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In vitro DIAAS of Swiss soybean cultivars using the INFOGEST model: Increase in protein quality from soybean to soymilk and tofu

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ABSTRACT

To support the transition towards more sustainable and healthy diets, viable alternatives to foods of animal origin need to be identified. Many plant-based protein sources are currently marketed with claims of minimal environmental impact, but very limited consideration has been given to their protein quality and bioavailable mineral content considering the fact that animal-based foods are typically the primary source of both in Western diets. In this study, traditionally consumed soy foods (cooked soybeans, soymilk, tofu) from different Swiss soybean cultivars were nutritionally characterized and the *in vitro* digestibility of individual amino acids and total protein were assessed using an *in vitro* model based on the static INFOGEST protocol; the protein quality was evaluated using the *in vitro* digestible indispensable amino acid score (DIAAS). The results reveal an increase in total protein *in vitro* digestibility across the traditional soy food production value chain: 52.1–62.7% for cooked soybeans, 84.1–90.6% for soymilk, and 94.9–98.4% for tofu. Protein quality, determined using the recommended amino acid pattern for 0.5–3 years old, was "low" (no claim) for cooked soybeans (DIAAS < 60), while soymilk (DIAAS $= 78$–88) and tofu products (DIAAS $= 79$–91) were of similar "good" protein quality, with considerably higher DIAAS values than those of cooked soybeans ($P < 0.001$). The iron and zinc contents in soy foods were substantial, but high molar ratios of phytic acid (PA) to iron (PA/Fe; >8) and PA to zinc (PA/Zn; >15) indicate a possible strong inhibition of iron and zinc bioavailability. Based on the DIAAS results, soymilk and tofu would be suitable plant-based alternatives to animal-based foods, while future efforts should focus on optimizing soybean preparation to overcome the negative effects of the plant tissue matrix as well as processing steps to reduce mineral absorption inhibiting substances.

1. Introduction

The call for an increased emphasis on plant-based protein sources for human consumption requires a detailed evaluation of their nutritional value, especially in terms of protein quality and bioavailable mineral content compared to animal-based foods, which are currently the major sources of both (Beal, Ortenzi, & Fanzo, 2023). The annual legume soybean (*Glycine* max (L.) Merr.) is one of the most widely cultivated legumes in the world (Karges et al., 2022). Soybeans play an important role in sustainability of agriculture worldwide, as they are only dependent on nitrogen (N) fertilization to a limited extent and can enrich the soil through biological nitrogen fixation (Mohammad Sohidul, Imam, & Rafiqul, 2022). Soybeans are rich in oil (≈20%) and protein (≈40%) (García, Torre, Marina, & Laborda, 1997). Soybeans also contain

Abbreviations: AA, amino acids; AAA, aromatic amino acids; DIAAR, digestible indispensable amino acid ratio; DIAAS, digestible indispensable amino acid score, lowest value of DIAAR; DM, dry matter; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; IAA, indispensable amino acid; IVD, *in vitro* digestion; LC-MS, liquid chromatography-mass spectrometry; PA, phytic acid; PP, polyphenols; proxy DIAAR, DIAAR based on total protein digestibility; SAA, sulfur-containing amino acids; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAA, total amino acids; TN, total nitrogen.

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significant amounts of minerals such as iron and zinc (Kassem, 2021) and vitamins (e.g., thiamin, riboflavin, niacin) (Kamboj & Nanda, 2018), as well as plant metabolites (e.g., isoflavones, saponins, lignans, phytic acid), which can affect nutritional quality and mineral bioavailability (Friedman & Brandon, 2001; Liener, 1994), and bioactive proteins (e.g., trypsin and chymotrypsin inhibitors, lectin, lipoxygenase, urease) (Friedman & Brandon, 2001; Gilani, Cockell, & Sepehr, 2005). Traditionally, soybeans are consumed in non-fermented (e.g., soymilk and tofu) and fermented forms (e.g., tempeh, miso and soy sauce) (Wilson, 1995). In Switzerland, the national agriculture research institute Agroscope runs a non-GMO (processed without genetic modification) soybean breeding program, with the aim to develop cultivars adapted to the Swiss climatic conditions (i.e., with low-temperature tolerance) and providing consistent yields (Klaiss et al., 2020). Currently available Swiss soybean cultivars have enhanced protein content, optimized flavor, and/or improved processability for soymilk and tofu production (Agroscope, 2016; Schori, 2003). The composition of soy foods is not only dependent on the cultivar (Karr-Lilienthal, Grieshop, Merchen, Mahan, & Fahey, 2004; Zarkadas et al., 2007) and environmental conditions, but also on food processing and preparation steps, which can have a substantial impact (García et al., 1997; Mojica, Dia, & de Mejía, 2014).

Since the cultivation and consumption of soybeans may play a greater role in human nutrition in the future (Klaiss et al., 2020), its adaptation to colder climate conditions would allow to expand the cultivation zones. Because very little is known about the newly developed GMO-free and cold-adapted Swiss varieties, the aim of this paper was, their characterization at different steps of transformation, such as cooked soybeans, tofu, and soymilk. Characterization included: (1) measuring concentrations of macronutrients, minerals, amino acids (AA), and antinutritional components; (2) identifying the main proteins; (3) analyzing the kinetics of peptides generated during *in vitro* digestion (IVD); (4) quantifying *in vitro* digestibility of total protein and of individual AA; and (5) estimating protein quality from *in vitro* digestible indispensable amino acid score (DIAAS) values. According to the Food and Agricultural Organization (FAO), the DIAAS method for assessing protein quality is established through human or pig *in vivo* experiments, and is calculated by considering the indispensable AA (IAA) composition of the food, true ileal digestibility of IAA within the food matrix, and age-specific dietary IAA requirements (Fao, 2013). In this work, DIAAS was assessed by applying an *in vitro* workflow (Sousa et al., 2022) based on the static INFOGEST digestion protocol (Brodkorb et al., 2019).

2. Material and methods

2.1. Chemicals and reagents

For this study, reagents were purchased from Merck (Zug, Switzerland). Enzymes and bile for IVD were α-amylase from human saliva (Sigma-Aldrich, product no. A1031, Lot SLCD9952, activity of 114.6 U/mg), pepsin from porcine gastric mucosa (Sigma-Aldrich, product no. P7012, Lot SLBW6530, activity of 3368 U/mg), pancreatin from porcine pancreas (Sigma-Aldrich, product no. P7545, Lot SLCD7175, trypsin activity of 6.6 U/mg), and bile extract porcine (Sigma-Aldrich, product no. B8631, Lot SLCC9272, bile acid concentration of 1.28 mmol/g). Trypsin Gold Mass Spectrometry Grade (Promega Corporation, product no. V5280, Lot 326060) was used for tryptic digestion for peptide mass fingerprinting. All other reagents are specified in the corresponding method section.

