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Steering protein and carbohydrate digestibility by food design to address elderly needs: The case of pea protein enriched bread

Martina Moretton, Marilisa Alongi *, Niccolò Renoldi, Monica Anese

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Italy

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ABSTRACT

A "source of protein" and a "high protein" bread intended for the elderly were obtained by replacing wheat flour in bread dough with 50 and 165 g/kg pea protein concentrate. Carbohydrate and protein digestibility was evaluated *in vitro* by mimicking adult and elderly digestive conditions. Protein digestibility was measured by the OPA assay. Carbohydrate digestibility was assessed by determining the incremental area under the glucose curve during the intestinal phase to estimate the glycaemic index (GI_e). Pea proteins negatively affected some key features accounting for elderly acceptability of bread, mainly the textural ones, with firmness increasing from 1.2 to 3.3 N. Proteolysis was not affected by physiological conditions but by reformulation, with "high protein" bread presenting the highest proteolysis, followed by "source of protein" and soft wheat bread (around 110, 80 and 70 mmol free NH_2/g_{dw}, respectively). Conversely, carbohydrate digestibility was restrained in elderly settings compared to adult ones, with glucose concentration during digestion reaching maximum values of 0.5 and 0.8 respectively, with no differences between enriched bread. Results may contribute to a better understanding of food digestibility under different gastrointestinal conditions and of its dependence on technological factors and would help to design age-tailored foods.

1. Introduction

Modern societies are facing socio-economic progress that is leading to a considerable increase in life expectancy (United Nations, 2019). In 2022, more than one-fifth of the EU population was aged 65 and over, and this ratio is expected to further increase in the coming decades (Eurostat, 2023). In developed countries, people aged over 65 years represent the so-called "elderly" (WHO, 2015). Ageing is associated with a decline in different physiological functions, including eating capability and gastrointestinal conditions, that can impact the nutritional status (Rémond et al., 2015). As a result, the elderly often face food-related chronic diseases, with sarcopenia and type 2 diabetes being among the most alarming (WHO, 2017). Sarcopenia is defined as the age-related decline in muscle functionality leading to an increased risk of falls, injury, disability, and mobility disorders (Fuggle et al., 2017). Type 2 diabetes is characterized by chronic postprandial hyperglycaemia leading to circulatory, nervous and immune system disorders (Kirkman et al., 2012). Preventing and managing the onset of these food-related diseases during ageing is urgently required to guarantee the elderly maintain a healthy and active status. Tackling sarcopenia and type 2 diabetes requires an increase in protein intake and digestibility and a reduction in the glycaemic response of carbohydrate-rich food. Besides identifying adequate dietary patterns is essential (Agarwal et al., 2013), designing food products tailored for the elderly becomes crucial (Chiara et al., 2019). These products must accomplish not only precise nutritional needs but also specific sensory requirements. The elderly generally prefer crumble, soft, easy-to-chew, and homemade-like food (Moretton, Cattaneo, et al., 2023). Currently, to our knowledge, no elderly-tailored foods able to concomitantly satisfy nutritional and sensory requirements are available on the market (Jędrusek-Golińska et al., 2020; Van Der Zanden et al., 2014) and only a few studies have addressed this topic so far (Assad-Bustillos et al., 2019).

The need to increase protein intake brings about the demand for a wider array of protein sources, with growing interest towards plant proteins, due to the urgency for a global transition from animal- to plant-based diets (European Commission, 2020). Still, very limited studies on the digestibility of plant proteins under elderly conditions are available in the literature (Melchior et al., 2023; Santos-Hernández et al., 2020). In these few studies, plant proteins showed a lower digestibility compared to animal ones. However, these results were acquired in

* Corresponding author.
E-mail address: marilisa.alongi@uniud.it (M. Alongi).

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model systems, while no information can be found yet on the digestibility of plant proteins when included in a complex food matrix. This requires the application of technological interventions that reasonably lead to interactions of proteins with other components, ultimately affecting their digestibility (Hiolle et al., 2020). Despite the urgency to disclose the actual digestibility of plant proteins in real food matrices, to our knowledge this has remained an unexplored field so far.

Increasing protein intake while restraining carbohydrate digestibility is a crucial challenge to be addressed by food researchers to concomitantly face sarcopenia and type 2 diabetes through technological solutions.

In the present research, bread was chosen as a representative case study of a real food extensively consumed by the elderly, with an estimated daily consumption of about 100 g per capita in European countries (Angelino et al., 2020). Although several bread formulations can be found on the market, the major ingredients are typically carbohydrate-rich cereal flour (*e.g.*, wheat, maize, rice and barley). Partially replacing these flours with protein-rich ones could represent a viable approach for increasing protein intake in the elderly diet (Garcia-Segovia et al., 2020). Among protein-rich flours, particularly interesting are plant-based ones that have been reported to lower the glycaemic index (GI) of bakery products (Burton & Lightowler, 2006).

Based on these assumptions, the present study aimed to develop a pea protein-rich bread by replacing wheat flour with 50 and 165 g/kg of pea protein concentrate in bread dough. These percentages were chosen to bear the claims "source of protein" and "high protein", respectively (Reg. EU No 1924/2006). The digestibility of proteins and carbohydrates was evaluated *in vitro* mimicking adult and elderly physiological conditions (Brodkorb et al., 2019; Melchior et al., 2023).

