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In vitro protein digestibility of plant-based foods and ingredients *vs* standardized values in growing pigs

Cristina Gómez-Marín [a,c], Natalia S. Fanelli [b], Beatriz Miralles [a], Hans H. Stein [b], Isidra Recio [a,*]

[a] Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), C. Nicolás Cabrera, 9, Madrid, 28049, Spain
[b] Department of Animal Sciences, University of Illinois, Urbana, IL, USA
[c] Escuela de Doctorado, Facultad de Ciencias, Universidad Autónoma de Madrid, Francisco Tomas y Valiente,7, Madrid, 28049, Spain

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ABSTRACT

In vitro methods for protein quality assessment have great potential, but to represent a real alternative, they need validation against *in vivo* data. In particular, there is a lack of data on plant protein sources, which are increasingly demanded as environmentally and ethically alternative to animal-based proteins. This study evaluated the digestibility and digestible indispensable amino acid score (DIAAS) of 17 plant-based substrates using an *in vitro* method based on an internationally harmonized protocol, and the results were compared with standardized ileal digestibility determined in pigs on the same substrates. Except for corn flakes, the *in vitro* protocol predicted the crude protein and amino acid digestibility, with absolute percent errors below 20 %. The effect of heat treatment on the rapeseed protein digestibility was also evidenced by the peptide profile of ileal contents, pointing to napin as the main contributor to the protein resistance. The presence of trypsin inhibitors in the sample could limit the use of *in vitro* methods; however, methodological adjustments such as the optimization of the substrate load can overcome this issue. These findings provide evidence of the applicability of *in vitro* digestion protocols for evaluating protein nutritional quality, advancing efforts to reduce the use of animals in nutrition science.

1. Introduction

Protein quality evaluation has been the subject of numerous studies and updates due to the importance of an accurate determination and the complexity of the digestion and absorption processes in the organism. To assess protein nutritional quality, the digestible indispensable amino acid score (DIAAS) was recommended as a replacement for protein digestibility corrected amino acid score (PDCAAS) (FAO, 2013). In this score, each indispensable amino acid (AA) is treated as an individual nutrient, and the ileal digestibility of each AA should be assessed in humans, but if this is not possible, in growing pigs or in growing rats, in that order (FAO, 2013). There are different protocols currently accepted to calculate true ileal protein and AA digestibility, based on the analysis of ileal contents or less invasive techniques to evaluate AA availability and metabolism, such as the dual isotope tracer approach or the indicator AA oxidation (FAO & IAEA, 2024). Since the adoption of DIAAS as a score, a significant number of foods and ingredients have been evaluated in humans using dual isotope tracer techniques (Kashyap et al., 2018; Shivakumar et al., 2019) or aspiration of ileal contents (Calvez et al., 2021; Itkonen et al., 2024). In animals, and especially growing pigs, important efforts have been made to evaluate the protein and AA digestibility of a variety of foods of plant and animal origin (Cervantes-Pahm et al., 2014; Hodgkinson et al., 2018; Mathai et al., 2017).

Although *in vivo* trials are the gold standard for assessing protein and AA digestibility, there is growing global and societal pressure to reduce the use of animals in research. Additionally, animal experiments are typically more time-consuming and costly than *in vitro* approaches, which has raised interest in developing *in vitro* methods with comparable accuracy in predicting AA digestibility of food proteins. If such methods can be successfully established, they could serve as a valuable complement to *in vivo* measurements in the future. In addition, because the food matrix or the technological treatment can affect AA digestibility, the need for assessing protein nutritional quality is a non-ending target that may require revision when food composition or processing parameters are changed. For these reasons, the need for

* Corresponding author.
E-mail address: i.recio@csic.es (I. Recio).

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standardized *in vitro* methods to complement *in vivo* assays is an old demand, but despite the efforts in this area, no *in vitro* method has been sufficiently validated to reach the necessary confidence.

The use of *in vitro* methods in the assessment of the nutritive value of feed and food has been reviewed by several authors (Boisen and Eggum, 1991; Butts et al., 2012; Moughan, 1999; Santos-Sánchez et al., 2024). Most of the *in vitro* methods to evaluate protein quality are enzymatic methods, using gastrointestinal enzymes of porcine origin, although protocols for feed may include a wide range of carbohydrates simulating microbial degradation (Boisen and Fernández, 1997). During protein hydrolysis, the pH changes as a result of proton and AA release, and therefore, protein digestibility has been widely evaluated by using pH-drop or pH-stat methods. However, these methods are strongly influenced by the buffering capacity of foods, which may result in poor correlations (Moughan et al., 1989). Other *in vitro* methods are based on the definition of a digestible or absorbable fraction by centrifugation, ultrafiltration, or precipitation with acids or solvents. Despite some of them showing good *in vivo-in vitro* correlations, these methods have not been widely accepted for the evaluation of the nutritional quality of proteins (Santos-Sánchez et al., 2024).

In the framework of the COST Action INFOGEST, an internationally harmonized protocol to simulate gastrointestinal digestion based on physiological parameters was proposed (INFOGEST protocol) (Brodkorb et al., 2019; Minekus et al., 2014). This protocol was later adapted to evaluate protein digestibility, defining an absorbable fraction by precipitation with methanol (Sousa et al., 2023). The method showed good comparability with *in vivo* protein and AA digestibility for seven substrates of animal and plant origin, with a mean difference of 1.2 %. In this context, the importance of running the *in vitro* assays under conditions as close as possible to the *in vivo* ones, and to take the actual food matrix and processing conditions into account, to validate the *in vitro* digestion protocol with *in vivo* data has been highlighted (FAO & IAEA, 2024).

To implement *in vitro* protocols as a screening tool or a complement to *in vivo* assays, it is necessary to evaluate their effectiveness against a wide range of foods in order to identify limitations and adapt the digestive conditions to overcome them, which is the main goal of this study. To achieve this goal, protein digestibility of 17 plant-based protein sources was assessed, including protein isolates, cooked legumes, and soy-derived products, as well as cereal-based foods, using an *in vitro* protocol based on the INFOGEST protocol. The results were compared with the previously determined standardized ileal protein digestibility in growing pigs. In addition, by analyzing the contents of the ileum of pigs, the effect of trypsin inhibitors in rapeseed protein isolate was investigated, and the conditions of digestion in the laboratory were optimized.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and enzymes used for *in vitro* digestion were purchased from Merck KGaA (Darmstadt, Germany). Chemicals and reagents used for total AA (TAA) analysis were from Waters Corporation (Milford, MA, USA).

2.2. Samples

Seventeen plant foods or ingredients were provided by the Monogastric Nutrition Laboratory at the University of Illinois (USA) in dried form. These ingredients were grouped into protein isolates and concentrates, legume-derived foods, and cereal food products. Protein isolates and concentrates included rapeseed protein isolate (RPI), heat-treated rapeseed protein isolate (RPI-HT), soy protein isolate (SPI), brown rice concentrate (BRC), pea protein concentrate (PPC), and pea protein isolate (PPI). Legume-derived foods comprised freeze-dried cooked navy beans, freeze-dried boiled green beans, soybean meal, fermented soybean meal, full-fat soybean meal, fermented full-fat soybean meal, and soybean milk. Cereal food products included corn flakes, quick rolled oats, whole wheat bread, and wheat bagels. Amino acid composition of these ingredients is represented in Fig. S1.

The protein and AA digestibility of these foods and ingredients were previously determined in ileal cannulated growing pigs, as previously described (Bailey et al., 2023; Baker et al., 2010; Espinosa et al., 2020, 2021; Fanelli et al., 2021). To accomplish further experiments needed for wheat-based products in a fresh and dried form, equivalent ingredients were acquired in a local supermarket. A description of the substrates and the form in which they were used is included in Table S1.

A protein-free cookie was used as a blank in the *in vitro* digestion (Moughan et al., 2005). The cookie was prepared by mixing 40.8 g of purified corn starch, 15.7 g of sucrose, 4.9 g of cellulose, 0.7 g of baking