2.2. Soybean cultivars and production of soymilk and tofu

Three Swiss soybean cultivars were investigated: Protéix (2009, Agroscope/DSP) was optimized for human consumption and features a high protein content, and good suitability for processing into soymilk and tofu, while Galice (2015, Agroscope/DSP) was developed to achieve high yields; Amandine (2012, Agroscope/DSP), a cultivar with an improved taste, does not contain the enzyme lipoxygenase 2, which reduces the grassy taste of soybeans resulting from the oxidation of fatty acids (Agroscope, 2016), and may increase the acceptance of soy-based foods by European consumers (Schori, 2003). All three soybean varieties (Amandine, Galice, and Protéix) were grown and harvested in Switzerland, and were further processed into dried soybeans, soymilk, and tofu at Agroscope in Changins, Switzerland. Dry soybeans were soaked for 16 h and subsequently cooked until the beans were tender (Amandine: 45 min; Galice: 25 min; Protéix: 25 min). Evaporated water was replaced during cooking and bean weight was monitored during soaking and cooking. Samples of soaking and cooking water were collected for analysis. For the production of soymilk and tofu, the soybeans were soaked overnight in fresh water. After rinsing, the beans were ground with water at a ratio of 12.5:1. The resulting "Go" (soup of raw soybeans) was first heated to 95 °C for in an oven under constant stirring for 20 min. Subsequently, it was hot-filtered using a commercial juice extractor to separate the okara from the soymilk. Then, the soymilk was heated again to 95 °C in a water bath for 15 min, and coagulated with a solution of Gluco-delta-lactone. After a resting period (2 h), the curds were pressed (2 kg) into a cheesecloth-covered mold using constant, even pressure. Finally, the tofu was removed from the mold (Bétrix et al., manuscript in preparation).

2.3. Product composition (macronutrients, amino acids, minerals, and antinutrients)

The dry matter (DM) contents of soybeans and tofu were calculated by subtracting the drying loss from the original weight, measured according to ISO standard 5534:2004 (ISO:5534, 2004). For the soymilk, the DM was determined as described in ISO 6731:2010 (ISO:6731, 2010). Fat content was analyzed according to Schmid-Bondzynski (ISO:1735, 2004). Total nitrogen (TN) was determined according to Kjeldahl, described in ISO 8968-3:2004 (ISO:8968-3, 2004). The protein contents were calculated by multiplying the TN by the nitrogen-to-protein conversion factor of 6.25 (TN × 6.25). The total AA were quantified with the Association of Official Analytical Chemists (AOAC) method 2018.06 for infant formula (Jaudzems, Guthrie, Lahrichi, & Fuerer, 2019), with modifications which have already been described (Hammer et al., 2023). Tryptophan content was determined according to ISO 13904:2014 (ISO:13904, 2014). Concentrations of minerals (Fe, Ca, Al, Cu, Mn, Zn) were measured by inductively coupled plasma mass spectrometry (ICP-MS iCap RQ, Thermo Scientific, Germany) in kinetic energy discrimination mode with helium as a collision gas, against a calibration curve prepared from single element standard solutions (Inorganic Ventures, Christiansburg, USA) using Sc and In as internal standards, after microwave assisted mineralization of the samples (Turbowave, MLS GmbH, Leutkirch, Germany). Sample preparation was performed as follows: to 250 mg of each sample, 4 ml of nitric acid (65%, sub-boiled) were added into 15 ml PTFE tubes. Tubes were closed with PTFE lids, placed onto the dedicated sample rack (MLS GmbH), and left for 15 min in a fume hood to react. The sample rack was then inserted into the Turbowave autoclave with a basic load made of 110 ml ultra-pure water (18.2 MΩ.cm) and 5 ml nitric acid (65%, sub-boiled). The samples were mineralized under a pressure of 35 bar nitrogen at 250 °C for 30 min, in addition to heating and cooling cycles of 15 min each. After mineralization, the samples were transferred to pre-weighed acid-washed 50 ml PE bottles (Semadeni, Ostermundigen, Switzerland), the tubes were rinsed three times with ultra-pure water, and the sample solutions were completed to 50 g with ultra-pure water. Phytic acid (PA) determination was performed according to a modified method by Makower (Makower, 1970), where iron was replaced by cerium during a precipitation step. After subsequent mineralization of precipitates, inorganic phosphate was measured according to Van Veldhoven and Mannaerts (Van Veldhoven & Mannaerts, 1987) and converted to PA concentrations. The total polyphenol (PP) concentration was

determined by adapting the Folin-Ciocalteu method (Singleton & Rossi, 1965).

All measurements were conducted at least in duplicate, with TAA, polyphenols, phytic acids, and mineral concentrations measured in triplicate.

2.4. Protein identifications of soy products (protein extraction, SDS-PAGE, peptide mass fingerprinting)

The proteins of soy products as well as the soaking and cooking water of soybeans were extracted from the food matrix and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the main gel bands were then identified by peptide mass fingerprinting.

To extract proteins, 100 mg of tofu and 200 μL of soymilk products were mixed with 200 μL buffer-1 (Tris 100 mmol/L, SDS 1%, 1,4-dithiothreitol 1%, pH = 7.4) and sonicated (3 × 6 pulses, power 60% for 2 sec, HTUSONI130, G. Heinemann, Germany) to facilitate dissolution. After protein precipitation by the addition of methanol, samples were centrifuged (17′949*g*, 4 °C, 10 min). The supernatants were discarded, and the pellets were first dried by evaporating the methanol under the fume hood (20 °C, 2 h) and then resuspended in 400 μL buffer-2 (Tris 100 mmol/L, SDS 1%, pH = 7.4). After 1 h at 20 °C, samples were sonicated (2 × 6 pulses) and centrifuged, and the supernatants were collected without lipid layer.