Protein digestibility was analysed by *o*-phthalaldehyde spectrophotometric assay (OPA) and carbohydrate digestibility was monitored to estimate the GI.

2. Materials and methods

2.1. Materials

Type '00' wheat flour (710 g/kg carbohydrates, 110 g/kg protein, 17 g/kg fat, 14 g/kg fibre, 149 g/kg moisture, from Barilla, Parma, Italy), pea protein concentrate previously characterized by Melchior et al. (2022) (800 g/kg protein, 80 g/kg fat, 49 g/kg carbohydrates, 42 g/kg fibre, 11 g/kg salt, 18 g/kg moisture), sunflower oil, dry yeast, sugar, and salt were purchased from local retailers and used for breadmaking.

White bread (623 g/kg flour, 346 g/kg water, 12 g/kg salt, 10 g/kg bakery yeast, 9 g/kg sugar) was purchased by a local producer (Udine, Italy) and used as a reference for GI estimation.

The following reagents were purchased from Sigma Aldrich (Milan, Italy): pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (8 × USP, P7545), porcine bile extract (B8631), amyloglucosidase from *Aspergillus niger* (A9913), ammonium carbonate ($(NH_4)_2CO_3$), sodium bicarbonate ($NaHCO_3$), ethanol 98%, L-(+)-arabinose, D-(−)-fructose, D-(+)-glucose, sucrose, *o*-phthaldialdehyde (OPA), dithiothreithol, sodium dodecyl sulfate (SDS), L-serine, trichloroacetic acid (TCA), sodium tetraborate.

Potassium chloride (KCl), sodium chloride (NaCl), and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Carlo Erba (Milan, Italy).

Magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen phosphate (K_2HPO_4), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were supplied by J. T. Baker (Phillipsburg, NJ, USA).

Total Starch Assay Kit (AA/AMG) was provided by Megazyme (Bray, Co. Wicklow, Ireland). Acetonitrile for HPLC gradient grade was purchased from VWR Chemicals (Radnor, Pennsylvania, USA). Deionized water (MilliQ System advantage A10®, Millipore S.A.S, Molsheim, France) was used.

2.2. Preparation of bread samples

Soft wheat bread was prepared with wheat flour (1 kg), sunflower oil (98 g/kg wheat flour), sugar (33 g/kg wheat flour), yeast (17 g/kg wheat flour) and salt (10 g/kg wheat flour). Other bread samples were obtained by partially replacing wheat flour with 50 and 165 g pea protein concentrate per kg wheat flour. In other words, considering 1 kg of wheat flour, bread samples were obtained by mixing 50 g pea protein concentrate with 950 g wheat flour, or 165 g pea protein concentrate with 835 g wheat flour. These replacement led to 30 and 100 g/kg proteins in the dough, respectively. Before mixing with other ingredients, pea protein concentrate was suspended in the water phase and stirred overnight at room temperature to allow protein hydration. All ingredients were mixed in a kneading machine (Kenwood, Chef XL Elite, Havant, UK) at speed 5 for 15 min, until the dough was completely stuck to the mixing hook, leaving the mixing bowl completely clean. Subsequently, dough portions (0.1 kg) were manually rounded, and leavened on a tray at 35 °C and 80% environmental relative humidity (ERH) for 60 min. The baking method was set based on preliminary experiments. The fermented dough was baked in a professional oven (Air-o-Steam Touchline, Electrolux, Pordenone, Italy) at 160 °C for 35 min, with a gradient of ERH set as follows: 80% for 5 min, 60% for 5 min, 40% for 5 min, 20% for 5 min, and 5% for 15 min. Finally, the bread was cooled at room temperature for 1 h and immediately subjected to the experiments.

2.3. Chemical and physical characterization of bread

2.3.1. Nutritional values

Nutritional values were estimated based on the labelled nutritional information of bread ingredients. The composition of samples was expressed as g/kg dry weight based on bread moisture.

2.3.2. Starch

The total starch assay procedure (AOAC Method 996.11) was applied to determine the starch content of freeze-dried (Epsilon 2–4 LSCplus, Martin Christ GmbH, Osterode am Harz, Germany) bread (McCleary et al., 1997).

2.3.3. Moisture

The moisture content of the crumb and crust was measured according to AOAC gravimetric method (AOAC, 2019). Samples were dried in a vacuum oven (1.32 kPa) (Vuotomatic 50, Bicasa, Milan, Italy) at 75 °C until constant weight (12 h) and moisture was expressed as the percentage ratio between the water content in the initial sample calculated as the difference between sample weight before and after drying (g), and the initial weight of the sample (g).

2.3.4. Water activity

Water activity (a_w) was measured at 25 °C using a hygrometer (Aqua Lab, Decagon Devices, USA).

2.3.5. Firmness

Slices (20 mm thick) were cut by hand from the central portion of the bread loaf. Uniaxial compression (4301, Instron LTD., High Wycombe, UK) was applied at two different points of the crumb, by a 12.7 mm diameter cylindrical probe attached to a 1 kN compression head at a 5 mm min^{-1} crosshead speed. Firmness was taken as the maximum force (N) for 5 mm sample penetration (Calligaris et al., 2013).

2.3.6. Color

The colour of bread was assessed on the loaf crust and the crumb of 20 mm-thick bread slices as reported by Moretton, Cattaneo, et al. (2023). A tristimulus colorimeter (CR-300, Chromameter-2-Reflectance, Minolta, Osaka, Japan) standardized against a white tile was used to collect at least ten measures on different points of samples and data were

expressed in CIE units as L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The hue angle (HA) was calculated as arctan (b*/a*) (Clydesdale, 1978).

