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## Effect of processing on the protein digestibility and mineral bioavailability of legumes

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### ABSTRACT

This study evaluates how processing methods, soaking/cooking, fermentation (e.g. tempeh), and protein coagulation (e.g. tofu), affect the nutritional profile, protein digestibility, and mineral bioavailability of faba beans, grey peas, yellow peas, and soybeans. Protein digestibility was assessed using *in vitro* digestion and o-phthalaldehyde (OPA) assay, whilst mineral bioavailability was estimated using phytate-to-mineral molar ratios and further evaluated using a Caco-2/HT29-MTX co-culture model measuring ferritin formation. Processing markedly influenced nutritional properties. Protein coagulation resulted in the highest protein content and degree of hydrolysis, indicating improved protein digestibility. Fermentation substantially reduced phytate levels across all crops, in some cases below detection limits, leading to lower phytate-to-mineral ratios compared with cooked and tofu products. Consistent with these estimates, higher ferritin formation was observed in cells exposed to digested fermented products than to tofu digesta. Processing also altered amino acid composition, reflecting structural modifications of proteins.

Overall, the results demonstrate that processing modulates protein digestibility and mineral bioavailability of legumes. Fermentation shows potential to enhance mineral availability, whereas protein coagulation improves protein digestibility. These findings are based on *in vitro* and cell-based models and provide guidance for the development of nutritionally improved plant-based foods.

### 1. Introduction

Compared to animal-based foods, plant-based proteins typically possess lower protein quality and digestibility (Day, 2013; Herreman et al., 2020). Additionally, anti-nutritional factors such as phytate can impair the bioavailability of essential minerals such as iron and zinc (Reddy et al., 1982; Wang & Guo, 2021).

To address these limitations, various processing methods can improve protein digestibility (Joye, 2019; Nosworthy et al., 2017) and reduce phytate content, thereby enhancing mineral bioavailability (Lestienne et al., 2005; Zhang, Stockmann, Ng and Ajlouni, 2022).

Fermentation, as applied in tempeh production, is well known to reduce anti-nutritional factors, particularly phytate, and to improve

protein digestibility and mineral availability in soy (Kumar et al., 2010; Sutardi and Buckle, 1988) and other legumes (Eklund-Jonsson et al., 2008; Fernandez Castaneda et al., 2024; Nishinari et al., 2018; Purwandari et al., 2024). However, the extent to which these improvements occur in non-soy legumes, particularly pea- and grey pea-based products, remains underexplored, and comparative data across legume species are limited.

Protein coagulation into tofu represents another established processing strategy that increases protein concentration and creates a versatile plant-based product (Hammer et al., 2024; Nishinari et al., 2018). However, this process may also retain or concentrate phytates, potentially limiting mineral bioavailability (Chigwedere et al., 2023). While soy-based tofu is widely studied and serves as a benchmark, there is

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limited information on the nutritional consequences of applying tofu-type processing to alternative legumes, despite growing interest in reducing reliance on soy.

Therefore, understanding how fermentation and protein coagulation affect protein digestibility and mineral bioavailability of non-soy legumes is essential for diversifying and improving plant-based protein sources. Furthermore, incorporating alternative legumes supports dietary diversity, promotes regional crop use, and helps reduce over-reliance on soy (EPRS, 2024; Karkanis et al., 2018; Watson et al., 2017). Evaluating these processing effects provides quantitative evidence of the nutritional performance of regionally adapted legumes and their potential to serve as nutritionally adequate alternatives to soy-based products. In addition, few studies have systematically compared

fermentation and protein coagulation across multiple legume species within the same experimental framework, or combined compositional analysis with *in vitro* digestion and cellular mineral uptake to evaluate functional nutritional outcomes.

The aim of this study was to compare the effects of fermentation (tempeh-type), and protein coagulation (tofu-type) with those of minimally processed legumes on protein digestibility and mineral bioavailability of soybean and selected non-soy legumes (faba bean, yellow pea, and grey pea). It was hypothesised that fermentation would enhance mineral bioavailability through phytate degradation and also improve protein digestibility due to enzymatic modification of protein structure, whereas protein coagulation would primarily increase protein digestibility as a result of increased protein concentration and structural

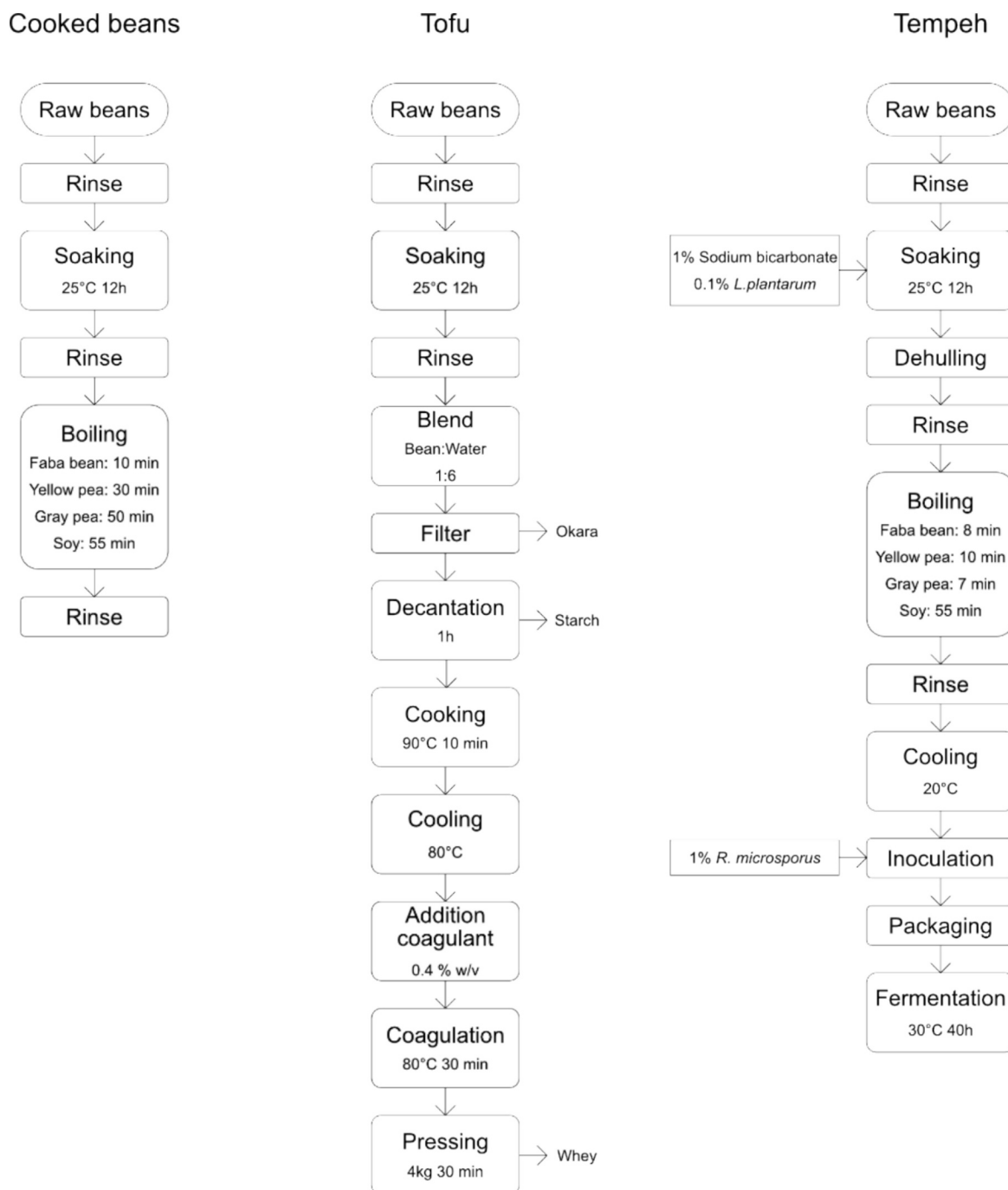


Fig. 1. Overview of the processing methods for cooked legumes, tofu, and tempeh.

changes.

## 2. Materials and methods

### 2.1. Materials

Faba bean (*Vicia faba* cv. Sampo, Sweden 2019), grey pea (*Pisum sativum* var. *arvense* cv. Rättviksärt, Sweden 2022), yellow pea (*Pisum sativum* cv. Ingrid, Sweden 2022), and soybean (*Glycine max* cv. ES Pallador, France 2023) were processed through soaking and cooking or used to prepare tofu and tempeh (Fig. 1).

### 2.2. Compositional analysis

#### 2.2.1. Protein content and amino acid composition

Crude protein content was determined using the Kjeldahl method with a conversion factor of 5.4 (FAO/WHO, 2011; Tomé et al., 2019). The measurements were performed in duplicate using a DT 220 Digester system followed by Kjeldahl protein-determining (Kjeltec 8200 system - Foss Analytical A/S, Hillerød, Denmark).