Protein extraction from raw and cooked soybeans was started by dissolving 100 mg of product in 900 μL of buffer-1. The samples were heated at 95 °C for 5 min, followed by an incubation at 25 °C for 14 h using an Eppendorf ThermoMixer C (Eppendorf AG, Germany) with constant shaking at 300 rpm. After sonicating samples (4 × 6 pulses), proteins were precipitated by methanol, centrifuged (17′949*g*, 4 °C, 10 min), and the supernatants discarded. The dry pellets were resuspended in 400 μL buffer-2, mixed, and incubated for 1 h at 20 °C. Subsequently, samples were sonicated (1 × 6 pulses) and centrifuged, and the resulting supernatants transferred into new tubes. After another centrifugation cycle, the supernatants were collected.

The sampled soaking and cooking water of the soybeans were mixed with methanol to precipitate proteins. After centrifugation (17′949*g*, 4 °C, 10 min), the supernatants were discarded. The pellets of the soaking and cooking water samples were resuspended in 200 μL and 500 μL buffer-1, respectively. The samples were incubated at 20 °C for 1 h and centrifuged, and the supernatants were collected.

Concentrations of extracted proteins were quantified using the Pierce® BCA Protein Assay Kit, Reducing Agent Compatible (ThermoFisher Scientific, USA). Protein solutions were mixed with 6x sample buffer (Tris-HCl 350 mmol/L, glycerol 50%, SDS 10%, 1,4-dithithreitol 100 mmol/L, pH = 6.8) and incubated at 95 °C for 5 min. Proteins were separated by SDS-PAGE (polyacrylamide 12%) by loading equal protein concentrations and volumes per sample and a molecular weight marker (Benchmark, Invitrogen, USA). Gels were stained with colloidal Coomassie, as described in Kang, Gho, Suh, and Kang (2002).

The individual proteins in main gel bands were identified by peptide mass fingerprinting (Egger, Ménard, & Portmann, 2018), which was performed according to Hammer et al. (2023). Briefly, after tryptic-in-gel digestion, peptides were separated and analyzed by a Rheos 2200 HPLC (Flux Instruments AG, Switzerland) with Xterra MS C18 column (Waters, USA), connected to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Switzerland) with an electron spray ionization interface. Fragmentation data were submitted to the Mascot search engine (Matrix Science, UK) using the Uniprot database (accessed 2019) to identify proteins.

2.5. In vitro digestion

Static INFOGEST IVD (Minekus et al., 2014) was performed with cooked soybeans, soymilk, and tofu from different cultivars. Briefly, foods were subjected to: (1) an oral phase (pH 7, 37 °C, 2 min) by addition of simulated salivary fluid and salivary amylase at 75 U/mL; (2) a subsequent gastric phase (pH 3, 37 °C, 120 min) involving dilution (1:1 vol/vol) with simulated gastric fluid and pepsin at 2000 U/mL; and (3) an intestinal phase (pH 7, 37 °C, 120 min) where the gastric chyme was diluted (1:1 vol/vol) with simulated intestinal fluid, a pancreatin suspension containing trypsin at 100 U/mL, and a bile suspension at 10 mmol/L. The preparation of the pancreatin suspension was adapted, as described in Sousa et al. (2022): Pancreatin and SIF were mixed, subjected to 5 min of ultrasonic treatment, centrifuged (2000*g*, 4 °C, 5 min), and supernatant was immediately added to IVD samples. During IVD, digestion samples were mixed by a rotating wheel (Stuart™ Rotator SB3, Bibby Scientific™, UK). Upon sampling, pepsin activity of gastric phase was stopped by adjusting the pH to 7, and intestinal phase digestion was stopped by addition of a protease inhibitor (1 mmol/L; AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Switzerland). After inactivation, digestion samples were immediately snap-frozen in liquid nitrogen.

As preparation for IVD, assays were performed to quantify the activities of digestive enzymes, the concentrations of bile salts (Minekus et al., 2014), and to adjust the pH of the digestive phases (Brodkorb et al., 2019). Before starting the IVD protocol, soymilk products were homogenized (2×, 10,000 rpm, 30 sec) with Omni-Prep Multi-Sample Homogenizer (Omni International, USA). The cooked soybeans and tofu products were cut into smaller pieces with a food processor (Moulinex DPA3, Moulinex, France) to simulate the effect of mastication. Soy products corresponding to 40 mg of protein were combined with water, resulting in a food sample of 1 g to start IVD. If soymilk products exceeded the required starting weight of 1 g, simulated salivary fluid was adjusted accordingly to obtain the required final volume for the oral phase. In each run, 1 g of a protein-free cookie, prepared as reported elsewhere (Sousa, Portmann, Dubois, Recio, & Egger, 2020), was digested in parallel to account for background in the subsequent analyses.

2.6. Digesta sampling and treatment

To investigate the IVD kinetics of peptides (amino acid counting, see Section 2.7), digesta of 21 IVD carried out in separate tubes were stopped at different times during the IVD protocol. After centrifugation (13,000*g*, 4 °C, 15 min) of digestion samples, supernatants and pellets were stored separately until analysis.

For the assessment of total protein *in vitro* digestibility (see Sections 2.8 and 2.9) and *in vitro* digestibility of individual AA (see Section 2.9), IVD was carried out until the end of the intestinal phase with at least three individual experiments. The procedure to separate digestible (potentially absorbable) and indigestible fractions was recently published by Sousa et al. (2022). Briefly, undigested proteins and larger peptides (>8–10 AA) in digesta were precipitated with methanol (80% vol/vol, final concentration) at −20 °C for 1 h, samples were centrifuged (2000*g*, 4 °C, 10 min), supernatants (digestible fraction) were collected, and 220 μL samples were dried in a CentriVap (Labconco, USA). To remove remaining supernatant, pellets (indigestible fraction) were washed twice with methanol and subsequently dried completely. Dried pellets and supernatants were spiked with internal standard L-Norvalin, flushed with nitrogen gas, and then hydrolyzed using hydrochloric acid 6 mol/L at 110 °C for 15 h.

2.7. Analysis and representation of peptide patterns (amino acid counting) (LC-MS)

Peptides in supernatants of digesta sampled at 21 different time points during IVD were analyzed as described in detail elsewhere (Egger, Schlegel, et al., 2018). The supernatants of the IVD kinetics were filtered through Amicon columns (Ultracel YM-30, Millipore, Zug, Switzerland). Peptides were separated by a Rheos 2200 HPLC (Flux Instruments AG, Switzerland), equipped with Xterra MS C18 column (Waters, USA), and

measured with coupled linear ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland). After an identification search with Mascot (Matrix Science, UK) using a database containing the main soy proteins (created from the Uniprot database, accessed in 2019), individual AA of all identified peptides of the protein of interest were summed up as previously described (Egger, Ménard, et al., 2018; Portmann et al., 2023). The resulting number, indicates how many times this amino has been detected within all identified peptides of this sequence of the protein. The abundance was color-coded from blue (low abundance) to red (high abundance). Unidentified sequences of the protein are indicated in white.

