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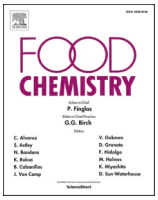

In vitro digestibility of dietary proteins and *in vitro* DIAAS analytical workflow based on the INFOGEST static protocol and its validation with *in vivo* data

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ABSTRACT

The FAO recommends the digestible indispensable amino acid score (DIAAS) to determine protein quality in foods, preferably tested *in vivo*. Here, the INFOGEST *in vitro* digestion protocol was applied and supplemented with an analytical workflow allowing the assessment of protein digestibility and DIAAS calculation. The protocol was applied to selected samples WPI, zein, collagen, black beans, pigeon peas, All-Bran®, and peanuts. The total protein digestibility, digestibility of individual amino acids (AA), and DIAAS values were established and compared with *in vivo* data for the same substrates. Total protein digestibility (total Nitrogen, $r = 0.7$, $P < 0.05$; primary amines (OPA), $r = 0.6$, $P < 0.02$; total AA, $r = 0.6$, $P < 0.02$) and digestibility of individual AA ($r = 0.6$, $P < 0.0001$) were in good agreement, between *in vitro* and *in vivo*, with a mean difference of 1.2 %. *In vitro* DIAAS was highly correlated with DIAAS obtained from *in vivo* true ileal digestibility values ($r = 0.96$, $R^2 = 0.89$, $P < 0.0001$) with a mean difference of 0.1 %.

1. Introduction

The nutritional quality of a protein depends on its amino acid composition, on the associated amino acid requirements, and on the digestibility of the amino acids in the upper gastrointestinal tract (Bessada, Barreira, & Oliveira, 2019; Havenaar et al., 2016). Digestibility of amino acids is the release of free amino acids from proteins and peptides (Stein, Sève, Fuller, Moughan, & de Lange, 2007). In 2013, the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) recommended the Digestible Indispensable Amino Acid Score (DIAAS), which is based on the true ileal digestibility of each indispensable amino acid (FAO, 2013) for protein quality evaluation. DIAAS replaces the previous protein digestibility corrected amino acid score (PDCAAS) method (FAO, 1991).

Ideally, the true ileal protein digestibility of foods should be determined in humans; for example, by sampling via a naso-ileal tube (Moughan & Wolfe, 2019). However, this approach is not compatible with the practical and ethical limits for routine studies; therefore, the current recommendation is to use ileum-fistulated growing pigs (Hodgkinson, Stein, de Vries, Hendriks, & Moughan, 2020) or growing rats (Moughan & Wolfe, 2019) as animal models. The growing pig model has recently been validated as a suitable *in vivo* model to establish

Abbreviations: AAA, aromatic amino acids; A, amino acids; DIAA, digestible indispensable amino acid; DIAAR, digestible indispensable amino acid ratio; DIAAS, digestible indispensable amino acid score, (lowest DIAAR); proxy *in vitro* DIAAR, DIAAR based on total digestibility; FAO, Food and Agriculture Organization of the United Nations; GLU, Glutamic acid; HPLC, High-performance liquid chromatography; IAA, indispensable amino acids; IVD, *In vitro* digestion; LC–MS, Liquid chromatography-mass spectrometry; MS, Mass spectrometry; OPA, o-phthalaldehyde; PDCAAS, protein digestibility-corrected amino acid scores; RT, Room temperature; SAA, sulphur containing amino acid; SD, standard deviation; *SEC*, size exclusion chromatography; SEM, standard error of the mean; TAA, Total amino acids; UHPLC, Ultra-high-performance liquid chromatography; UV/VIS, Ultraviolet–visible; WPI, Whey protein isolate.

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human DIAAS values using substrates with expected variable digestibilities, as a first goal of the PROTEOS project. This ongoing international scientific collaboration, coordinated by the Global Dairy Platform and financed by a consortium of food companies and sectors, aims at the implementation of the DIAAS measure by establishing a large data set of AA digestibilities for human foods (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). These invasive animal studies however are costly and raise ethical issues, and a reproducible and validated *in vitro* protocol would be useful, especially for screening purposes in the development of novel foods or for testing specialty (e.g. vegan) food products. This is supported by the FAO/WHO, who specifically recommended the development and validation of *in vitro* methods for predicting true ileal amino acid digestibility and bioavailability in humans (FAO, 2013).

The aims of the present work were first to establish an *in vitro* workflow based on the static INFOGEST *in vitro* digestion protocol that would allow the determination of predicted true ileal protein digestibility at the level of individual amino acids and the calculation of *in vitro* DIAAS values; and second to compare the *in vitro* values obtained with *in vivo* digestibilities for seven protein sources for which *in vivo* protein digestibility values are available (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b; Rutherfurd, Fanning, Miller, & Moughan, 2014).

The static model of *in vitro* digestion was developed in 2014 by the COST Action INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014), with the aim of closely mimicking human physiology, and was validated for its biological relevance in the case of protein with milk proteins fed to the growing pig as the animal model (Egger et al., 2017), and by comparison with human jejunal effluents (Sanchón et al., 2018). Until now however, the INFOGEST protocol has lacked of a reproducible experimental and analytical workflow allowing a quantitative analysis of protein digestibility and subsequent determination of *in vitro* digestible indispensable amino acid ratio (DIAAR) and digestible indispensable amino acid score (DIAAS). DIAAR refers to the ratio of the absorbed indispensable AA represented relative to the requirements, and DIAAS is the lowest DIAAR of a protein, also called the limiting AA. Describing such a workflow was the first aim of this study.

Furthermore, the same seven protein sources as used for the validation of the pig model (PROTEOS project), were assessed here for total protein *in vitro* digestibility, *in vitro* digestibility of individual AA, and *in vitro* DIAAS values and were compared to the *in vivo* values (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). These protein sources included four foods (wheat bran cereal [All-Bran®], pigeon peas, black beans, and peanuts) and three isolated proteins (zein, whey protein isolated [WPI], and collagen) (Sousa, Portmann, Dubois, Recio, & Egger, 2020).

Moreover, because the analysis of individual amino acids is costly and not universally available for every-one, an approximation of DIAAR and DIAAS (proxy *in vitro* DIAAR and DIAAS), based on total protein *in vitro* digestibility (using total Nitrogen or OPA) was calculated for the same seven substrates and compared to the *in vivo* DIAAR and DIAAS values. This approximation is an improvement on the previous method of Protein Digestibility Corrected Amino Acid Score (PDCAAS, (FAO, 1991)), as it is based on simulated ileal digestibility as opposed to fecal digestibility. The proxy DIAAS was calculated in a similar way to the PDCAAS-like values (based on the standardized total tract digestibility (STTD) of crude protein (CP)), as reported by Mathai et al. (Mathai, Liu, & Stein, 2017). The proxy DIAAS could replace it as a faster screening method.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and enzymes used in the present study were purchased from MERCK (Zug, Switzerland).

2.2. Sample preparation for in vitro digestion (IVD)

The seven protein sources (three isolated proteins [zein, WPI, and collagen] and four foods [peanuts, All-Bran® wheat bran cereal, pigeon peas, and black beans]; Supplemental Table 1) were prepared and characterized as previously described (Sousa et al., 2020) prior to IVD according to the static INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). Briefly, to simulate real ingested foods, 40 g of black beans and pigeon peas were soaked overnight (18 h), cooked (200 mL of water, 288 mg salt) for 20 min (black beans) or 10 min (pigeon peas), and disintegrated to an approximate particle size of 2–3 mm using a fork. The All-Bran® and peanuts were ground with mortar and pestle (particle size 2–3 mm) before IVD, while collagen, WPI, and zein were used without further preparation. As a blank digestion, a protein-free cookie (Moughan, Butts, van Wijk, Rowan, & Reynolds, 2005), containing only fat and carbohydrates was digested in parallel to the test foods, as previously described (Sousa et al., 2020). The cookie was prepared from 40.8 g purified corn starch, 15.7 g sucrose 4.9 g cellulose, 0.7 g baking powder, 0.5 g ground ginger and 36.9 g margarine and baked at 175 °C in portions of ~ 35 g for 30 min. All cookie ingredients were bought at a local supermarket (Coop, Switzerland), except the cellulose (Merck, Zug, Switzerland). For IVD, the cookie was disintegrated manually to crumbs of 2–3 mm in size. The protein sources were normalized according to their protein content, and 0.04 g of total protein (based on total nitrogen multiplied by a conversion factor of 6.25 (FAO, 2013)) per gram of food were used for *in vitro* digestion. The influence of other nutrients on *in vitro* digestion of protein was tested by mixing the collagen, WPI, and zein (normalized to 0.04 g protein) with different quantities of the ground cookie (0.1, 0.2, 0.25, 0.5 g) to simulate a meal composition, as previously described (Moughan et al., 2005).

