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Abstract

Nowadays, consumers are increasingly inclined toward plant-based meat analogues for sake of food security, safety, and sustainability. This growing interest, not only from consumers but also from food companies, brought the offer on the market to be wide and vast. From our previous study it emerged that the market supply, especially the Italian one, is diversified both in terms of protein sources and nutrient content. Although these products are increasingly consumed, for most of the meat analogues today on the market, little is still known about their actual protein quality and digestibility. To fill this gap, in this study different commercial plant-based burgers (2 soy-based and 2 pea-based) were selected and compared to two beef burgers, as controls, in terms of protein quality and digestibility. The findings of this study demonstrated the essential amino acidic profile lacks lysine for almost all burgers (including the meat-based ones) compared to the amino acid scoring pattern set by FAO/WHO (for older children and adults), even if the sum of essential amino acids was within the range of sufficiency. All samples showed good initial protein integrity with low hydrolysis (above 6%) and percentage of D-enantiomers (above 15%). The study of the digestibility, performed by the validated INFOGEST *in vitro* model, showed better protein solubilisation in the case of meat burgers (63 ± 3% and 61 ± 8%), but a good digestibility also in the case of plant-based ones (from 55% to 40%). The degree of hydrolysis of the solubilised proteins was very high in all samples (from 65% to 40%) indicating a very good protein accessibility to digestive enzymes. The analysis of the peptide fraction of digestates indicated a high prevalence of collagen proteins in beef burgers and of reserve proteins in plant-based burgers. This study showed that the differences between these products are mostly dependent on the quality of the raw materials used, rather than on the vegetal or animal protein source. Therefore, to have a product with a good protein quality and digestibility, independently from the protein origin, the consumer needs to make an accurate choice, carefully reading the ingredient list.

Keywords: meat analogs, protein digestibility, protein quality, amino acids, plant proteins, plant-based burgers

1. Introduction

When looking at meat production, food security is an essential concern considering the population growth: 2.3 billion population growth is expected by 2050, bringing the total of human beings on earth to 9.1 billion, with a predicted 70% more food requirement (FAO, 2009). This will lead to an enormous increase in the use of natural resources (i.e., feed, water, energy, land), mainly related to meat production (Kumar et al., 2017). Indeed, the consumption of natural resources used for meat production was estimated to be from 2 to 7 kg of grain per kg of meat, while the water consumption goes between 3.9-15.5 m^3/ton depending on the livestock (Kumar et al., 2017). It is estimated that meat-based human diets require 2 million L of water per person per year vs the 1 million L needed for vegetarian diets (Smil, 2002). Furthermore, for the breeding of animals, 70% of the arable land is used and, in addition, 40% and 75% of the total grain and soy produced is used for animal feeding (Kumar et al., 2017). Environmental concern is only one of the major issues related to meat production and consumption. Other important factors to take into account are animal welfare and health concern (Kumar et al., 2017). In particular, high consumption of red processed meat can occur in health problems such as cardiovascular disease and colorectal cancer (Chan et al., 2011).

For the above-reported concerns related to meat production and consumption, consumers are increasingly inclined to switch to more vegetable-based diets, which leads to increased production of plant-based meat analogues. Anyway, not all consumers are willing to introduce meat analogues into their diet. Four principal groups of consumers have been identified as favourable to consuming these products: i) flexitarians that want to reduce their meat consumption and search for a balanced and healthy diet; ii) consumers interested in animal welfare and food sustainability; iii) convenience-conscious consumers; iv) consumers oriented toward innovation (Sun et al., 2021).

Meat analogues, which can be defined as food products formulated to mimic meat in nutrients, texture, flavour, and appearance, appear to be the better solution to introduce alternative proteins in human food choices (Kumar et al., 2017). There is a huge selection of vegetable protein sources suitable for this aim. In particular, soy, pea, and wheat proteins are the most commonly used (Kyriakopoulou et al., 2019). To provide a similar nutritional profile in terms of Essential Amino Acids (EAA), usually, a combination of vegetable foods based on cereals and legumes together is also used (Aschemann-Witzel et al., 2021). Soy and pea proteins, in particular, are cheap and easily available, but they cannot naturally resemble the fibrous structure of meat (Kołodziejczak et al., 2021). To achieve a good structure, two different approaches can be used in the making of meat analogues: the bottom-up and the top-down (Dekkers et al., 2018). The top-down approach is the most efficient, being robust and scalable: it involves the formation of the desired fibrous structure by applying shear stress. In this context, extrusion is the most widespread method used (Kyriakopoulou et al., 2019). For this reason, the most common ingredients for meat analogues are Textured Vegetable Proteins (TVPs) and High Moisture Extrudates (HMEs) (Kołodziejczak et al., 2021).

These intensive processes make it possible to obtain a meat-like structure, but they can affect the protein quality and integrity. Briefly, the process needed to form a viscoelastic mass in the extruder – which will be then aligned inside the cooling die – resulting in an increment of the temperature that can reach 140-180 °C (Kyriakopoulou et al., 2019). Depending on the temperature reached, the product structure can be affected – mostly due to the decrease of disulphide linkages induced by the increase in the temperature (Arêas, 1992). Besides this, other protein modifications can occur during the texturization, such as changes in solubility and protein oxidation and denaturation, resulting in a decreased quality of proteins (Kyriakopoulou et al., 2019).

The nutritional characteristics of meat analogues are different from those of meat products. Generalising, meat analogues have a lower proportion of saturated fats and a higher content of calcium, phosphorus, and potassium, while red meat products have a higher content of methionine and bring vitamin B12, iron, and zinc to the diet (Kołodziejczak et al., 2021; Kumar et al., 2017). In addition, meat analogues contain carbohydrates since fibres, polysaccharides, and starches are commonly used to improve texture and water retention (Kołodziejczak et al., 2021). When meat analogues were compared to commercial meat products sold in the Italian market (Cutroneo et al., 2022), results highlighted that various categories of plant-based analogues presented some valuable nutritional aspects, including having a comparable amount of proteins.