2.3.7. Image analysis

The image of bread slices was acquired in an image acquisition cabinet (Immagini & Computer, Bareggio, Italy) equipped with four 23-W frosted photographic floodlights, in a position allowing minimum shadow and glare, using a professional digital camera Canon reflex EOS 550D (Canon Inc., Tokyo, Japan) equipped with an EF-S 60 mm f/2.8 Macro USM lens. The lens-to-object distance was fixed at 52 cm. For each bread loaf, central slices of 1.3 cm thickness were used for image analysis. Crumb porosity was evaluated by using the Image-Pro Plus 6.3 software (Media Cybernetics Inc., USA) according to Sapirstein et al. (1994) with some modifications. Images were converted to 8-bit grey-scale and segmented to a binary image. A squared field of view (18 × 18 mm) at 300 dots per inch (dpi) resolution from the central part of the slice was assessed. Spatial calibration was carried out using a digital image of a ruler. The software provided cell density (cell cm^{-2}), mean cell area (MCA, mm^2), and cell to total area (CTA).

2.4. In vivo oral processing and static in vitro digestion

The oral phase was performed *in vivo*, following the procedure proposed by Gao et al. (2019). The use of a single subject with consistent mastication behaviour as a chewing "device" has been reported in previous studies (Freitas et al., 2018; Gao et al., 2019; Hutchings et al., 2012). Therefore, an adult volunteer (female, 25 years old) and an elderly volunteer (female, 62 years old) having a full dentition, normal occlusion, no mastication or salivation problems (self-reported), were asked to chew a piece of bread crumb (2.5 g) for 15 s (Mosca et al., 2022). Boluses were collected in a sample holder and frozen at −20 °C until analysis. The volunteers were asked to rinse their mouths with mineral water between each sample. This study has been approved by the Institutional Review Board of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine (protocol n. 0003401). Cash incentives were not provided.

Bread bolus was *in vitro* digested by applying the static protocols simulating adult (Brodkorb et al., 2019) and elderly (Melchior et al., 2023) gut conditions. The composition of simulated gastric (SGF) and intestinal (SIF) fluids, stock solutions of pepsin in water (1×10^{-6} and 3×10^{-6} kat/mL for adult and elderly conditions, respectively), and pancreatin (1.3×10^{-5} kat/mL) and bile (134 mol/L) in SIF was based on the static *in vitro* digestion protocols. Boluses were defrosted at room temperature immediately before analysis and water was added to a final volume of 5 mL to guarantee the 1:1 (w/v) fluid-to-meal ratio. The gastric phase was started by adding SGF, $CaCl_2(H_2O)_2$ and a volume of pepsin solution providing an activity in the final gastric mixture of 3.3×10^{-5} kat/mL for adults and 2.5×10^{-5} kat/mL for the elderly, respectively. The pH was adjusted with HCl (6 mol/L) to 3 under adult conditions and to 4.5 under elderly ones, and water was added to maintain a 1:1 (v/v) fluid-to-bolus ratio. The gastric phase was simulated by maintaining the sample at 37 °C in a thermostat (Thermocenter TC-40T, SalvisLab, Rotkreuz, Switzerland) under stirring with a rotatory shaker (F205, Falc Instruments s.r.l., Treviglio, Italy) for 2 h at 15 rpm, and for 3 h at 7 rpm under adult and elderly conditions, respectively. The intestinal phase was started by mixing the chyme with a volume of pancreatin solution providing an activity in the final intestinal mixture of 1.6×10^{-6} kat/mL for adults and 7.6×10^{-7} kat/mL for the elderly, respectively; bile salts (10 mmol/L for adults and 6.6 mmol/L for elderly conditions); SIF; and $CaCl_2(H_2O)_2$. The pH was adjusted with NaOH (1 mol/L) to 7.0 or 6.6 under adult or elderly conditions, respectively, and water was added to maintain a 1:1 (v/v) fluid-to-chyme ratio. The sample was stirred at 37 °C for 2 h at 15 rpm, and for 3 h at 7 rpm, under adult or elderly conditions, respectively.

2.4.1. Protein digestibility

After the gastric and intestinal phases, samples were collected and heated at 100 °C before centrifugation at 7,000 × *g* for 10 min at 4 °C (Avanti Centrifuge™ J-25, Beckman Coulter, Indianapolis, IN, USA). The levels of free NH_2 groups and protein hydrolysis were determined using the OPA spectrophotometric assay (Bavaro et al., 2021) with minor modifications according to Moretton, Alongi, et al. (2023).

Protein digestibility (%) was calculated as the percentage ratio between the concentration of free NH_2 in the digested sample at the end of gastric or intestinal phases, corrected by subtracting the contribution of free NH_2 from enzymes, and the total content of free NH_2 in the undigested bread samples upon acid hydrolysis with 6 mol/L HCl at 110 °C for 24 h, and the proteolysis as mmol/L free NH_2/g_{dw}.