2.3. Pancreatin suspension preparation

It was found that pancreatin formed a suspension with undissolved particles, resulting in non-reproducible measurements. Therefore, some modifications were made to the INFOGEST IVD protocol to improve the repeatability of the measurements. Trypsin activity was measured according to the previous protocol (Brodkorb et al., 2019) using a pancreatin suspension that was prepared as follows: Shortly before the digestion experiment (or for activity measurement), the pancreatin was first suspended by mixing in simulated intestinal fluid at a concentration of 100 U trypsin activity/mL of digest, then vortexed for 10 s, followed by ultrasound treatment (45 Hz, 130 W) at room temperature for 5 min. Thereafter, the suspension was centrifuged (2000 g, at RT, for 5 min), and the supernatant was transferred into a new tube, placed on ice, and immediately used for the digestion experiment (or for trypsin activity measurements). For the digestion experiment, the trypsin activity of the pancreatin supernatant (according to the here described preparation) was adjusted to 100 U/ mL of digesta. The impact of ultrasound and centrifugation on pancreatin activity was tested and no statistical difference was found (data not shown).

2.4. In vitro digestion with the INFOGEST static model for protein digestibility assessment

For calculation of protein digestibility, it is necessary to monitor the weights of the different fractions of food and cookie digests at different time points during the experiment: W1 = total digest, including MeOH, W2 = weight of the dried pellet, W3 = weight of the supernatant (including MeOH, = W1-W2), therefore at the very beginning of the digestion experiment, the weight of each individual tube (with cap) is monitored. Enzyme activities and bile concentrations were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). All digestion experiments were performed with the same batch of enzymes, with an amylase activity of 51.55 U/mg (Sigma-

Aldrich, Lot SLCD1111), pepsin activity of 3368 U/mg (Sigma-Aldrich, Lot SLBW6530), trypsin activity in pancreatin of 6.6 U/mg (Sigma-Aldrich, Lot SLCD7175), and a bile acid concentration of 0.72 mmol/g (Sigma-Aldrich, Lot SLBT0867). All substrates were digested *in vitro* using the INFOGEST protocol (Minekus et al., 2014) with the above described adaptation for pancreatin solubilization. In brief, the substrates were disintegrated, normalized to 0.04 g protein, and diluted with 1 mL of water. Previous experiments with higher protein amounts led to lower digestibility of specific substrates and higher variability in experiments (data not shown). Therefore, in order to ensure that the digestive enzymes were in excess, the digestions were performed with 0.04 g of protein in 1 g of food as previously shown with SMP (Egger et al., 2017). 1 mL of the prepared substrates were mixed with 1 mL simulated salivary fluid (pH 7, 37 °C) containing amylase (300 U/mL of digesta), for 2 min. A 2 mL volume of simulated gastric juice (pH 3, 37 °C) containing pepsin (2000 U/mL of digesta) was then added and incubated at 37 °C for 120 min. A 4 mL volume of simulated intestinal juice (pH 7, 37 °C) containing pancreatin (100 U trypsin activity/mL of total digesta) and bile (10 mmol/L of total digesta) was then added and incubated at 37 °C for 120 min. The entire digestion was performed under constant gentle mixing (16 rpm) on a rotating wheel (Bibby Scientific™ Stuart™ Rotator SB3). Digestion was stopped after 120 min of gastric digestion by increasing the pH to 7 with NaOH (1 mol/L) and adding the protease inhibitor 4-(2 aminoethyl) benzensulfonylfluoride (AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland) after 120 min of the intestinal phase. All the samples were immediately snap frozen in liquid nitrogen. For each set of samples digested in each experiment, a protein-free enzyme blank (cookie) was digested in parallel (Fig. 1). Each set of seven different substrates plus one cookie was at least performed in three individual experiments, yielding at least three independent values (n = 3).

2.5. Sample separation into digestible and indigestible fractions

After defrosting, the digested samples were separated into digestible (potential absorbable) and un-digestible (non-absorbable) fractions by precipitation with MeOH (80 % (v/v), final concentration) at −20 °C for 1 h and subsequent centrifugation (2000*g* at 4 °C for 15 min). For both the foods and cookie blank, the supernatants (food supernatant: Fs, cookie supernatant: Cs) were collected in new tubes without taking the interface (a representative aliquot of the total), and the pellets (food pellet: Fp, cookie pellet: Cp) were washed twice with MeOH (100 %), centrifuged between the washing steps (2000 g at 4 °C for 5 min), and then dried (the pellet was used as whole and not separated in aliquots) in a CentriVap (Labconco, Kansas City, Missouri USA) (Fig. 1). The weights of the tubes (with lid), the total digests with added MeOH (Fs, Cs plus MeOH in gram), and the dried pellets (Cp, Fp in gram) were monitored to allow the calculation of protein digestibility at the level of individual AA at the end of the experiment. Amino acids recorded from the cookie blank were considered enzyme background: values below background (due to analytical bias) were set to zero.

2.6. High performance size exclusion chromatography (SEC) for determination of the bioavailable fraction

In order to calculate the protein digestibility at the level of individual AA, the potential bioavailable fraction needed to be separated from the indigestible part. Therefore, after the intestinal phase of IVD, the digesta from the food or cookie were precipitated with MeOH (80 %), centrifuged, and separated into a supernatant (S) and a pellet (P). The peptide size distribution in both fractions was verified by a *SEC* determination, as described previously (Johns et al., 2011). The *SEC* system was calibrated with 13 molecular mass markers (Supplemental Table 1, Supplemental Fig. 1). Based on the standards, the molecular weight (x-axis, Da) was plotted against the retention time (y-axis, min) and a logarithmic (ln) regression curve was generated, to illustrate their dependence (Supplemental Fig. 2). According to this equation, the retention time of 40 min corresponded to a molecular weight slightly above 1000 Da, which was between the peptide standards [D-Ala^2]-Deltrophin II (7 AA, 782.88 Da) and Angiotensin II (8 AA, 1046.18 Da). The non-precipitated digesta, supernatant, and the pellet resulting from the MeOH (80 %) precipitation were injected onto a Superdex Peptide 10/300 GL high performance gel filtration column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mobile phase was H_2O/ACN/TFA (70/30/0.1 (v/v/v)) at a flow rate of 0.35 mL/min. The detection wavelength was 205 nm and the injection volumes were 10 µL for supernatant and pellet. The solid samples were resuspended in the mobile phase for injection. The cookie and a water blank were analyzed for background characterization (Supplemental Fig. 2).

2.7. Analysis of total nitrogen by Kjeldahl in foods and digesta (pellet and supernatant)

The total nitrogen present in the foods was quantified using the Kjeldahl method, according to ISO 8968–3:2007/IDF 20–3: 2007 (ISO 8968-3, 2007). The same method was used for total nitrogen in the pellet (P), and in the supernatant (S) after precipitation with MeOH 80 %. Solid pellet samples were quantitatively solubilized by addition of 2 mL of H_2SO_4 (96 %), followed by vigorous mixing for 1 min, subsequent addition of 2 mL of H_2O_2, and was again followed by vigorous mixing until complete solubilization (Foods and Cookie: 1 min; WPI and Collagen 5–10 min; Zein 20 min). The addition of H_2O_2 leads to an exothermic reaction with foam production. In order to avoid sample loss, large vials were used.