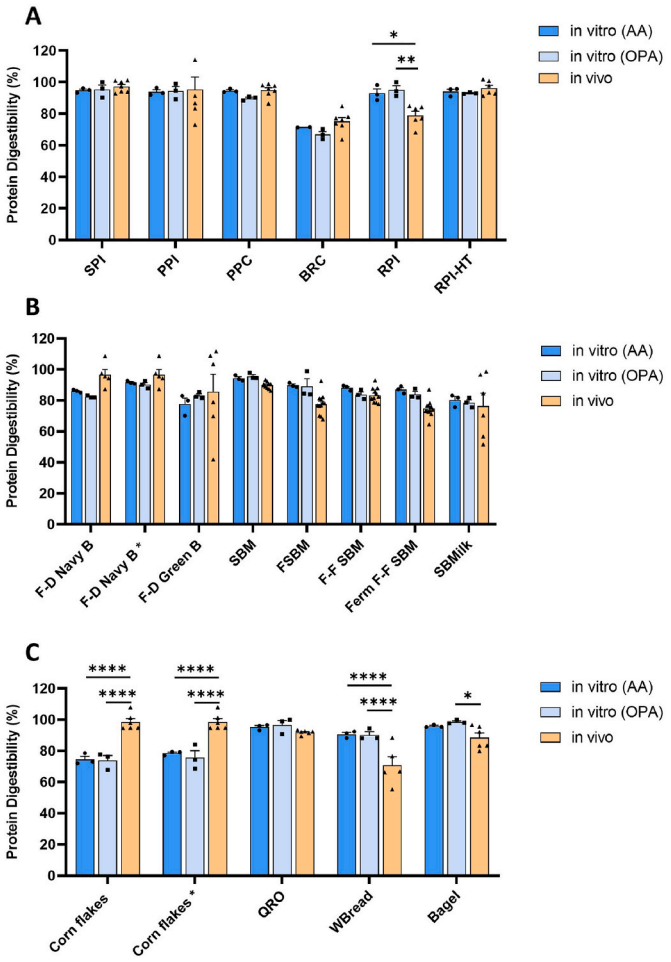

Fig. 1. Protein digestibility of different plant-based isolates/concentrates (A), legume-derived foods (B), and cereal-derived foods (C) after *in vitro* digestion, quantified by total amino acids analysis (*in vitro* AA) and total amino groups analysis (*in vitro* OPA), compared to ileal crude protein digestibility. Number of biological replicates *in vivo* n = 8; *in vitro* n = 3. Error bars indicate SEM. Statistical significance compared between *in vitro* AA, *in vitro* OPA, and *in vivo* (two-way Anova with Sidak's post-hoc test) is indicated by * $p < 0.03$, ** $p < 0.002$, *** $p < 0.0002$, **** $p < 0.0001$. SPI: Soy Protein Isolate, BRC: Brown Rice Concentrate, PPC: Pea protein concentrate, PPI: Pea protein isolate, RPI: Rapeseed protein isolate, RPI-HT: Rapeseed protein isolate-Heat Treated, F-D Navy B: Freeze-Dried Cooked Navy Beans, F-D Green B: Freeze-Dried Boiled Green Beans, SBM: Soybean meal, FSBM: Fermented Soybean meal, F-F SBM: Full-Fat Soybean meal, Ferm F-F SBM: Fermented Full-fat Soybean meal, SBMilk: Soybean Milk, WBread: Wholemeal Bread, QRO: Quick Rolled Oats. Samples marked with an asterisk indicate digestion with 1 mL of additional H_2O in the oral phase.

powder, 0.5 g of ground ginger, and 36.9 g of margarine (Sousa et al., 2020). After proper mixing, individual portions of approximately 35 g were baked at 175 °C for 30 min. The portions were manually ground. All ingredients needed to prepare the protein-free cookie were purchased in a local supermarket, except cellulose (Sigma-Aldrich, Merck, Germany).

The nitrogen content of the foods was determined by the Dumas method (AOAC 968.06–1969) using an elemental microanalyzer (LECO CHNS-932; Leco, St. Joseph, MI, USA). The AA composition of the foods was determined as described below and used for *in vitro* DIAAS calculations.

2.3. Evaluation of the in vitro protein and amino acid digestibility

The INFOGEST static *in vitro* digestion protocol (Brodkorb et al., 2019) was used following the modifications described by Sousa et al. (2023) for the assessment of protein digestibility. Three biological replicates were performed for each ingredient. Prior to *in vitro* digestion, enzyme activities and bile salt concentration were measured as specified in the harmonized protocol (Brodkorb et al., 2019). The same batch of each enzyme and bile salts was used for all digestions: amylase with an activity of 327.1 U/mg (Sigma-Aldrich; batch 0000316687), pepsin of 2393 U/mg (Sigma-Aldrich; Lot SLCN5563), pancreatin of 9.585 U/mg (Sigma-Aldrich; Lot SLCM8903), and bile salts concentration of 2.29 mmol/g (Sigma-Aldrich; Lot SLCN7357).

Pancreatin was ultrasound treated (45 Hz, 130 W) for 5 min at room temperature and centrifuged (2000×*g*, for 5 min) to improve enzyme solubilization and reduce protein background. Protein isolates were digested together with the protein-free cookie (40 mg protein +250 mg protein-free substrate) to simulate the composition of a complex meal. Due to the low protein concentration of freeze-dried cooked navy beans and corn flakes, it was difficult to achieve the recommended texture by the INFOGEST static *in vitro* digestion protocol during the oral phase, and the digestions were conducted without and with an extra 1 mL of H_2O.

To determine protein and AA digestibility in RPI and RPI-HT, these substrates were digested using different protein inputs and different trypsin concentrations. As protein input, these ingredients were assayed at 40, 80, 120, or 160 mg of protein. On the other hand, pancreatin in the intestinal phase was adjusted to achieve trypsin concentrations 100, 80, or 60 U/mL.

2.4. Total amino acid analysis by Ultra high-Performance Liquid Chromatography (UPLC)

The samples were subjected to acid hydrolysis according to AOAC 2018.06 method (Jaudzems et al., 2019) to quantify both TAA and total amino groups by the o-phthalaldehyde (OPA) method. The soluble and insoluble digested fractions were incubated in 6 M HCl at 110 °C for 15 h, and the non-digested food products for 24 h. The non-digested food products were analyzed in triplicate.

Hydrolysates were neutralized by adding 6 M NaOH 1:1 (v:v), and then resuspended 1:1 (v:v) in 0.1 M HCl. Diluted samples were filtered through 0.45 μm pore size filters. The samples were derivatized using the AccQ-Tag™ Ultra Derivatization Kit. The calibration curve used for quantification was composed of 1:10 (v:v) of 2.5 mM AA Standard (Sigma-Aldrich), 1:10 (v:v) of 2.5 mM Asparagine and 1:10 (v:v) of 2.5 mM Norvaline in 0.1 M HCl as internal standard, and diluted with 0.1 M HCl to reach a concentration of 250 pmol/μL for the highest concentration calibration point and its respective serial dilutions in 0.1M HCl. As standard of the hydrolyzed form of Cystine, 10 mM and 1 mM of Cystine stock solutions at 0.05M in NaOH were hydrolyzed in 6 M HCl as described above. TAA analysis was carried out in an Acquity ultra-high-performance liquid chromatography system (Waters Corp. Eschborn, Germany) with an ACCQ-TAG™ ULTRA C18 1.7 μm, 2.1 x 100 mm column, coupled to a UV detector (Acquity™ Ultra Performance LC). Standards and samples were analyzed according to the following conditions: 1 μL injection volume, flow rate of 0.7 mL/min, column temperature of 55 °C, and UV detection at 260 nm. Because Cystine and Methionine eluted at similar retention times, accurate quantification of both AA was not possible for all substrates, and calculation of the digestible indispensable amino acid ratio (DIAAR) was done with the sum of the sulfur AA.

2.5. Calculation of protein-, amino acid- and OPA-based digestibility, DIAAR and DIAAS

Total protein digestibility determined by OPA or TAA analysis, and individual AA digestibility were calculated as described by Sousa et al. (2023). The DIAAS was calculated according to the FAO (FAO, 2013) for young children (6 months–3 years), but substituting the standardized ileal digestibility by the value of the *in vitro* AA digestibility.

$$\text{DIAAS} = 100 \times \text{lowest value}\left[\frac{mg\ of\ digestible\ dietary\ indispensable\ aminoacid\ in\ 1\ g\ of\ the\ dietary\ protein}{mg\ of\ the\ same\ dietary\ indispensable\ amino\ acid\ in\ 1\ g\ of\ the\ reference\ protein}\right]$$

In addition, DIAAR was also calculated by using the value of total *in vitro* protein digestibility determined by OPA, and this is referred to as "*proxy*DIAAR", as previously defined by Sousa et al. (2023).

$$proxy\text{DIAAR} = 100 \times \left[\frac{mg\ of\ indispensable\ aminoacid\ in\ 1\ g\ of\ the\ dietary\ protein \times total\ protein\ digestibility}{mg\ of\ the\ same\ dietary\ indispensable\ amino\ acid\ in\ 1\ g\ of\ the\ reference\ protein}\right]$$

2.6. Liquid chromatography-high resolution tandem mass spectrometry (LC-MS/MS) analysis of the ileal contents

Freeze-dried ileal contents (n = 5) after RPI and RPI-HT intake were analyzed by nanoLC-MS/MS using an Orbitrap Exploris 240 coupled to a Vanquish Neo nano UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an EASY-Spray™ PepMap™ Neo UHPLC columns (Thermo Fisher Scientific) with a *m/z* scan range of 350–1500. Peptide identification was performed with Peaks Studio 11.0 (Bioinformatics Solutions, Waterloo, ON, Canada). A homemade database was