2.4.2. Carbohydrate digestibility and glycaemic index estimation

Samples were collected at increasing times during the intestinal phase (*i.e.*, 20, 60, 90, 120 min under adult conditions and 20, 60, 90, 120 and 180 min under elderly conditions) and kept on ice. Samples were then added with 0.1 mL amyloglucosidase and stirred for 2 h at 37 °C (F205, Falc Instruments s.r.l., Treviglio, Italy). The reaction was stopped by adding ethanol 98% (1:4 v/v) and samples were centrifuged at 7,000 × *g* for 10 min at 4 °C. The supernatant was filtered (0.45 μm cut-off PVDF membrane) and injected in a High-Performance Liquid Chromatography system (HPLC) (Agilent 1260 Infinity Quaternary LC, Agilent Technologies, Germany) to quantify glucose (Alongi et al., 2019). Glucose release was plotted against time to obtain the incremental area under curve (IAUC) (Matthews et al., 1990). The estimated glycaemic index (GI_e) was calculated as the percentage ratio between the IAUC of the sample and the IAUC of the reference food (*i.e.*, white bread), under adult or elderly conditions (Brouns et al., 2005).

2.5. Statistical analysis

Results are expressed as mean ± standard deviation (SD) of at least three measurements on two replicated samples. One-way ANOVA was carried out and the Tukey test was used to determine statistically significant differences among means ($p < 0.05$) and Bartlett's test was used to check the homogeneity of variance, using R software (v. 4.2.0) for Windows (The R foundation for statistical computing, 2022).

3. Results and discussion

3.1. Chemical composition of bread

Wheat flour in bread dough was partially replaced with 50 and 165 g pea protein concentrate per kg wheat flour. Based on the labelled nutritional information of the ingredients used for the preparation of bread and on their formulation (Paragraph 2.2), the protein content was calculated. Soft wheat bread contained around 110 g/kg_{dw} of proteins and this value increased to 150 and 230 g/kg_{dw} when wheat flour was partially replaced by respectively 50 and 165 g pea proteins per kg wheat flour. The energy content of bread samples was calculated based on the labelled nutritional information of the ingredients. Soft wheat bread provided 2650 kcal/kg and this value remained almost unchanged when wheat flour was partially replaced with pea protein concentrate. However, the addition of this protein-rich source determined a change in the main nutrient providing energy. In the case of soft wheat bread, 10% of energy was provided by proteins, while this value increased to 130 and 200 g/kg when 50 and 165 g pea proteins/kg of wheat flour were respectively replaced with pea protein concentrate. This change in energy source allows reformulated bread to be claimed as a "source of protein" and "high protein", respectively. According to Regulation (EU) No 1924/2006, these claims are allowed when at least 12 and 20% of the total energy of the food is provided by proteins, respectively (Table 1).

Carbohydrate content was also computed and decreased from 747 in soft wheat bread, to 705 and 618 g/kg_{dw}, when wheat flour was replaced

Table 1
Nutritional values of soft wheat, "source of protein", and "high protein" bread.

	Soft wheat bread	"Source of protein" bread	"High protein" bread
Energy (kcal/kg)	2656	2678	2730
of which from protein (kcal/kg)	100	130	200
Composition (g/kg $_{\text{dry weight}}$)			
Total carbohydrates	747	705	618
of which starch	683 ± 42[a]	681 ± 8[a]	601 ± 6[b]
Protein	116	151	230
Fat	112	115	120
Fiber	15	16	19
Salt (NaCl)	10	11	12

[a, b]: in the same row, means indicated by different letters are significantly different ($p < 0.05$).
* Calculated based on the labelled nutritional information (g/kg fresh weight) and the experimental moisture content.

with increasing amounts of pea protein concentrate. As expected, starch was the main component of bread carbohydrates, accounting for over 900 g/kg of total carbohydrates in soft wheat bread. The addition of 50 g/kg of pea protein concentrate did not produce a significant change in starch content, whereas the highest amount resulted in a significant decrease in this value (Table 1). Finally, only little changes were calculated for fat, fibre, and salt contents upon bread reformulation, due to the contribution of pea protein concentrate (Paragraph 2.1).

The differences in the nutritional composition within the bread types under investigation are expected to affect their physical properties, given that starch and proteins are not only important nutrients, but they also play a key structural role.

3.2. Chemical and physical properties of bread

The differences in the nutritional composition within the bread types under investigation are expected to affect their physical properties, given that starch and proteins not only are important nutrients but also play a key structural role. In this regard, Table 2 shows some of the physical and chemical properties of bread.

The optimized steam baking method for breadmaking enabled gradual dehydration of the loaf, preventing crust cracking and improving its appearance and texture (Ahrné et al., 2007; Altamirano-Fortoul et al., 2012). These baking conditions produced in all cases bread with a homemade appearance, which represents one of the key features for the acceptability of this product by the target consumers, *i.e.*, the elderly (Moretton, Cattaneo, et al., 2023).