2.8. Acid hydrolysis

In order to obtain quantitative results of the total amino acid residues

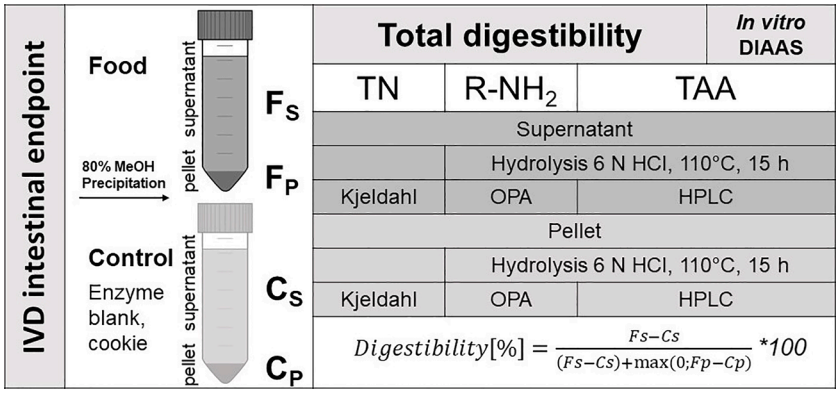


Fig. 1. Sample preparation work flow. One protein-free cookie was digested in parallel with one or more foods in a set. After intestinal digestion, MeOH precipitation, and centrifugation, the samples were separated into supernatant (S) and pellet (P) and treated independently for analysis. Three different analytical endpoints were performed: Total N (TN) with Kjeldahl, total free primary amines (R-NH_2) with o-phthalaldehyde (OPA), and individual amino acids with HPLC. For OPA and HPLC, an acidic hydrolysis with 6 N HCl was performed for 15 h prior to analysis. Digestibilities were calculated with the same formula for all three methods. Fs = food supernatant, Fp = food pellet, Cs = cookie supernatant, Cp = cookie pellet.

present in the sample as small peptides, the samples were subjected to acid hydrolysis with 6 mol/L HCl prior to the total amino acid (TAA) and total amino group (OPA) analyses. Briefly, 220 μL of the supernatant was dried in glass vials in a CentriVap (Labconco, Kansas City, Missouri USA) and resuspended in 220 μL H_2O, 120 μL 3,3′-dithiodipropionic acid (DDP)/0.1 % NaOH (0.2 mol/L), 120 μL HCl (0.2 mol/L), 40 μL norvaline (NVa; 10 mmol/L), and 500 μL HCl (37 %). The whole digesta pellet was directly weighed into a vial and resuspended with 1760 μL H_2O, 960 μL DDP 0.1 %/NaOH (0.2 mol/L), 960 μL HCl (0.2 mol/L), 320 μL NVa (10 mmol/L) and 4 mL HCl (37 %). All the samples were placed in a 110 °C oven for 15 h.

2.9. Quantification of total amino groups (R-NH_2, OPA method)

The total amino groups (R-NH_2) in the supernatant and pellets of the precipitated samples after acid hydrolysis (section 2.8) were measured using the o-phthalaldehyde (OPA) method. In brief, the samples were diluted 10 times with perchloric acid (0.5 mol/L) to precipitate proteins and longer peptides. After derivatization with OPA and in the presence of 2-mercapto-ethansulfonic acid, the resultant 1-alkylthio-2-alcylisonindol compounds were measured by UV/VIS photometry at 340 nm. The results were calculated based on a glutamic acid standard curve (Kopf-Bolanz et al., 2012). A protein-free cookie (blank digestion) was used as a measure of background.

2.10. Determination of individual amino acids in substrates

Sample preparation was based on a documented procedure (Waters, 2007). Briefly, hydrolyzed samples were obtained after 24 h of acid hydrolysis (HCl 6, mol/L) at 110 °C. Then, 100 μL of the hydrolysate was evaporated in a Speed-Vac (UVS 400A, Savant) for 2 h and reconstituted with 20 mmol/L HCl, which contained α-aminobutyric acid (AAbA) as the internal standard. To convert the amino acids into highly stable derivatives, 20 μL of the reconstituted sample was added to 60 μL of borate buffer and 20 μL of the derivatization reagent (6-aminoquinolyl-Nhydroxysuccinimidyl carbamate). The amino acid profile was analyzed using a Vanquish UHPLC system (Thermo Fisher Scientific, Reinach, Switzerland) equipped with an ultraviolet (UV) detector. Chromatographic separation was carried out on an AccQ-Tag Ultra analytical column (2.1 × 100 mm, 1.7 μm) (Waters, Baden, Switzerland) using the mobile phase and gradient described by Waters (Waters, 2007). Chromatographic conditions were: a flow rate of 0.7 mL/min, an injection volume of 0.5 μL a column temperature of 55 °C and and UV chromatograms were recorded at 260 nm. Cysteine and methionine were oxidized before hydrolysis, by adding to 1 g of sample 5 mL of oxidation solution (78 % formic acid, 3 % H_2O_2, 54 mmol/L phenol), followed by an incubation at 0 °C for 16 h. The reaction was stopped by addition of an excess of sodium disulfite (e.g. 0.84 g to 1 g of sample). Tryptophan content was quantified by HPLC (LC 1290 Infinity II LC System, Agilent Technologies, USA) after alkaline hydrolysis, according to ISO 13,904 (ISO/DIS-13904, 2014). Briefly, to 250 mg of sample, 8.4 g of $Ba(OH)_2$, then 16 mL of H_2O were added and gently mixed. The screw cap tubes were not completely closed, so as to avoid pressure during hydrolysis. The tubes were placed in a water bath at 110 °C for 20 h, samples were cooled to 80 °C, then 30 mL of H_2O, 2 mL of internal standard (α-méthyl-DL-tryptophane, Sigma M8377-1G), 0.5 gm/mL) and 13 mL of acid mix (0.16 mol/L phosphoric acid, 3 mol/L HCl) were added, samples were mixed. pH was adjusted to 3.25 with HCl (1 mol/L) or NaOH (1 mol/L), 35 mL of MeOH (43 %) were added and completed to 100 mL, the samples were filtered through a filter (595 ½, 150 mm, Schleicher&-Schuell 311645) prior to analysis. Chromatographic separation was carried out on a Lichrospher 100–5 RP-18 CC column. Analysis conditions were a flow rate of 0.8 mL/min, an injection volume of 5 μL a column temperature of 40 °C, time of 11 min, and fluorescence settings ex: 274 nm, em: 356 nm. All analyses were done with at least two technical replicates.

2.11. Determination of individual amino acids of in vitro digesta

The TAA in the *in vitro* intestinal digests were analyzed with the adapted AOAC method 2018.06 for infant formula (Jaudzems, Guthrie, Lahrichi, & Fuerer, 2019). Briefly, after acid hydrolysis (Section 2.8), all the samples were derivatized with AccQ-Tag Ultra reagent (Waters, 2007), and the amino acid profile was determined by ultra-high-performance liquid chromatography (UHPLC) (Acquity UPLC BEH C18 2.1 × 150 mm, 1.7 μm, Waters, Baden, Switzerland) coupled with a UV detector (Vanquish, Thermo Scientific, Reinach, Switzerland). The UHPLC conditions were as follows: 2 μL injection volume, column temperature of 50 °C, UV detection at 260 nm, and a flow rate of 0.4 mL/min.

2.12. In vitro total digestibility, DIAAR, DIAAS, and proxy DIAAS calculation

Total digestibilities and digestibility of individual AA of the *in vitro* digested substrates were calculated by dividing the digestible part (supernatant) of the food through the total of the food (supernatant + pellet), by using the formula (1) also shown in Fig. 1.

$$\textit{in vitro}\ \text{digestibility}\ [\%] = (\text{Fs} - \text{Cs}) / ((\text{Fs} - \text{Cs}) + \max(0; \text{Fp} - \text{Cp})) \times 100 \quad (1)$$

Fs = Food supernatant, Cs = Cookie supernatant, Fp = Food pellet, Cp = Cookie pellet.