Amino acid composition was determined using the method described by Özcan and Şenyuva (2006) with minor modifications. In brief, proteins were hydrolysed by adding 8 ml 6 mol/l HCl to 0.1 g of freeze-dried product, followed by incubation for 24 h at 110 °C. Samples were filtered using 33 mm syringe filters (PES 0.2 µm, sterile, 15206869; Fisher Scientific, New Hampshire, USA) and injected into the LC-MS system [Agilent 1260–1290 Infinity LC System with a Phenomenex (Phenomenex Inc., Torrance, USA) column (C18 (2) 250 mm × 4.6 mm, 3 µm), coupled to an Agilent 6120 single Quadrupole MS in the SIM-positive mode] (Agilent Inc., Santa Clara, CA, USA), using an injector volume of 2 µl. Mobile phase A consisted of 30 ml/l MeOH, 2 ml/l formic acid, and 0.1 ml/l acetic acid (HAc) whilst mobile phase B consisted of 500 ml/l MeOH, 2 ml/l formic acid, and 1 ml/l HAc. To derive the standard curve, 18 amino acids (20,088 Amino Acid Standard H, Thermo Scientific™, Waltham, USA), supplied at 2.5 mmol/l (except cysteine, 1.25 mmol/l) and each in 0.1 mol/l HCl, were used. Each measurement was performed in triplicate.

#### 2.2.2. Lipid content

Lipid extraction was performed as described by Hara and Radin (1978). Briefly, a solvent mixture of hexane and isopropanol (3:2, v/v) was used to extract the total lipid content. Extracted lipids were subsequently dried under a stream of nitrogen and weighed to determine the total lipid yield (Hara & Radin, 1978). All samples were analysed in triplicate.

#### 2.2.3. Ash and moisture

Ash content was measured according to AOAC official method 942.05. In short, samples were weighed, incinerated in a muffle furnace (Model 62,700, Barnstead Thermolyne Corporation, Ramsey, Minnesota, United States) at 550 °C for 12 h, cooled in a desiccator for 1 h, and re-weighed. Dry matter content was determined according to AOAC official method 934.01 by drying the samples to a constant weight (>20 h) in a convection oven (Model 2,000,655, J:P: Selecta, Barcelona, Spain) at 105 °C. Both analyses were performed in duplicate.

#### 2.2.4. Sugar residues from carbohydrates

Monosaccharide content was determined using a modified version of the existing method from Sluiter et al. (2008). Briefly, the sample was mixed with 72% sulfuric acid. The mixture was kept under vacuum for 15 min, then incubated at 30 °C for 1 h with stirring occurring every 20 min. Afterward, deionised water was added, and the sample was autoclaved at 125 °C for 1 h before being vacuum-filtered. Fucose (400 mg/l) served as the internal standard. The samples were filtered through 0.45 µm filters into HPLC vials for analysis. Monosaccharide composition was assessed via high-performance anion exchange chromatography

(HPAEC) with pulsed amperometric detection (PAD) using an ICS 3000 system (Dionex, Sunnyvale, USA) and an AEC column (CarboPac PA 1 analytical 4 × 250 mm). Milli-Q water was used as the solvent and a cleaning step with 60% v/v 200 mM NaOH and 40% v/v 200 mM NaOH +170 mM NaOAc was performed between injections. Standards included D (+)-glucose, D (+)-xylose, D (+)-galactose, L-(+)-arabinose, L (+)-rhamnose, and D (+)-mannose. All samples were analysed in triplicates.

#### 2.2.5. Mineral content

Iron and zinc were determined in triplicate through atomic absorption spectrometry (240/280 Series AA Systems; Agilent, Santa Clara, USA). For the calibration, a standard curve with a concentration of 0.125–0.5 mg/l was used for iron (iron standard for AAS, 16596 Supelco, Bellefonte, USA) and a concentration of 0.2–0.8 mg/l for zinc (Zinc 2% HNO<sub>3</sub>, P10010532, CAS 7440-66-6, SPEX CertiPrep™, Metuchen, USA). All measurements were carried out in accordance with the manufacturer recommendations. Prior to measurement, the samples were microwave-digested at 180 °C for 20 min (Milestone Microwave Laboratory System, EthosPlus, Sorisole, Italy) under acidic conditions, as described by Fredrikson, Carlsson, Almgren and Sandberg (2002).

#### 2.2.6. Phytate content

Phytate (inositol hexakisphosphate, IP6) concentrations were measured using high-performance ion chromatography (HPIC) coupled with a UV–vis detector (UV-4075; Jasco, Oklahoma City, OK, USA), as described previously (Carlsson et al., 2001). In the extraction step, freeze-dried powder was mixed with 0.5 mol/l HCl for 3 h. The extract was then centrifuged at 12,000 ×g for 5 min and the supernatant was transferred to an HPLC vial. To elute IP6, an isocratic eluent (800 ml/l 1 mol/l HCl, 200 ml/l Milli-Q water) was used (HPLC pump: 14.5 MPa; model PU-4000i; Jasco Inc., Easton, MD, USA) at a flow rate of 0.8 ml/min. The injection volume was 50 µl. The eluent was mixed with ferrous nitrate at 14.5 MPa, flow rate 0.4 ml/min, using an HPLC pump (model PU-4180; Jasco, Oklahoma City, OK, USA) equipped with a PA-100 guard column and a DIONEX CarboPac PA-100 column (Thermo Scientific™, Waltham, USA). After the post-column reaction, IP6 was detected at 290 nm in a UV–visible HPLC detector. IP6 concentration was calculated using external standards with a concentration range 0.1–1 mmol/l. The analysis was performed in triplicate.

### 2.3. Calculation of iron and zinc bioavailability

To estimate the relative bioavailability of iron and zinc within the products, the molar ratios of phytate to minerals, specifically phytate to iron (Phy:Fe) and phytate to zinc (Phy:Zn), were calculated. For iron, Phy:Fe is suggested to be <1, or preferably <0.4, to significantly improve non-haem iron absorption from plant-based meals (Hurrell & Egli, 2010). According to the European Food Safety Authority (EFSA), Phy:Zn <5 corresponds to high zinc absorption, Phy:Zn = 5–15 is defined as moderate absorption, and ratios >15 represent low bioavailability (Efsa., 2014).

### 2.4. In vitro digestion

#### 2.4.1. Chemicals and enzymes

Chemicals and enzymes were purchased from Sigma-Aldrich, St. Louis, USA and Thermo Fisher Scientific, Massachusetts, USA, including bile extract bovine (A0436166, CAS 8008-63-7), α-Amylase from human saliva (A1031, CAS 9000-90-2), pancreatin from porcine pancreas 8xUPS (P7545, CAS 8049-47-6), and pepsin from porcine gastric (P7012, CAS 9001-75-6). To determine enzyme activity, assays were carried out as described in the supplementary information provided by Brodtkorb et al. (2019). However, to measure trypsin activity, adjustments were made as described by Sousa et al. (2023). The concentration of bile salts in the bile extract was determined using a Bile Acid Assay Kit

(Sigma-Aldrich MAK309).

#### 2.4.2. Sample preparation and *in vitro* digestion

To estimate the protein digestibility, fresh products were masticated (human mastication) for 15 s to allow saliva and salivary amylase to mix with the sample before being weighed into 15 ml polypropylene tubes for further digestion. The amount of product used for the digestion was normalised according to the protein content of the fresh product (0.04 g of protein per gram of food). Lastly, water was added to the masticated product to reach an initial weight of 1 g of food. For each digestion, a blank digestion, containing the same batch of enzymes and 1 g of protein-free cookie was carried out (Sousa et al., 2023).

To estimate the mineral bioavailability, 1 g of freeze-dried (FD) product was weighed into 15 ml polypropylene tubes and mixed with 3 ml of water to achieve an acceptable viscosity of the bolus. The digestion was conducted in the same manner as for the protein digestion; however, no human mastication was performed (instead, human salivary amylase was added during the oral phase) and for the blank digestion, water was added instead of the cookie. An overview of the resulting iron concentrations in the different digesta is provided in Table S3. Furthermore, to validate the iron uptake methodology a reference digestion was conducted using 50 µM ferrous sulphate (FeSO<sub>4</sub>) dissolved in 0.1 M HCl (Rodriguez-Ramiro et al., 2019). The *in vitro* digestion was carried out as described previously (Brodkorb et al., 2019) with minor adjustments as described by Sousa et al. (2023) for the pancreatin preparation. All digestion experiments were performed in triplicate, including appropriate blanks (cookie or water blank) for each digestion cycle. For all digestions, the same batch of enzymes, consisting of amylase (109.8 U/mg), pepsin (2677.2 U/mg), pancreatin (trypsin activity 6.33 U/mg), and bile acid (1.18 mmol/g) was used. Digestions were conducted at 37 °C under continuous mixing (40 rpm) using a Tube Revolver Rotator (Thermo Scientific™). After completion of the intestinal phase, samples intended for protein characterization (degree of hydrolysis and amino acids from the soluble fraction) were stored at -20 °C until analysis, whereas samples for mineral uptake experiments were freeze-dried and stored at room temperature until further use.

#### 2.4.3. Degree of hydrolysis

The protein digestibility of the products was assessed by measuring free amino groups in the intestinal digestes (degree of protein hydrolysis, DH) after *in vitro* digestion. DH was determined in triplicate, using the o-phthalaldehyde (OPA) method (Nielsen et al., 2001). For the serine standard, a concentration range of 0.185–0.95 mmol/l (DL-Serine, LOT SLBK6776V, CAS 302–84-1) was prepared. For the calibration curve, 400 µL of standard solution was added to a flow-cuvette with 3 ml OPA reagent and the solution was incubated for 120 s at room temperature, after which the absorbance was measured at 340 nm. To measure DH in the digesta, the samples were centrifuged at room temperature for 10 min at 10,000 ×g (Heraeus Pico and Fresco 17, Thermo Fisher Scientific, Waltham, USA) and the absorbance was then measured as described for the standard. DH was calculated as:

$$DH (\%) = \frac{NH_2 (\text{Sample})}{\text{Total } NH_2 (\text{Acid hydrolysate})} \times 100$$

where  $NH_2$  (sample) is the concentration of free amino groups in each digested sample after blank correction, expressed as serine equivalents/g protein. Total  $NH_2$  (acid hydrolysate) is the total amount of free amino groups after acid hydrolysis. Acid hydrolysis was conducted at 100 °C for 24 h using 6 mol/l HCl.