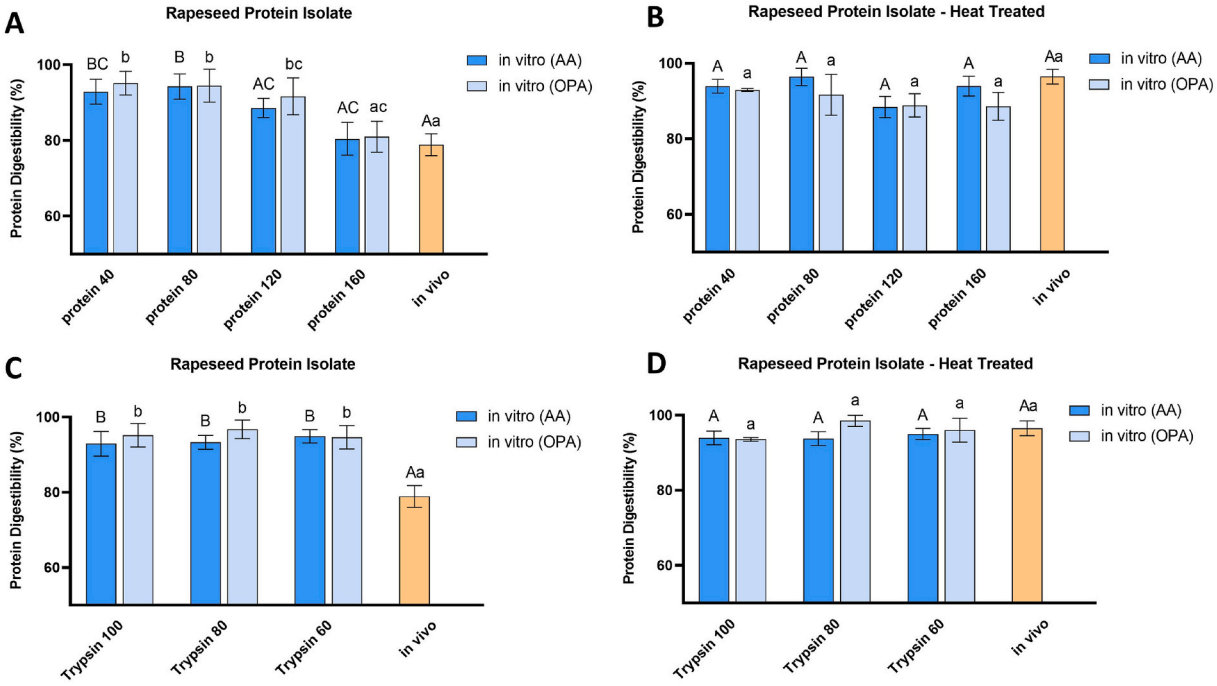

Fig. 2. Protein digestibility of rapeseed protein isolate (A, C) and heat-treated rapeseed protein isolate (B, D) after *in vitro* digestion performed at different input protein and trypsin concentrations in comparison with *in vivo* crude protein digestibility. Input protein in mg: 40, 80, 120, and 160. Trypsin concentrations 100, 80, and 60 units per mL during *in vitro* intestinal digestion. Number of biological replicates *in vivo* n = 8; *in vitro* n = 3. Error bars indicate SEM. Statistical significance comparing between *in vitro* AA, *in vitro* OPA, and *in vivo* protein digestibility was analyzed by two-way ANOVA with Tukey's post-hoc test. Statistical significance ($p < 0.05$) in the comparison between different protein input or trypsin concentrations is indicated by different upper case (AA) or lower case (OPA) letters.

generated according to the most abundant proteins found in rapeseed. No specific enzyme cleavage was used.

2.7. Statistical analysis

Total protein digestibility determined by *in vitro* TAA or OPA analysis, and *in vivo* protein digestibility were compared using a two-way ANOVA with Tukey's post-hoc test. The absolute percent error (APE) and mean absolute percentage error (MAPE) of the *in vitro* protein and AA digestibility value compared with the *in vivo* value were calculated. For the APE calculation, the absolute difference between the *in vitro* and the *in vivo* value was divided by the *in vivo* value. For the MAPE calculation, the sum for APE data pairs between *in vitro* and *in vivo* is divided by the number of data pairs (Nadia et al., 2024). Individual *in vitro* AA digestibility was also compared with its respective *in vivo* AA digestibility value using two-way ANOVA with Sidak's post-hoc test. The difference between *in vitro* and *in vivo* DIAAR was calculated by conducting a Bland-Altman analysis (Martin Bland and Altman, 1986). The number of biological replicates *in vivo* was n = 8 for protein isolates/concentrates; n = 5–12 for legume-derived foods; n = 6 for cereals or derivatives; and n = 3 for the *in vitro* digestions.

3. Results and discussion

3.1. In vitro protein digestibility. Comparison with standardized ileal digestibility in pigs

The *in vitro* protein digestibility was calculated following two different analytical procedures, i.e., TAA analysis and total amino groups by OPA. No significant differences between the two *in vitro* values were observed. The *in vitro* protein digestibility by TAA and OPA analysis was compared with the *in vivo* standardized ileal digestibility of AA determined in growing pigs (Fig. 1). No differences between the *in vitro* and *in vivo* values were observed except for corn flakes, where the *in vitro* digestibility was lower (p-value <0.0001), and for RPI (p-value <0.05 for TAA; p-value <0.01 for OPA) and wheat bread (p-value <0.0001 for both TAA and OPA), where the *in vitro* digestibility was greater than the *in vivo* value. For bagels, *in vivo* protein digestibility agreed with *in vitro* digestibility determined by TAA analysis, but not by OPA (p-value <0.05).

In the group of protein isolates and concentrates (Fig. 1A), the standardized ileal protein digestibility values were correctly predicted by TAA analysis, except in the case of RPI, where the *in vitro* value was 17.9 % greater than the *in vivo* value (Table S2). It was proposed that the presence of trypsin inhibitors in RPI (1.8 %) was responsible for the lower *in vivo* digestibility compared with the heated sample (Bailey et al., 2023). However, the concentration of trypsin inhibitors did not affect *in vitro* digestibility, probably due to an excess of trypsin under our conditions. When the concentration of trypsin inhibitors was reduced to 0.06 % in the heat-treated sample, the *in vitro* values agreed with the *in vivo* samples (APE 2.3 %). Other authors have demonstrated the resistance of trypsin inhibitors in the presence of low pH and pepsin, under conditions similar to ours (2000 U pepsin/ml), and only heat treatments at temperatures ≥100 °C could inactivate these inhibitors by affecting their structural rigidity provided by the disulfide bridges (Takács et al., 2022).

In the case of whole foods (Fig. 1B and C), the *in vitro* protein digestibility values agreed with the *in vivo* values except for corn flakes and wheat bread. In some cases, such as navy beans and corn flakes, the low protein concentration and elevated particle density may have prevented the complete moisturization in the initial steps of the *in vitro* gastrointestinal digestion. To address this, additional digestions were conducted for navy beans and corn flakes, where the volume in the oral phase was increased to obtain a paste-like consistency as recommended by the INFOGEST 2.0 protocol (Brodkorb et al., 2019), by adding an extra 1 mL of water. The APE between the *in vivo* and *in vitro* values decreased from 10.9 to 5.2 % in navy beans and from 24.4 to 20.4 % in cornflakes (samples highlighted with an asterisk in Fig. 1). The

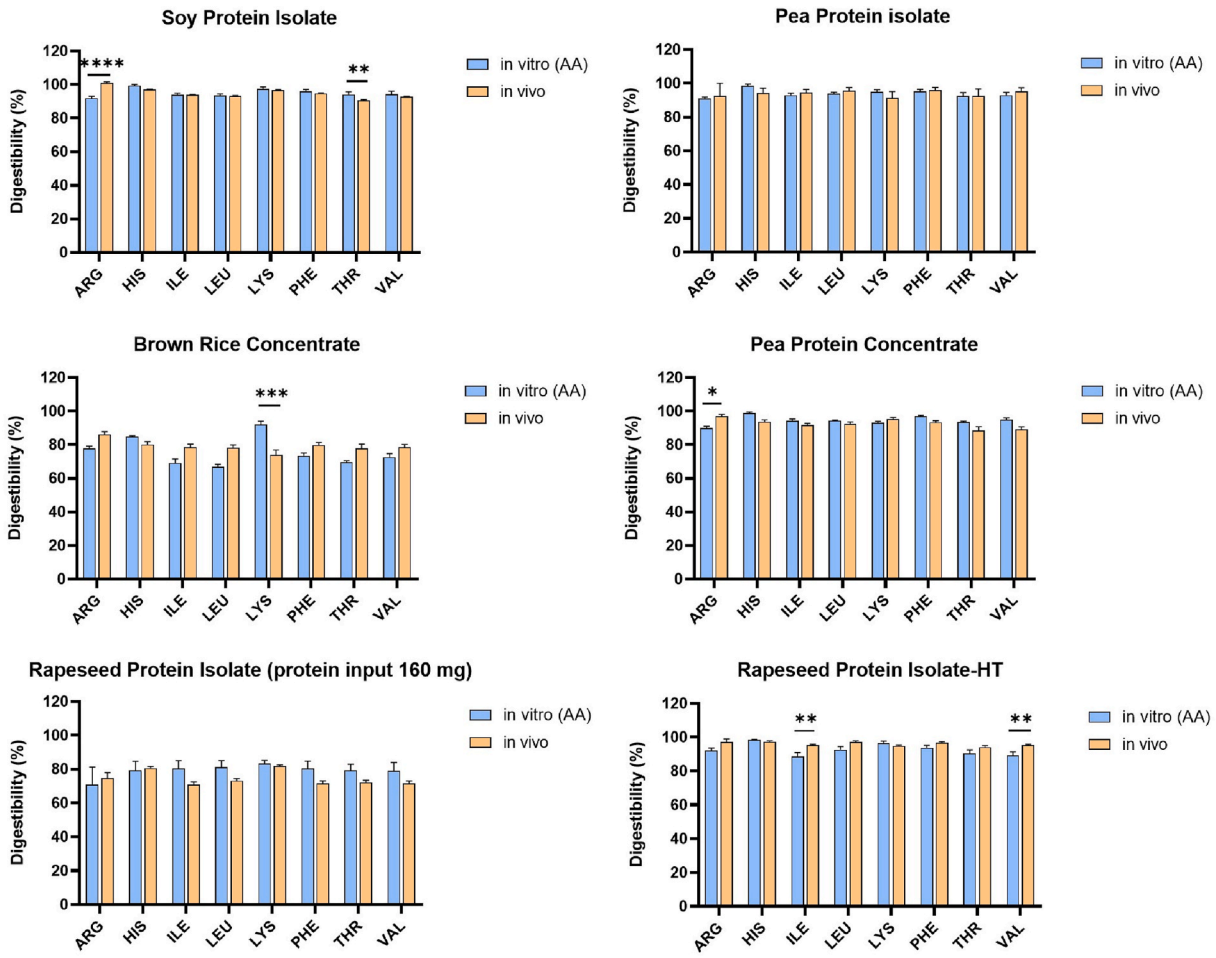

Fig. 3. Digestibility of individual amino acids of six plant-based protein isolates and concentrates after *in vitro* digestion protocol compared to *in vivo* data. Number of biological replicates *in vivo* n = 8; *in vitro* n = 3. Error bars indicate SEM.

remaining difference in the case of corn flakes might result from the low protein concentration of this substrate (5.9 % protein). Reduced true ileal digestibility of corn in rats (66.7 %) has been reported (Rutherfurd et al., 2015), which is closer to the *in vitro* values (74.5 % or 78.5 % with the addition of water) obtained in this work.

