and 0.5 mg/mL of protein in the digestion supernatant (B) of soy meal and protein extract. The enzymatic reference was used as a negative control while Diprotin A was used as a positive control. Samples: Energy soy meal (E+), protein extract in mild condition from Energy defatted meal (EM+), protein extract in rough condition from Energy defatted meal (ER), Namaste soy meal (N+), protein extract in mild condition from Namaste meal (NM+), protein extract in rough condition from Namaste meal (NR+), negative control (negative CTR). Different letters indicate statistically different samples ($p < 0.05$) (one-way ANOVA, Tukey's test).

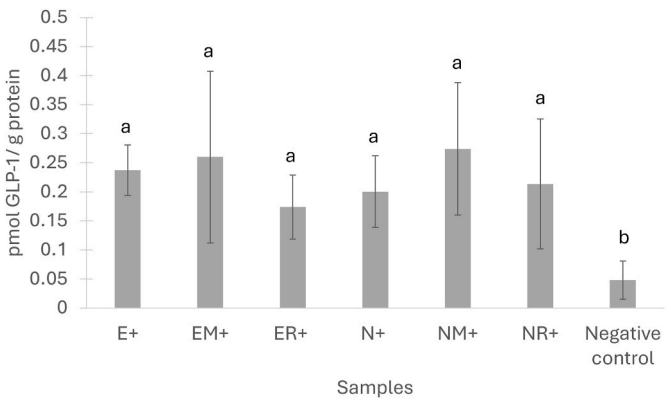

Fig. 7. Total GLP-1 release assessed in digested soy meal and protein extract under mild and rough conditions in both Energy and Namaste varieties. Values show the pmol of total GLP-1 in the medium of treated samples normalised by protein content in the wells. Samples: Energy soy meal (E+), protein extract under mild condition from Energy defatted meal (EM+), protein extract under rough condition from Energy defatted meal (ER), Namaste soy meal (N+), protein extract under mild condition from Namaste meal (NM+), protein extract under rough condition from Namaste meal (NR+), negative control. Different letters mean statistically different samples ($p < 0.05$) (one-way ANOVA, Tukey test).

for extracting plant proteins were performed. Overall, the two soybean varieties showed differences in raw protein content, amino acid profile and protein solubilisation after gastrointestinal digestion. Moreover, different extraction conditions showed significant differences confirming that the rough basic treatment, which presents the highest extraction yield and true protein content can hinder the protein quality (D-amino acid formation) and protein digestibility, even if it improves the digestibility of recalcitrant proteins. In addition, we tested whether the different varieties and treatments showed effects on bioactivity. Our results suggest that the different methods do not strongly modify GLP-1 release even if, at lowest concentration tested, the DPP-IV inhibitory capacity seems more pronounced when the rough conditions were applied, suggesting that promoting the hydrolysis of the whole protein pattern leads to a higher concentration of bioactive peptides. Taken all together, soy demonstrated at intestinal level, a high capacity to stimulate the GLP-1 release and to inhibit the activity of the DPP-IV enzyme even though some alteration of solubilisation and D-amino acid formation were observed in rough conditions. On this ground, further studies can be dedicated at a more molecular level to ensure the production of high-quality protein extracts, to preserve their nutritional value. Besides nutritional quality, these analyses could help us to understand which molecules are responsible for this bioactivity. In a future prospect this knowledge could potentially be used to produce new protein mixture or innovative products with tailored characteristics that preserve nutritional value and promote the secretion of bioactive compounds.

CRediT authorship contribution statement

Francesca Accardo: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Barbara Prandi:** Writing – review & editing, Conceptualization. **Tullia Tedeschi:** Writing – review & editing. **Esther Rodríguez-Gallego:** Writing – review & editing. **Anna Ardévol:** Writing – review & editing, Conceptualization. **Stefano Sforza:** Writing – review & editing, Supervision, Conceptualization. **Montserrat Pinent:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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