Bread appearance was also affected by the presence of pea protein concentrate in the formulation, as can be visually observed in Table 2. In particular, the crust browning considerably increased when the highest pea protein concentrate was used. This colour change was thus quantified by measuring the colour parameters, *i.e.*, luminosity (L*) and hue angle (HA). Overall, these parameters progressively decreased with increasing pea protein concentrate, in both crust and crumb. HA decrease indicates a shift from yellow to orange-brown, in agreement with other authors studying the chemical and physical properties of bread enriched with pea proteins (Garcia-Segovia et al., 2020). Browning can be associated not only with the colour of pea protein concentrate but also with a greater occurrence of the Maillard reaction during baking. The addition of pea proteins results in a higher lysine content that reacts with reducing carbohydrates during the Maillard reaction, leading to the formation of brown pigments (Gómez et al., 2008; Mohammed et al., 2012). Even though colour significantly changed upon reformulation, this is not expected to compromise bread acceptability by elderly consumers (Moretton, Cattaneo, et al., 2023). On the contrary, a crucial feature to be considered in this regard is the product texture, due to the chewing and swallowing problems typically affecting the elderly (Cichero, 2017). Considering that an adequate development of the gluten network plays a key role in allowing the formation of an aerated structure in bread (Renoldi et al., 2022), image analysis on bread crumb was performed and mean cell area (MCA), cell density, and cell to total area (CTA) were calculated (Table 2).

Bread reformulation with pea proteins produced an increase in the MCA from 0.25 ± 0.03 to 0.87 ± 0.01 mm^2 and a concomitant decrease in cell density from 44.1 ± 3.5 to 20.5 ± 0.7 cell cm^{-2}, respectively for wheat and "high protein" bread. In other words, this sample presented fewer bubble cells that were characterized by a higher volume. Reformulating bread with the highest percentage of pea proteins caused a

Table 2
Appearance, color parameters (lightness, L*; and hue angle, HA), mean cell area (MCA), cell density, cell to total area (CTA), firmness, moisture, and a_w, of soft wheat, "source of protein", and "high protein" bread.

			Soft wheat bread	"Source of protein" bread	"High protein" bread
Appearance		Crust			
		Crumb			
Color	L*	Crust	60.3 ± 4.9[a]	56.4 ± 4.3[b]	48.3 ± 3.3[c]
		Crumb	72.6 ± 0.9[a]	71.2 ± 3.3[a]	65.4 ± 1.1[b]
	HA	Crust	69.4 ± 4.5[a]	65.6 ± 3.1[b]	60.2 ± 3.4[c]
		Crumb	92.6 ± 1.2[a]	89.0 ± 0.4[b]	82.4 ± 1.2[c]
MCA (mm^2)		Crumb	0.25 ± 0.03[b]	0.37 ± 0.12[b]	0.87 ± 0.01[a]
Cell density (cells/cm^2)		Crumb	44.1 ± 3.5[a]	27.2 ± 3.1[b]	20.5 ± 0.7[b]
CTA		Crumb	0.11 ± 0.01[b]	0.10 ± 0.02[b]	0.18 ± 0.01[a]
Firmness (N)		Crumb	1.2 ± 0.2[c]	1.7 ± 0.3[b]	3.3 ± 0.4[a]
Moisture (%)		Crust	7.0 ± 0.9[b]	6.8 ± 1.4[b]	9.6 ± 0.4[a]
		Crumb	31.5 ± 0.7[b]	33.6 ± 0.5[a]	34.4 ± 0.9[a]
a_w		Crust	0.57 ± 0.01[b]	0.59 ± 0.06[b]	0.72 ± 0.01[a]
		Crumb	0.92 ± 0.01[b]	0.93 ± 0.01[ab]	0.95 ± 0.01[a]

[a,b,c]: in the same row, means indicated by different letters are significantly different ($p < 0.05$).

significant CTA increase (from 0.11 ± 0.01 to 0.18 ± 0.01 for wheat and "high protein" bread, respectively), indicating that a larger area was occupied by air bubbles in the "high protein" bread compared to the other samples. Given that the distribution of air bubbles and their size is known to affect the mechanical behaviour of bread, firmness was also measured (Table 2). Differently from what was expected (Renoldi et al., 2022), the highest MCA and CTA observed in the "high protein" bread were associated with the highest firmness (3.3 ± 0.4 N), the value of which was 3-fold higher compared to that of soft wheat bread (1.2 ± 0.2 N). These results suggest that, despite pea proteins produced a more aerated structure, their presence also increased network stiffness. This can be explained by the nature of the interactions occurring between pea proteins and gluten. Pea proteins have been reported to form aggregated structures at the boundary of the gluten network, while no specific interaction through weak and covalent bonds has been observed (Ducrocq et al., 2020). As a result, a structural reinforcement due to a steric hindrance, rather than a network weakening, is expected to occur. Such a "scaffolding" effect has been observed also in other vegetable protein-enriched bakery products (Stamatie et al., 2022).

As the presence of pea proteins was previously reported to affect water retention due to their water holding capacity (Melchior et al., 2022), moisture was assessed. Although the overall moisture content did not differ among samples (accounting for 685 ± 7, 664 ± 05 and 656 ± 9 g/kg for soft wheat, "source of protein" and "high protein" bread, respectively), significant differences emerged when it was determined separately on crust and crumb (Table 2). Higher values were found in the crumb of reformulated bread types (336 and 344 g/kg) compared to the soft wheat one (315 g/kg). As expected, this effect was less pronounced in the crust, in which only the highest pea protein content led to a significant increase in moisture (96 g/kg), given that water evaporation occurs more easily on the bread surface. Results relevant to the water activity (a_w) (Table 2) reflected the same trend observed for moisture, with a significant a_w increase in the crumb and crust of "high protein" bread (0.72 and 0.95 respectively). This could contribute to an increase in the chewiness of bread, thus counterbalancing its highest firmness, ultimately increasing elderly acceptance towards the product.