The same formula (1) is used for calculation of total digestibility and digestibility of individual AA, by considering the total amounts of N (mg), R-NH_2 (mmol glutamic acid equivalents), total AA (mg), or individual AA (mg), in the supernatants of food (Fs) and cookie (Cs) (=W3, according to section 2.4); and pellets of food (Fp) and cookie (Cp) (=W2, according to section 2.4), accounting for all dilution steps performed during the analysis. Depending on the analysis, either N, R-NH_2, total AA, or individual AA in the supernatant (Cs) and pellet (Cp) of the protein-free cookie, corresponding to the enzyme background, were subtracted from the corresponding fractions (Fs and Fp) of the food digests to account for the autolysis of the digestive enzymes. In highly digestible foods, the pellet of the foods (Fp) only contains digestive enzymes (and is equal to the cookie pellet, Cp) and it could occur that due to analytical bias, the term Fp-Cp turns negative. In order to avoid negative values, the minimal value of this term was set to 0, which is expressed by: max(0;Fp-Cp) and results in a digestibility of 100 % for the corresponding food.

The *in vitro* digestible indispensable amino acid (*in vitro* DIAA) per gram of food protein was calculated according to the formula 2):

$$\textit{in vitro}\ \text{DIAA} = \text{mg of IAA per g of food protein} \times \textit{in vitro}\ \text{digestibility of IAA} \quad (2)$$

The *in vitro* DIAAR per gram of food protein was calculated, considering the reference protein for preschool children (6 month to 3 years), given by FAO (FAO, 2013), according to the formula 3):

$$\textit{invitro}\text{DIAAR}(\%) = 100 \times \frac{\text{mg of } \textit{invitro}\text{ DIAA in 1 g of dietary protein}}{\text{mg of the same dietary IAA in 1 g of the reference protein}} \quad (3)$$

The DIAAR was calculated for all individual indispensable amino acids, and the DIAAS of a food is the lowest DIAAR as defined by FAO (FAO, 2013).

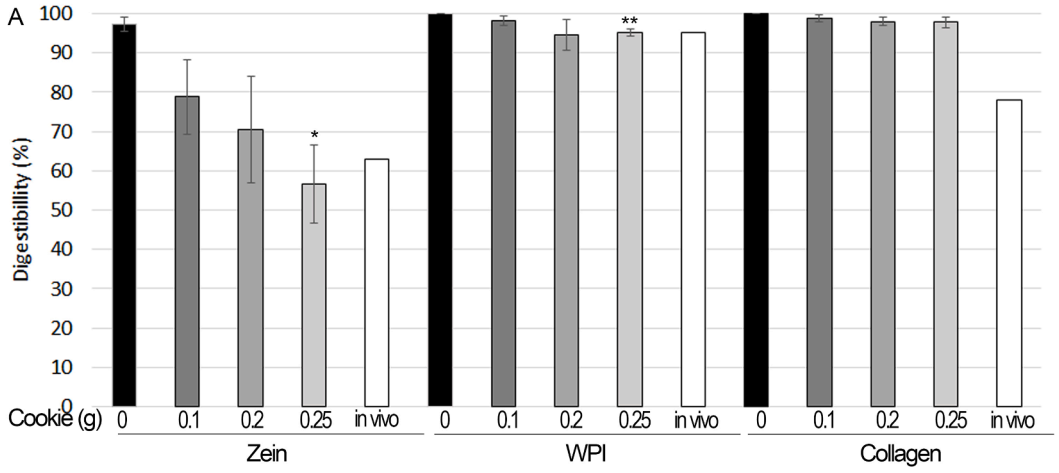
Proxy *in vitro* DIAAR values were determined in a similar way as previously described (Mathai et al., 2017), by using the total *in vitro* ileal digestibility obtained by TN, OPA, or TAA analysis (Formula Fig. 1), instead of the standardized total tract digestibility (STTD, %). As a reference protein, the FAO recommendation for preschool children (6 month to 3 years) was considered (FAO, 2013), values higher than 100 % were not truncated to 100 %.

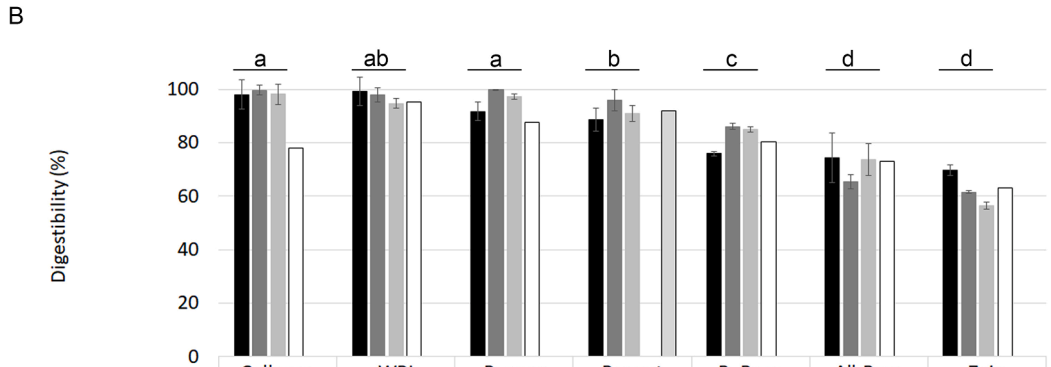
Proxy *in vitro* DIAA: For each indispensable amino acid, the proxy *in vitro* digestible indispensable amino acid (proxy DIAA) per gram of food was calculated by multiplying the mg of indispensable amino acid per g of food protein by the total ileal *in vitro* digestibility value, according to the formula (4):

$$\text{proxy } \textit{in vitro} \text{ DIAA} = \text{mg of IAA per g of food protein} \times \text{total } \textit{in vitro} \text{ digestibility} \quad (4)$$

The proxy *in vitro* DIAAR per gram of food protein was calculated, considering the reference protein for preschool children (6 month to 3 years), given by FAO (FAO, 2013), according to the formula (5):

$$\text{proxy } \textit{in vitro} \text{ DIAAR}\,(\%) = 100 \times \frac{\text{mg of proxy } \textit{in vitro} \text{ DIAA in 1 g of dietary protein}}{\text{mg of the same dietary IAA in 1 g of the reference protein}} \quad (5)$$





	Collagen	WPI	P. peas	Peanut	B. Bean	All-Bran	Zein
TN	98	99	92	89	76	74	70
OPA	100	98	100	96	86	65	62
TAA	98	95	97	91	85	74	57
in vivo hum/pig	78	95	88		80	73	63
in vivo rat				92			

Fig. 2. Total Protein digestibility of the pure proteins (zein, whey protein isolate [WPI], and collagen) in the absence (0) or presence of increasing amounts (0.1, 0.2, 0.25 g) of protein-free cookie. *In vivo*: mean digestibility in humans and pigs (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). Significant differences ($P < 0.05$) are indicated with * for zein plus 0.25 g of cookie and ** for WPI plus 0.25 g of cookie, respectively (A). Mean values of total protein digestibility based on released TN (Kjeldahl), R-NH_2 (OPA), and TAA (HPLC). The *in vitro* results were compared with *in vivo* (mean human and pig values) results from the PROTEOS study (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b) and rat values for peanuts (Rutherfurd, Fanning, et al., 2014). The different letters indicate significant differences in average digestibilities considering the average of all three methods, where a to b: $P < 0.05$, a, b to c: $P < 0.01$, and a, b to d: $P < 0.0001$ (B). Error bars represent SEM of $N \leq 3$ experiments, (A, B). Method comparisons between *in vitro* and *in vivo* results, according to previous work (Bland & Altman, 1986) showing the average digestibility (x-axis) versus the differences in total digestibilities (y-axis) of all individual amino acids of the seven substrates. The mean bias between methods was 1.2 %, and the upper and lower limits indicate ± 2 * SD of the average difference (C).