2.8. Quantification of primary amines in digesta (R-NH_2 method)

Acid-hydrolyzed IVD supernatants and pellets (see Section 2.6) were diluted fivefold and tenfold, respectively, by the addition of 500 mmol/L perchloric acid, and derivatized with o-phthaldialdehyde (Church, Swaisgood, Porter, & Catignani, 1983). The absorbance of the resulting 1-alkylthio-2-alcylisonindol compound was measured at 340 nm with a UV/VIS spectrophotometer, in parallel with a glutamic acid standard curve (Kopf-Bolanz et al., 2012), to quantify the total amount of primary amines in digestible (supernatant) and indigestible (pellet) fractions.

2.9. Quantification of individual amino acids in digesta (Total amino acids method)

The total amount of individual AA present in IVD supernatants (digestible fraction) and pellets (indigestible fraction) after acid hydrolysis (see Section 2.6) was measured with the AOAC method 2018.06 for infant formula (Jaudzems, Guthrie, Lahrichi, & Fuerer, 2019), using modifications described elsewhere (Sousa et al., 2022). Briefly, after derivatization of hydrolyzed samples with AccQ-Tag Ultra reagent (Waters, USA), amounts of individual AA were quantified by ultra-high performance liquid chromatography (UHPLC, Acquity UPLC BEH C18 2.1 × 150 mm, 1.7 µm, Waters) coupled with a UV detector (Vanquish, Thermo Scientific, Switzerland).

2.10. Calculation of in vitro digestibility, in vitro DIAAR, and proxy in vitro DIAAR

The total amount of individual AA (mg), measured in hydrolyzed IVD supernatants and pellets (total amino acids (TAA) method, Section 2.9), was used to determine the *in vitro* digestibility of each AA, from which the *in vitro* DIAAR values were determined. The mean *in vitro* digestibility of all AA is referred to as the total protein *in vitro* digestibility. As an alternative analytical method, the total protein *in vitro* digestibility was calculated with the total amount of primary amines (mmol glutamic acid equivalents) in hydrolyzed IVD supernatants and pellets (R-NH_2 method, Section 2.8). With the total protein *in vitro* digestibility, which cannot distinguish between individual AA, an approximation of DIAAR (called proxy *in vitro* DIAAR) was calculated with the sole difference from the *in vitro* DIAAR being that total protein *in vitro* digestibility (average digestibility obtained either from TAA or R-NH_2 method) was used for all IAA, instead of their individual digestibility.

Calculations were performed as previously reported by Sousa et al. (2022). Briefly, digestibility of individual and total protein (Eq. (1)) was calculated by the division of the cookie-corrected digestible fraction (supernatants; Cookie supernatant = Cs; Food supernatant = Fs) by the cookie-corrected total of digestible (supernatants) and indigestible (pellets; Cookie pellet = Cp; Food pellet = Fp) fractions. To determine *in vitro* DIAAR, the *in vitro* digestible IAA content (DIAA) for each IAA in one gram of food protein (TN × 6.25) was first calculated with Eq. (2) and subsequently divided by the AA pattern of a reference protein provided in the FAO report (Fao, 2013) (Eq. (3)). Proxy *in vitro* DIAAR was calculated based on Eqs. (4) and (5).

$$invitro\text{digestibility}\,[\%] = \frac{(Fs - Cs)}{((Fs - Cs) + \max(0; Fp - Cp))} \times 100 \quad (1)$$

$$invitroDIAA = mgofIAApergoffoodprotein \times invitrodigestibilityofIAA \quad (2)$$

$$invitroDIAAR[\%] = \frac{invitroDIAA(Eq.2)}{\text{mg of the same dietary IAA in 1 g of the reference protein}} \times 100 \quad (3)$$

$$proxyinvitroDIAA = mgofIAApergfoodprotein \times totalinvitrodigestibility \quad (4)$$

$$proxyinvitroDIAAR = \frac{proxyinvitroDIAA(Eq.4)}{mgofthesamedietaryIAAin1gofthereferenceprotein} \times \quad (5)$$

In vitro DIAAR and proxy *in vitro* DIAAR were calculated by considering the AA pattern of a reference protein, reflecting the IAA requirements for either (1) infants (birth to 6 months), (2) young children (6 months to 3 years), or (3) older children, adolescents, and adults (Fao, 2013). For a given reference pattern, the lowest of the nine calculated DIAAR is reported as the *in vitro* DIAAS of a food and the corresponding IAA as the first limiting IAA. For legal purposes, the FAO requests the use of the reference pattern for young children (Fao, 2013).

2.11. Statistical analysis

Data analyses were performed using IBM SPSS Statistics 28.0.1.1 (SPSS Inc., Chicago, IL), R statistical programming environment (R Version 4.1.3, 2022) and Microsoft Office Excel (Version 2210, Microsoft, Redmond, WA). The results are indicated as means ± standard deviations (SDs). Statistical differences in total protein *in vitro* digestibility, *in vitro* DIAAR of each AA, and *in vitro* DIAAS values between products were analyzed using two-way analysis of variance (ANOVA), considering the effect of product type and soybean cultivar, followed by Bonferroni's test as a post-hoc test with the level of significance set at $P < 0.05$.

3. Results

3.1. Soy food characterization

The dried raw soybeans from the Galice, Amandine, and Protéix soybean cultivars contained between 36.5% and 44.1% of protein and between 11.4% and 13.3% of fat, with the highest protein and the lowest fat contents found in the Protéix cultivar. Cooked soybeans, soymilk, and tofu were produced from raw soybeans, which yielded cooked soybeans and tofu products with approximately 15% protein and fat contents below 10% in fresh matter. Soymilk products had a protein content of 2.4–2.8% and a fat content of 1.2–1.4% (Table 1, Suppl. Table B, values based on dry matter).