For wheat bread and bagels, the difference between the *in vitro* protein digestibility, using TAA analysis as analytical method, and the *in vivo* value was greater than for other ingredients, reaching APEs of 28.1 % and 8.3 %, respectively (Table S2), although differences were not significant for bagels (Fig. 1C). This effect was attributed to differences in the physical form of the foodstuffs, i.e freeze-dried for the *in vitro* procedure, and fresh bread for the *in vivo* experiment. Therefore, an experiment was conducted to assess the influence of bread moisture on protein digestibility. For this purpose, locally purchased whole wheat bread and wheat bagels, in both fresh and freeze-dried forms, were subjected to simulated gastrointestinal digestion. Results demonstrated that when these foods were subjected to *in vitro* digestion in their fresh form, the difference relative to *in vivo* data was reduced from 28.1 to 24.1 % in wheat bread and from 8.3 to 5.3 % in bagels (Table S3). To note, these fresh wheat-based products were purchased in a local supermarket, and therefore, they were not the same as those tested *in vivo*. Other authors have emphasized the importance of the food matrix (Rieder et al., 2021) or the meal to digestive fluid ratio (Martineau-Côté et al., 2024) in the application of the INFOGEST *in vitro* protocol. In addition, concerning the differences in these bakery foods, it has to be taken into account that reactive Lysine was used to calculate ileal digestibility *in vivo* whereas total Lysine was determined in the *in vitro* digests. The difference between the *in vivo* protein digestibility of whole wheat bread (70.7 % ± 6.2) and bagels (88.5 % ± 3.2) may be a result of the greater concentration of fiber in the whole meal bread compared with the bagels, because fiber and plant tissue structures can reduce protein digestibility (FAO, 1991). Specifically, for bread, differences in protein digestibility have been demonstrated because of differences in flour type and in the percentage of high molecular weight-glutenins (Lavoignat et al., 2022).

To test whether the differences between *in vitro* and *in vivo* digestibility of RPI were due to a surplus of trypsin activity, the trypsin-protein ratio in the *in vitro* digestion of RPI and RPI-HT was reduced by two approaches. Protein in the digestion was increased from 40 mg to 160 mg (Fig. 2A and B), and the digestibility of protein in the unheated product decreased with the increase in protein concentration, and approached the *in vivo* values at 120 or 160 mg (three and four times the initial amount). However, the increase in protein did not affect the digestibility of protein in RPI-HT, neither in terms of TAA nor free amino groups, which indicates that an increase in the protein input in samples containing trypsin inhibitors affects the activity of trypsin in the *in vitro* digestion without compromising the digestibility of protein in the sample deprived of enzyme inhibitors. On the other hand, a step-wise decrease in the trypsin concentration in the *in vitro* experiment was also assessed (Fig. 2C and D). In this case, decreasing the trypsin activity did not result in reduced digestibility in RPI-HT or untreated RPI. Because this is a protein isolate with good protein digestibility, it is possible to reduce the enzyme-substrate ratio without affecting the final value for the digestibility of protein. However, in more complex food matrices or ingredients with limited digestibility, the decreased enzyme/substrate ratio may not always be advisable. The presence of

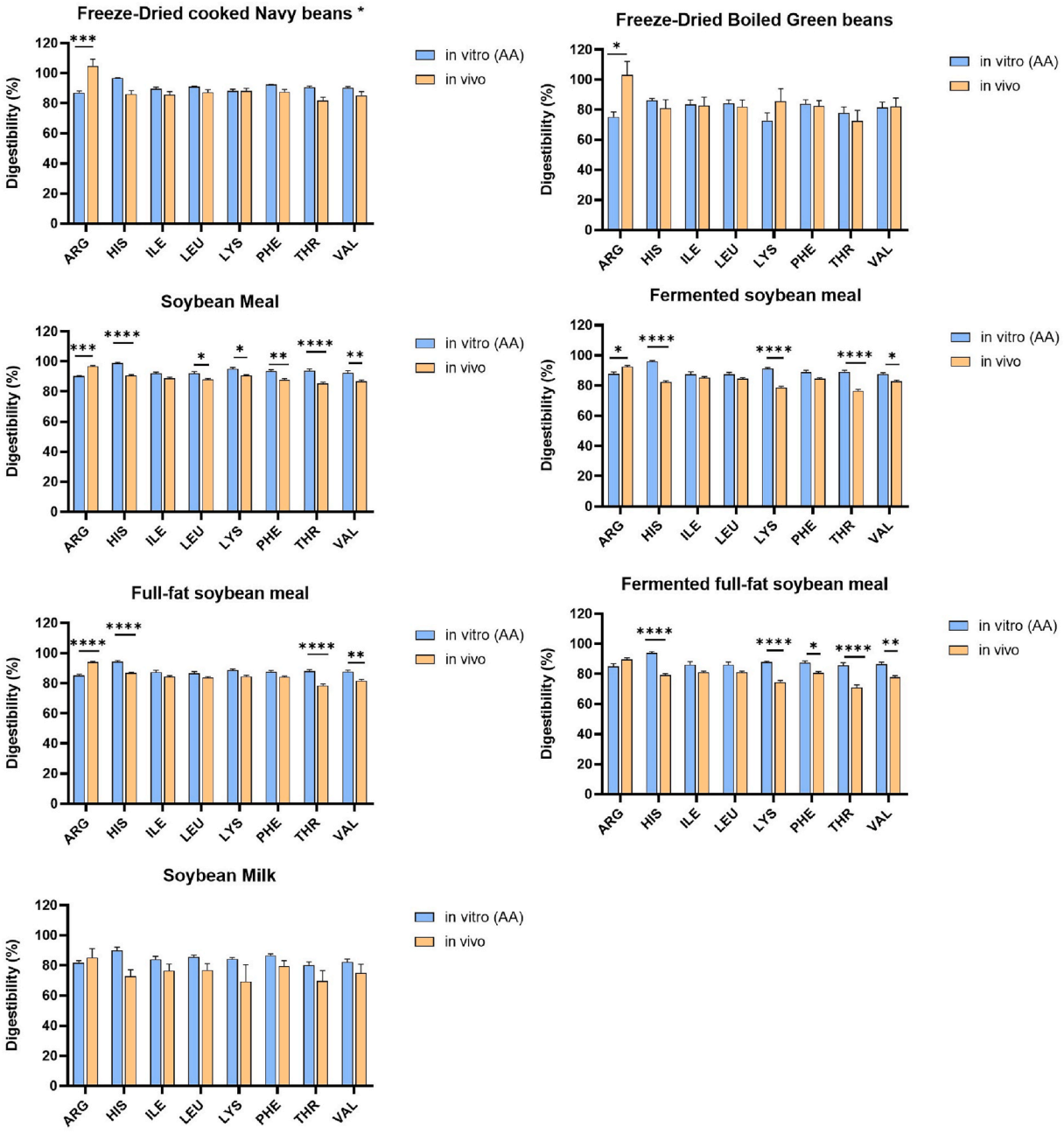

Fig. 4. Digestibility of individual amino acids of eight legume-derived foods after *in vitro* digestion protocol compared to *in vivo* data. Number of biological replicates *in vivo* n = 5–12; *in vitro* n = 3. Error bars indicate SEM. (*) Digested with 1 mL of additional H_2O in the oral phase.

anti-nutritional factors has smaller effects on the *in vitro* digestibility of protein in grain legumes compared with the *in vivo* standardized ileal digestibility (Jezierny et al., 2010). This represents a challenge for *in vitro* methods that can be overcome by a stricter control of the enzyme-substrate ratio.

3.2. Amino acid digestibility

Digestibility of individual AA was consistent with results obtained for total protein digestibility. *In vitro* digestibility of indispensable AA was compared with *in vivo* values, and the APE and MAPE were calculated (Table S4). For *in vitro-in vivo* comparison of gastric digestion, MAPE values < 20 % indicate that a specific *in vitro* approach accurately simulates the *in vivo* food gastric digestion processes, whereas a MAPE value greater than 20 % but less than 50 % indicates reasonable simulation (Nadia et al., 2024). Following this framework, data points with APE <50 % indicate reasonable similarity to corresponding *in vivo* data, and APE >50 % can be utilized to identify limitations in the *in vitro* approach. All studied substrates had MAPE values below 20 %, except for corn flakes (Table S4).