Anyhow, very little information about the actual protein quality of the plant-based meat analogues usually found in the market is known. Hence, the present study aims at characterizing this aspect. In particular, the actual protein quality and digestibility of commercial meat analogues will be evaluated, and compared to the relative commercial meat references.

2. Materials and methods

2.1. Samples selection

In this study, four commercial plant-based burgers – two based on soy and two based on peas – were selected and compared with two meat burger products, used as comparison samples.

To select the plant-based burgers to be analysed, the 105 commercial burgers surveyed in our previous study (Cutroneo et al., 2022) were considered and a cut-off of 15 g of protein per portion was used. This cut-off was selected based on the content of commercial meat burgers retrieved in the same study. Among the 29 products with at least 15 g of protein content per portion, two based on soy and two based on peas were chosen.

For each burger (A, B, C, D, E, and F) the protein source, the protein content for the portion (g), the number of ingredients (n), and the ingredients that provide proteins were reported (Table S. 1).

2.2. Samples preparation

Before the analyses, all samples were cooked in a non-stick pan without oil. The plant-based burgers were cooked – following the instruction on the label – for 3 min per side, while for the beef burgers approximately 4 min per side, until completely cooked at the core – that was confirmed cutting in half the product.

The burgers, both raw and cooked, were minced (3 sec each) – using the IKA® A11 basic analytical mill (IKA®-Werke GmbH & Co, Staufen, FR, Germany) – to make the samples homogeneous before subjecting them to subsequent analyses.

All the analyses carried out for this work were performed in duplicate unless specifically noted otherwise.

2.3. Proximate analysis

Total nitrogen was determined with the Kjeldahl method according to the European Regulation (EC) No 152/2009 (European Commission, 2009), using a DKL Heating Digester and UDK 139 Semi-Automatic Distillation Unit (VELP SCIENTIFICA, Usmate Velate, MB, Italy). The nitrogen content was multiplied by a conversion factor – 5.57 for beef burgers, 5.77 for soy-based burgers and 5.86 for pea-based ones (Krul, 2019; Mariotti et al., 2008) – to estimate the protein content (g/100 g).

The total fat (g/100 g) was determined according to the AOAC standard procedure (AOAC, 2002), using a Semi-Automatic Soxhlet Extractor SER 148/3 (VELP SCIENTIFICA, Usmate Velate, MB, Italy) and diethyl ether as extraction solvent.

The dry matter and ash analysis were conducted following the protocol indicated in the ISTISAN 1996/34 report (Baldini et al., 1996).

2.4. Amino acid profile determination

2.4.1. Total amino acid determination

Total amino acid determination was performed, on raw and cooked products, following the standard procedure reported by Prandi et al. (2021) with some modifications. 0.5 g of sample underwent ad acid hydrolysis (23 h at 110 °C) with 6 N hydrochloric acid (HCl) (Sigma Aldrich, St. Louis, MO, USA). Nor-leucine (Sigma Aldrich, St. Louis, MO, USA) was used as an internal standard. For methionine ad cysteine, the acid hydrolysis was preceded by performic acid oxidation. Briefly, 50 mg of sample were added with freshly prepared performic acid (9 volumes of 95% formic acid mixed with hydrogen peroxide) and kept at 0 °C for 16 h. After that, the excess of performic acid was removed with hydrobromic acid and samples were dried under nitrogen flow. Subsequently, acid hydrolysis was performed as already described.

A calibration standard solution was also prepared mixing a standard mixture 2.5 mM (Thermo Scientific, Waltham, MA, USA) with a mixture of amino acids 2.5 mM (nor-leucine, hydroxyproline, cysteic acid,

133 methionine sulfone - Sigma Aldrich, St. Louis, MO, USA) in ratio 1:1. Five different concentration points
134 (1.25 mM, 0.625 mM, 0.3125 mM, 0.156 mM, 0.078 mM) were obtained in duplicate by subsequential
135 dilutions.

136 Samples were then derivatised, according to the manufacturer's instructions, using the AccQ-Fluor reagent kit
137 (Waters, Milford, MA, USA) and analysed using a UPLC ACQUITY system coupled with an ACQUITY SQ
138 ESI-MS system (Waters, Milford, MA, USA). The analysis was performed with an ACQUITY UPLC Peptide
139 BEH C18 (300Å, 1.7 µm, 2.1mm 170 × 150mm) column (Waters, Milford, MA, USA) with an ACQUITY
140 UPLC Peptide BEH C18 VanGuard™ (300Å, 1.7 µm, 2.1mm × 5mm) pre-column (Waters, Milford, MA,
141 USA). The analysis was performed as reported by Buhler et al. (2019).

142 The software used for data acquisition and processing was MassLynx™ V4.0 (Waters, Milford, MA, USA).
143 The amino acid contents (mM) were estimated by using the calibration curves performed, normalizing all the
144 areas with the internal standard (nor-leucine).

145 *2.4.2. Total tryptophan determination*

146 Tryptophan determination was performed, on raw and cooked products, with alkaline hydrolysis. 150 mg of
147 dried sample were placed in 8 mL Pyrex with a Teflon-lined screw cap. The samples were added with 4 mL
148 of 4 N sodium hydroxide (NaOH) (Carlo Erba, Milan, MI, Italy) and 150 µL of α-methyl-tryptophan (50
149 mg/100 mL) (Sigma Aldrich, St. Louis, MO, USA). Then samples were placed in an oil bath at 100 °C for 6
150 h. After that, the samples were cooled at room temperature and neutralized with 6 N HCl. Then samples were
151 centrifuged at 4 °C and 3220 *g* for 45 min. The supernatant was filtered and brought to 10 mL in a volumetric
152 flask with Milli-Q water.

153 To quantify tryptophan, a response factor was performed: 150 µL of tryptophan (50 mg/100 mL) (Sigma
154 Aldrich, St. Louis, MO, USA) were placed in a 10 mL volumetric flask, added with 150 µL of α-methyl-
155 tryptophan (50 mg/100 mL), and brought to volume with Milli-Q water. Both the response factor and the
156 samples were performed in duplicate.