Soy foods of all cultivars had an AA profile rich in IAA (Suppl. Table 1) with molar ratios of indispensable to dispensable AA (IAA/DAA) between 0.56 and 0.61 (Table 2). Protéix products generally had slightly lower IAA/DAA ratios, indicating their higher protein content resulting from an increase in dispensable AA rather than IAA (Table 2). The IAA content per gram of dietary protein, as presented in Table 2, indicates that most of the soy foods would meet the required amino acid scoring pattern for preschool children (Fao, 2013), if digestibility is not taken into account. For tofu products, the levels of sulfur-containing AA (SAA), leucine and/or lysine were below the FAO recommendations (Fao, 2013) (Table 2).

Polyphenols (PP) and phytic acid (PA) were lowest in tofu products, when normalized for protein content. Soaking and cooking of the raw soybeans resulted in cooked soybeans with reduced PP contents per gram protein but not reduced PA contents, while soymilk products had

Table 1
Characterization of the soy products of the soybean cultivars Galice, Amandine, and Protéix. Values are means ± SDs in fresh matter. DM = dry matter, Zn = zinc, Fe = iron, Ca = calcium, PP = polyphenols, PA = phytic acid, GAE = gallic acid equivalents.

		DM [g/100 g]	Fat [g/100 g]	Protein [g/100 g]	Zn [mg/kg]	Fe [mg/kg]	Ca [g/kg]	PP [mg GAE/ 100 g]	PA [g/100 g]	PA:Fe molar ratio	PA:Zn molar ratio
Galice	Raw soybeans	92.10 ± 0.05	13.32 ± 0.22	36.5 ± 1.1	49.61 ± 0.38	69.13 ± 1.45	2.01 ± 0.05	187.6 ± 6.3	0.80 ± 0.06	9.8	16.0
	Cooked soybeans	34.56 ± 0.11	9.72 ± 0.02	14.3 ± 0.4	15.94 ± 0.26	17.94 ± 0.19	0.73 ± 0.01	54.6 ± 1.3	0.33 ± 0.02	15.5	20.4
	Soymilk	4.94 ± 0.04	1.42 ± 0.01	2.4 ± 0.0	2.89 ± 0.08	3.59 ± 0.05	0.08 ± 0.00	11.0 ± 0.1	0.06 ± 0.03	14.6	21.3
	Tofu	23.65 ± 0.21	7.89 ± 0.05	12.5 ± 0.5	8.69 ± 0.12	13.26 ± 0.17	0.49 ± 0.01	32.1 ± 0.9	0.17 ± 0.01	10.5	18.8
Amandine	Raw soybeans	92.40 ± 0.04	13.32 ± 0.18	41.3 ± 0.5	37.76 ± 1.12	80.20 ± 5.49	2.36 ± 0.08	166.9 ± 3.4	1.07 ± 0.01	11.3	28.1
	Cooked soybeans	34.29 ± 0.01	9.24 ± 0.01	17.4 ± 0.2	15.67 ± 0.46	27.03 ± 0.63	0.32 ± 0.00	37.8 ± 0.2	0.52 ± 0.01	16.4	33.1
	Soymilk	5.11 ± 0.00	1.42 ± 0.05	2.7 ± 0.0	2.60 ± 0.19	3.52 ± 0.11	0.08 ± 0.00	10.6 ± 0.1	0.09 ± 0.00	21.4	33.9
	Tofu	24.85 ± 1.48	8.96 ± 0.03	14.4 ± 3.2	9.64 ± 0.30	13.78 ± 0.12	0.61 ± 0.01	26.2 ± 0.3	0.21 ± 0.00	13.0	21.8
Protéix	Raw soybeans	92.05 ± 0.08	11.43 ± 0.14	44.1 ± 0.3	40.21 ± 1.51	75.28 ± 2.15	2.05 ± 0.06	145.4 ± 7.4	1.04 ± 0.03	11.7	25.7
	Cooked soybeans	32.44 ± 0.08	7.49 ± 0.29	17.2 ± 0.1	15.20 ± 0.12	21.48 ± 0.15	0.34 ± 0.00	41.0 ± 0.6	0.43 ± 0.04	17.0	28.2
	Soymilk	4.92 ± 0.03	1.22 ± 0.01	2.8 ± 0.0	2.25 ± 0.13	2.87 ± 0.36	0.07 ± 0.01	6.4 ± 0.1	0.09 ± 0.00	26.8	40.0
	Tofu	27.70 ± 0.57	8.46 ± 0.18	16.3 ± 2.3	10.62 ± 0.06	23.59 ± 0.32	0.21 ± 0.00	30.8 ± 0.3	0.25 ± 0.00	8.8	23.0

Table 2
IAA composition of soy products from the soybean cultivars Galice, Amandine, and Protéix. Values are means ± SDs in mg AA/g protein and are presented in comparison to recommended amino acid scoring pattern used in DIAAS calculation.