#### 2.4.4. Amino acids from the soluble fraction

The amount of free amino acids *i.e.*, the amino acids and small peptides released from the food matrix that are available for absorption, was determined using a method adapted from Sousa et al. (2023) with minor modifications. Briefly, methanolic precipitation was employed to separate proteins into digestible (potentially absorbable) and

indigestible (non-absorbable) fractions by adding methanol to a final concentration of 80% (v/v), followed by incubation at -20 °C for 1 h. Samples were then centrifuged at 2000 ×g for 15 min at 4 °C. For both the food samples and the blank (cookie control), 220 µl of the supernatant was collected and dried using a CentriVap system (Eppendorf® Concentrator Plus EP5305000304, Hamburg, Germany). The dried proteins were then resuspended in 6 M hydrochloric acid and hydrolysed for 15 h at 110 °C. Following hydrolysis, the samples were filtered and diluted in 0.2 M acetic acid before being injected into the LC-MS system, as described in section 2.2.2.

#### 2.5. *In vitro* iron uptake

##### 2.5.1. Cell lines and culture conditions

Iron uptake in intestinal cells was studied with an *in vitro* model of the human intestine, consisting of human intestinal epithelial Caco-2 cells (HTB37; ATCC, Manassas, VA, USA) and mucus-producing HT29-MTX-E12 cells (ECACC 12040401; ATCC, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, 41966029), supplemented with 10% heat-inactive foetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA), 1% non-essential amino acids (Fisher Scientific Waltham, MA, USA, 11140-035), and 1% penicillin/streptomycin (Sigma, P0781). Cells were kept in a humidified incubator at 37 °C and 5% CO<sub>2</sub> and the medium was refreshed every two to three days. The cells were passaged at approx. 80% confluence and cells up to a passage number of 50 were used. Two individual experiments were conducted on cells with less than 10 passages difference.

##### 2.5.2. Sample preparation

To assess iron uptake, freeze-dried digested samples were resuspended in 10 ml of iron-free minimal essential medium (MEM; Thermo Fisher Scientific, Waltham, MA). Prior to resuspension, the resuspension medium was adjusted to pH 7.0 and supplemented with 10 mM PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]), 4 mg/l hydrocortisone, 5 mg/l insulin, 5 mg/l selenium, 34 mg/l triiodothyronine, 1% antibiotic-antimycotic solution, and 20 mg/l epidermal growth factor (Sigma-Aldrich, St. Louis, USA), hereafter referred to as MEM+ (Glahn et al., 1998).

Additionally, a freeze-dried blank digestion (water control) containing only digestive enzymes, as well as a blank digestion containing digestive enzymes and 50 µM FeSO<sub>4</sub>, were prepared in the same manner to evaluate the effects of the digestive enzymes on the cell monolayer and to assess iron uptake in the absence of phytate and other iron inhibitors. For each sample, including the digestion blanks and controls (only medium), three replicates were performed per experiment.

The resuspended samples were placed in an ultrasonic water bath (Branson 5510E-DTH, CT, USA) for 10 min to ensure complete solubilisation of the digestes. Samples were then deproteinised by ultrafiltration using Vivaspin 20 ultrafiltration units (10,000 MWCO; Sartorius, Göttingen, Germany) at 6000 rcf and 20 °C for 1 h. To evaluate concentration-dependent iron uptake in the cells, the deproteinised samples were sterilised using 0.22 µm filters and diluted in MEM+ at 2-fold, 5-fold, and 10-fold concentrations before being introduced to the fully differentiated cell monolayer. A schematic overview of the *in vitro* digestion setup, sample preparation, and cell cultivation is provided in Fig. S1.

##### 2.5.3. Cell uptake experiments

To evaluate iron uptake, Caco-2 and HT29-MTX cells were co-cultured in 12-well cell culture inserts with polyester membranes (0.4 µm pore size, Corning, New York, NY). Cells were seeded at a ratio of 9:1 (Caco-2:HT29-MTX) and a density of  $4 \times 10^5$  cells/cm<sup>2</sup> per Transwell insert. The medium was refreshed every 2 to 3 days. Cells were differentiated over a period of 21 days and monolayer integrity was assessed by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA), according to

Nolleaux et al. (2006). TEER was measured twice per week during differentiation on Transwells (24 h after medium renewal), and just prior and after pre-incubation in MEM+ and exposure to the digested samples. TEER values were corrected for the resistance of a Transwell insert without cells.

Twenty-four hours prior to exposure to the digested samples, cells were cultured in MEM+ to reduce intracellular iron levels (Glahn et al., 1998; Rodriguez-Ramiro et al., 2019). Subsequently, 0.5 ml of the digested, filtered, and diluted samples (Puradisc 30 syringe filters, 0.2 µm, sterile, Whatman®, Maidstone, UK) was added to the apical compartment, and cells were incubated for 24 h. Following incubation, the apical and basolateral phases were collected, and the cell monolayers were harvested by adding CelLytic™ M (Sigma Aldrich, C2978) directly to the membrane inserts. The apical medium was further analysed for extracellular lactate dehydrogenase (LDH) as an indicator of cell viability. LDH levels were measured according to the manufacturer's protocol (Promega, Leiden, the Netherlands). The sample LDH release was expressed relative to the Triton-induced maximum. Therefore, 1% Triton X-100 was used as a positive control to induce complete cell lysis, representing 100% LDH release, whilst MEM+ only served as the negative control for baseline LDH levels.

#### 2.5.4. Ferritin analysis

To estimate the cellular iron uptake, the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) was determined in cell lysates (Tako et al., 2011) using an enzyme-linked immunoassay (Eagle Biosciences, Amherst, NH, Product number FRR31-K01) according to the instructions provided. The only exception was the increased incubation time from 30 min to 2 h for the mouse anti-ferritin antibody-horseradish peroxidase (HRP) conjugate (Glahn, 2022). The total cell protein concentrations were quantified using the Bio-Rad DC™ protein assay kit (500–0116, Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's protocol.

#### 2.6. Statistics

The results are presented as mean and standard deviation or pooled standard deviation and were further analysed by one-way analysis of variance (ANOVA, Type I), followed by Tukey's post-hoc test. All statistical analyses were performed with R Studio (Version 4.3.0, RStudio Inc., Boston, USA).

### 3. Results and discussion

#### 3.1. Compositional analysis

The changes in chemical and nutritional composition resulting from the different processing methods are presented in Table 1. Protein coagulation methods (e.g. tofu production) consistently yielded the highest protein levels, particularly for faba bean (74.7%), grey pea (72.6%), and yellow pea (69.4%). This increase largely reflects a concentration effect during curd formation and whey removal rather than an actual increase in protein yield. Fermentation also led to an increase in protein content, though the effect was less pronounced compared to that of tofu. Soy-based products stood out in terms of fat content with significantly higher levels (17.8–18.3%) compared to the other crops, which generally remained below 9%. Fermentation caused a moderate increase in fat in some cases, notably in yellow pea tempeh, likely due to the relative concentration of lipids following microbial utilisation of carbohydrates during fermentation which exhibited a clear rise compared to its cooked legume and tofu counterparts. Sugar residues were highest in the flour samples of yellow pea, grey pea, and faba bean, but were substantially reduced in tofu products. This reduction is consistent with the removal of soluble carbohydrates during processing (Chua & Liu, 2019; van der Riet et al., 1989).

Aside from the overall differences in total sugar content, the sugar

**Table 1**

Chemical and nutritional composition (in g/100 g DM) of the different crops and processing methods.