Consistent with the values for protein digestibility, *in vitro* AA digestibility values for RPI at a protein input of 40 mg exceeded the *in vivo* ones, demonstrating a lack of effect of trypsin inhibitors on the indispensable AA release under the *in vitro* conditions used in this work (Table S4). Therefore, AA digestibility values obtained with a protein input of 160 mg are shown in Fig. 3. By using this lower enzyme/substrate ratio for RPI, APE values for AA digestibility ranged from 1.5 to 13.4 % (Table S4). For corn flakes and corn flakes with 1 mL additional water, MAPE values were 30.2 and 25.0 %, respectively, and APE for individual indispensable AA were above 50 % only for Arginine and

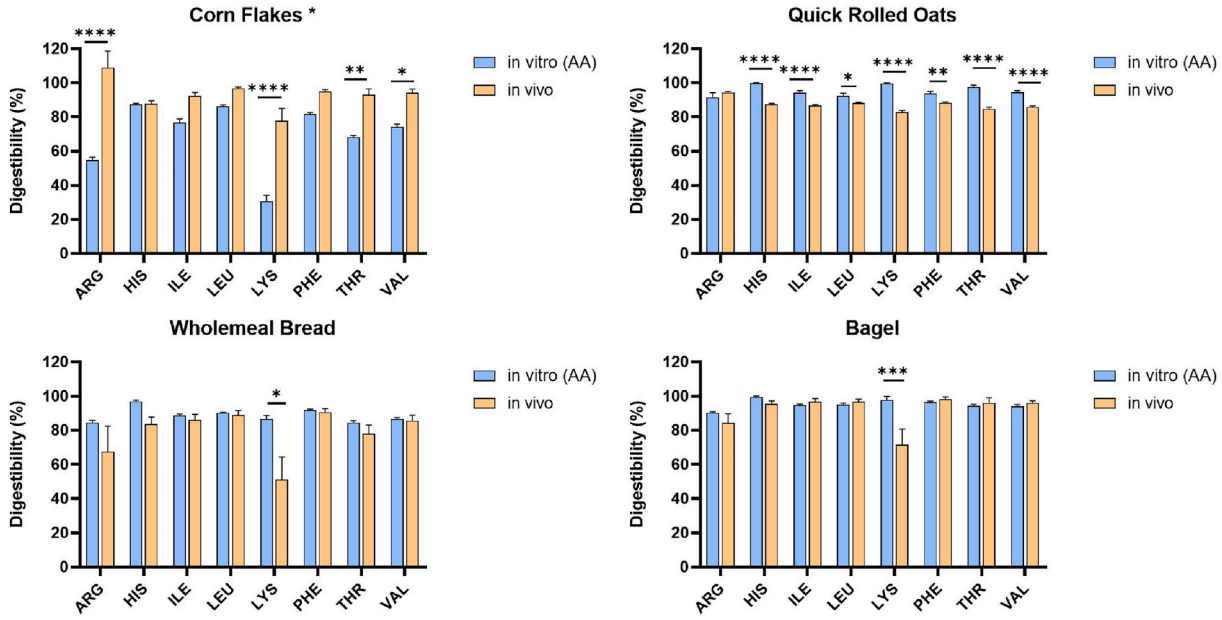

Fig. 5. Digestibility of individual amino acids of four whole cereal-based foods after *in vitro* digestion protocol compared to *in vivo* data. Number of biological replicates *in vivo* n = 6; *in vitro* n = 3. Error bars indicate SEM (*) Digested with 1 mL of additional H2O in the oral phase.

Lysine (Table S4).

Differences between *in vivo* and *in vitro* digestibility of some AA were observed for legumes and soy-derived foods (Fig. 4), but the calculated APE was less than 30 % in all cases (Table S4). Individual AA digestibility of navy beans did not differ with or without the addition of water, with MAPE values of 7.6 and 6.1, respectively.

No differences were observed between *in vivo* and *in vitro* digestibility of AA in wheat bread and bagels, except that Lysine digestibility was higher in the *in vitro* procedure than *in vivo* (p-value <0.001) (Fig. 5). *In vitro* AA digestibility in quick rolled oats was also greater than *in vivo* (p-value <0.05), although the APE values were ≤20 %. Corn flakes had reduced *in vitro* AA digestibility compared with the *in vivo* digestibility of AA, and the APE was >50 % for Lysine and Arginine (Table S4).

Among the essential AA, Lysine exhibited the greatest difference between *in vitro* and *in vivo* values among several of the foods included in the present work. The *in vitro* digestibility of Lysine was markedly greater than *in vivo* in soybean-derived products (p-value <0.05) and wheat-based products (p-value <0.05) because reactive Lysine was determined in the ileal contents, whereas total Lysine was quantified in the *in vitro* assays. In agreement with our results, reactive Lysine was consistently lower than total Lysine in the ileal contents of humans consuming cooked black beans, toasted wheat bread, and wheat bran (Hodgkinson et al., 2023). Because modified Lysine can be absorbed, but may not be utilized for protein synthesis (Hurrell and Carpenter, 1977), it is important to determine reactive Lysine and total Lysine in the absorbable and non-absorbable fractions after simulated digestion.

3.3. Digestible indispensable amino acid ratio (DIAAR) and digestible indispensable amino acid score (DIAAS)

The DIAAR was calculated with respect to the AA requirements for young children (6 months–3 years), and the digestible indispensable AA score (DIAAS), which corresponds to the lowest DIAAR value, was calculated as well for all foods included in the present work (Tables 1–3) (Consultation FAO, 2013). The difference between *in vivo* and *in vitro* values for DIAAR was calculated for concentrates/isolates (Fig. 6A), legumes and soy-derived foods (Fig. 6B), and cereal-based foods (Fig. 6C), and expressed as a *bias* value, to assess how closely the *in vitro* method predicts the *in vivo* value. The *bias* between methods for isolates/concentrates, legumes and soy-derivatives, and cereals or derivatives were −1.6, 10.2, and −0.08 %, respectively. Although for legumes and soy-derived foods, the mean *bias* indicates an overestimation by the *in vitro* method according to Nadia et al. (2024), these values are within the range considered to be an accurate prediction, namely $-20\,\% \leq bias \leq 20\,\%$.

Table 1 shows the DIAAR values obtained in ileal cannulated pigs in comparison with the *in vitro* DIAAR calculated with the AA digestibility of each indispensable AA and the *in vitro proxy* DIAAR calculated with the value of protein digestibility determined by OPA in the isolates/concentrates group. The *in vitro* method based on AA or OPA predicted the *in vivo* DIAAR value and the limiting AA with reasonable accuracy except for RPI and RPI-HT. In these foods, the limiting AA in the *in vitro* model was Threonine, calculated by TAA, and Leucine, calculated by OPA, whereas the *in vivo* model demonstrated that the limiting AA in RPI was Leucine, and Lysine was limiting in RPI-HT. In these foods, the *in vitro* DIAAR values for Leucine, Lysine, and Threonine were close, and small differences in AA quantification may have affected the identification of the limiting AA. For the remaining protein concentrates, the *in vitro* DIAAS and the *proxy* DIAAS values were not different from the *in vivo* value for DIAAS. Using the INFOGEST static protocol, but with different precipitation conditions (trichloroacetic acid 12 %), *in vitro* protein digestibility values for several legume and cereal protein materials were not different from *in vivo* values, with the greatest discrepancies for DIAAS in substrates such as wheat and zein, where Lysine was the limiting AA (Komatsu et al., 2023).

Soy foods and soy derivatives (Table 2) had sulfur AA as the limiting AA, which was also consistent with the *in vivo* data. Although cooked and freeze-dried green and navy beans were used *in vitro*, our results showed comparable DIAAR values to those obtained *in vivo*, not being affected by the drying process in these substrates. Although differences between *in vitro* and *in vivo* were observed for DIAAR values of some soybean meal products, AA digestibility values were close, and differences were acceptable with APE values < 24 %. Among the different soy-based products, soybean milk had the least *in vitro* DIAAS, and full-fat soybean meal had the greatest. It has been reported that heat processing or moisture conditions can lead to differences in protein quality scores in soy-based products (Van Den Berg et al., 2022). For instance, digestibility values (92.2 %) higher than those reported in the present study (76.4 %) have been reported for UHT soy milk in cannulated minipigs (Reynaud et al., 2021).

Table 1
Comparison of digestible indispensable amino acid ratio (DIAAR) determined in ileal cannulated pigs (*in vivo* DIAAR), with *in vitro* and *in vitro proxy* DIAAR of soy protein isolate (SPI), pea protein isolate (PPI), pea protein concentrate (PPC), brown rice concentrate (BRC), rapeseed protein isolate (RPI), and rapeseed protein isolate-heat treated (RPI-HT). *In vitro* DIAAR is determined by using AA digestibility, while *in vitro proxy* DIAAR is calculated with the value of protein digestibility obtained by OPA. DIAAS value for each substrate is coloured in blue. Number of biological replicates *in vivo* n = 8; *in vitro* n = 3.