	FAO	Raw soybeans			Cooked soybeans			Soymilk			Tofu		
	Child[1]	Galice	Amandine	Protéix	Galice	Amandine	Protéix	Galice	Amandine	Protéix	Galice	Amandine	Protéix
HIS	20	26.7 ± 0.6	25.7 ± 1.1	25.6 ± 0.6	30.7 ± 0.1	27.6 ± 0.4	26.4 ± 0.3	25.3 ± 1.0	25.9 ± 1.1	25.4 ± 0.2	24.1 ± 5.0	24.5 ± 4.4	22.0 ± 2.6
ILE	32	48.5 ± 1.6	46.1 ± 2.8	46.2 ± 1.1	56.6 ± 0.2	50.8 ± 0.8	48.3 ± 0.5	47.3 ± 2.0	48.1 ± 2.4	47.0 ± 0.4	47.0 ± 9.3	45.6 ± 7.7	40.1 ± 3.2
LEU	66	76.3 ± 1.9	73.3 ± 2.5	72.5 ± 1.4	87.5 ± 0.2	78.7 ± 1.0	75.2 ± 0.7	75.2 ± 1.7	76.9 ± 3.4	75.1 ± 2.4	79.2 ± 15.6	62.1 ± 15.0	55.3 ± 9.5
LYS	57	65.1 ± 1.5	61.9 ± 2.4	59.8 ± 0.9	74.4 ± 0.4	66.6 ± 1.0	63.0 ± 0.6	63.2 ± 1.8	63.6 ± 2.3	61.3 ± 0.5	58.7 ± 11.9	59.3 ± 10.3	51.6 ± 5.2
SAA	27	31.9 ± 0.7	31.4 ± 0.7	29.4 ± 0.2	35.0 ± 0.1	33.2 ± 0.1	30.4 ± 0.1	31.1 ± 0.0	32.2 ± 0.0	29.6 ± 0.0	24.3 ± 0.4	27.0 ± 0.6	23.3 ± 0.4
AAA	52	84.9 ± 1.3	81.9 ± 1.1	80.0 ± 0.5	92.0 ± 0.2	80.3 ± 0.6	78.4 ± 0.4	85.5 ± 0.1	87.6 ± 0.1	85.4 ± 0.1	82.4 ± 2.0	104.4 ± 4.2	93.3 ± 4.1
THR	31	40.0 ± 0.8	38.0 ± 1.1	36.7 ± 0.6	46.2 ± 0.2	41.1 ± 0.7	38.4 ± 0.3	39.2 ± 0.9	39.0 ± 1.5	37.6 ± 0.5	37.5 ± 7.6	36.9 ± 6.4	32.8 ± 3.5
TRP	8.5	13.9 ± 0.0	12.9 ± 0.0	12.8 ± 0.0	15.0 ± 0.0	12.4 ± 0.0	12.7 ± 0.0	13.1 ± 0.2	11.0 ± 0.1	11.0 ± 0.0	11.0 ± 0.1	10.0 ± 0.0	10.1 ± 0.2
VAL	43	51.3 ± 1.1	48.0 ± 2.0	47.9 ± 1.3	58.5 ± 0.2	52.0 ± 0.6	49.6 ± 0.6	49.2 ± 1.0	49.2 ± 1.7	48.4 ± 1.0	49.0 ± 10.4	49.1 ± 9.1	43.8 ± 4.7
Ratio[2]	na	0.60	0.60	0.58	0.61	0.61	0.59	0.58	0.57	0.56	0.61	0.60	0.58

[1] FAO report 2013, page 29, recommended amino acid scoring pattern for children aged 6 months to 3 years (Fao, 2013).
[2] Indispensable to dispensable amino acids molar ratio (IAA/DAA).

similar PP and PA contents compared to raw soybeans (Table 1).

The mineral content is presented in Table 1 (zinc, iron, calcium) and Suppl. Table 2 (aluminum, copper, manganese). Soy foods were found to be a source of calcium, iron, and zinc. Raw Amandine soybeans had the highest iron and calcium contents, with 8 mg and 240 mg per 100 g of food, respectively (Table 1). Zinc content was highest in raw soybeans of the Galice cultivar, with a value of 5 mg per 100 g of food (Table 1). Nonetheless, very high molar ratios of PA to iron (PA/Fe; >8), and PA to zinc (PA/Zn; >15) were observed for all soy foods, indicating a possible marked inhibition of bioavailability overall, whereas PA/Zn and PA/Fe ratios tended to be slightly lower in tofu than in soymilk and cooked soybeans (Table 1).

3.2. Soy protein identifications

Protein patterns were visualized by SDS-PAGE using cooked soybeans from the three soybean cultivars and their respective soaking and cooking water (Fig. 1), as well as of the respective tofu and soymilk products (Suppl. Fig. 1). All samples were normalized according to their protein concentrations. Selected gel bands were excised, and soy

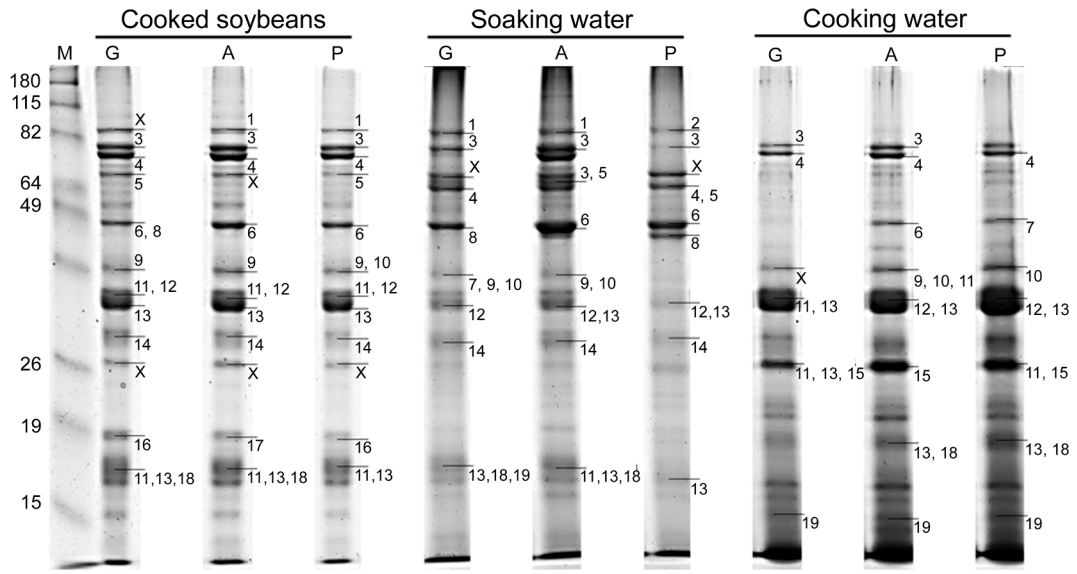


Fig. 1. Gel with cooked soybeans of the three soybean cultivars Galice (G), Amandine (A), and Protéix (P), in comparison to soaking and cooking water. Gel bands are labeled with numbers that correspond to identified proteins by peptide mass fingerprinting, listed in Table 3.

proteins were identified by tryptic in-gel digestion and subsequent peptide mass fingerprinting, as listed in Table 3.

The majority of the identified soy proteins belong to the two multi-subunit storage proteins glycinin (Table 3: No. 9–13) and β-conglycinin (Table 3: 3, 4, 6–8). Moreover, other seed storage proteins (Table 3: No. 15, 19), lipoxygenases (Table 3: No. 1, 2), lectin, and trypsin inhibitor A (Table 3: No. 14, 18), and the proteins of cellular components (Table 3: No. 5, 16, 17) were identified in soy foods.

The relative protein composition of cooked soybeans, tofu, and soymilk from the three soybean cultivars indicated few differences, as all soy foods had similar gel band patterns and almost identical proteins identified (Suppl. Fig. 1, Table 3). Almost all proteins identified in cooked soybeans were also found in the soaking and cooking water, except for lipoxygenases, which were absent in all cooking water samples (Fig. 1). Gel bands for soaking water were most intense for subunits of β-conglycinin, whereas gel bands for glycinin's subunits were more pronounced in cooking water.