Product		Composition				
		Protein	Fat	Sugar residues	Ash	Dry matter
Soy	Flour	40.4 <sup>de</sup>	12.0 <sup>b</sup>	32.3 <sup>efgh</sup>	4.7 <sup>de</sup>	92.8 <sup>f</sup>
	Cooked	43.7 <sup>d</sup>	17.8 <sup>a</sup>	25.3 <sup>fghi</sup>	3.0 <sup>fg</sup>	32.6 <sup>b</sup>
	Tofu	55.4 <sup>c</sup>	18.3 <sup>a</sup>	17.5 <sup>hi</sup>	6.7 <sup>c</sup>	26.0 <sup>g</sup>
	Tempeh	56.2 <sup>c</sup>	18.1 <sup>a</sup>	19.6 <sup>ghi</sup>	3.4 <sup>ef</sup>	29.1 <sup>m</sup>
Yellow Pea	Flour	22.0 <sup>h</sup>	1.8 <sup>c</sup>	67.9 <sup>ab</sup>	3.0 <sup>fg</sup>	89.1 <sup>l</sup>
	Cooked	22.7 <sup>h</sup>	1.9 <sup>c</sup>	65.6 <sup>abc</sup>	2.1 <sup>gh</sup>	43.7 <sup>c</sup>
	Tofu	69.4 <sup>b</sup>	2.3 <sup>c</sup>	9.8 <sup>i</sup>	13.6 <sup>a</sup>	21.8 <sup>j</sup>
Grey Pea	Flour	37.8 <sup>ef</sup>	8.7 <sup>b</sup>	42.5 <sup>def</sup>	5.0 <sup>di</sup>	37.8 <sup>d</sup>
	Cooked	28.1 <sup>g</sup>	2.0 <sup>c</sup>	61.7 <sup>abc</sup>	2.6 <sup>gh</sup>	85.2 <sup>p</sup>
	Tempeh	26.0 <sup>gh</sup>	1.9 <sup>c</sup>	72.5 <sup>a</sup>	1.4 <sup>h</sup>	37.6 <sup>a</sup>
Faba Bean	Flour	72.6 <sup>b</sup>	2.4 <sup>c</sup>	15.6 <sup>hi</sup>	10.9 <sup>b</sup>	22.2 <sup>n</sup>
	Cooked	38.4 <sup>e</sup>	3.3 <sup>c</sup>	48.8 <sup>cde</sup>	2.6 <sup>gh</sup>	32.8 <sup>h</sup>
	Tempeh	36.0 <sup>ef</sup>	2.5 <sup>c</sup>	50.9 <sup>bcd</sup>	3.5 <sup>ef</sup>	86.0 <sup>o</sup>
Pooled standard deviation	Flour	33.1 <sup>f</sup>	1.7 <sup>c</sup>	54.9 <sup>abcd</sup>	3.0 <sup>fg</sup>	44.0 <sup>e</sup>
	Cooked	74.7 <sup>a</sup>	0.9 <sup>c</sup>	8.9 <sup>j</sup>	9.8 <sup>b</sup>	25.4 <sup>k</sup>
	Tempeh	45.0 <sup>d</sup>	3.3 <sup>c</sup>	37.4 <sup>defg</sup>	2.0 <sup>gh</sup>	31.8 <sup>i</sup>
Pooled standard deviation		1.26	1.07	5.98	0.35	1.06

One-way ANOVA was used to determine differences between the raw materials. Lowercase letters indicate significant differences between samples per column according to Tukey's test ( $p < 0.001$ ).

residue profile varied notably depending on both the crop type and processing method that were used (Table S2). Glucose was the predominant sugar in most samples, especially the flours, with the highest levels observed in faba bean flour (85.0%) and yellow pea flour (83.2%). In contrast, tofu products consistently displayed the lowest glucose content across all crops, with faba bean tofu showing the lowest value (12.9%). Arabinose and galactose levels were generally elevated in fermented products, particularly in soy and grey pea tempeh, which contained arabinose levels of 12.5% and 11.4%, respectively. Soy tempeh had the highest galactose content (28.9%). Rhamnose, mannose, and xylose remained relatively low across most samples, with only slight increases observed in tempeh compared to flour and cooked forms. These compositional changes are structurally relevant, as the removal of soluble carbohydrates and modification of cell wall polysaccharides may reduce matrix complexity and improve enzyme accessibility. In contrast, higher levels of non-starch polysaccharides and fibre-derived sugars may contribute to physical barriers that limit protein digestion (Rousseau et al., 2020).

The overall ash content was elevated in tofu made from yellow pea, grey pea, and faba bean, indicating mineral concentration during tofu processing. However, this increase should be interpreted with caution, as minerals are co-precipitated together with phytate during protein coagulation (see Table 3). Consequently, higher mineral levels in tofu do not necessarily indicate improved nutritional quality or bioavailability.

The results on the overall composition align with previously reported values. For soy, dried raw soybeans have been shown to contain between 36.5% and 44.1% protein and 11.4% to 13.3% fat (Hammer et al., 2024). Faba beans typically contain around 28.0% protein and 1.6% fat, whilst yellow pea has been reported to contain approximately 22.3% protein and 3.5% fat (Millar et al., 2019). Changes in carbohydrate content and composition between raw and cooked beans, as well as tofu products, can be attributed to the removal of soluble carbohydrates during cooking and tofu making (Hammer et al., 2024; Wight & Cilliers, 1989). Aside from dehulling, fermentation can lead to a reduced overall sugar content and increased protein content (Damanik et al., 2018; Purwandari et al., 2024; Raob, 2014). Previous studies have demonstrated that fermentation can result in an approximate 50% decrease in stachyose, an oligosaccharide composed of two galactose units, one glucose and the other fructose. This reduction is caused by fermenting

microorganisms, such as *Rhizopus oligosporus*, which produce  $\alpha$ -galactosidase enzymes that hydrolyse stachyose into simpler sugars and these are then utilised as an energy source during microbial growth ((Suparmo, and Markakis, P., 1987); van der Riet et al., 1989).

Whilst dietary fibre offers numerous health benefits (Dhingra et al., 2012; Gidley, 2024), it can also hinder protein digestibility. Fibres and complex polysaccharides may form physical barriers that block enzyme access, encapsulate proteins within the cell wall matrix, or increase gastrointestinal viscosity, thereby slowing enzymatic activity and protein breakdown (Karim et al., 2024). The extent of this inhibition is dependent on factors such as polysaccharide concentration, viscosity, and molecular structure (Dhital et al., 2017; Gidley, 2024; Karim et al., 2024; B. Zhang et al., 2015).

### 3.2. Amino acid composition

To further characterise the effect of processing on nutritional composition, the amino acid composition was analysed and is presented in Table 2. Overall, high levels of aspartic and glutamic acid were found across all products. Moreover, arginine was particularly abundant in all faba bean products and in grey pea tofu compared to the soy and yellow pea products. Beyond the crop-specific differences, processing methods, such as tofu and tempeh production, influenced the amino acid profiles. The most notable effect in faba bean, yellow pea, and grey pea products was a reduced glutamic and/or aspartic acid content in tempeh compared to tofu. Faba bean and yellow pea tempeh showed a simultaneous increase in alanine levels. These changes indicate that processing modifies protein composition through selective protein retention, protein losses during processing, and microbial metabolism, rather than reflecting changes in total protein quantity. Despite these variations, the relative distribution of essential amino acids remained largely within the range required for human nutrition, suggesting that processing-induced shifts had only a limited impact on overall essential amino acid adequacy. A change in amino acid profile arising from fermentation with the fungal strain *Rhizopus oligosporus* has been previously observed for different agro-industrial by-products (Ibarruri &

Hernández, 2018) and also soybeans, pigeon peas, and chickpeas (Toor et al., 2022). During fermentation, proteolytic activity of *Rhizopus* species has been reported to contribute to protein hydrolysis and the release of free amino acids (Ikasari & Mitchell, 1994). However, enzymatic activity was not directly measured in the present study, and the observed changes should therefore be interpreted in the context of previously reported mechanisms. However, during fermentation, certain amino acids can also be synthesised by glycolysis or transamination, degraded via a range of catalysing reactions, and enter the tricarboxylic acid cycle or urea cycle (Polanowska et al., 2020) resulting in altered amino acid profiles. The present results, therefore, reflect metabolic modification of amino acids during fermentation rather than a direct improvement in protein nutritional quality.

Differences in amino acid composition between tofu and cooked beans may also stem from processing-related protein losses, as certain proteins are removed with the whey during pressing (Chua & Liu, 2019; Hammer et al., 2024). All processed raw materials met or exceeded the adult essential amino acid reference pattern defined by FAO/WHO/UNU (2007). This assessment is based solely on amino acid composition and does not account for protein digestibility, amino acid bioavailability, or the presence of antinutritional factors. Therefore, protein quality should be interpreted in conjunction with *in vitro* digestion and bioavailability results.

### 3.3. In vitro digestion and degree of protein hydrolysis

The protein digestibility was estimated by determining the degree of hydrolysis (DH) after *in vitro* digestion (Fig. 2). Unprocessed legumes are not intended for human consumption and thus the dry products were excluded from this analysis. The DH reflects the extent of protein breakdown under the applied *in vitro* conditions and should therefore be interpreted as a measure of protein susceptibility to enzymatic hydrolysis rather than true protein digestibility.

The lowest DH was observed in the cooked products, with an average of 37%. Fermented products showed a higher average DH (47%); however, a significant difference ( $p < 0.001$ ) between cooked and

**Table 2**  
Amino acid composition of the different crops and processed products compared to the recommended protein intake for adults (FAO/WHO/UNU, 2007).