Ingredient		*in vivo* DIAAR	*in vitro* DIAAR	*in vitro proxy* DIAAR
SPI [a]	HIS	124.7 ± 0.3 [a]	127.6 ± 0.8 [a]	121.7 ± 1.1 [b]
	ILE	145.4 ± 0.4 [a]	145.1 ± 1.9 [a]	146.7 ± 1.3 [a]
	LEU	109.2 ± 0.3 [a]	109.6 ± 1.2 [a]	111.1 ± 1.0 [a]
	LYS	105.8 ± 0.3 [a]	106.4 ± 1.5 [a]	103.6 ± 0.9 [a]
	SAA	**82.9 ± 0.3** [a]	**75.0 ± 2.8** [b]	**84.5 ± 0.8** [a]
	AAA	165.6 ± 0.4 [a]	160.5 ± 1.9 [b]	164.7 ± 1.5 [a]
	THR	103.9 ± 0.8 [a]	108.0 ± 1.9 [b]	108.8 ± 1.0 [b]
	VAL	110.5 ± 0.5 [a]	112.2 ± 2.2 [a]	112.9 ± 1.0 [a]
PPI [b]	HIS	118.8 ± 3.7 [a]	123.7 ± 1.4 [a]	117.9 ± 1.7 [a]
	ILE	148.2 ± 2.9 [a]	145.3 ± 2.0 [a]	146.6 ± 2.1 [a]
	LEU	120.3 ± 2.3 [a]	117.6 ± 1.2 [a]	117.7 ± 1.7 [a]
	LYS	120.7 ± 5.3 [a]	112.1 ± 1.6 [a]	110.9 ± 1.6 [a]
	SAA	**59.5 ± 3.4** [ab]	**48.2 ± 1.4** [a]	**64.7 ± 0.9** [b]
	AAA	170.0 ± 3.6 [a]	161.9 ± 1.7 [a]	165.6 ± 2.4 [a]
	THR	104.7 ± 4.8 [a]	104.6 ± 2.2 [a]	105.9 ± 1.5 [a]
	VAL	117.7 ± 2.8 [a]	114.6 ± 2.2 [a]	115.7 ± 1.7 [a]
PPC [a]	HIS	116.2 ± 1.5 [a]	122.7 ± 0.7 [a]	117.3 ± 0.8 [a]
	ILE	145.7 ± 1.7 [a]	149.8 ± 1.9 [a]	150.4 ± 1.0 [a]
	LEU	114.7 ± 1.4 [a]	117.0 ± 0.6 [a]	117.3 ± 0.8 [a]
	LYS	127.1 ± 1.2 [a]	124.2 ± 1.0 [a]	126 ± 0.9 [a]
	SAA	**59.6 ± 1.8** [a]	**61.8 ± 4.0** [a]	**70.1 ± 0.5** [b]
	AAA	169.5 ± 2.1 [a]	167.5 ± 1.9 [a]	173.1 ± 1.2 [a]
	THR	98.9 ± 2.6 [a]	104.5 ± 0.5 [ab]	105.6 ± 0.7 [b]
	VAL	112.8 ± 2.0 [a]	120.1 ± 1.3 [b]	119.6 ± 0.8 [b]
BRC [a]	HIS	87.1 ± 1.8 [ab]	92.4 ± 0.5 [a]	77.6 ± 0.1 [b]
	ILE	109.0 ± 2.7 [a]	95.9 ± 3.5 [b]	99.2 ± 0.2 [ab]
	LEU	95.5 ± 2.2 [a]	81.5 ± 1.9 [b]	87.2 ± 0.1 [ab]
	LYS	**35.0 ± 1.5** [a]	**43.6 ± 1.0** [a]	**33.9 ± 0.1** [a]
	SAA	122.1 ± 2.9 [a]	110.4 ± 6.6 [a]	124.7 ± 0.2 [a]
	AAA	156.0 ± 3.5 [a]	139.3 ± 3.0 [b]	144.9 ± 0.2 [ab]
	THR	81.5 ± 2.7 [a]	73.1 ± 0.7 [a]	74.8 ± 0.1 [a]
	VAL	114.5 ± 2.7 [a]	106.0 ± 3.1 [a]	104.1 ± 0.2 [a]
RPI (protein input 160 mg) [a]	HIS	125.7 ± 1.4 [a]	129.2 ± 3.9 [a]	125.4 ± 5.5 [a]
	ILE	85.1 ± 1.8 [a]	100.2 ± 2.1 [b]	96.2 ± 4.2 [b]
	LEU	**76.3 ± 1.4** [a]	87.2 ± 2.4 [b]	**84.0 ± 3.7** [ab]
	LYS	85.9 ± 0.8 [a]	88.3 ± 1.4 [a]	84.4 ± 3.7 [a]
	SAA	166.0 ± 0.9 [a]	134.5 ± 12.6 [b]	162.2 ± 7.1 [a]
	AAA	78.4 ± 1.9 [a]	90.6 ± 3.0 [b]	90.5 ± 4.0 [b]
	THR	76.8 ± 1.3 [a]	**86.2 ± 2.5** [a]	85.6 ± 3.8 [a]
	VAL	83.3 ± 1.5 [a]	96.0 ± 2.5 [b]	93.5 ± 4.1 [ab]
RPI-HT [a]	HIS	152.4 ± 0.8 [ab]	154 ± 0.5 [a]	147.1 ± 2.4 [b]
	ILE	113.5 ± 0.7 [a]	105.6 ± 2.8 [b]	112 ± 1.8 [ab]
	LEU	101.8 ± 0.6 [a]	97.1 ± 1.9 [a]	**98.4 ± 1.6** [a]
	LYS	**99.9 ± 0.5** [a]	101.6 ± 1.4 [a]	99.1 ± 1.6 [a]
	SAA	187.2 ± 1.2 [a]	178.3 ± 6.5 [b]	184.4 ± 3.0 [ab]
	AAA	108.4 ± 0.7 [a]	97.7 ± 3.1 [b]	105.5 ± 1.7 [a]
	THR	100.8 ± 0.9 [a]	**96.9 ± 2.2** [a]	100.7 ± 1.7 [a]
	VAL	110.8 ± 0.7 [a]	103.9 ± 2.4 [b]	109.4 ± 1.8 [ab]

[a] HM Bailey et al. (2023).
[b] Data non-published.

Table 2
Comparison between *in vivo*, *in vitro,* and *in vitro proxy* digestible indispensable amino acid ratio (DIAAR) of freeze-dried cooked navy beans (F-D Navy B), freeze-dried boiled green beans (F-D Green B), soybean meal (SBM), fermented soybean meal (FSBM), full-fat soybean meal (F-F SBM), and soybean milk (SBMILK) for young children (6 months–3 years old). DIAAS value for each substrate is coloured in blue. Number of biological replicates *in vivo* n = 5–12; *in vitro* n = 3.

Ingredient		*in vivo* DIAAR	*in vitro* DIAAR	*in vitro proxy* DIAAR
F-D Navy B [a b]	HIS	148.9 ± 3.8 [a]	167.3 ± 0.1 [b]	158.3 ± 1.0 [ab]
	ILE	159.4 ± 4.1 [a]	166.9 ± 2.0 [a]	170.7 ± 1.0 [a]
	LEU	126.5 ± 2.7 [a]	132.0 ± 0.7 [a]	132.8 ± 0.8 [a]
	LYS	130.8 ± 2.8 [a]	131.1 ± 1.6 [a]	136.1 ± 0.8 [a]
	SAA	**74.5 ± 3.8** [a]	**83.3 ± 1.4** [ab]	**95.2 ± 0.6** [b]
	AAA	171.0 ± 3.5 [a]	175.6 ± 0.1 [a]	180.9 ± 1.1 [a]
	THR	137.2 ± 4.1 [a]	152.5 ± 1.2 [b]	154.0 ± 0.9 [b]
	VAL	131.0 ± 4.0 [a]	138.7 ± 1.5 [a]	140.9 ± 0.9 [a]
F-D Green B [b]	HIS	109.9 ± 7.7 [a]	116.8 ± 2.0 [a]	105.4 ± 5.2 [a]
	ILE	132.7 ± 8.8 [a]	133.9 ± 4.6 [a]	124.5 ± 6.2 [a]
	LEU	104.8 ± 5.7 [a]	107.6 ± 3.0 [a]	99.4 ± 4.9 [a]
	LYS	116.0 ± 11.4 [a]	98.4 ± 7.2 [a]	105.2 ± 5.2 [a]
	SAA	**86.0 ± 8.1** [a]	**91.2 ± 8.3** [a]	**86.8 ± 4.3** [a]
	AAA	133.1 ± 7.6 [a]	133.8 ± 4.9 [a]	129.8 ± 6.4 [a]
	THR	105.8 ± 10.2 [a]	113.5 ± 5.7 [a]	113.4 ± 5.6 [a]
	VAL	115.0 ± 8.1 [a]	114.2 ± 5.0 [a]	109.0 ± 5.4 [a]
SBM [c,d]	HIS	120.4 ± 0.7 [a]	130.9 ± 1.0 [b]	125.1 ± 1.4 [c]
	ILE	140.2 ± 1.1 [a]	145.2 ± 1.5 [b]	148.8 ± 1.6 [b]
	LEU	104.2 ± 0.8 [a]	108.9 ± 1.4 [b]	111.6 ± 1.2 [b]
	LYS	106.3 ± 0.7 [a]	111.8 ± 1.0 [b]	110.8 ± 1.2 [b]
	SAA	**88.3 ± 1.0** [a]	**91.4 ± 0.4** [a]	**97.9 ± 1.1** [b]
	AAA	151.8 ± 1.1 [a]	152.7 ± 1.4 [a]	161.8 ± 1.8 [b]
	THR	100.1 ± 1.0 [a]	116.0 ± 1.4 [b]	116.5 ± 1.3 [b]
	VAL	102.0 ± 0.9 [a]	108.6 ± 1.8 [b]	110.7 ± 1.2 [b]
FSBM [d]	HIS	102.5 ± 1.0 [a]	119.2 ± 0.6 [b]	111.4 ± 1.0 [c]
	ILE	131.4 ± 1.2 [a]	134.5 ± 2.5 [ab]	138.1 ± 1.3 [b]
	LEU	97.9 ± 0.9 [a]	100.9 ± 1.6 [ab]	103.8 ± 1.0 [b]
	LYS	84.2 ± 1.0 [a]	97.8 ± 0.6 [b]	96.1 ± 0.9 [b]
	SAA	**74.9 ± 1.4** [a]	**83.7 ± 1.5** [b]	**89.4 ± 0.8** [b]
	AAA	143.0 ± 1.0 [a]	142.4 ± 2.7 [a]	150.7 ± 1.4 [b]
	THR	92.6 ± 1.3 [a]	107.4 ± 1.4 [b]	108.4 ± 1.0 [b]
	VAL	95.4 ± 1.0 [a]	100.6 ± 1.1 [b]	103.2 ± 1.0 [b]
F-F SBM [e]	HIS	115.7 ± 0.8 [a]	126 ± 0.9 [b]	117.6 ± 1.3 [a]
	ILE	137.1 ± 1.2 [a]	141.8 ± 2.1 [ab]	143.2 ± 1.6 [b]
	LEU	100.6 ± 0.9 [a]	103.9 ± 1.4 [ab]	105.8 ± 1.2 [b]
	LYS	99.0 ± 0.9 [a]	103.4 ± 1.0 [a]	102.9 ± 1.2 [a]
	SAA	**86.3 ± 1.1** [a]	**92.4 ± 2.4** [b]	**97.0 ± 1.1** [b]
	AAA	143.3 ± 1.1 [a]	143.2 ± 1.9 [a]	149.7 ± 1.7 [b]
	THR	96.6 ± 1.2 [a]	107.7 ± 1.3 [b]	107.9 ± 1.2 [b]
	VAL	98.9 ± 1.1 [a]	106.0 ± 1.2 [b]	106.6 ± 1.2 [b]
Ferm F-F SBM [b]	HIS	100.3 ± 1.2 [a]	119.4 ± 1.1 [b]	110.5 ± 1.7 [b]
	ILE	129.8 ± 1.3 [a]	138.5 ± 2.9 [b]	139.6 ± 2.1 [b]
	LEU	94.6 ± 1.1 [a]	100.9 ± 2.1 [ab]	101.9 ± 1.5 [b]
	LYS	81.2 ± 1.4 [a]	96.0 ± 0.8 [b]	95.2 ± 1.4 [b]
	SAA	**75.7 ± 1.9** [a]	**88.0 ± 2.5** [b]	**92.8 ± 1.4** [b]
	AAA	132.8 ± 1.6 [a]	137.8 ± 3.5 [ab]	143.3 ± 2.2 [b]
	THR	84.7 ± 2.1 [a]	103.0 ± 2.1 [b]	104.4 ± 1.6 [b]
	VAL	93.7 ± 1.3 [a]	104.4 ± 1.6 [b]	104.9 ± 1.6 [b]
SBMILK [b]	HIS	103.1 ± 6.2 [a]	127.5 ± 3.1 [b]	113.8 ± 3.3 [ab]
	ILE	128.6 ± 7.3 [a]	141.1 ± 3.3 [a]	134.8 ± 3.9 [a]
	LEU	95.7 ± 5.4 [a]	106.6 ± 1.5 [a]	99.8 ± 2.9 [a]
	LYS	86.8 ± 13.7 [a]	105.5 ± 1.0 [a]	100.3 ± 2.9 [a]
	SAA	**64.2 ± 7.4** [a]	**70.4 ± 4.3** [a]	**86.2 ± 2.5** [a]
	AAA	140.4 ± 8.0 [a]	149.6 ± 2.9 [a]	146.0 ± 4.2 [a]
	THR	91.1 ± 9.1 [a]	104.9 ± 3.0 [a]	105.0 ± 3.0 [a]
	VAL	92.6 ± 7.2 [a]	101.6 ± 2.4 [a]	99.1 ± 2.8 [a]