157 Finally, an aliquot of each sample was transferred in vials for the UPLC-MS analysis. Samples were analysed
158 using a UPLC ACQUITY system coupled with an ACQUITY SQ ESI-MS system (Waters, Milford, MA,
159 USA). The analysis was performed with an ACQUITY UPLC Protein BEH C18 (300Å, 1.7 µm, 2.1mm 170
160 × 150mm) column (Waters, Milford, MA, USA) with an ACQUITY UPLC Peptide BEH C18 VanGuard™
161 (130Å, 1.7 µm, 2.1mm × 5mm) pre-column (Waters, Milford, MA, USA). The mobile phase was made by
162 Milli-Q water with 0.2% of ACN and 0.1% of Formic acid (eluent A) and ACN with 0.1% of Formic acid
163 (eluent B). Analysis conditions were as follows: column temperature: 35 °C, injection volume: 4 µL, flow:
164 0.25 mL/min, gradient: 0-1.8 min isocratic 100% A, 1.8-13.2 min linear from 100% A to 50% A, 13.2-14 min
165 50% A, 14-14.5 min linear from 50% A to 0% A, 14.5-16.1 min 0% A, 16.1-16.5 min linear from 0% A to

100% A, 16.5-29.5 min 100% A. Detection conditions were as follows: polarity: ES+, capillary: 3.20 kV, cone: 30.00 V, extractor: 4.00 V; RF: 0.20 V, source temperature: 150 °C, desolvation temperature: 300 °C, cone gas flow: 100 L/h, desolvation gas flow: 650 L/h, LM 1 resolution: 14.60, HM 1 resolution: 15.20, ion energy 1: 0.30; gain: 1.00, acquisition mode: SIR (m/z 188.0; 202.1; 205.0; 219.1), scan duration: 1 s.

The software used for data acquisition and data processing was MassLynxTM V4.0 (Waters, Milford, MA, USA). Tryptophan content (mM) was estimated by multiplying the response factor by the normalized area of the compound and the concentration (mM) of the internal standard (α-methyl-tryptophan).

2.5. Protein integrity

2.5.1. Degree of hydrolysis

The determination of the degree of hydrolysis was performed on the cooked samples. For the protein extraction, 100 mg of each sample were added with 1 mL of extraction buffer containing 4 M Urea (VWR Chemicals, Radnor, PA, USA), 100 mM Ammonium bicarbonate (NH_4HCO_3) (Sigma Aldrich, St. Louis, MO, USA), and 5 mM Dithiothreitol (DTT) (PanReac AppliChem, Darmstadt, DA, Germany).

The degree of hydrolysis (DH) was calculated using the o-phthaldialdehyde (OPA) method described by Spellman et al. (2003) with some modifications. Briefly, the assay was performed by adding 20 µL of the sample - suitably diluted - to 2.4 mL of OPA/N-acetylcysteine (NAC) reagent, composed of: 5 mM OPA (Sigma Aldrich, St. Louis, MO, USA), 5 mM NAC (Merck Millipore, Burlington, MA, USA), 2% sodium dodecyl sulphate (SDS) (Sigma Aldrich, St. Louis, MO, USA), and 75 mM borate buffer, in 1:9 Methanol: Milli-Q water, pH 9.5.

The measurement of absorbance was carried out at 340 nm using a JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklahoma City, OK, USA). For each sample, the measurement was taken three times and the mean was used for the calculation. The intrinsic absorbance of samples was also measured by adding 20 µL of the sample (diluted 1:20 with Milli-Q water) to 2.4 mL of Milli-Q water. To determine the DH a calibration curve was performed using L-isoleucine (2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL). The calibration curve was analysed in the same way as the samples. The DH% was calculated as the percentage of the ratio between free amino groups determined by the procedure and the total amino groups of the sample.

2.5.2. Amino acid enantiomeric purity determination

The amino acid enantiomeric purity was determined, on raw and cooked products, following the standard procedure already reported by Prandi et al. (2021) with some modifications. Briefly, 0.5 g of the sample underwent acid hydrolysis (6 h at 110 °C) with 6 N HCl. The hydrolysates were dried using a Rotavapor R-215 (BÜCHI, Flawil, SG, Switzerland). The solid residue was resuspended in 2 mL of 2 M HCl in 2-propanol (Sigma Aldrich, St. Louis, MO, USA) freshly prepared, and samples were derivatised using Trifluoroacetic

198 anhydride (Sigma Aldrich, St. Louis, MO, USA). Samples were then dried under nitrogen flow and
199 resuspended in 1 mL of Dichloromethane (Carlo Erba, Milan, MI, Italy) before GC/MS analysis.

200 The analysis was carried out using an Agilent Technologies 7820° gas-chromatograph (Agilent Technologies,
201 Palo Alto, CA, USA) coupled to an Agilent Technologies 5977B mass spectrometer (Agilent Technologies,
202 Palo Alto, CA, USA), as described by Anzani et al. (2017).

203 The software used for data acquisition was MassHunter (Agilent Technologies, Palo Alto, CA, USA), while
204 for data processing ChemStation software (Agilent Technologies, Palo Alto, CA, USA) was used. The
205 enantiomeric purity, calculated as the percentage of D-enantiomers (D%), was estimated as the percentage of
206 the ratio between the amino acid D-enantiomer and the sum of D- and L- enantiomers.