The differences among bread types observed in terms of nutritional composition, and chemical and physical properties are expected to produce a different digestive behaviour, particularly regarding the macronutrients of major interest in bread, *i.e.*, proteins and carbohydrates.

3.3. Protein digestibility

The fate of proteins during *in vitro* digestion was investigated employing two variables, namely the percentage ratio of free NH_2 groups released during the gastric or intestinal phase to the total free NH_2 groups, which indicates protein digestibility, and the quantity of free NH_2 groups per mass of dry weight, which is indicative of proteolysis (Table 3).

At the end of the gastric phase, protein digestibility was negligible, accounting for less than 4% in all cases, independently from the physiological conditions applied (Table 3). Results are in agreement with Hiolle et al. (2020) who studied the digestibility of proteins in bakery products rich in proteins (*e.g.*, pudding, sponge cake and biscuit) during the digestion simulation based on the INFOGEST protocol. These authors observed that proteolysis mostly occurred at the intestinal stage, while during the gastric phase, the mechanical disintegration of food prevails.

Moving to the intestinal phase, protein digestibility considerably increased (Table 3). The extent of protein digestibility during the intestinal phase was affected by the amount of pea proteins added to bread formulation. In particular, protein digestibility decreased when pea protein content increased, with a significant reduction (from 90 to 60%) when soft wheat flour was replaced by 165 g/kg of pea protein concentrate to obtain the "high protein" bread. These results indicate that the efficiency of protein digestibility decreased when protein concentration was higher. Such a decrease could be attributed to structural changes occurring upon reformulation. It is noteworthy that bread structure mainly relies on the formation of a gluten network with voids in between bond nodes that ease the access of proteases to their substrate (Hiolle et al., 2020). The presence of pea proteins in reformulated bread produced a stiffer network (Table 2) that reasonably represented a physical hindrance for proteases.

Table 3
Protein digestibility (%) and proteolysis (mmol free NH_2/g_{dw}) of soft wheat, "source of protein", and "high protein" bread at the end of the gastric and intestinal phases, under adult and elderly conditions.

Phase	Bread type	Protein digestibility (%)		Proteolysis (mmol free NH_2/g_{dw})	
		Adult	Elderly	Adult	Elderly
Gastric	Soft wheat bread	2.9 ± 0.7^{b}	3.1 ± 0.6^{ab}	2.3 ± 0.5^{c}	2.4 ± 0.5^{c}
	"Source of protein" bread	4.2 ± 0.9^{a}	3.4 ± 0.6^{a}	4.1 ± 0.9^{b}	3.3 ± 0.6^{b}
	"High protein" bread	$3.1 \pm 0.6^{ab,*}$	2.7 ± 0.4^{b}	$5.8 \pm 1.1^{a,*}$	5.0 ± 0.7^{a}
Intestinal	Soft wheat bread	$90.0 \pm 5.3^{A,*}$	84.0 ± 4.4^{A}	$71.6 \pm 4.2^{B,*}$	66.7 ± 3.5^{C}
	"Source of protein" bread	84.3 ± 11.5^{A}	86.6 ± 11.9^{A}	84.7 ± 8.9^{B}	81.0 ± 14.1^{B}
	"High protein" bread	59.6 ± 6.1^{B}	62.5 ± 4.5^{B}	108.6 ± 11.2^{A}	114.1 ± 8.3^{A}

[a, b, c]: indicate statistically significant ($p < 0.05$) difference between bread types at the end of gastric phase for both adult and elderly gastrointestinal conditions. [A, B, C]: indicate statistically significant ($p < 0.05$) difference between bread types at the end of intestinal phase for both adult and elderly gastrointestinal conditions. *: indicate statistically significant ($p < 0.05$) difference between adult and elderly gastrointestinal conditions within bread type and digestion phase.

Since protein digestibility does not provide any information regarding the actual amount of free amino acids that are found at the intestinal level and could be absorbed, Table 3 also reports proteolysis data that refer to the free amino groups quantified at the end of gastric and intestinal phases of digestion per gram of bread on a dry basis. At the end of the gastric phase, a low proteolysis was detected, confirming that protein hydrolysis only marginally occurs during this phase. Conversely, proteolysis increased considerably after the intestinal phase and was affected once again by the addition of pea proteins. However, different from protein digestibility, the proteolysis significantly increased from 71 to 109 mmol free NH_2/g_{dw} from the soft wheat to the "high protein" bread. This means that, considering the almost negligible moisture differences among bread samples (Table 2), consuming a portion of "high protein" bread instead of soft wheat would increase by 30% the number of free amino acids potentially available for absorption.

When gastrointestinal conditions were changed from the adult to the elderly setting, protein digestibility and proteolysis were almost unaffected, probably as a result of counterbalancing effects of changed physiological conditions. On the one hand, during the gastric phase a reduction in protein digestibility would be caused by the use of a pH (*i.e.*, 4.5) further from that of pepsin optimum (*i.e.*, 3), and a 25% reduction in enzyme concentration under elderly conditions compared to adult ones. On the other hand, protein digestibility would be increased by the longer gastric phase used in elderly settings and by the breakdown of the starchy matrix operated by the salivary α-amylase that probably favoured protease accessibility (Freitas et al., 2018). Similar considerations can be made regarding the intestinal phase, during which the lower pancreatin concentration was probably counterbalanced by the longer duration of this phase under elderly conditions compared to adult ones.