Product		Amino Acids composition (mg/g protein)														
		Essential amino acids							Non-essential amino acids							
		His	Ile	Leu	Lys	Phe	Thr	Val	Ala	Arg	Asp	Glu	Gly	Pro	Ser	Tyr
Soy	Flour	18 <sup>b</sup>	42	82	65	46	38 <sup>b</sup>	45	44 <sup>b</sup>	70 <sup>a</sup>	117 <sup>b</sup>	197	39	48	51	21 <sup>b</sup>
	Cooked	22 <sup>ab</sup>	46	85	71	50	42 <sup>ab</sup>	49	46 <sup>b</sup>	68 <sup>a</sup>	137 <sup>b</sup>	207	41	53	53	25 <sup>ab</sup>
	Tofu	21 <sup>ab</sup>	48	89	68	54	41 <sup>ab</sup>	50	44 <sup>b</sup>	65 <sup>ab</sup>	177 <sup>a</sup>	212	40	55	56	27 <sup>ab</sup>
	Tempeh	23 <sup>a</sup>	48	88	72	49	44 <sup>a</sup>	52	59 <sup>a</sup>	57 <sup>b</sup>	176 <sup>a</sup>	184	41	49	53	29 <sup>a</sup>
	<i>p-Value</i> <sup>1</sup>	*	0.164	0.321	0.434	0.079	0.066	0.069	**	**	***	0.067	0.531	0.101	0.484	*
Yellow pea	Flour	10 <sup>b</sup>	35 <sup>b</sup>	74 <sup>b</sup>	78	38 <sup>bc</sup>	33 <sup>b</sup>	42 <sup>b</sup>	42 <sup>b</sup>	60 <sup>ab</sup>	62 <sup>c</sup>	170	38	36 <sup>b</sup>	45 <sup>b</sup>	14 <sup>c</sup>
	Cooked	10 <sup>b</sup>	35 <sup>b</sup>	74 <sup>b</sup>	74	36 <sup>c</sup>	33 <sup>b</sup>	42 <sup>b</sup>	40 <sup>b</sup>	52 <sup>b</sup>	60 <sup>c</sup>	165	36	35 <sup>b</sup>	45 <sup>b</sup>	10 <sup>c</sup>
	Tofu	22 <sup>a</sup>	50 <sup>a</sup>	92 <sup>a</sup>	85	58 <sup>a</sup>	43 <sup>a</sup>	53 <sup>a</sup>	48 <sup>b</sup>	71 <sup>a</sup>	226 <sup>a</sup>	177	41	46 <sup>a</sup>	56 <sup>a</sup>	30 <sup>a</sup>
	Tempeh	19 <sup>a</sup>	45 <sup>a</sup>	83 <sup>ab</sup>	79	47 <sup>b</sup>	44 <sup>a</sup>	52 <sup>a</sup>	59 <sup>a</sup>	54 <sup>b</sup>	111 <sup>b</sup>	161	40	43 <sup>a</sup>	52 <sup>ab</sup>	23 <sup>b</sup>
	<i>p-Value</i> <sup>1</sup>	***	**	*	0.184	***	**	**	**	*	***	0.253	0.156	**	**	***
Grey pea	Flour	11 <sup>c</sup>	29 <sup>c</sup>	66 <sup>c</sup>	63 <sup>c</sup>	29 <sup>c</sup>	29 <sup>b</sup>	38 <sup>c</sup>	37 <sup>b</sup>	62 <sup>bc</sup>	73 <sup>b</sup>	163 <sup>b</sup>	33 <sup>b</sup>	29 <sup>c</sup>	39 <sup>bc</sup>	11 <sup>b</sup>
	Cooked	15 <sup>bc</sup>	38 <sup>b</sup>	82 <sup>b</sup>	77 <sup>ab</sup>	39 <sup>b</sup>	34 <sup>b</sup>	48 <sup>b</sup>	40 <sup>b</sup>	72 <sup>b</sup>	72 <sup>b</sup>	176 <sup>b</sup>	38 <sup>b</sup>	38 <sup>b</sup>	47 <sup>b</sup>	12 <sup>b</sup>
	Tofu	27 <sup>a</sup>	52 <sup>a</sup>	103 <sup>a</sup>	88 <sup>a</sup>	57 <sup>a</sup>	46 <sup>a</sup>	58 <sup>a</sup>	53 <sup>a</sup>	102 <sup>a</sup>	256 <sup>a</sup>	219 <sup>a</sup>	45 <sup>a</sup>	50 <sup>a</sup>	59 <sup>a</sup>	31 <sup>a</sup>
	Tempeh	17 <sup>b</sup>	34 <sup>bc</sup>	64 <sup>c</sup>	72 <sup>bc</sup>	32 <sup>bc</sup>	34 <sup>b</sup>	41 <sup>c</sup>	58 <sup>a</sup>	46 <sup>c</sup>	80 <sup>b</sup>	117 <sup>c</sup>	34 <sup>b</sup>	31 <sup>c</sup>	38 <sup>c</sup>	16 <sup>b</sup>
	<i>p-Value</i> <sup>1</sup>	***	***	***	**	***	***	***	***	***	***	***	***	***	***	***
Faba bean	Flour	21 <sup>b</sup>	38 <sup>b</sup>	81 <sup>bc</sup>	70 <sup>b</sup>	36 <sup>b</sup>	36 <sup>b</sup>	45 <sup>b</sup>	42 <sup>c</sup>	98 <sup>a</sup>	105 <sup>b</sup>	187 <sup>b</sup>	40 <sup>ab</sup>	43 <sup>bc</sup>	49 <sup>b</sup>	16 <sup>c</sup>
	Cooked	23 <sup>b</sup>	41 <sup>b</sup>	89 <sup>b</sup>	74 <sup>ab</sup>	39 <sup>b</sup>	36 <sup>b</sup>	50 <sup>b</sup>	46 <sup>bc</sup>	100 <sup>a</sup>	98 <sup>b</sup>	202 <sup>b</sup>	40 <sup>b</sup>	46 <sup>b</sup>	53 <sup>b</sup>	12 <sup>c</sup>
	Tofu	30 <sup>a</sup>	53 <sup>a</sup>	109 <sup>a</sup>	83 <sup>a</sup>	54 <sup>a</sup>	44 <sup>a</sup>	58 <sup>a</sup>	52 <sup>b</sup>	116 <sup>a</sup>	278 <sup>a</sup>	234 <sup>a</sup>	46 <sup>a</sup>	56 <sup>a</sup>	65 <sup>a</sup>	35 <sup>a</sup>
	Tempeh	22 <sup>b</sup>	39 <sup>b</sup>	77 <sup>c</sup>	72 <sup>b</sup>	36 <sup>b</sup>	38 <sup>b</sup>	47 <sup>b</sup>	65 <sup>a</sup>	64 <sup>b</sup>	110 <sup>b</sup>	142 <sup>c</sup>	37 <sup>b</sup>	40 <sup>c</sup>	45 <sup>c</sup>	21 <sup>b</sup>
	<i>p-Value</i> <sup>1</sup>	***	***	***	*	***	**	**	***	***	***	***	*	***	***	***
Pooled standard deviation		2	3	5	5	3	3	3	4	6	7	9	3	3	3	3
mg/kg BW per day <sup>2</sup>		10	20	39	30	25	15	26								
mg/g protein <sup>3</sup>		15	30	59	45	30	23	39								

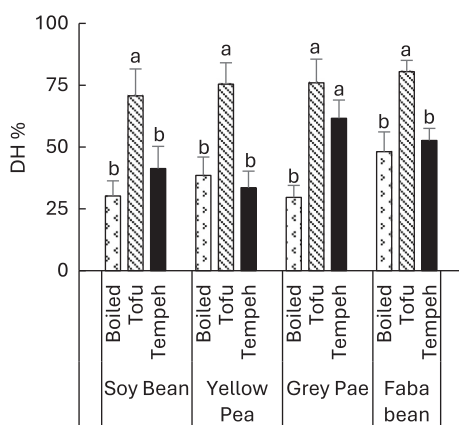
Results are expressed as mg/g protein based on dry weight  $\pm$  SD. <sup>1</sup>One-way ANOVA was used to determine differences between the raw materials. Different superscript letters indicate significant differences according to Tukey's test (0.001 \*\*\*, 0.01 \*\*, 0.01\*). <sup>2</sup>mg protein required by an adult per kg body weight. <sup>3</sup>Mean nitrogen requirement of 105 mg/kg per day (0.66 g protein/kg per day) (FAO/WHO/UNU, 2007). Due to the use of acid hydrolysis during sample preparation, tryptophan and, to some extent, cysteine and methionine could not be detected (Ozols, 1990).

**Table 3**

Mineral (iron, zinc) and phytate content found in the raw materials, compared to the recommended daily intake of iron and zinc (Nordic Council of Ministers, 2023) and the calculated molecular ratio of phytate (Phy) to iron/zinc.

Product		Composition			Molecular ratio	
		Iron	Zinc	Phytate	Phy:Fe <sup>3</sup>	Phy:Zn <sup>4</sup>
Soy	Flour	99 <sup>a</sup>	50 <sup>b</sup>	10.9 <sup>e</sup>	9 <sup>f</sup>	22 <sup>c</sup>
	Cooked	55 <sup>c</sup>	41 <sup>d</sup>	13.0 <sup>d</sup>	20 <sup>a</sup>	32 <sup>a</sup>
	Tofu	81 <sup>b</sup>	56 <sup>a</sup>	15.5 <sup>c</sup>	16 <sup>c</sup>	27 <sup>b</sup>
	Tempeh	63 <sup>c</sup>	44 <sup>c</sup>	3.0 <sup>h</sup>	4 <sup>i</sup>	7 <sup>e</sup>
Yellow Pea	Flour	45 <sup>c</sup>	32 <sup>c</sup>	6.7 <sup>f</sup>	13 <sup>e</sup>	21 <sup>c</sup>
	Cooked	36 <sup>c</sup>	29 <sup>c</sup>	7.8 <sup>f</sup>	18 <sup>b</sup>	26 <sup>b</sup>
	Tofu	164 <sup>a</sup>	104 <sup>a</sup>	27.5 <sup>b</sup>	14 <sup>d</sup>	26 <sup>b</sup>
	Tempeh	51 <sup>b</sup>	38 <sup>b</sup>	3.6 <sup>h</sup>	6 <sup>h</sup>	9 <sup>e</sup>
Grey Pea	Flour	47 <sup>c</sup>	50 <sup>b</sup>	4.4 <sup>g</sup>	8 <sup>g</sup>	9 <sup>e</sup>
	Cooked	47 <sup>c</sup>	37 <sup>c</sup>	4.6 <sup>g</sup>	8 <sup>g</sup>	12 <sup>d</sup>
	Tofu	160 <sup>a</sup>	119 <sup>a</sup>	15.0 <sup>c</sup>	8 <sup>g</sup>	13 <sup>d</sup>
	Tempeh	59 <sup>b</sup>	39 <sup>b</sup>	–	0 <sup>j</sup>	0 <sup>f</sup>
Faba Bean	Flour	73 <sup>b</sup>	65 <sup>b</sup>	12.7 <sup>d</sup>	15 <sup>d</sup>	19 <sup>c</sup>
	Cooked	67 <sup>b</sup>	53 <sup>c</sup>	11.4 <sup>e</sup>	14 <sup>d</sup>	21 <sup>c</sup>
	Tofu	171 <sup>a</sup>	142 <sup>a</sup>	30.8 <sup>a</sup>	15 <sup>cd</sup>	21 <sup>c</sup>
	Tempeh	91 <sup>b</sup>	61 <sup>bc</sup>	–	0 <sup>j</sup>	0 <sup>f</sup>
Pooled standard deviation		6	4	0.4	0.8	0.9
Recommended intake <sup>1</sup>						
Male 18–50 years		9	12.7			
Male 51–70 years		9	12.4			
Females 18–50 years		15 <sup>2</sup>	9.7			
Females 51–70 years		8 <sup>3</sup>	9.5			