2.13. Statistical evaluation for the comparison between in vitro and in vivo results

The *in vitro* experiments were performed with 0.04 g of protein in 1 g of food. In order to ensure that this amount represented the whole food, a minimum of three replications at different days were performed per substrate. In the *in vivo* experiments performed in the frame of the PROTEOS project (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), no statistical differences were observed between the human and the pig model. Therefore, as the best estimate for true ileal digestibility, the average values of the pig and human results were used for the comparison with *in vitro* digestibilities analyzed in this work. These average *in vivo* results were statistically compared to *in vitro* results by a Bland-Altman graphical representation (Bland & Altman, 1986). The average of the two methods are plotted on the x-axis and the difference between the two methods on the y-axis. The average difference between methods,

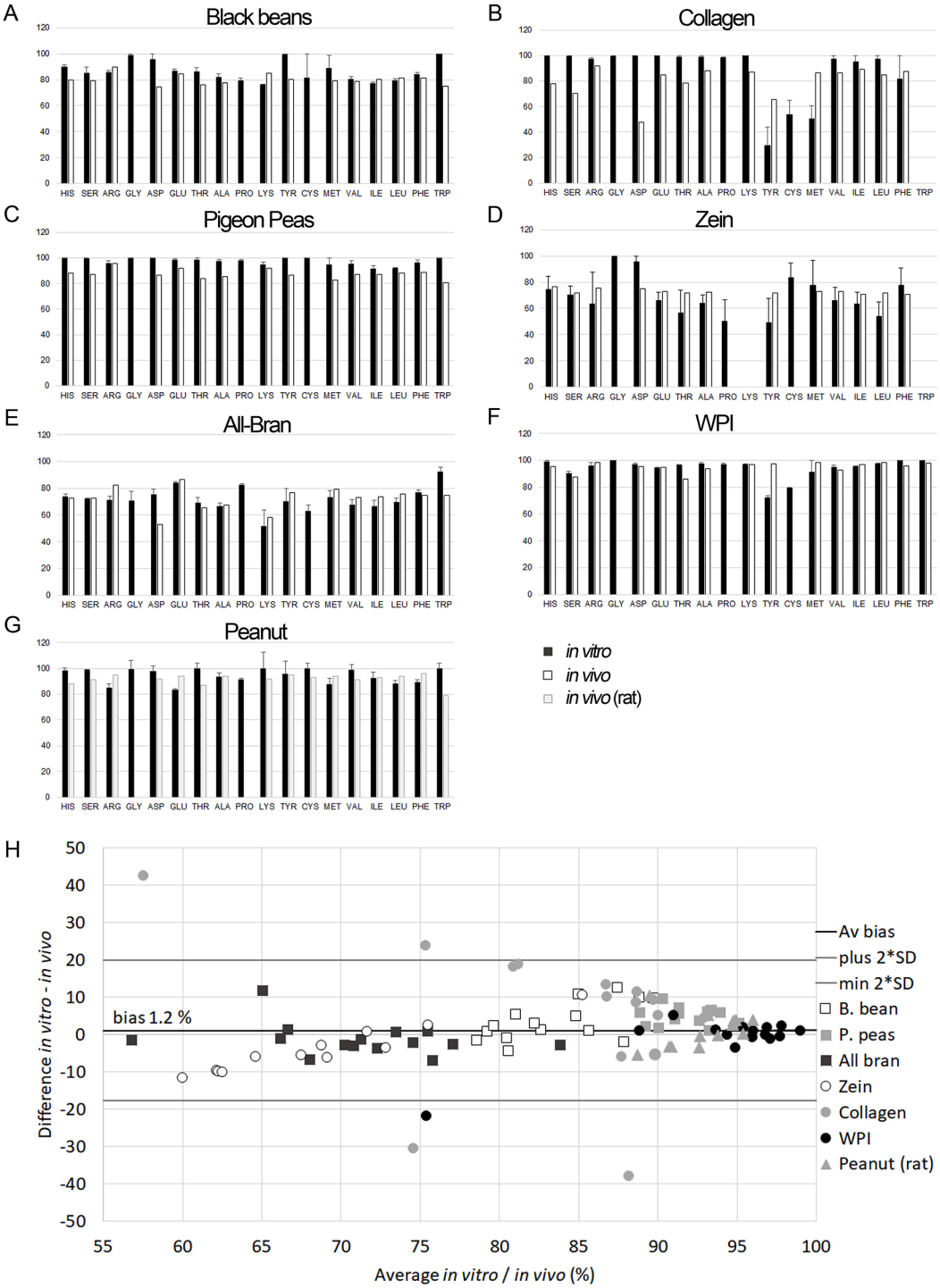


Fig. 3. *In vitro* digestibility of individual amino acids (black) after IVD compared with *in vivo* data for B. bean (A), Collagen (B), Pigeon pea (C), Zein (D), All-Bran (E), WPI (F) (mean pig and human values, white) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), or Peanut (G) (rat, light grey). Error bars are SEM of three individual *in vitro* experiments.

called bias indicates the expected difference between the methods and the upper and lower limits are ± 2*SD of the differences between the methods. Correlation coefficients (r) and P values for the comparison of *in vitro* versus *in vivo* for total digestibility (Fig. 2B), individual AA (Fig. 3H), and DIAAR (Fig. 5A) were calculated in Excel performing a linear regression with the Analysis ToolPak. Statistical differences between the three analytical methods (TN, R-NH_2, and TAA) were calculated with ANOVA for repeated measures and total digestibilities of the different substrates were calculated by applying paired t-tests, using the Analysis ToolPak in Excel.

3. Results

3.1. General procedure for digestibility assessment based on the static INFOGEST protocol

After completion of the intestinal phase of the static INFOGEST protocol, all components, food and enzymes are present in the test tube. For quantitative assessment of digestibility, it was therefore necessary to subtract the background enzymes from the food proteins. This background enzyme material was determined by digesting a sample of protein-free cookie, in parallel with each set of food sources (Fig. 1). A cookie digest was preferred over a water blank digest because previous experiments with water had shown a high autolysis of enzymes in the absence of food substrate, which led to an underestimation of food digestion (data not shown). In consequence, together with each set of foods, a cookie digest was performed and weights were monitored as described (section 2.4). Moreover, calculation of protein digestibility required separation of the bioavailable fraction from the undigested proteins and longer peptides of the tested food, after completion of IVD. After testing different precipitation agents MeOH 80 % was selected because it yielded the highest recovery and the most reproducible results (data not shown). After precipitation and centrifugation the supernatant corresponding to the potential digestible fraction and the pellet containing the indigestible part were treated separately.

3.2. Characterization of the bioavailable fraction and enzyme background by SEC

After intestinal IVD, a protein precipitation with MeOH 80 % was performed to separate bioavailable from non-bioavailable material. Both fractions, supernatant and pellet were analyzed by *SEC* to verify the peptide size distribution. The majority of the peaks in the supernatant eluted after 40 min, corresponding to a molecular weight below 1000 Da, whereas the most intense peaks in the pellet eluted before 40 min. In the *SEC* chromatograms the limit of 1000 Da is indicated by the vertical line (Supplemental Fig. 3), located between angiotensin II (MW 1046 Da) and the peptide D-Ala^2-deltrophin (MW 783 Da), which was calculated with the equation established with the standards (Supplemental Fig. 1B). The *SEC* chromatogram of the cookie digest (Supplemental Fig. 3, cookie) was analyzed as a baseline for the digestive enzymes. An additional water digest confirmed that the main peak in the chromatogram of the cookie supernatant (Supplemental Fig. 2, cookie) was not from proteins of the digestive enzymes, because it was absent in the supernatant of the water digest which as well contained the same amount of enzymes (Supplemental Fig. 2).