Even though the INFOGEST protocol here applied has been specifically developed to assess protein digestibility *in vitro*, it does not take into account gastric emptying, which represents an important factor affecting protein digestion and uptake (Ariëns et al., 2021). Acquired

results thus provide a first insight into the conjoint effect of bread formulation and physiological factors on protein digestibility, while further research based on human trials is still required to validate the observed outcomes.

3.4. Carbohydrate digestibility and GI_e

Bread samples were assessed for carbohydrate digestibility to evaluate the effect of wheat flour replacement with pea proteins, as well as the impact of different physiological digestion conditions. Fig. 1 shows the kinetics of glucose release during the intestinal phase of *in vitro* digestion of soft wheat, "source of protein", and "high protein" bread under adult (Fig. 1a) or elderly (Fig. 1b) conditions.

In all cases, glucose concentration increased steeply after 20 min from the beginning of the intestinal phase and remained almost unchanged over the entire course of the intestinal phase (Fig. 1).

Differently from proteins, Fig. 1 shows that carbohydrate digestibility was not affected by bread reformulation. Overall, no differences were found among bread types neither under adult (Fig. 1a), nor under elderly (Fig. 1b) conditions.

Conversely, by comparing the different gastrointestinal conditions, results indicate that in the elderly setting carbohydrate digestibility was significantly compromised. After 20 min from the onset of the intestinal phase, a glucose concentration of around 70 g/kg was found when bread was digested under adult conditions. On the contrary, the elderly setting led to a significantly lower ($p < 0.05$) glucose concentration (< 0.5 g/g) even though the physiological conditions (*e.g.*, higher stomach pH) were more favourable for salivary α-amylase activity (Freitas et al., 2018).

The considerable impact of physiological conditions is probably due to the reduction in pancreatin activity and to the slower intestinal peristalsis typically resulting from the ageing process (Laugier et al., 1991; Rémond et al., 2015). To measure such a difference, the IAUC was computed (Table 4).

Even though no significant differences were detected among the glycaemic curves of different bread types (Fig. 1), soft wheat bread digested under adult conditions presented the highest IAUC (Table 4), while under elderly conditions all samples presented comparable IAUC values.

Moreover, by comparing adult and elderly digestive conditions, statistically significant differences ($p < 0.05$) were only found in the case of soft wheat bread. In this regard, it must be pointed out that the intestinal phase under elderly conditions was longer (180 min) compared to that under adult conditions (120 min). As a result, the lower glucose concentration released during the intestinal digestion under elderly settings compared to adult ones was counterbalanced by the longer duration of the intestinal phase under elderly settings compared to adult ones.

The IAUC of different bread types were then combined with those of reference white bread digested under adult and elderly conditions (accounting for 91.5 ± 14.9 and 100.2 ± 12.3, respectively) to estimate the glycaemic indices (GI_e).

Under adult conditions, the higher GI_e was observed in the case of soft wheat bread, followed by "high protein" and with the lowest value in "source of protein" bread. It is known that soft wheat bread is one of

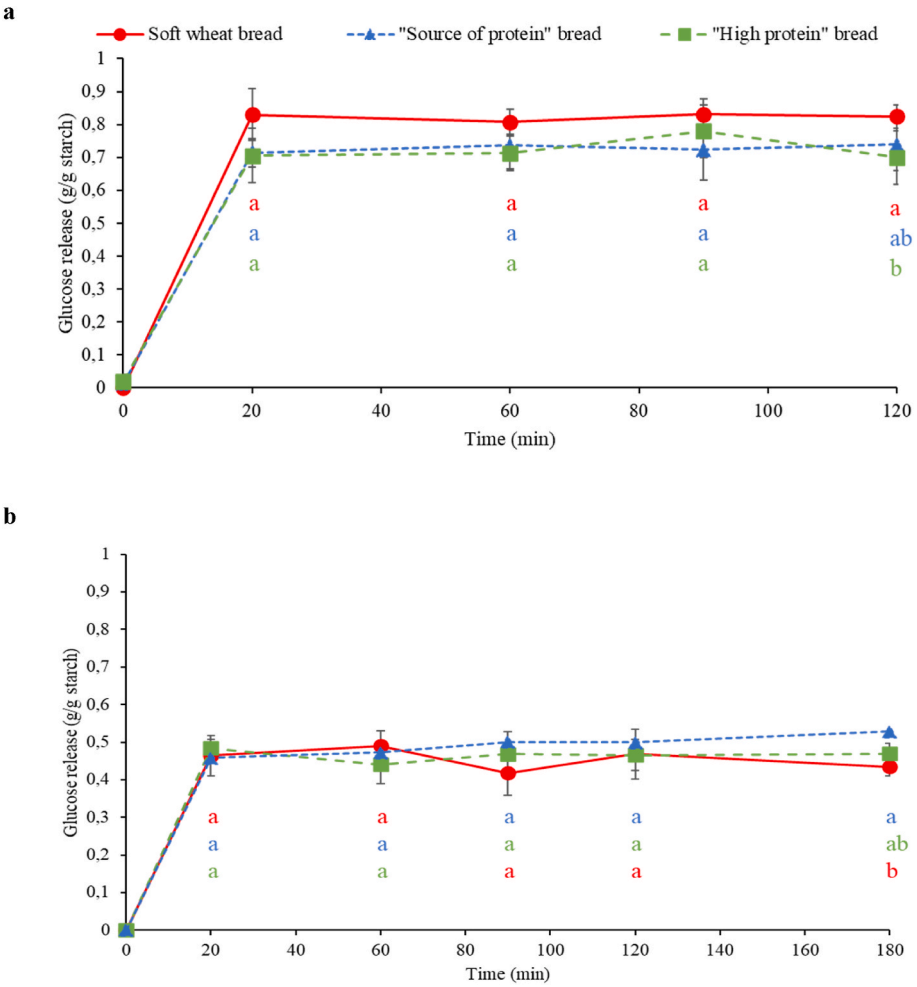

Fig. 1. Glucose release as a function of time during the intestinal phase of *in vitro* digestion of soft, "source of protein", and "high protein" bread under adult (a) and elderly conditions (b). [a, b]: indicate statistically significant ($p < 0.05$) differences among bread types at the same time point.