Iron/ zinc are expressed as mg/kg dry weight. Phytate is expressed as g/kg dry weight. <sup>1</sup>Recommended intake (RI) in mg/day, according to the Nordic Nutrition Recommendation 2023, assuming a mixed animal/vegetable diet with a phytic acid intake of about 600 mg/day. <sup>2</sup>If there are large menstruation bleedings, screening of iron status and supplementation is indicated. <sup>3</sup>If still menstruating, the RI for 25–50 y (15 mg/day) should be used (Nordic Council of Ministers, 2023). <sup>4</sup>Molar ratio of phytate to iron (Phy:Fe), where Phy:Fe < 1 is needed for adequate iron absorption from plain cereal or legume-based meals. Phy:Fe = 6 can be considered adequate in composite meals high in ascorbic acid and meat (Hurrell & Egli, 2010). <sup>5</sup>Molar ratio of phytate to zinc (Phy:Zn) where Phy:Zn < 5 corresponds to high zinc absorption and Phy:Zn ≤ 15 corresponds to moderate absorption (Efsa, 2014). Lowercase letters indicate significant differences between samples (p < 0.001).



**Fig. 2.** Degree of protein hydrolysis (DH) in % in regard to processing (Boiled beans, Tofu, Tempeh). Lowercase letters indicate significant differences between the different processing methods (p < 0.001).

fermented products was only observed for grey peas. These results indicate that the effect of fermentation on protein hydrolysis was crop-dependent and not consistently observed across all legumes. Previous results on tempeh showed a DH of around 30% for fermented jack bean (Purwandari et al., 2024) and 24% for black bean tempeh (Wang et al., 2022) and are therefore within a similar range to our results (Fig. 2). Fermentation has been reported to enhance protein hydrolysis through

the secretion of proteolytic enzymes (Zhang et al., 2022). In the present study, increased DH was observed for some crops; however, enzymatic activity was not directly assessed. Although an increased DH was observed in both faba bean and soy tempeh, only the grey pea tempeh showed a significant improvement compared to the cooked beans. This can possibly be attributed to the hull removal of the grey peas in the tempeh product compared to the cooked beans. Although all tempeh products contained dehulled legumes, grey pea tempeh showed lower levels of indigestible sugars such as galactose, xylose, mannose, and klon lignin compared to, for example, yellow pea tempeh, which exhibited a similar total sugar content.

Overall, tempeh products were found to have a high content of arabinose (Table S2), which, despite not being directly associated with reduced protein digestion, can be part of larger fibre structures such as arabinoxylans or pectins. These fibres may physically or chemically interfere with protein digestibility (Karim et al., 2024; Zhang & Varadhanabhuti, 2014).

This is further supported by the fact that tofu, which contained significantly fewer sugar residues and shows a less complex food matrix, yielded the highest average degree of hydrolysis (DH) of 75% compared to the corresponding boiled and fermented pulses. The higher DH observed for tofu is therefore primarily attributed to the reduced matrix complexity and lower levels of structural polysaccharides, which improve enzyme accessibility, rather than reflecting intrinsically superior protein quality.

A similar pattern has been previously reported, where soybeans and the corresponding tofu showed an *in vitro* digestibility of 58% and 95%, respectively (Hammer et al., 2024). The generally higher digestibility found by Hammer et al. (2024) can be attributed to the fact that they terminated the *in vitro* DIAAS, which also includes di- and tri peptides in the digesta after an additional hydrolysis step of the soluble fraction (Sousa et al., 2023). Based on the results presented by Marinea et al. (2021) for a firm tofu-like model system, the average DH was 70%, similar to observations from the current study.

#### 3.4. Amino acids from the soluble fraction

The relative distribution of amino acids in the soluble fraction after *in vitro* digestion was determined and is presented in Fig. S2. The distribution of individual amino acids varied significantly depending on both the type of legume and the processing method. Tofu and tempeh products exhibited higher levels of essential amino acids, such as leucine, isoleucine, and lysine, compared to their cooked counterparts, indicating enhanced protein hydrolysis and greater transfer of amino acids into the soluble fraction rather than directly implying increased bioaccessibility.

However, the release of threonine, alanine, and arginine remained low across all samples, despite their sufficient presence in the undigested products. This pattern may reflect differences in protein structure, peptide stability, or enzymatic cleavage specificity rather than indicating a nutritional limitation. Therefore, the lower proportion of threonine in the soluble fraction should be interpreted cautiously and does not necessarily imply reduced nutritional availability *in vivo*. Moreover, non-essential amino acids such as glutamic acid and aspartic acid were found in high concentrations across all samples, particularly in cooked legumes such as faba bean and soy. These results highlight that processing not only alters the structure of legume proteins but can also influence the release and availability of individual amino acids, which ultimately impacts their nutritional quality.

These findings align with previous results on garden peas, grass peas, soybeans, and lentils, where leucine, lysine, and phenylalanine were identified as the most abundant essential free amino acids released after intestinal digestion. In contrast, threonine was released in relatively lower amounts, comparable to that of methionine (Santos-Hernández et al., 2020). However, a less pronounced limitation in threonine release was observed among black beans, pigeon peas, and wheat bran.

Nonetheless, the digestibility of threonine was generally lower compared to the other amino acids (Hodgkinson et al., 2022; Sousa et al., 2023).

### 3.5. Minerals and phytate

The concentrations of iron/ zinc, along with phytate, are presented in Table 3. Significant differences were found between the individual crops as well as the different processes ( $p < 0.001$ ). Among all crops, the tofu products showed significantly ( $p < 0.001$ ) higher amounts of iron, zinc, and phytate than the uncooked, cooked, or fermented products. This simultaneous increase indicates a concentration effect during protein coagulation, and therefore the higher mineral contents in tofu should be interpreted with caution, as they do not necessarily reflect improved nutritional quality. The fermentation caused an iron increase in the grey pea and yellow pea tempeh compared to the uncooked and cooked products; however, the observed trend was less dominant than the one observed for the tofu product. The fermentation, however, led to a significant ( $p < 0.001$ ) reduction in phytate across all products, with none detected in the faba bean and grey pea tempeh.

The amounts of iron/ zinc and phytate found in the different crops are compatible with previously presented results for faba bean (Mayer Labba et al., 2021; Sinković et al., 2023) pea (Auer et al., 2024; Tulbek et al., 2016) and soy (Akande et al., 2010; Hammer et al., 2024). The overall higher levels of iron, zinc, and phytate in the tofu can be attributed to the protein increase during the coagulation (Chigwedere et al., 2023; Wang & Guo, 2021). Ishiguro et al. (2008) demonstrated that during the curd formation, phytate binds to particulate protein and gradually increases as the curd formation progresses (Ishiguro et al., 2008).

The reduction in phytate observed after fermentation is consistent with previous studies reporting phytase activity from both endogenous legume enzymes and *Rhizopus* species. Although phytase activity was not directly measured in this study, the observed decrease is in line with the well-documented role of microbial and endogenous phytases during tempeh fermentation (Bhalla et al., 2022; Sudarmadji & Markakis, 1977; Sutardi and Buckle, 1988). This has been observed during the fermentation of soy (Egounlety & Aworh, 2003; Urbano et al., 2000), but also for other legumes (Chitra et al., 1996; Coda et al., 2015) which highlights the important role of fermentation in improving nutritional properties of plant-derived foods (Sá et al., 2019).

Comparing the mineral content in 100 g of fresh products (Table S3) to the recommended daily intake of iron and zinc revealed that tofu possesses higher levels of these minerals than both tempeh and boiled products. For instance, 100 g of faba bean-based tofu can provide approximately half of the daily iron requirement for men and one-fourth for women. Similarly, tofu made from yellow pea and grey pea contains around 3.6 mg of iron per 100 g, making these products potential dietary sources of iron based on composition. The highest zinc content was found in faba bean tempeh, with 3.6 mg per 100 g, which would meet one-third of the daily zinc requirement for women and one-fourth for men.

These estimates are based on total mineral content and do not account for the inhibitory effects of phytate and other matrix components on mineral absorption.