3.3. Digestibility of protein and determination of individual amino acids

For the determination of individual AA and protein digestibility calculations, supernatants and pellets from MeOH precipitated intestinal digests were analyzed using three different analytical approaches: total nitrogen (TN) by Kjeldahl, total primary amines (R-NH_2) by OPA after acid hydrolysis, and AA by HPLC after acid hydrolysis. For all three analytical endpoints, total digestibility was calculated using the formula (1) given in Fig. 1 and described in section 2.12. The *in vitro* digestibility results were compared with *in vivo* digestibility data obtained from growing pigs cannulated at the terminal ileum (*T*-cannula) and from adult human ileostomates (PROTEOS project), for the same protein sources (same batch of material) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). In the PROTEOS study, there was no statistically significant effect of species ($P > 0.05$), therefore the *in vivo* values are means of the human and pig data (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). Digestibility of peanut protein was not reported in the PROTEOS *in vivo* study because of a high variability in digestibility most probably due to the large particle size found in the ileal digesta of the pigs. Therefore, instead, protein digestibility and DIAAS values established in a rat ileal digestibility model were taken for *in vivo* comparison for the peanut data (Rutherfurd, Fanning et al., 2014). Among the substrates, WPI, zein, and collagen, with different expected protein digestibilities, had been chosen for the *in vivo* experiments. No differences in digestibility were observed *in vitro,* as all three substrates were highly digestible when digested as pure proteins (Fig. 2A, bars 0 g of cookie). However, in the *in vivo studies,* the isolated proteins had not been given alone. These substrates had been combined with other macronutrients in order to be better accepted by the participants or pigs and to better mimic a whole meal. The pigs were fed additional fat, carbohydrates (starch and sugars), vitamins, and minerals in their diet, while the human subjects had received 25 g of the tested isolated proteins together with a protein-free cookie (Moughan et al., 2005). Therefore, in the present study, and in order to better simulate the "whole meal" *in vivo* conditions, the three isolated protein powders (0.04 g protein) were digested *in vitro* together with different amounts of the same protein-free cookie as used for the enzyme blanks (Fig. 2A). The results showed a clear decrease in the *in vitro* total protein digestibility of zein by 30 % ($P < 0.05$), a poorly soluble protein with a low *in vivo* digestibility in the presence of 0.25 g of cookie in the digestion. By contrast, digestibility of WPI decreased only by 3 % ($P < 0.05$) and collagen by 2 % (not significant), which were both highly digestible *in vivo,* remained high. Taking into account that 0.25 g protein-free cookie condition gave results that more closely resembled those of the *in vivo* assay, in all subsequent experiments, the isolated proteins (0.04 g) were combined with 0.25 g of protein-free cookie (Fig. 2A).

The *in vitro* digestibility of protein based on TN, R-NH_2 or TAA was subsequently calculated for all of the protein substrates. No significant differences were found between the three methods and the results were generally in line with the respective *in vivo* digestibilities obtained in the PROTEOS project in humans and pigs (Fig. 2B) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). Correlation coefficients (r) and P values were $r = 0.7$, $P < 0.05$ for total Nitrogen, $r = 0.6$, $P < 0.02$ for primary amines (OPA), and $r = 0.6$, $P < 0.02$ for total AA. The different substrates had different total digestibilities (as indicated with different letters in Fig. 2B) with average digestibilities over 96 % for WPI, Collagen, and P. peas; 92 % for Peanut; 71 % for All-Bran, and 64 % for Zein, respectively.

In addition, the results for analysis of TAA by HPLC were used to calculate the digestibilities based on all individual amino acids for each substrate, according to the formula (1) (section 2.12, Fig. 1); the *in vitro* digestibilities are given in Supplemental Table 3. For each substrate, *in vitro* values were compared to the *in vivo* mean of the human and pig values (or rats for peanut, respectively) (Fig. 3A-G). No *in vivo* digestibility data from humans and pigs were available for cysteine, glycine, and proline. A method comparison (Bland et al., 1986) was also performed to show the average *in vitro* and *in vivo* digestibilities (x-axis) versus the differences between *in vitro* and *in vivo* digestibilities (y-axis) for each individual amino acid for the seven substrates. The *in vitro* digestibilities were compared with data from humans and pigs (average digestibility) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b) or rats (triangles) (Rutherfurd, Fanning et al., 2014), resulting in a correlation of $r = 0.6$ and a P value $P < 0.0001$. The mean bias between methods was 1.2 % and the upper and lower limits indicated ± 2 * SD of the average difference between methods (Fig. 3H).

3.4. Calculation of *in vitro* DIAAR values and comparison with *in vivo* DIAAR

The DIAAR and DIAAS values based on using the *in vitro* AA digestibilities were calculated based on the digestibility of each individual indispensable amino acid, the amount of that AA in the food and the reference requirement values for that AA for the three age groups (FAO, 2013) (Supplemental Table 4). The *in vitro* values were compared to values based on *in vivo* true ileal digestibility data from pigs and humans (mean value across species, white bars) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), or rats (peanut) (Rutherfurd, Fanning et al., 2014) (Fig. 4A-G). The DIAAS, corresponding to the lowest *in vitro* and *in vivo* DIAA ratios for preschool children (6 month to 3 years) for each investigated substrate were in agreement between the different models, where available (*in vivo* SAA data are missing at this time point), and are listed in Supplemental Table 5. A correlation graphic of *in vitro* (x-axis) versus *in vivo* (y-axis) digestibility based DIAAR values was calculated by comparing the *in vitro* data for all indispensable amino acids in each substrate with *in vivo* data (mean values from human and pig) or rat (triangles) data for the age group of preschool children (6 month – 3 years), yielding a slope of 0.96 and an R^2 of 0.89 (Fig. 5A). For the same age group, the DIAAR values obtained *in vitro* were also compared statistically with *in vivo* data for each indispensable amino acid for all the substrates and were represented as a Bland-Altman graph (Bland et al.,