Table 4
Incremental area under curve (IAUC) and estimated glycemic index (GI_e) of soft wheat, "source of protein", and "high protein" bread under adult and elderly conditions.

Gastrointestinal conditions	IAUC			GI_e		
	Soft wheat bread	"Source of protein" bread	"High protein" bread	Soft wheat bread	"Source of protein" bread	"High protein" bread
Adult	$94.9 \pm 4.5^{a, *}$	79.4 ± 3.9^{b}	84.9 ± 4.7^{b}	$103.7 \pm 4.9^{A, *}$	86.7 ± 7.6^{B}	$92.7 \pm 5.1^{B, *}$
Elderly	78.0 ± 5.4^{a}	82.9 ± 6.0^{a}	80.9 ± 3.5^{a}	77.9 ± 5.4^{B}	86.8 ± 5.3^{A}	81.2 ± 3.1^{AB}

$^{a, b}$: in the same row, indicate statistically significant ($p < 0.05$) differences in the IAUC between bread types. $^{A, B}$: in the same row, indicate statistically significant ($p < 0.05$) differences in the GI_e between bread types. *: in the same column, indicate statistically significant ($p < 0.05$) difference between adult and elderly gastrointestinal conditions within each bread type.

the highest glycaemic index products and the replacement of wheat flour with legume-based ones is among the main strategies currently adopted to reduce the glycaemic response of bakery products (Burton & Lightowler, 2006). The ability of legumes to reduce the glycaemic response lies in some of the intrinsic features of their cellular structure. Legume cells present a thick wall that reduces the starch gelatinization and amylase permeability and therefore its access to starch granules (Burton et al., 2011; Burton & Lightowler, 2006; Fardet et al., 2006). In addition, the inclusion of proteins in the formulations led to a firmer network (Table 2) able to entrap and physically protect starch granules from amylases during digestion (Ge et al., 2021).

Under elderly gastrointestinal conditions, the GI_e was lower as compared to what was observed in adults, with no significant differences between "high protein" and "source of protein" bread. These GI_e values can thus only be attributed to the reduced efficacy of amylolytic activity in the elderly intestinal digestive phase compared to adults (Melchior et al., 2023).

Besides increasing protein intake, the reformulation with legume proteins represents a successful strategy to reduce the GI of bread (Burton & Lightowler, 2006). This capacity has been attributed to the higher thickness of the cell wall of legumes compared to that of wheat, which reduces amylase permeability and lowers water absorption thus limiting starch gelatinization (Bajka et al., 2021; Edwards et al., 2021).

As previously mentioned, it must be kept in mind that the static *in vitro* digestion protocol here applied does not account for any absorption step during the intestinal phase, even though this is a crucial factor affecting carbohydrate digestion (Mackie et al., 2020). Therefore, future research will be required to validate acquired results through controlled human bioavailability trials. Moreover, until now no consensus on a standardised *in vitro* model to assess carbohydrate digestibility has been reached. The results acquired in the present study could contribute to the development of harmonized *in vitro* static digestion protocols dedicated to the assessment of carbohydrate digestibility.

4. Conclusion

In the present study, a staple food widely consumed by the elderly population such as bread was reformulated by partially replacing wheat flour with pea protein concentrate to concomitantly tackle sarcopenia and type 2 diabetes.

Results showed that the replacement of wheat flour did not produce any difference in terms of glycaemic response. Still, it must be pointed out that carbohydrate digestibility was lower in elderly gastrointestinal settings compared to adult ones. These results could be regarded as positive considering that type 2 diabetes typically onsets during ageing.

The enrichment of bread with pea proteins substantially contributed to protein digestibility: regardless of the gastrointestinal setting (*i.e.*, adult *vs.* elderly), at the end of digestion the number of free amino acids available for absorption was progressively higher in the "source of proteins" and "high proteins" bread types, compared to the conventional white bread. However, it must be pointed out that the addition of pea proteins led to a decrease in digestion efficiency, meaning that a high ratio of the proteins added in the formulation remained undigested. This outcome suggests that further research is required to steer technological interventions to maximize the efficiency of food design intended for the elderly. The possibility to improve nutrient digestibility by applying unconventional processing interventions could open up new opportunities. Matching the enrichment of nutrients carried out by targeted formulation with the application of processing interventions able to maximize nutrient digestibility could guarantee a more efficient use of resources. This would concomitantly improve the nutritional profile and the sustainability of food intended for the elderly, ensuring that resources are used more efficiently and effectively.

CRediT authorship contribution statement

Martina Moretton: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Marilisa Alongi:** Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Niccolò Renoldi:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Monica Anese:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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