[a] Digested with 1 mL of H_2O additional before the oral phase.
[b] Data non-published.
[c] C.D. Espinosa et al. (2021).
[d] C.D. Espinosa et al. (2020).
[e] K.M. Baker et al. (2010).

For cereal-based products (Table 3), the limiting AA was Lysine in all cases, and the *in vitro* method identified the limiting AA in all substrates. In corn flakes, the *in vitro* DIAAS value determined by TAA analysis was lower than the *in vivo* value due to the low digestibility observed for Lysine. Nevertheless, in the *proxy* DIAAR calculation, where the determination is based on total protein digestibility, the Lysine value obtained is 16.5, which is in agreement with the *in vivo* value (16.3). The *in vitro* DIAAR values for quick rolled oats were greater than *in vivo* values, which is likely due to the high AA digestibility for all AA, except Arginine (Fig. 6). However, *in vivo* values were obtained from pigs that were fed quick oats prepared as a porridge (Fanelli et al., 2021). The influence of cooking on AA digestibility was reported by Nosworthy et al. (2023), who observed a decrease in AA scores of Navarro oat after cooking. However, the *in vitro* DIAAR values for wheat bread and bagels were not

Table 3
Comparison between *in vivo*, *in vitro,* and *in vitro proxy* digestible indispensable amino acid ratio (DIAAR) of corn flakes, quick rolled oats (QRO), wholemeal bread (WBread), and bagel for young children (6 months–3 years old). DIAAS value for each substrate is coloured in blue. Number of biological replicates *in vivo* n = 6; *in vitro* n = 3.

Ingredient		*in vivo* DIAAR	*in vitro* DIAAR	*in vitro proxy* DIAAR
Corn flakes [a] [b]	HIS	127.4 ± 2.6 [a]	127.3 ± 0.7 [a]	114.1 ± 1.1 [b]
	ILE	118.6 ± 2.5 [a]	98.5 ± 2.7 [b]	100.6 ± 0.9 [b]
	LEU	230.8 ± 1.8 [a]	206.0 ± 1.4 [b]	187.1 ± 1.8 [c]
	LYS	**16.3 ± 1.5** [a]	**6.4 ± 0.7** [b]	**16.5 ± 0.2** [a]
	SAA	132.5 ± 3.3 [a]	98.2 ± 1.5 [b]	109.3 ± 1.0 [c]
	AAA	187.5 ± 2.6 [a]	159.3 ± 1.4 [b]	154.8 ± 1.4 [b]
	THR	97.5 ± 3.6 [a]	71.6 ± 1.1 [b]	82.3 ± 0.8 [c]
	VAL	104.9 ± 2.4 [a]	82.6 ± 1.8 [b]	87.4 ± 0.8 [b]
QRO [b]	HIS	94.7 ± 0.6 [a]	108.1 ± 0.2 [b]	103.2 ± 1.2 [b]
	ILE	102.7 ± 0.8 [a]	111.6 ± 1.4 [b]	112.9 ± 1.3 [b]
	LEU	95.5 ± 0.6 [a]	100.4 ± 1.5 [b]	103.4 ± 1.2 [b]
	LYS	**56.7 ± 0.8** [a]	**68.4 ± 0.2** [b]	**65.4 ± 0.8** [b]
	SAA	151.2 ± 1.0 [a]	155.8 ± 6.0 [b]	161.4 ± 1.9 [c]
	AAA	144.9 ± 0.6 [a]	148.9 ± 2.5 [a]	156.6 ± 1.8 [b]
	THR	83.8 ± 1.1 [a]	96.5 ± 1.1 [b]	94.4 ± 1.1 [b]
	VAL	99.7 ± 0.8 [a]	109.8 ± 1.1 [b]	110.7 ± 1.3 [b]
WBread [c]	HIS	92.4 ± 4.5 [a]	107.0 ± 0.8 [a]	100.0 ± 1.4 [a]
	ILE	105.7 ± 4.0 [a]	108.9 ± 0.9 [a]	111.1 ± 1.5 [a]
	LEU	89.3 ± 2.7 [a]	90.5 ± 0.5 [a]	90.9 ± 1.2 [a]
	LYS	**22.8 ± 5.9** [a]	**38.5 ± 0.9** [a]	**40.3 ± 0.5** [a]
	SAA	102.2 ± 12.6 [a]	110.5 ± 2.7 [a]	120.7 ± 1.6 [a]
	AAA	125.8 ± 10.0 [a]	128.8 ± 1.2 [a]	133.9 ± 1.8 [a]
	THR	69.8 ± 4.7 [a]	75.8 ± 0.9 [a]	81.2 ± 1.1 [a]
	VAL	86.3 ± 3.3 [a]	87.4 ± 0.8 [a]	91.3 ± 1.2 [a]
Bagel [c]	HIS	103.9 ± 2.0 [a]	108.4 ± 0.6 [a]	104.4 ± 0.6 [a]
	ILE	114.7 ± 2.1 [a]	112.2 ± 0.8 [a]	113.5 ± 0.6 [a]
	LEU	98.7 ± 1.4 [a]	97.0 ± 0.8 [a]	97.7 ± 0.5 [a]
	LYS	**28.5 ± 3.7** [a]	**39.0 ± 0.8** [a]	**38.2 ± 0.2** [a]
	SAA	123.3 ± 2.2 [a]	115.1 ± 1.4 [a]	124.4 ± 0.7 [a]
	AAA	140.8 ± 10.3 [a]	141.0 ± 0.1 [a]	146.6 ± 0.8 [a]
	THR	82.1 ± 2.7 [a]	80.6 ± 0.8 [a]	82.0 ± 0.4 [a]
	VAL	93.0 ± 1.4 [a]	91.2 ± 1.0 [a]	92.9 ± 0.5 [a]

[a] Digested with 1 mL of H_2O additional before the oral phase.
[b] Fanelli et al. (2021).
[c] Data non-published.

different from values obtained *in vivo*.