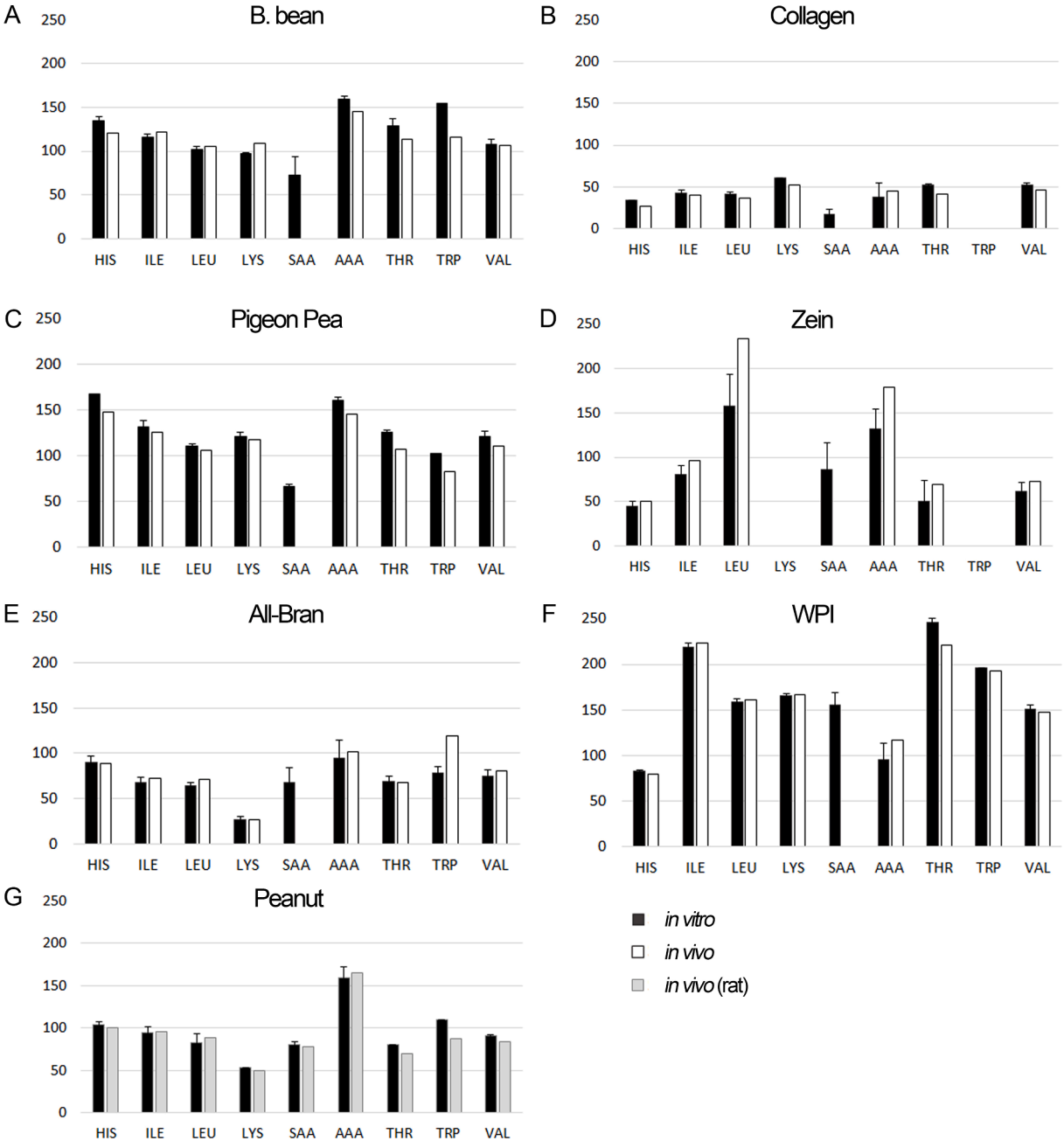


Fig. 4. *In vitro* AA digestibility based DIAAR values compared to *in vivo* AA digestibility based data from pigs and humans (average values, white) for black bean (A), collagen (B), pigeon pea (C), zein (D), All-Bran® wheat cereal (E), and whey protein isolate (WPI, F) (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), or from rats for peanut (G) (Rutherfurd, Fanning, et al., 2014). Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. Isolated proteins (collagen, zein, and WPI) were digested together with 0.25 g of a protein-free cookie. Samples were analyzed at least in triplicate, error bars represent SD (A-G).

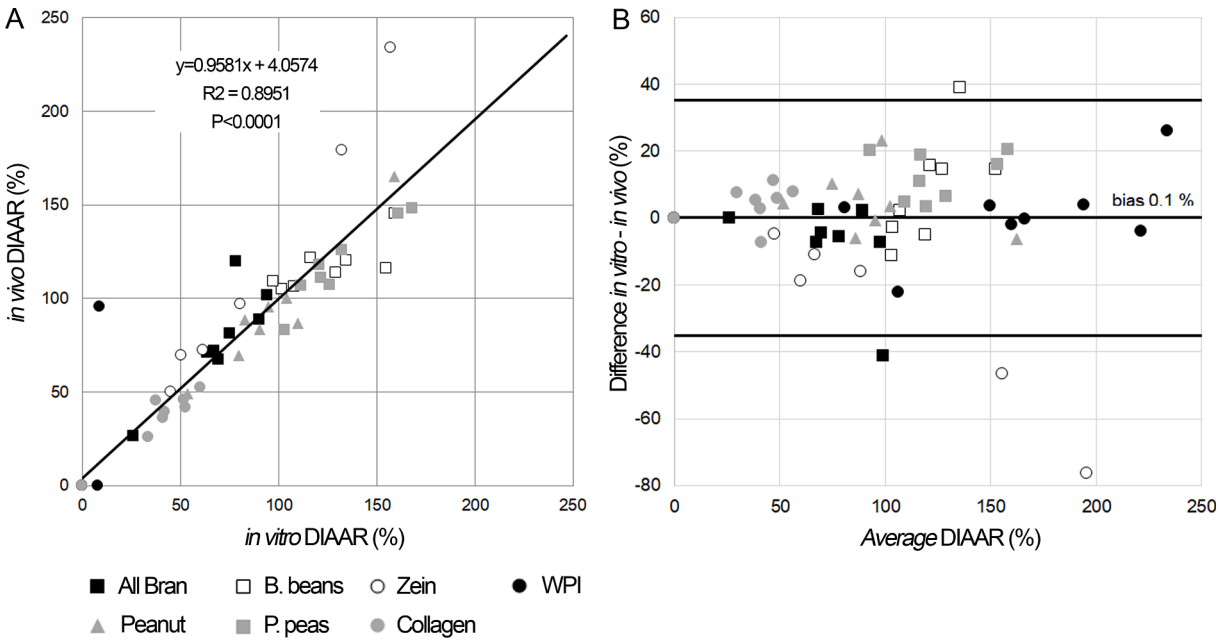


Fig. 5. Correlation of *in vitro* DIAAR values with average *in vivo* data from pigs and humans for black bean, pigeon pea, All-Bran® wheat cereal, collagen, zein, and WPI or from rats for peanut (Rutherfurd, Fanning, et al., 2014) (A). Statistical comparison between *in vitro* and *in vivo* DIAAR results (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), according to previous work (Bland & Altman, 1986), show the average DIAAR (x-axis) versus the differences between *in vitro* and *in vivo* DIAAR (y-axis) of all essential amino acids of the same comparisons, as described in Fig. 2C (B). The mean bias between methods was 0.1 % and upper and lower limits indicate ± 2 * SD of the average difference. Except for peanut, the comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values. (A, B).

1986) (Fig. 5B). The DIAAR for the indispensable AA for each substrate was represented on the x-axis versus the difference (*in vitro* – *in vivo*) on the y-axis. The average difference between the two methods (bias) was 0.1 %, indicated by the middle line and the upper and lower limits are ± 2 * SD of the average difference. The *in vivo* DIAA values for the sulphur containing amino acid cysteine were not available, therefore SAA values could not be reported and no comparisons were possible (Fig. 5). For the age group for older children, adolescents, and adults a similar method comparison resulted in a correlation slope of 0.94 and a R^2 of 0.89 between *in vitro* and *in vivo* DIAAR (Supplemental Fig. 4A) and an average bias of 0.4 % in the Bland-Altman graphic (Supplemental Fig. 4B).

3.5. Approximation of in vitro DIAAR based on total in vitro digestibility

An approximation of *in vitro* DIAAR based on total protein *in vitro* digestibility, therefore called proxy *in vitro* DIAAR, was calculated for the indispensable amino acids in a similar manner to that described by (Mathai et al., 2017), using the FAO reference protein for preschool children (FAO, 2013), with the following adjustment. Total *in vitro* digestibility values from all three analytical approaches (TAA, R-NH_2 (OPA), and TN) were used instead of standardized total tract digestibility (STTD) (Supplemental Table 5). All *in vitro* DIAAR values and proxy *in vitro* DIAAS values were compared with *in vivo* data (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b) (Supplemental Fig. 5), by calculation of correlation lines between *in vivo* DIAAR (mean human and pig) (y-axis) versus *in vitro* DIAAR (slope 0.96, R^2 0.89, white circles) or proxy *in vitro* DIAAR values, based on TAA (slope 1.02, R^2 0.95, black), OPA (slope 0.96, R^2 0.96, gray), or TN (slope 0.98, R^2 0.97, white) (x-axis), respectively (Supplemental Fig. 6).

4. Discussion

4.1. Definition and characterization of the bioavailable fraction

After *in vitro* digestion, all components of the digested foods as well as the enzymes remain in the test tube. This is unlike the *in vivo* situation, where the true ileal protein digestibility is determined by subtracting the total ingested food by the fraction remaining in the terminal ileum after absorption of free amino acids and small peptides through the brush border cells (Hodgkinson et al., 2020, Hodgkinson et al., 2022a; Hodgkinson et al., 2022b; Moughan & Wolfe, 2019). Moreover, the static *in vitro* model used here not only lacks the absorption step, but also lacks the brush border enzymes that are responsible for additional hydrolysis in the small intestine, where the final stage of peptide digestion takes place, by reducing poly- and oligopeptides to free amino acids, and di- and tripeptides (Holmes & Lobley, 1989). Therefore, one of the first goals for the development of the *in vitro* method for protein digestibility, was to separate the fraction that can be considered bioavailable after intestinal *in vitro* digestion from the indigestible part. *In vivo*, the size of the absorbed peptides is still debated and remains a matter of ongoing research (Ozorio et al., 2020; van der Wielen, Moughan, & Mensink, 2017; Wang, Xie, & Li, 2019). Protein digestion in the gut lumen, resulting in the release of free amino acids is reported to be incomplete, representing only 20–30 % of the total nitrogen at the level of the ileum (Adibi & Mercer, 1973; Santos-Hernández et al., 2020). Because the *in vitro* model lacks the brush border enzymes, the peptides in the digesta were expected to be longer than those found *in vivo* and therefore, the bioavailable fraction was considered as comprising free amino acids and peptides up to 8–10 AA. *In vivo*, these oligopeptides would be further degraded by the brush border enzymes and intra-cellularly, before uptake into the portal vein (absorption). To separate between the digestible and non-digestible fractions, after IVD, all the digesta were precipitated with MeOH 80 %. MeOH precipitation was selected for the separation of fractions, because it was the most reliable method, yielding the highest recovery and easy to evaporate. *SEC* was performed in both fractions (supernatant and pellet), as a verification for peptide length and the results for all substrates confirmed that in the supernatant, the main peaks eluted after 40 min, corresponded to peptides below 800–1000 Da, according to the standards used (e.g. angiotensin II, 8 AA). In contrast to this, the peaks in the pellet that eluted before that

time point, demonstrated that the separation used was efficient and reproducible. However, as to whether this applies for all substrates, especially for heated foods, where substantial amounts of "limit peptides" may be present (Rutherfurd, Montoya, & Moughan, 2014), remains to be verified.