To estimate the bioavailability of iron and zinc in different products, molar ratios of Phy:Fe and Phy:Zn were calculated (Table 3). Among these, yellow pea and soy tempeh showed the lowest Phy:Fe, at 6.7 and 4.6, respectively. Overall, grey pea products displayed a lower Phy:Fe compared to yellow pea and faba bean products, likely because of the lower phytate content in grey peas. It should be noted that phytate-to-mineral molar ratios provide predictive indicators of mineral absorption potential rather than direct measures of bioavailability under physiological conditions.

Comparing the effects of processing on estimated bioavailability revealed that neither cooking nor tofu production leads to improved iron

bioavailability. In fact, an opposite trend was observed, where the estimated iron bioavailability in soy and yellow pea products decreased after cooking and tofu processing.

A similar overall trend was seen for zinc bioavailability calculations, with cooking and tofu processing failing to generate any improvement. As with iron, the estimated zinc bioavailability was highest in soy and yellow pea tempeh, followed by grey pea products.

An increase in Phy:Fe and Phy:Zn arising from the cooking and tofuming process has been previously observed in soy (Hammer et al., 2024), faba beans (Dhull et al., 2022; Luo et al., 2009), and other legumes (Lestienne et al., 2005) where the cooking process led to a decrease in iron and zinc. This could be a result of the cooking process, where certain water-soluble compounds (Hammer et al., 2024), including fibres, leach into the cooking water, leading to a loss in dry matter (Dellavalle et al., 2013; Lestienne et al., 2005; Luo et al., 2009).

The overall low estimated bioavailability of both minerals is commonly observed in legumes. Previous studies have reported similar findings for soy (Hammer et al., 2024; Ishiguro et al., 2008), faba beans (Mayer Labba et al., 2021), and peas (Auer et al., 2024; Fredrikson et al., 2001). In contrast, grey showed a favourable composition characterised by relatively low phytate levels combined with appreciable iron and zinc contents. However, a potential nutritional advantage cannot be concluded based on phytate content alone and requires confirmation through bioavailability assessments. In this study, fermentation significantly enhanced the estimated bioavailability of iron and zinc in both soy and yellow peas. In the case of faba beans and grey peas, fermentation resulted in the complete degradation of phytate, suggesting a reduced inhibitory effect of phytate and a higher potential for mineral absorption rather than confirming increased absorption.

### 3.6. Cell uptake and ferritin formation

To evaluate if the phytate reduction in tempeh led to increased mineral bioavailability, *in vitro* experiments using the Caco-2/HT29-MTX co-culture model were performed to assess cellular iron uptake. For this, intestinal cells were exposed to three concentrations of the digested tempeh or tofu for 24 h, and ferritin formation in the cells was determined as an indicator for iron bioavailability. The experiments were conducted in two independent rounds. The results from Experiment 1 are presented and discussed below, whilst the findings from Experiment 2 are included in the supplementary materials. Both experiments showed similar trends, supporting the reliability of the results.

#### 3.6.1. Monolayer integrity and cell viability

Monolayer integrity was monitored by measuring transepithelial electrical resistance (TEER) as a function of the culture period prior to the exposure to digested food samples (Fig. 3A). The TEER showed a gradual increase over 19 days and stabilised when the cell layer was fully differentiated on around day 21, resulting in an average TEER value of 718  $\Omega\cdot\text{cm}^2$  in Experiment 1 and 673  $\Omega\cdot\text{cm}^2$  in Experiment 2 (Fig. S3).

Previously reported TEER values for Caco-2 and HT29-MTX co-cultures were between 300 and 400  $\Omega\cdot\text{cm}^2$  after 21 days (García-Rodríguez et al., 2018; Nolleveaux et al., 2006). However, in general, TEER values between 500 and 1100  $\Omega\cdot\text{cm}^2$  are considered acceptable for fully differentiated and functionally intact epithelial monolayers (Kus et al., 2023).

After a 21-day differentiation period (Fig. 3B) the cells were exposed to different dilutions of digesta (2, 5, and 10  $\times$  diluted Digesta). Following a 24 h incubation, a significant ( $p < 0.001$ ) decrease in TEER, compared to the control (cells incubated in MEM+), was observed for tofu made from faba bean and tempeh made from faba bean, soy, and grey pea at a 2 $\times$  dilution. Additionally, the 5 $\times$  diluted soy tempeh resulted in a significant ( $p < 0.001$ ) TEER decrease in both experiments. This indicates a reduction in the barrier integrity of the epithelial monolayer (Narai et al., 1997), which may be linked to the high iron

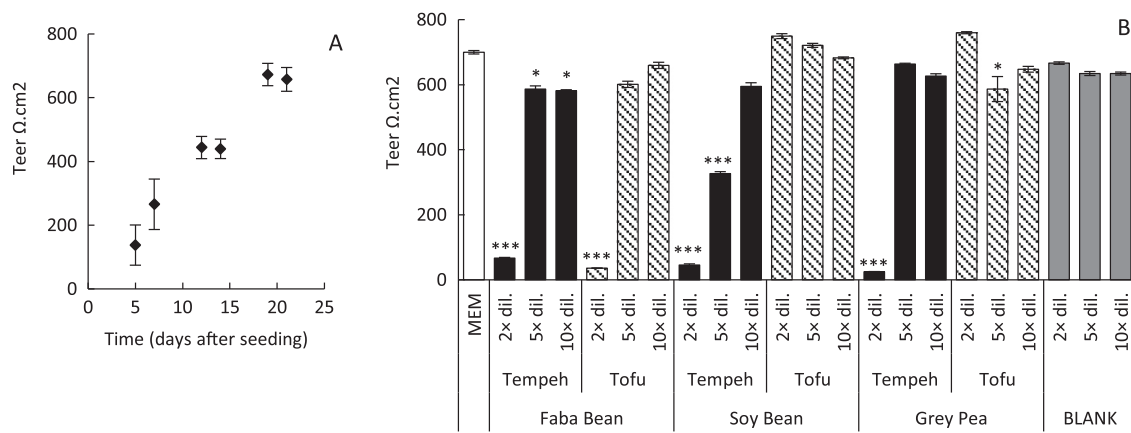


Fig. 3. Changes in TEER throughout the 21-day differentiation period (A) and after exposure to the 2, 5, and 10× dilutions of digesta (Tempeh, Tofu, Blank, Control) to evaluate concentration-dependent effects on cell monolayer integrity. The asterisk indicates significant differences between the different samples/ concentrations and the MEM+ with \*  $p < 0.5$ ; \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

concentrations in the medium (Alvarez-Hernandez et al., 1991). This effect is primarily due to the generation of reactive oxygen species (ROS) caused by excess iron, which induces oxidative damage to tight junction proteins such as occludin and claudins. The oxidative stress leads to reduced expression and mislocalisation of these proteins, thereby weakening tight junctions and disrupting epithelial barrier function (Ferruzza et al., 2002). Since the 2× dilution samples contained the highest amount of iron, this could potentially have caused or contributed to the decrease in TEER. These findings indicate that high digesta concentrations may induce cellular stress and compromise monolayer integrity, which could influence nutrient transport and uptake measurements.

To assess cell viability, extracellular lactate dehydrogenase (LDH) release was analysed in the apical medium relative to the triton-induced maximum. Dying cells generally lose their membrane integrity and release LDH into the medium, which therefore directly correlates to cell death. Thus, an increase of 20–30% of cell death compared to the negative control (MEM) can be considered cytotoxic.

Overall, a significantly higher LDH release ( $p < 0.001$ ; Fig. 4) was observed in cells incubated with 2× diluted digested samples (excluding grey pea tofu in Experiment 2, Fig. S4) as well as the blank digestion, compared to the control (>20% increase). This suggests that the digestive enzymes, and potentially the digested samples, exert cytotoxic effects on the cells.

This supports previous findings that digestion fluids from the INFOGEST protocol are cytotoxic to intestinal cell models, largely due to the presence of bile salts and enzymes which can damage cell membranes or alter osmolality, especially at high concentrations (Antal et al., 2024; Kondrashina et al., 2023; Vital et al., 2024). However, because LDH release remained within a 20% increase from the baseline, the 2× dilutions were included in the ferritin measurements, however results obtained at this concentration should be interpreted with caution due to potential cytotoxic effects.

### 3.6.2. Ferritin

Cellular iron uptake from the digested food products was assessed by measuring cellular ferritin (Fig. 5; Fig. S5). The baseline cell ferritin levels were on average 3.0 and 2.3 ng/mg of protein, which is in agreement with previously reported results by Mahler et al. (2009) and Laparra et al. (2009) for different beans. A significantly higher ferritin formation was observed for 5× and 10× diluted digested tempeh products, whereas no significant difference was found between the control and tofu samples. Under conditions where monolayer integrity was maintained (5× and 10× dilutions), ferritin formation was significantly higher for digested tempeh compared to tofu, suggesting a greater cellular iron uptake potential for fermented products.