3.4. Peptidomic analysis of the ileal contents after RPI and RPI-HT digestion

As previously mentioned, the difference in the *in vivo* digestibility of RPI and RPI-HT was attributed to the presence of trypsin inhibitors. The peptide profile of the ileal remnants after the digestion of RPI or RPI-HT could provide evidence on the actual protein fraction involved in this difference. Peptidomic analysis of the ileal contents (5 subjects × 2 substrates) was carried out. The identified peptides corresponded to the main rapeseed storage proteins, cruciferin 4 and napin, and are displayed in the corresponding protein sequence (Fig. 7). Of 272 distinct sequences, 262 were identified upon RPI intake, while only 27 peptides were identified in the RPI-HT ileal contents, which supports the greater protein digestibility of RPI-HT as a result of the effectiveness of heat treatment in reducing trypsin inhibitors. The great number of peptides in RPI-HT for napin suggests that its digestibility is not strongly affected by the presence of trypsin inhibitors, whereas the influence of heating on the digestibility of cruciferin is noteworthy. In agreement with our results, the limited digestibility of the napin fraction from rapeseed in minipigs has been recently reported, where the different heat treatment or extraction/purification processes applied showed a low impact on its susceptibility to digestion (Kapel et al., 2025).

4. Conclusions

The *in vitro* digestion method based on the INFOGEST protocol

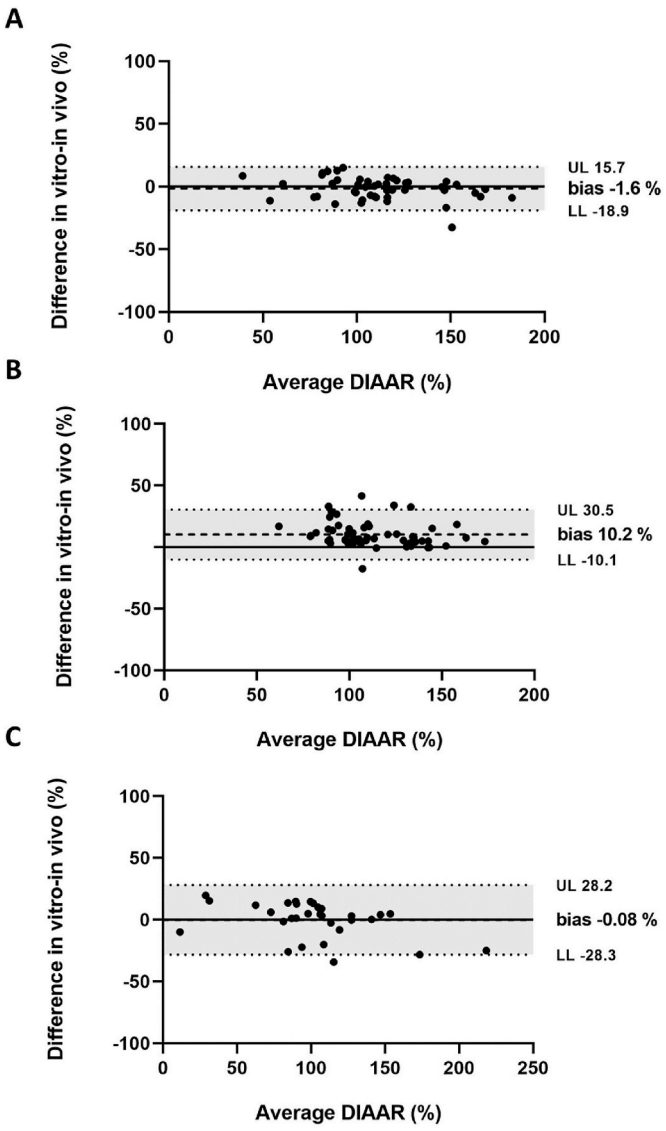

Fig. 6. Difference between *in vivo* DIAAR and *in vitro* DIAAR of plant-based isolates/concentrates (A), legume-derived foods (B) and cereals or derivatives (C). *Bias* value, lower and upper limits at 95 % of agreement for A, B or C were calculated by performing a Bland-Altman analysis.

predicted protein digestibility of various plant-based foods with APE values below 30 % for all substrates. Regarding the *in vitro-in vivo* comparisons of AA digestibility, all studied substrates had MAPE values below 20 %, except for corn flakes. The effect of heat treatment on RPI digestibility was evidenced by the distinct peptide profile of ileal contents, pointing to napin as the main contributor to the protein resistance. The *in vitro* protocol is less sensitive to the presence of trypsin inhibitors at the enzyme/substrate ratio initially proposed, but increasing the amount of sample in the simulated digestion resolved this discrepancy. DIAAR values were calculated according to the FAO requirements for young children (6 months–3 years), and *bias* from the *in vivo* values ranged from −1.6 % for protein concentrates and cereal products to 10.2 % in the legume group. Some of the limitations encountered in this study have served to propose strategies to overcome them. For instance, it would be important to define the level of enzyme inhibitors driving a decrease in the *in vivo* digestibility of specific substrates and adjust the enzyme/substrate ratio in these simulated digestions. Different analytical procedures for the quantitative determination of AA can also result in disagreements between *in vivo* and *in vitro* procedures for the calculated DIAAS values. For instance, for wheat-based products, differences

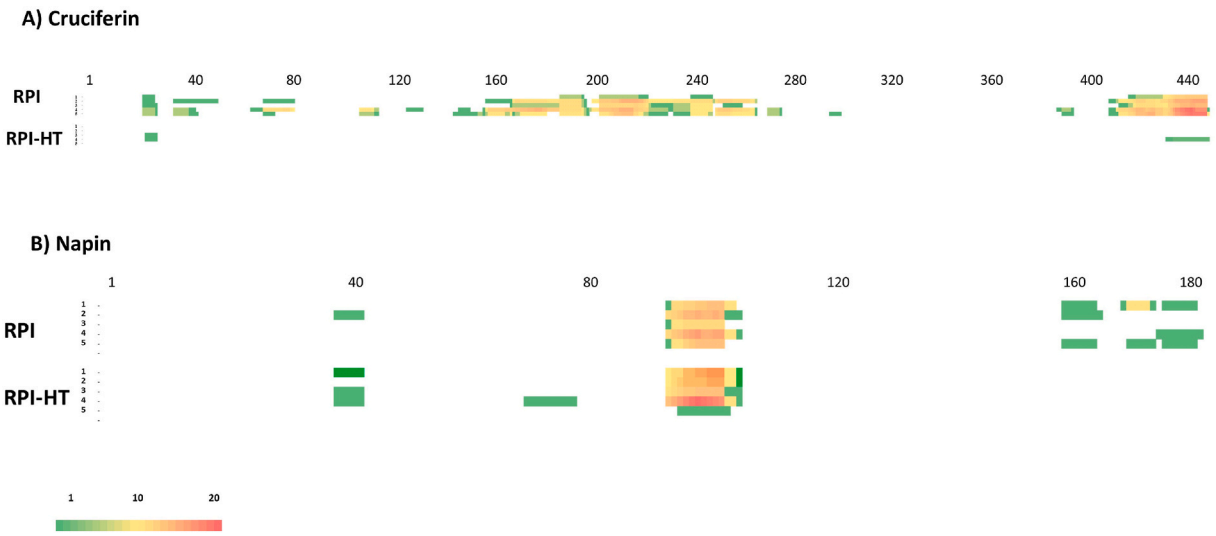

Fig. 7. Heat map according to the frequency of appearance of amino acids corresponding to peptide sequences from cruciferin (A) and napin (B) found in the ileum after the digestion of rapeseed protein isolate (RPI) and rapeseed protein isolate heat-treated (RPI-HT). Each row corresponds to the biological replicates for each sample (n = 5). Green and red colour corresponds to low and high frequency, respectively.

in AA digestibility of Lysine are attributed to the determination of reactive Lysine (*in vivo*) vs total Lysine (*in vitro*). However, other substrates, such as corn flakes, require further research to match *in vitro* protein and AA digestibility with *in vivo* data. In comparative experiments, it is important to consider the food matrix assayed (moist *vs* dried) in each case, as well as the particle size of the sample (milling degree) and the changes it may undergo as a result of storage or handling. These factors may have a critical impact on protein and AA digestibility values and consequently on the DIAAS value. In summary, the results of this work indicated that an *in vitro* digestion procedure may be used to predict protein and AA digestibility in a variety of plant proteins in different foodstuffs with reasonable accuracy. In addition, future research is needed to explore the use of ex vivo testing which could be useful to bridge the gap between *in vitro* and *in vivo* studies.

CRediT authorship contribution statement

Cristina Gómez-Marin: Writing – original draft, Investigation, Formal analysis, Data curation. **Beatriz Miralles:** Writing – review & editing, Supervision. **Natalia S. Fanelli:** Formal analysis *in vivo*, Data curation, review & editing. **Hans H. Stein:** review & editing, Resources, Conceptualization. **Isidra Recio:** Writing – review & editing, Supervision, Resources, Conceptualization, Funding acquisition.

Declaration of competing interest

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

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Abbreviations Used

APE	absolute percent error
AA	amino acid
BRC	brown rice concentrate
DIAAR	digestible indispensable amino acid ratio
DIAAS	digestible indispensable amino acid score
LC-MS/MS	Liquid chromatography-high resolution tandem MS
MAPE	mean absolute percentage error
PDCAAS	protein digestibility-corrected amino acid score
PPI	pea protein isolate
PPC	pea protein concentrate
RPI	rapeseed protein isolate
RPI-HT	rapeseed protein isolate heat-treated
SPI	soy protein isolate
TAA	total amino acid

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2026.101311.

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