207 **2.6. Digestibility of proteins**

208 *2.6.1. In vitro gastro-intestinal digestion procedure*

209 The *in vitro* gastro-intestinal digestion protocol was carried out following the standard procedure "INFOGEST
210 static *in vitro* simulation of gastrointestinal food digestion" (Brodkorb et al., 2019). As the first step, test
211 digestion was carried out to verify the pH stability during all digestive phases. Then, the cooked burgers
212 underwent the *in vitro* gastro-intestinal digestion procedure using an Orbital Shaker-Incubator ES-20 (Biosan,
213 Riga, Latvia) at 37 °C. Briefly, 1 g of minced sample - to simulate chewing - was added with the salivary phase
214 solution (pH 6, containing 75 U/mL of α-Amylase - Sigma Aldrich, St. Louis, MO, USA), in a ratio 1:1 w/v.
215 After 2 min, the bolus was added with the gastric phase solution (pH 3, containing 2000 U/mL of Pepsin -
216 Sigma Aldrich, St. Louis, MO, USA), in a ratio of 1:1 v/v. After 2 h, the chyme was added with the intestinal
217 phase solution (pH 7, containing 100 U/mL of Pancreatin, expressed as trypsin activity, and 10 mM Bovine
218 Bile - Sigma Aldrich, St. Louis, MO, USA), in a ratio 1:1 v/v. At the end of the 2 h, the chyle was heated in
219 an oil bath at 90 °C for 15 min to stop the reaction. Samples were then centrifuged at 4 °C and 3220 *g* for 45
220 min and the supernatant was collected.

221 Furthermore, the digestion procedure was conducted on 1 mL of Milli-Q water as blank. Controls were also
222 performed: 1 g of minced sample underwent the same digestion procedure without the use of enzymes.

223 From the supernatant collected from all samples (digestates, blanks, and controls) the percentage of soluble
224 proteins was estimated. The digestibility, of soluble proteins, was evaluated by the degree of hydrolysis (DH%)
225 and compared also to a standard protein from chicken egg (ovalbumin). This protein was digested, in a
226 concentration comparable to that naturally present in egg white, under the same conditions as the samples.

227 *2.6.2. SDS-PAGE analysis of digestates*

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on the digestates. To better compare samples the analysis was carried out on extracted raw and cooked samples and the supernatant after digestion of digestates and digestion blanks. The extraction of proteins, for raw and cooked burgers, was carried out as previously described (paragraph 2.5.1 Degree of hydrolysis).

The protein content of all samples was determined, using the Qubit Fluorometer™ with the Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, CA, USA), following the guidance material. The SDS-page analysis was performed, as described by Prandi et al. (2021). For each sample, 40 µg of protein were used. The analysis was performed using a Criterion XT Bis-Tris Gel at 10% (BIO-RAD, Hercules, CA, USA) and the gel was scanned using a GS-800 calibrated imaging densitometer (BIO-RAD, Hercules, CA, USA).

2.6.3. Solubilised protein determination after digestion

The soluble protein content of samples after the *in vitro* gastro-intestinal digestion was estimated on the supernatant of digestates, blanks, and controls. For all samples, the total nitrogen content was estimated by performing Kjeldahl analysis – as already described (paragraph 2.3 Proximate analysis) – on 1 mL of supernatant. The soluble protein content in percentage was then estimated by the ratio between the real protein content – net of blank – of the supernatants and the estimated content of the total protein of the same starting sample before digestion.

2.6.4. Degree of hydrolysis of digested samples

The determination of the degree of hydrolysis was performed, as previously described (paragraph 2.5.1 Degree of hydrolysis), on the supernatant obtained from the digestion procedure - of digestates, blanks, and controls - properly diluted.

The DH% was calculated as the percentage of the ratio between free amino groups after digestion and the total amino groups of the sample.

2.6.5. LC-HR-MS analysis

Digestates were analysed in High Resolution Mass Spectrometry. The separation and the detection – performed with a µHPLC (Dionex Ultimate 3000, Sunnyvale, CA, USA) coupled to a mass spectrometer Orbitrap LTQ XL (Thermo Scientific, Waltham, MA, USA) – were performed as described by Prandi et al. (2020).

The software used for data acquisition was Xcalibur 2.0.7 (Thermo Scientific, Waltham, MA, USA), while for data processing Peaks Studio (Bioinformatics Solutions, Waterloo, ON, Canada) was used. The database search was carried out – depending on the sample – on *BOS TAURUS* (samples A and B), *GLYCINE MAX SOYABEAN* (samples C and D), and *PISUM* (samples E and F). Positive hits for protein identification were

arbitrarily set for all those proteins identified by the program with a coverage >20%, (−10lgP) > 100, and all those peptides with a score (−10lgP) > 20, since such value should reduce the risk of false positives to zero.

2.7. Statistical analysis

The statistical analysis was carried out using the Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM Corp., Chicago, IL) and performed at $\rho < 0.05$ of the significance level.

To investigate the differences between samples for the total fat and protein content (Table 1), the differences between samples for the protein content determined with the Kjeldahl method and the sum of total amino acids (Table 2), the difference in the sum of EAA among raw and among cooked burgers (Figure 2, panel A and B), the difference among samples in the degree of hydrolysis % and the percentage of D-enantiomers % (Figure S. 1, panel A and B), and the difference between digestates in the solubilisation of proteins and degree of hydrolysis estimated on the soluble protein (Figure 3 panel A and B), the one-way ANOVA with multiple pairwise comparisons test ($\rho < 0.05$) was used.

To investigate the difference in the amino acidic profile of the samples compared based on the protein base, the Independent Samples t-Test ($\rho < 0.05$) was used.

3. Results and discussion

3.1. Sample description

The four analogue burgers – two soy-based and two pea-based – were first characterised in terms of the proximate composition. Then the actual protein quality was evaluated by: i) the identification of the composition of the protein fraction; ii) the determination of the protein quality in terms of total amino acids composition; iii) the study of digestibility.

All these aspects were compared to the meat products they intend to mimic. For this purpose, two different beef burgers were chosen as the control.

All the above analyses were performed on the cooked burgers; thus, a burger of each type was cooked following the instruction on the label (in a non-stick pan without oil).

3.2. Proximate analysis

The composition of beef and plant-based burgers – stated on the nutritional label – for the raw products is reported in the supplementary material (Table S. 2). All the samples appear to be similar in composition, with the main differences found, as expected, in the content of total carbohydrates. Comparing the labels of plant-based burgers with the median data retrieved from a previous study (Cutroneo et al., 2022), similar contents in nutrients can be found, with some differences due to the wide variability of these commercial products. The

same variability, albeit with the median nutrient content very similar to that of the products here selected, was also seen in other surveys conducted for the plant-based commercial products sold in the United Kingdom and Ireland (Alessandrini et al., 2021; Safefood, 2021).