In contrast, the 2× diluted digested tempeh products did not show an increase in ferritin formation despite possessing the highest iron

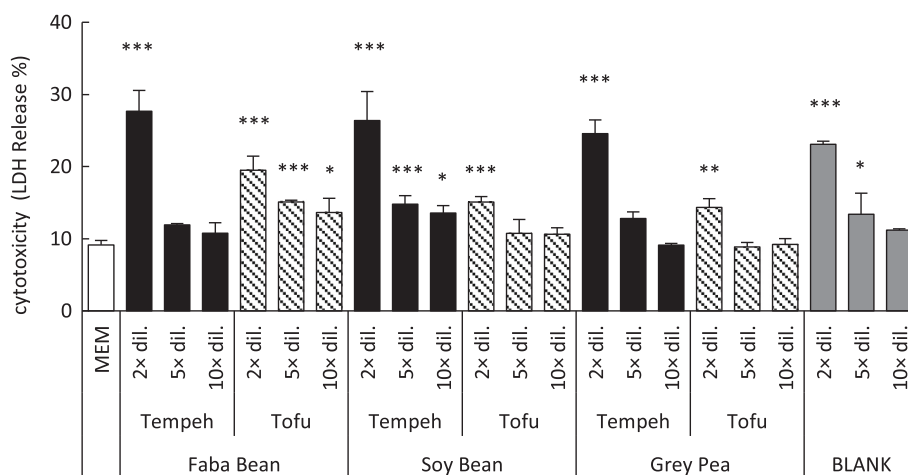
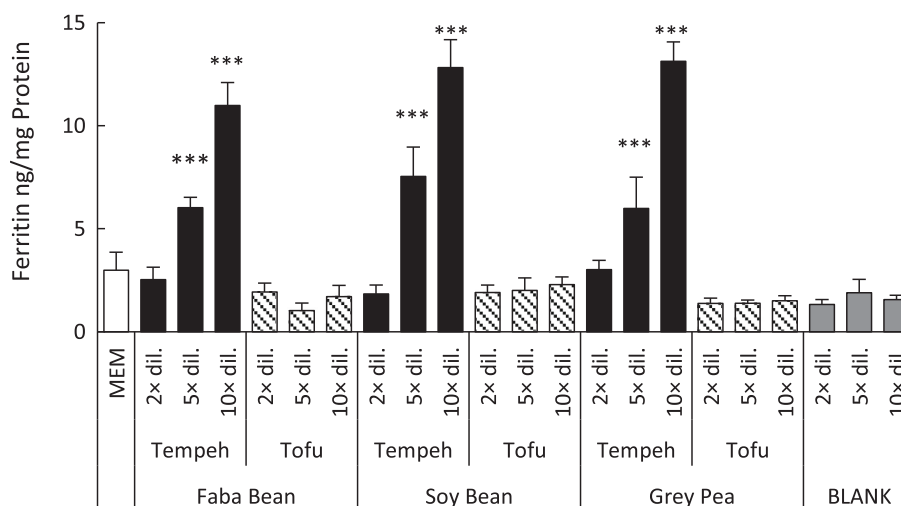


Fig. 4. Cytotoxicity after exposure to the 2, 5, and 10× dilutions of digesta (Tempeh, Tofu, Blank, Control) to evaluate concentration-dependent LDH release expressed relative to the Triton-induced maximum (100%). The asterisk indicates significant differences between the different samples/ concentrations and the MEM with \*  $p < 0.5$ ; \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .



**Fig. 5.** Ferritin formation in the cells after exposure to the 2, 5, and 10× dilutions of digesta (Tempeh, Tofu, Blank, Control) to evaluate concentration-dependent uptake. The asterisk indicates significant differences between the different samples/ concentrations and the MEM with \*  $p < 0.5$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

concentration. This may be related to reduced monolayer integrity and increased cytotoxicity in the 2× dilutions, as indicated by the LDH and TEER measurements, which could confound the ferritin response despite higher iron concentrations. The general trend of the 10× diluted samples which shows a significantly higher ferritin formation than the 5× diluted samples may reflect differences in cellular stress or matrix-related effects; therefore, concentration-dependent differences should be interpreted cautiously. This was particularly reported for soy tempeh in Experiment 1 and grey pea tempeh in Experiment 2 (Figs. 3 and 4).

Overall, ferritin results were interpreted primarily under exposure conditions that maintained acceptable cell viability and barrier integrity. Results obtained at higher digesta concentrations should be considered exploratory, as cellular stress and monolayer disruption may influence iron uptake measurements.

However, despite the concentration-dependent differences, ferritin formation is a reliable indicator of cellular iron uptake (Glahn, 2022). In the present study, this was further confirmed by the performed reference *in vitro* digestions using  $\text{FeSO}_4$ , a highly bioavailable iron source free from anti-nutritional factors. As shown in Fig. S6, ferritin formation significantly increased when cells were exposed to a 5× diluted  $\text{FeSO}_4$  sample compared to baseline iron uptake. This increase demonstrates that the model's ferritin response accurately reflects cellular iron uptake capacity when no inhibitors are present. Therefore, using  $\text{FeSO}_4$  as a positive control confirms that ferritin formation in this system is a sensitive and robust indicator of iron absorption in intestinal cells, independent of confounding effects from anti-nutritional compounds.

Nevertheless, comparing results between studies remains challenging due to the substantial variations in experimental conditions. Firstly, the type of cells used can affect overall iron uptake. Whilst Caco-2 cells alone are often used to study iron bioavailability (Au & Reddy, 2000; Dellavalle et al., 2013; R. P. Glahn, 2022; Oliveira et al., 2023; Rodriguez-Ramiro et al., 2019), the addition of mucus-producing cells can influence uptake (Mahler et al., 2009). An increasing ratio of HT29-MTX cells, for example, may lead to a decrease in ferritin levels. Although a physiologically relevant 90:10 ratio was used in this study (Mahler et al., 2009), the ratio can vary among different studies, with a 75:25 ratio often being used (Laparra et al., 2009; Mahler et al., 2009; Sandberg et al., 2018). Secondly, the differentiation time of the cells varies among experiments, commonly ranging between 13 and 16 days (Glahn, 2022; Mahler et al., 2009; Sandberg et al., 2018). Whilst full differentiation is generally achieved after 21 days, shorter culture durations may result in incomplete differentiation, potentially affecting monolayer functionality and cellular responses such as nutrient uptake (Kus et al., 2023). This, together with the use of different plate setups,

can lead to differences among experiments, highlighting the need for a standardised protocol when determining iron bioavailability. In 2022, Glahn published a bioassay for the measurement of food iron bioavailability, which aimed to provide a more standardised method. This protocol only uses Caco-2 cells and also requires performing the *in vitro* digestion on the same day as the uptake experiment (Glahn, 2022), which consequently limits the sample size depending on the number of digestions that can be performed in one day. Further, the protocol uses a different *in vitro* digestion method than the commonly used Infogest protocol, which has already been established in many labs, and requires additional preparations.

Although methodological differences can complicate direct comparisons across studies, the consistent link between ferritin formation and iron uptake reinforces its value as a practical and reliable indicator of intestinal iron bioavailability *in vitro*. In this context, ferritin allows for comparisons of iron uptake between samples to identify which crop or treatment yields the greatest amount of bioavailable iron.

#### 4. Conclusion

This study analysed the effects of different processing techniques on the nutritional properties of faba bean, yellow pea, grey pea, and soy. Overall, the results demonstrate that processing modifies the food matrix in ways that differentially influence protein digestibility and mineral availability, highlighting that nutritional quality depends on the interaction between processing method and crop type rather than a single universally optimal treatment. Fermentation altered the amino acid composition of faba bean, yellow pea, and grey pea products, notably reducing glutamic and aspartic acid levels compared to tofu. Fermentation increased the average degree of protein hydrolysis (DH), although a statistically significant improvement for cooked legumes was only observed for grey pea tempeh. Tofu, on the other hand, consistently exhibited the highest DH values across all crops. These findings indicate that protein coagulation improves protein accessibility primarily through matrix simplification and increased protein concentration, whereas the effects of fermentation on protein digestibility are limited and crop-dependent. Despite the limited effect on protein digestion, fermentation led to a reduction of phytate content in all products, with complete phytate degradation observed among both faba bean and grey pea tempeh. This phytate reduction, alongside retained or elevated levels of iron and zinc, led to markedly improved estimated mineral bioavailability in the fermented products. However, these improvements are based on predictive indicators and compositional changes and should be interpreted as potential rather than absolute nutritional

advantages.

The combined *in vitro* digestion and Caco-2/HT29-MTX cell model further supported these trends. Ferritin formation, used as an indicator of cellular iron uptake, was higher in cells exposed to digested tempeh compared to tofu under conditions where monolayer integrity was maintained.

While these results support the potential of fermentation to enhance iron uptake, the findings should be interpreted cautiously, given the physiological simplifications and experimental limitations inherent to *in vitro* cell models.

Overall, the findings partially support the study hypothesis. Fermentation improved mineral accessibility through phytate degradation, whereas improvements in protein digestibility following fermentation were limited. In contrast, protein coagulation consistently enhanced protein hydrolysis, confirming its effectiveness in improving protein digestibility.

Together, these results indicate that fermentation is particularly relevant for improving mineral accessibility, while protein coagulation is more effective for enhancing protein digestibility. Rather than identifying a single superior processing method, the findings emphasize the importance of tailoring processing strategies to the desired nutritional outcome and to the specific legume matrix. This matrix-dependent perspective is important for the development of nutritionally optimized plant-based foods.

#### CRedit authorship contribution statement

**Jaqueline Auer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Loes Duivenvoorde:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Meike van der Zande:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **Marie Alminger:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Laura Alejandra Fernandez Castaneda:** Writing – review & editing, Resources, Formal analysis. **Jing Lu:** Writing – review & editing, Resources, Formal analysis. **Giovanni Tizzanini:** Writing – review & editing, Investigation, Formal analysis. **Galia Zamaratskaia:** Writing – review & editing, Supervision. **Anders Högberg:** Writing – review & editing, Supervision. **Maud Langton:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

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#### 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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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2026.118938>.

#### Data availability

Data will be made available on request.

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