Table 3
List of main identified soy proteins by LC-MS analysis after tryptic in-gel digestion. In Fig. 1 and Suppl. Fig. 1, gel bands are labeled with number of protein to indicate that the protein was identified in the gel band.

No.	Mass	Protein [Uniprot]	Protein name
1	94 kDa	LOX1_SOYBN	Seed Lipoxygenase 1
2	97 kDa	LOX2_SOYBN	Seed Lipoxygenase 2
3	70 kDa	Q84UB3_SOYBN	Beta-Conclycinin alpha
4	70 kDa	GLCA1_SOYBN[1]	Beta-Conglycinin alpha Subunit 1
5	61 kDa	SBP_SOYBN	Sucrose-binding Protein
6	48 kDa	Q50JD8_SOYBN	Beta-Conglycinin beta Subunit
7	50 kDa	GLCB1_SOYBN	Beta-Conglycinin beta Subunit 1
8	50 kDa	GLCB2_SOYBN	Beta-Conglycinin beta Subunit 2
9	58 kDa	A3KEY8_GLYSO	Glycinin A3B4
10	58 kDa	GLYG5_SOYBN	Glycinin G5
11	64 kDa	GLYG4_SOYBN	Glycinin G4
12	54 kDa	GLYG2_SOYBN	Glycinin G2
13	56 kDa	GLYG1_SOYBN[1]	Glycinin G1
14	31 kDa	LEC_SOYBN	Lectin
15	46 kDa	7SB1_SOYBN	Basic 7S Globulin
16	24 kDa	OLEO2_SOYBN	P24 Oleosin Isoform B
17	24 kDa	OLEO1_SOYBN	P24 Oleosin Isoform A
18	24 kDa	ITRA_SOYBN[1]	Trypsin Inhibitor A
19	18 kDa	2SS_SOYBN	2S Albumin
X	na	na	No proteins identified

[1] Proteins selected for evaluation of kinetics of peptides generated during IVD, shown as peptide patterns in Fig. 2 for Protéix soy foods.

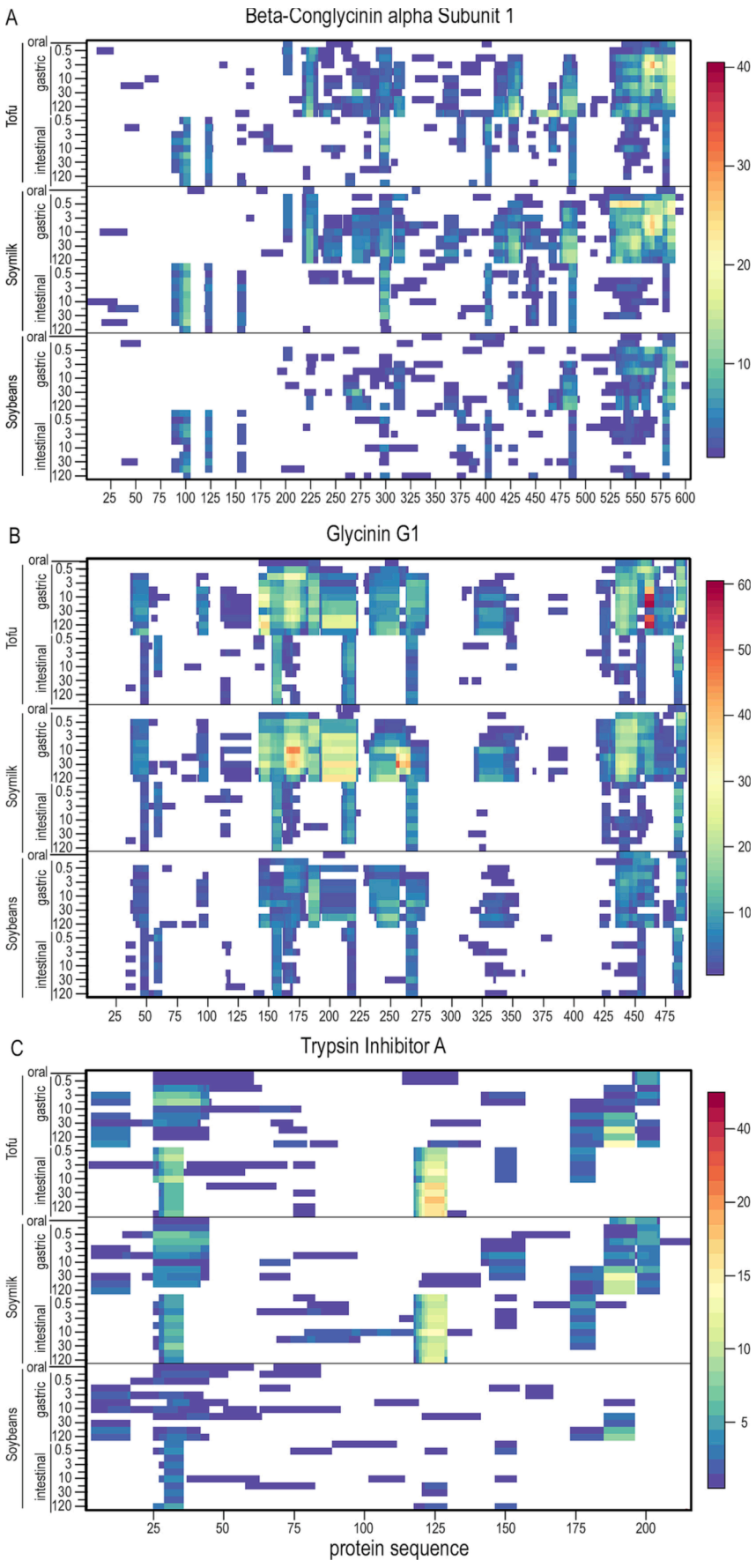
3.3. Kinetics of peptide generation

At the end of the intestinal phase, no soy proteins could be detected in the digesta of any of the soy foods by peptide mass fingerprinting (data not shown). Peptide release kinetics during IVD of selected proteins of cooked soybeans, soymilk, and tofu were visualized by aligning the identified peptides along the protein sequence for each selected time point during gastric and intestinal digestion, as described in Section 2.7. Peptide release kinetics are shown for A) *Beta-Conglycinin alpha Subunit 1* (GLCA1_SOYBN); B) *Glycinin G1* (GLYG1_SOYBN); and C) *Trypsin Inhibitor A* (ITRA_SOYB), from the Protéix (Fig. 2), Amandine, and Galice cultivars (Suppl. Fig. 2, Suppl. Fig. 3).