Due to the fact that these products are consumed after cooking, the proximate analysis was carried out on the cooked samples. The results, shown in Table S. 3, highlighted that, as expected, the highest protein and fat content can be found in beef burgers, while plant-based burgers showed a higher total carbohydrates and salt content. The total fat and protein content of cooked products is shown in more detail in Table 1. The total fat was statistically higher ($\rho < 0.05$) in beef burgers. The lowest content of total fat was found in sample E (6.21 ± 0.24 g/100 g), being the only plant-based burger that statistically differs ($\rho < 0.05$) from the other analogues. However, although the lipid content of these products is lower than that of their corresponding animal products, these commercial plant-based products show a high lipid content if compared with traditional analogues (such as tofu and seitan) (Bohrer, 2019). This is due to the fact that, to obtain the desired flavour and texture attributes, the formulations are added with a wide variety of lipid-rich ingredients. The most commonly used are rapeseed, coconut, sunflower, corn, sesame oil and cocoa butter (Bohrer, 2019). The role of lipids in manufacturing meat analogues is essential for both technological and sensorial aspects. Indeed, they contribute to lubrification and texture during the processing, but they also improve the mouthfeel of these products – better juiciness, tenderness, and flavour release (Bohrer, 2019).

<<Insert Table 1>>

As shown in Table 1, the protein content of products showed a statistically higher ($\rho < 0.05$) value in beef burgers. Sample F was observed to be the only plant burger with a comparable protein amount to meat burgers. However, the comparison of these samples based only on the protein content is not sufficient. The determination of the amino acids profile is essential to estimate the actual protein quality of food. It is well known that only the proteins having all the nine EAA in a sufficient amount can be considered complete proteins, as in the case of milk and dairy, eggs, meat, and fish. On the contrary, even if the total protein content is comparable with the animal products, plant proteins are considered often incomplete proteins because of the lower amount of EAA and the higher of non-EAA (Yu & Fukagawa, 2020).

3.3. Amino acid profile determination

The total amino acids of the raw and cooked products were then determined. The sum of total amino acids was in the first place compared with the protein content determined with the Kjeldahl method, and the results are shown in Table 2, revealing a good agreement between the two values. As expected, in both analyses, the protein content of raw and cooked products showed a statistically higher ($\rho < 0.05$) protein content in beef burgers. Sample F (pea-based) was the only plant burger with a comparable amount of proteins to meat ones when the sum of total amino acids was considered.

<<Insert Table 2>>

<<Insert Figure 1>>

The total amino acid profile reported for cooked products (Figure 1) for beef burgers showed a similar amino acidic profile between the two samples, even with some amino acids being statistically different ($\rho < 0.05$) – i.e., alanine, asparagine, cysteine, glutamic acid, glycine, hydroxyproline, lysine, and proline, albeit the absolute values were anyway very close. Almost the same trend can be found in soy-based products, where the amino acidic profile resulted very similar between the two samples. Statistical differences ($\rho < 0.05$) were found, also in this case, for some amino acids having slightly different values, such as alanine, histidine, leucine, serine, and valine. In this case, however, some amino acids seemed to be quite different between the two burgers, in particular aspartic acid, lysine, and methionine. The last case of the pea-based burgers was quite peculiar, since the amino acidic profile of the two samples presented notable statistical differences ($\rho < 0.05$) in the amount of almost all amino acids, with the exception of histidine. This variability can be possibly due to the processing carried out to obtain the protein concentrate/isolate used in the formulation of the analogue burgers or the mixing with other protein sources. Alternatively, the variability in the amino acid composition can also be possibly ascribed to the different origins of the plant base used (variety, cultivation, etc.) (Bou et al., 2022).

As for the differences existing between the three protein bases (beef, soy, and pea), they are the ones expected according to their origin. In particular, beef burgers showed a higher content of cysteine than plant-based burgers. Plant proteins, on the other side, lack some EAA, in particular sulphur amino acids (Gorissen et al., 2018). To improve the amino acidic profile, plant proteins are usually blended with other protein sources. The best blends relate to the mix of pulse proteins (rich in lysine) with cereal proteins (rich in methionine and cysteine) (Dimina et al., 2022; Sudheesh et al., 2022). This can be seen, for example, in sample F (pea-based) where, probably due to the addition of rice protein, the methionine content is higher if compared to sample E (pea-based).

As expected, other differences were found in the content of glycine and in the presence of hydroxyproline in beef burgers, which are typical amino acids of collagen (Listrat et al., 2016). A difference in the content of lysine, which anyways resulted low in all burgers, was also observed.

These differences are extremely important in order to define the nutritional value of the protein sources. Several studies showed how the vegan diet – depending on the protein sources chosen – can be lacking in protein amount. Even when the recommended protein intake is fulfilled, there is the risk of occurring in a deficiency of some specific EAA (Bakaloudi et al., 2021). Among EAA, lysine, methionine, tyrosine, and tryptophan can be insufficient in vegan diets (Schmidt et al., 2016). The sufficient supply of EAA – in the case of vegan diets – mainly depends on the type of protein consumed. Soy proteins contain a sufficient amount of EAA, whereas

non-soy protein-based diets, or most generally diets with a restricted intake of pulses, seeds, and nuts, can give a lower content of EAA (Bakaloudi et al., 2021; Mariotti & Gardner, 2019).

For the above-reported considerations, the content of EAA of the burger samples was compared with the amino acids scoring pattern reported by FAO for children – from 6 years to 18 – and adults (WHO, 2011). The comparison was also done by using egg protein, which is well-known for its complete profile in essential amino acids (CREA Centro Alimenti e Nutrizione, 2019). As can be seen from Figure 2, no statistical differences ($\rho < 0.05$) were found in the sum of EAA within the samples. Furthermore, in both raw and cooked products, the sum of EAA seems to satisfy the requirements for children and adults, despite being, as expected, not comparable with the egg reference protein one.