An additional difficulty with the *in vitro* digestion models is the quantification of the background protein, which consists of enzymes. In the *in vivo* experiments within the PROTEOS project, the pigs received a protein-free diet, while the human subjects were fed a protein-free cookie, and the AA's measured at the terminal ileum after ingestion of these products were considered to represent the baseline for endogenous material. Consistent with this, a protein-free cookie was digested *in vitro* in parallel and used as an enzyme blank for all foods. For the low digestible Zein, the significant decrease ($P < 0.05$) in digestibility in presence of 0.25 g of cookie was 30 %, whereas for WPI it was only 3–4 %. The significant difference for WPI can be explained by the small standard deviation for WPI, compared to the higher variability in Zein, which is a less soluble substrate. The digestion of a water blank was also tested but without any substrate. In the latter case, the hydrolysis of proteins (i.e., those present in pancreatin) and autolysis of digestive enzymes was higher, leading to an underestimation of digestibility compared to the *in vivo* data (data not shown).

4.2. Digestibility of proteins and determination of individual amino acids

With the aim to develop a method being technically feasible at a lab scale and keeping experimental costs low, the IVD experiments were performed with 0.04 g of dietary protein corresponding to 1 g of food. To ensure that the results were representative for the specific food, each food was at least digested three times independently, resulting in a standard deviation for total digestibility between 4 and 8 % between replicates for all three analytical methods (TN, R-NH_2, and TAA). In order to further confirm the *in vitro* results for a specific substrate, a higher number of repetitions could be performed. After the intestinal digestion, three different analytical approaches were performed and used to calculate the digestibilities of total proteins and individual amino acids. The *in vitro* digestibility based on TN, primary amines (OPA, R-NH_2), and TAA showed similar tendencies compared to the equivalent *in vivo* digestibilities. Each of the methods has advantages, and selection of the preferred method depends on the substrate and the methods available in the particular laboratory. Analysis of TAA should be the method of choice because it allows the calculation of DIAAR for individual AA as recommended by FAO (FAO, 2013). The analysis of primary amines by OPA is more specific for amino acids and suited for substrates with expected high non-protein nitrogen content. The TN analysis is a standardized and highly automated method, accepted and broadly available around the world. A trend to overestimate the total digestibility of pure proteins was evident, as shown in Fig. 3A, where pure proteins without the cookie had a digestibility of 100 %. For the *in vivo* experiments, the pure proteins were combined with diets containing fat, carbohydrates, sugars, and other nutrients as used in the pig trials and with a protein-free cookie as used in the human experiments (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b). Consequently, this was simulated *in vitro* by adding the same protein-free cookie used as a baseline to the protein sources (Fig. 3A) to provide a closer simulation of the *in vivo* approach.

Interestingly, the digestibility of zein, a poorly soluble protein that had a significantly lower digestibility *in vivo* (Calvez et al., 2019; Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), was reduced by 30 % ($P < 0.05$) together with 0.25 g of cookie (Fig. 2A). This effect was much less pronounced for WPI (decrease in 3 %, $P < 0.05$) and collagen (decrease 2 %, not significant), where digestibilities remained high even in the presence of increasing amounts of cookie (Fig. 2A). Therefore, the *in vitro* digestion of pure proteins or ingredients should always be measured in combination with other nutrients representing a food or even a whole meal, as recommended in the protocol (Brodkorb et al., 2019).

In vitro digestibility of collagen overestimated the *in vivo* digestibility to some extent. Earlier studies with rats showed that collagen had a fecal digestibility of 95 % (Laser-Reuterswärd, Asp, Björck, & Ruderus, 1982). Possible explanations could be differences in the composition of the test meal (addition of other ingredients *in vivo*), influencing the pH of the meal, or differences in pH during the digestion process, impacting protein solubility and digestibility (Reuterswärd & Fabiansson, 1985). Therefore, for optimal comparison, the exact same meal composition should be selected. Nevertheless, evaluating all of the investigated substrates together showed a high comparability between *in vivo* and *in vitro* digestibilities of individual amino acids in the seven substrates, with an average bias of only 1.2 % according to the Bland-Altman comparison (Bland & Altman, 1986) (Fig. 2C).

4.3. In vitro DIAAR and proxy in vitro DIAAR comparability with in vivo experiments

The *in vitro* DIAAR values for all of the indispensable amino acids in the seven investigated substrates were highly correlated ($R^2 = 0.96$, $P = <0.0001$) with the *in vivo* data (Hodgkinson et al., 2022a; Hodgkinson et al., 2022b), with a mean bias between *in vitro* and *in vivo* data of 0.1 %, according to the Bland-Altman plot (Bland & Altman, 1986) (Fig. 4B). This analysis showed that > 95 % of all the data points lay between ± 2 * SD of the average of the two methods. The calculation of proxy *in vitro* DIAAR values with the adapted calculation from Mathai et al. (2017), using the total *in vitro* ileal digestibility instead of STTD could partially overcome earlier described limitations of the PDCAAS (Mathai et al., 2017; Schaafsma, 2012) (e.g., the overestimation due to consideration of fecal instead of ileal digestibility and the truncation to 100 % (FAO, 1991). However, our results confirm previous observations that proxy *in vitro* DIAAS, especially when based on TN or OPA values, are crude estimates (Mathai et al., 2017), neglecting differences in the digestibility of individual amino acids, and not considering the FAO recommendation to treat each indispensable amino acid as an individual nutrient (FAO, 2013). Moreover, and when an estimate of TAA is available, the calculation of *in vitro* DIAAR using the digestibilities of individual indispensable AA is preferred over the proxy DIAAR based on overall TAA digestibility.

5. Conclusion

The *in vitro* protein digestibility, *in vitro* DIAAR and *in vitro* DIAAS values of seven foods were established with the INFOGEST static IVD protocol and compared with results from human and pig *in vivo* experiments. Overall, the total *in vitro* digestibilities (TN: $r = 07$, $P < 0.05$; OPA: $r = 0.6$, $P < 0.02$; TAA: $r = 0.6$, $P < 0.02$) were in good agreement with the *in vivo* results. The *in vitro* digestibilities of individual AA ($r = 0.6$, $P < 0.0001$) and *in vitro* DIAAR ($r = 0.96$, $P = 6E\text{-}30$), were highly correlated with *in vivo* results, with a tendency toward overestimation with the *in vitro* approach. The digestibilities of pure proteins should be assessed in combination with other nutrients to simulate the digestion of a real food. The present data clearly show that the INFOGEST static IVD protocol has great potential as a tool for the calculation of protein digestibility at the level of individual AA needed to determine *in vitro* DIAAS values. However, at present, only seven substrates have been investigated. More *in vitro* and *in vivo* comparability data will be needed in the future to further validate this newly developed *in vitro* workflow and allow its implementation as a robust and reproducible method for digestibility and DIAAS predictions. The protocol also needs further testing with highly transformed products, such as extruded proteins or heated foods. Nevertheless, once broadly validated, this protocol could represent an ideal tool for the screening of new products and could be helpful for producers when evaluating and screening protein sources at the level of product development. An additional advantage of *in vitro* digestibility systems is their fast adaptation to conditions of special age

groups or health conditions, such as infants, the elderly, or health-impaired persons, for which *in vivo* studies are difficult due to ethical constraints.

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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134720.

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