<<Insert Figure 2>>

The profile of the specific EAAs showed lysine as the only limiting amino acid compared to the scoring pattern required for both children and adults. This was found for both raw and cooked products and in the case of all samples, with the only exception of samples D and F – showing an adequate content. It might appear odd that even beef samples lacked lysine, but it must be considered that the amino acidic composition of meat strictly depends on the type of cut used. The different amounts of muscle fibres, connective and adipose tissue determine the quality of meat. As shown above, the high content of hydroxyproline hints at a composition of beef burgers quite rich in collagen. Collagen-rich muscles are higher in glycine and non-EAA (Listrat et al., 2016). It follows that products obtained from cuts of meat richer in connective tissue – such as the burgers analysed in this work – have a worse amino acidic profile when compared with products – such as slice meat – obtained from cuts mostly made of muscle fibre (Lee et al., 1978).

In the comparison with the egg reference protein, almost all samples showed a lower amount for each essential amino acid. Only histidine was found in a sufficient amount compared to egg proteins, highlighting again, as expected, the higher nutritional value of the latter.

3.4. Protein integrity

The goal in the making meat analogues, besides a good nutritional target, is also to obtain a meat-like structure. To have this structure, intensive processes are needed. These processes result in a structure of plant proteins that better mimic the structure of meat, but can also affect the quality of proteins.

For this reason, the quality and integrity of proteins were investigated estimating their degree of hydrolysis (DH%) and the percentage of D enantiomers (D%) of cooked samples. Results are graphically shown in Supplementary material (Figure S. 1). DH% were found to be quite low in general. The statistically highest ($\rho < 0.05$) DH% was shown for sample E (5.33 ± 0.48), while the statistically lower values ($\rho < 0.05$) were found

for samples C and F (respectively 2.69 ± 0.14 and 1.77 ± 0.24). In any case, all samples showed low DH% after the cooking treatment (DH < 5.3%), indicating a fairly high protein integrity.

As well as for the DH%, the total D% determined for all cooked samples was low (D < 14.3%). The statistically higher ($\rho < 0.05$) degrees were found in samples C and E (respectively 14.0 ± 1.15 and 14.3 ± 1.12), while F was the one, as for the DH%, showing the statistically lower ($\rho < 0.05$) D% (8.83 ± 0.93). For all the samples, the amino acid with the higher D% was aspartic acid.

The D-aspartic acid – which is known to be the most sensitive amino acid in extreme treatments of pH and temperature (Prandi et al., 2019) – appears to be higher in the samples C (soy-based) and E (pea-based). Indeed, it can be noticed that samples C and E are made with rehydrated proteins (Table S. 1), which underwent an extrusion process. The other amino acids showing a percentage of detectable D enantiomers were the ones also known to be sensitive to processing: alanine, glutamic acid, lysine, and phenylalanine (Anzani et al., 2017; Prandi et al., 2022).

It is well known that heat treatments used in food production can affect protein structure, functionality, and quality. The entity of the modification that occurred depends mostly on the temperature and the time applied for the treatment (Mejia et al., 2020). In this case, comparing the data observed for the DH% and the D%, it was possible to assume that the cooking treatment applied did not affect the protein integrity of the products, thus the effects observed must be due to the treatment applied in the making of the products.

3.5. Digestibility

To evaluate the digestibility of the plant-based products in comparison with meat burgers, the INFOGEST *in vitro* static gastro-intestinal digestion procedure (Brodkorb et al., 2019) was carried out.

The huge number of publications involving *in vitro* gastro-intestinal digestion of food is an indication of the current interest of the scientific community in this procedure (Lucas-González et al., 2018). These protocols are more and more used in different fields due to the indicative and comparable (with *in vivo* data) information that they can give, especially when human studies are ethically questionable or too expensive and resource-consuming (Minekus et al., 2014). A lot of work was carried out performing static digestion models where the digestion is performed in three successive phases (oral, gastric, and intestinal) with precise incubation time, temperature, simulated fluids (including specific enzymes), and pH (Lucas-González et al., 2018). The protocol chosen in the present paper, in particular, is a static *in vitro* procedure that allows determining the digestion products of foods (as peptides and amino acids, fatty acids, and sugars) to the endpoints of the procedure (at the end of the oral, gastric or intestinal phase) (Brodkorb et al., 2019). The authors of the protocol demonstrated good reproducibility of the profile of digestates obtained with the INFOGEST procedure through inter-laboratory trials (Brodkorb et al., 2019). Furthermore, the protocol was already used in literature for the

assessment of the digestibility of both meat and legume protein isolates (Ariëns et al., 2021; Santos-Hernández et al., 2020; Zhou et al., 2021).

The digestates obtained with the INFOGEST procedure were characterized by performing an SDS-PAGE analysis, and then the soluble proteins and the degree of hydrolysis were estimated. Finally, the peptide profile of digestates was analysed by LC-HR-MS.

<<Insert Figure 3>>

To compare the protein profile before and after digestion, an SDS-page analysis was performed. Figure 3 shows the proteins for each protein base (one representative sample showed) before digestion, and the corresponding band identification is reported in Table S. 4. Myosin, actin, and tropomyosin in beef burgers (Farouk et al., 2014), β-conglycinin, glycinin, and lectin in soy burgers (Prandi et al., 2021), convicilin, legumin, and vicilin in pea burgers (Prandi et al., 2021) were the major proteins identified (for comparison with the literature) in the corresponding burgers. Comparing raw and cooked samples, the same profile was observed, indicating that the cooking treatment did not affect the protein profile. The only exception was found in samples A (Figure 3 panel A) and B (beef-based), where a loss in myosin was observed. In digested samples, on the other side, no protein bands were detected, indicating an apparent complete digestibility of the proteins.

To determine the solubilisation of the proteins upon digestion, the soluble proteins in digestate solutions were estimated by Kjeldahl analysis. Moreover, the degree of hydrolysis (DH%) of the solubilised protein fraction, which can be considered as the ratio between the hydrolysed proteins and the total protein in solution, allowed us to determine the breakdown level of the solubilized proteins providing information on the amount of free amino groups released after the digestion process.

<<Insert Figure 4>>

The statistically highest ($\rho < 0.05$) value of protein solubilisation after digestion – reported in Figure 4 panel A – was the one of ovalbumin (our reference protein), indicating for that protein, as expected, an optimal digestibility. Comparing the solubilisation of proteins within the burgers, the meat ones (A and B) showed higher degrees: 63 ± 3 and 61 ± 8 respectively. Even if, as expected, the meat products showed better digestibility than the plant-based ones, the protein solubilised after digestion resulted slightly lower than what was expected for a meat-based matrix, which usually is around 80% (Accardo et al., 2022). This is likely due to the already mentioned (paragraph 3.3 Amino acid profile determination) high composition in collagen protein observed for the meat burgers. The lowest ($\rho < 0.05$) degree was instead observed for sample F (41 ± 5), which resulted statistically different from all the other burgers. Sample F was the one having the highest amount of amino acids, if compared to the other pea-based burger, and the lowest amount of D-amino acids in percentage, all features indicating that proteins have been minimally pre-treated, which might also explain the lower digestibility.

Concerning the DH%, results (Figure 4 panel B) showed, as expected, the statistically highest ($\rho < 0.05$) DH% for the ovalbumin, confirming the optimal digestibility. Anyway, among burgers, generally also pretty high values were observed, indicating in all cases very good accessibility to proteins from the digestive enzymes. Plant-burger D was the one showing the statistically higher ($\rho < 0.05$) DH% (65 ± 2) compared to beef burgers. The other plant-based burgers, instead, had comparable DH% to beef burgers. The higher degrees of hydrolysis reported for the analogue burgers can be ascribed to the treatments that proteins underwent during their making. The different treatments, in fact, could have possibly affected the protein integrity (Prandi et al., 2022), with subsequential better accessibility to the protein for the digestive enzymes. Anyhow, above the difference intercurring between each sample, the degree of hydrolysis showed high hydrolysis of the protein solubilised after the *in vitro* digestion procedure, indicating a huge release of free amino acids and small peptides (di- and tri-peptides).

The present results seem in disagreement with the data observed in the literature. From our knowledge, only a similar study is present in the literature (Zhou et al., 2021). However, Zhou et al. (2021) compared an extra lean grounded beef (protein content: 18.6 g/100 g) with a plant-based grounded analogue (protein content: 20.7 g/100 g) made mainly with soy. Indeed, they observed better hydrolysis of grounded meat in comparison with a grounded plant-based meat analogue. The DH% determined with the OPA method showed 85.2 % of the degree of hydrolysis in meat samples versus 69.8 % of the analogue counterpart. The digestibility of both products resulted higher than the one estimated in this study. As already said, this is probably due to the type of product analysed. The plant-based burgers contain binders (Sha & Xiong, 2020) – as methylcellulose or gums – in their formulations, and these binders are indigestible and their presence in the formulation can lead to the lower digestibility of the products. On the other hand, the ground meat degree of hydrolysis was almost double the one estimated for our control beef burgers. But this can be easily explained, as already highlighted with the amino acidic profile, that the commercial burgers here analysed showed high content of glycine and hydroxyproline – amino acids typical of collagen (Listrat et al., 2016). This leads to the conclusion that our beef burgers are rich in connective tissue, with a consequent decrease in the degree of hydrolysis of proteins, and a lower digestibility (Farouk et al., 2019; Mitchell et al., 1927).

3.6. Peptide content of the digested samples

All the digestates were analysed by LC-HR-MS spectrometry according to the method described in the material and methods, in order to define their peptide content. To reduce the presence of false positives, data filtering was performed by arbitrarily setting threshold cut-offs at 20 (−10lgP parameter in the PEAKS software®, measuring the statistical significance of peptide-spectrum match) for the score and at 20% for the protein coverage. Indeed, the application of such restricted parameters reduced the number of identified peptides but also allowed us to focus our characterization on the more confident hits and most abundant proteins. Figure 5 shows the distribution of peptides for each sample. Results highlighted a wide variability within the samples of the same protein base. Looking at the plant-based samples, it is curious to notice that the ones made with

rehydrated protein concentrate (C and E) show a lower number of peptides than the ones made of protein isolate (D and F). This result confirmed that the type of treatment that the protein underwent – in particular, extraction and extrusion for the rehydrated protein concentrate vs the sole extraction for the protein isolate – influences their hydrolysis.

<<Insert Figure 5>>

Going more into detail, the peptides identified in the beef burgers (samples A and B) were mainly from collagen (Table S. 5), confirming a high connective tissue content in the meat cuts that make up these burgers.

The high presence of collagen in the samples had already been hypothesized by analysing the amino acid profile of the animal-based burgers. At any rate, it was not possible to confirm this with an SDS-page analysis of the raw and cooked samples due to the collagen's high molecular weight. This is also in accordance with the ratio of proteins solubilised after digestion (Figure 4 panel A) and the DH% that showed low hydrolysis of proteins, as expected, of the meat samples. Besides, for what concerns the plant-based burgers, the proteins identified both for soy and pea can be classified as storage proteins. Furthermore, the protein identified in the digestates were mostly the same as already identified in the protein profile of the raw and cooked products (Table S. 4). The only protein that was not solubilised after digestion in soy-based burgers was, as expected, lectin – which is known to be an antinutritional factor (López-Moreno et al., 2022). Another aspect that can be noticed is that most of the proteins identified in the digestates are known to be allergens. In particular, β-conglycinin and glycinin are well known and deeply characterized allergens in soy (L'Hocine & Boye, 2007) and for this reason are also regulated in the Regulation (EU) 1169/2011 on the provision of food information to consumers (European Union, 2011).

4. Conclusions

The study highlighted the differences in the composition, protein quality, and digestibility of plant- and meat-based burgers. Looking at the amino acidic profile, it can be concluded that there is a high variability of the products having the same protein base. Plant-based burgers showed a comparable protein quality and integrity to beef burgers. Beef burgers showed better digestibility than the analogue ones, as indicated by the protein solubilisation, even if their composition limited the digestibility. Indeed, the high presence of glycine and hydroxyproline in the beef burgers, the lower amount of lysine, the lower digestibility than expected, and the relevant presence of peptides from collagen in the digestates indicated a low quality of the meat used for the production of these burgers. Conversely, plant-based burgers showed a good profile in EAA and a good digestibility – both comparable with the results retrieved for beef burgers. It is worth emphasizing that the protein content of the plant-based products selected for this study (higher than 15 g for portion) is not representative of the overall availability present on the market. Indeed, from our previous survey on meat

517 analogues in the Italian market they represent just 27.6% of the total meat analogue burgers (Cutroneo et al.,
518 2022).

519 The findings of this study highlighted that there is variability in the digestibility of all the products (both beef-
520 and plant-derived) when compared to the literature. This indicates that digestibility is related not only to the
521 source of protein (vegetable or meat) but mainly to the quality of the raw materials. It follows that, beyond the
522 choice of animal or analogue products, there is a need for the consumer of a careful choice for high quality
523 products.

524 ## Authorship contribution statement

525 **Sara Cutroneo**: Investigation, Formal analysis, Writing – original draft. **Barbara Prandi**: Investigation,
526 Formal analysis, Writing – review & editing. **Andrea Faccini**: Formal analysis. **Nicoletta Pellegrini**:
527 Conceptualization, Writing – review & editing. **Stefano Sforza**: Conceptualization, Writing – review &
528 editing. **Tullia Tedeschi**: Supervision, Conceptualization, Writing – review & editing.

529 ## Declaration of competing interest

530 The authors declare that they have no known competing financial interests or personal relationships that could
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Tables

Table 1 *Total fat and protein content of cooked beef and plant-based burgers, reported as g/100 g of dry matter (DM)*

CODE	PROTEIN BASE	TOTAL FAT (g/100 g DM)	PROTEIN (g/100 g DM)
A	Beef	49.9 ± 0.17 [a]	47.0 ± 0.07 [c]
B	Beef	34.4 ± 0.33 [b]	52.2 ± 0.47 [a]
C	Soy	23.4 ± 0.45 [c]	34.1 ± 0.03 [f]
D	Soy	23.8 ± 0.42 [c]	37.7 ± 0.14 [d]
E	Pea	15.4 ± 0.24 [d]	35.6 ± 0.03 [e]
F	Pea	23.2 ± 0.42 [c]	49.7 ± 0.12 [b]

Data are expressed as mean ± standard deviation. Letters in columns refer to differences between samples, for which one-way ANOVA with multiple pairwise comparisons test was used ($\rho < 0.05$).

Table 2 *Protein content of raw and cooked beef and plant-based burgers, reported as g/100 g of dry matter (DM), determined with Kjeldahl method and the sum of total amino acids determined by UPLC-MS*

	CODE	PROTEIN BASE	KJELDAHL METHOD (g/100 g DM)	TOTAL AMINO ACIDS (g/100 g DM)
RAW	A	Beef	46.8 ± 0.37 [b]	42.5 ± 0.52 [b]
	B	Beef	48.7 ± 0.01 [a]	49.3 ± 0.42 [a]
	C	Soy	35.2 ± 0.89 [d]	35.8 ± 0.55 [c]
	D	Soy	34.1 ± 0.50 [d]	32.4 ± 0.59 [d]
	E	Pea	31.9 ± 0.02 [e]	30.5 ± 0.15 [e]
	F	Pea	44.1 ± 0.46 [c]	42.4 ± 0.54 [b]
COOKED	A	Beef	47.0 ± 0.07 [c]	45.4 ± 0.81 [b]
	B	Beef	52.2 ± 0.47 [a]	50.6 ± 0.64 [a]
	C	Soy	34.1 ± 0.03 [f]	33.4 ± 0.28 [d]
	D	Soy	37.7 ± 0.14 [d]	38.0 ± 1.08 [c]
	E	Pea	35.6 ± 0.03 [e]	33.9 ± 0.39 [d]
	F	Pea	49.7 ± 0.12 [b]	48.9 ± 1.05 [a]

Data are expressed as mean ± standard deviation. Letters in columns refer to differences between samples, for which a one-way ANOVA with multiple pairwise comparisons test was used ($\rho < 0.05$).

Figure captions

Figure 1 *Total amino acid profile of cooked beef and plant-based burgers divided by protein base: A) beef burgers (samples A and B); B) soy-based burgers (samples C and D); C) pea-based burgers (samples E and F). Asterisks on bars refer to differences, determined with the Independent Samples t-Test ($\rho < 0.05$), between samples with the same protein base.*

Figure 2 *Sum of EAA reported for raw (panel A) and cooked (panel B) beef and plant-based burgers compared with amino acids scoring pattern reported by FAO for children - from 6 years to 18 - and adults (WHO, 2011) (reference lines) and with the egg reference protein (CREA Centro Alimenti e Nutrizione, 2019) (black bar). Letters on bars refer to the difference between samples, for which a one-way ANOVA with multiple pairwise comparisons test was used ($\rho < 0.05$).*

Figure 3 *SDS-PAGE analysis of beef (panel A, sample A), soy (panel B, sample C), and pea (panel C, sample E) burgers: M) Marker; R) Raw sample; C) Cooked sample; D) Digested sample; Db) Digestion blank; Eb) Enzymatic blank.*

Figure 4 *Soluble proteins % estimated after in vitro gastro-intestinal digestion procedure (panel A) and Degree of hydrolysis % estimated on soluble proteins (panel B) reported for digested beef (A, B), soy (C, D) and pea (E, F) burgers compared with ovalbumin (in black). Letters on bars refer to the difference among samples, for which a one-way ANOVA with multiple pairwise comparisons test was used ($\rho < 0.05$).*

Figure 5 *Distribution of peptides for beef (panel A), soy (panel B), and pea (panel C) burger digestates determined with LC-HR-MS analysis.*