The peptide pattern of *Beta-Conglycinin alpha Subunit 1* (Fig. 2a) was very similar for tofu and soymilk; however, fewer peptides were identified in cooked soybeans. Identified peptides were primarily towards the C-terminus of the protein sequence (approximate positions: 200–590). In the intestinal phase, fewer peptides were identified towards the C-terminus of the protein sequence and large segments towards the N-terminus had no identified peptides in both the gastric and intestinal phases.

The peptides of *Glycinin G1* (Fig. 2b) were released along the whole protein sequence in a block-wise manner (approximate positions: 145–220, 230–280, and 425–485) with smaller segments without any identifications in between. Similar to the peptides of *Beta-Conglycinin alpha Subunit 1*, *Glycinin G1* peptides were more abundant in the gastric phase compared to the intestinal phase, suggesting that the main digestion occurred throughout the gastric phase or in the very beginning of the intestinal phase, as soon as pancreatin was introduced. For Amandine and Galice products (Suppl. Fig. 2, Suppl. Fig. 3), more peptides were identified for tofu than for soymilk, whereas no clear difference was observed between tofu and soymilk in Protéix products (Fig. 2b). *Glycinin G1* was least degraded in cooked soybeans compared to soymilk and tofu of all cultivars.

Released peptides of *Trypsin inhibitor A* (Fig. 2c) did not clearly differ in abundance between the gastric and intestinal phases, though peptides were concentrated in two vs three sequences of the protein (approximate positions: 25–40, 115–130, and 170–180) in the gastric phase and the intestinal phase, respectively. In Protéix products, *Trypsin inhibitor A* was more degraded in soymilk than in tofu (Fig. 2c). However, tofu was



(caption on next page)

Fig. 2. Peptide patterns of the soy proteins **(A)** *Beta-Conglycinin alpha Subunit 1* (GLCA1_SOYBN); **(B)** *Glycinin G1* (GLYG1_SOYBN), and **(C)** *Trypsin Inhibitor A* (ITRA_SOYBN), are shown for soy foods from soybean cultivar Protéix. *In vitro* digesta were sampled at multiple times during IVD and are presented in chronological order from the top to the bottom (y-axis). Peptide patterns of samples are shown for 2 min of oral phase; 0, 0.5, 1, 3, 5, 10, 20, 30, 60, and 120 min of gastric, and intestinal phase, respectively. Identified peptides were aligned along the protein sequence (x-axis); amino acids within identified peptides were summed up and are shown in color codes according to their abundance as described in Section 2.7. White areas indicate that no peptides were measured.

degraded to a higher extent than soymilk in Amandine and Galice products (Suppl. Fig. 2, Suppl. Fig. 3). In all three peptide patterns (Fig. 2), proteins in cooked soybeans were degraded the least compared to tofu and soymilk, which was true for all three cultivars.

3.4. In vitro digestibility of AA and of total protein

Assessment of total protein *in vitro* digestibility by TAA (Fig. 3) and R-NH_2 analyses (Suppl. Fig. 4) resulted in considerably lower values for cooked soybeans than for soymilk and tofu products ($P < 0.001$). Tofu products were found to be the most digestible compared to all investigated soy foods ($P < 0.001$). Moreover, total protein *in vitro* digestibility of tofu products did not differ from the highly digestible cooked chicken breast (Hammer et al., 2023).

Total amino acid analysis (Fig. 3) revealed differences between cultivars: Cooked soybeans of the Protéix cultivar (62.7% ± 2.5%) were found to be more digestible than those of the Amandine (52.1% ± 2.1%; $P < 0.001$) and Galice (56.8% ± 0.8%; $P < 0.05$) cultivars. The soymilk of the cultivar Protéix (90.6% ± 1.6%) had a higher total protein *in vitro* digestibility than soymilk of the cultivar Amandine (84.1% ± 3.2%; $P < 0.05$) but was not different from soymilk and tofu of the Galice cultivar.

In soymilk, *in vitro* digestibility was lowest for tryptophan, tyrosine, and cysteine, with values ranging from 60% to 75% across cultivars, whereas values for several AA were in the 90–100% range. Tofu products had some *in vitro* digestibility values between 85% and 90%, specifically for methionine and tyrosine, but the majority of AA had values between 95% and 100%, except for the Galice cultivar. *In vitro* digestibility of individual AA for the three variants of cooked soybeans ranged from 30% to 40% for tryptophan to 80% for methionine (Suppl. Table 3).

3.5. In vitro DIAAR and DIAAS

In vitro DIAAR values of cooked soybeans, soymilk, and tofu from the Protéix cultivar were compared to DIAAR values of chicken breast (Hammer et al., 2023) (Fig. 4a). Cooked soybeans had the lowest *in vitro* DIAAR values for all AA ($P \leq 0.002$), except for sulfur-containing AA (SAA). For histidine, leucine, and lysine, soymilk had higher *in vitro* DIAAR values than tofu ($P < 0.01$), but lower values for AAA ($P < 0.001$). *In vitro* DIAAR values were higher for chicken than soymilk and tofu ($P < 0.001$), except for tryptophan and AAA.

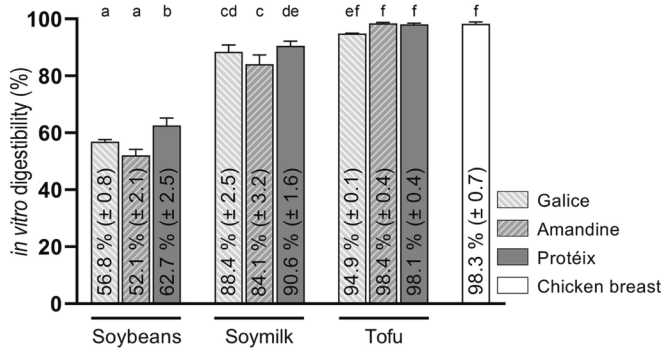


Fig. 3. Total protein *in vitro* digestibility determined by TAA analysis of cooked soybeans, soymilk, and tofu from soybean cultivars Galice, Amandine, and Protéix, in comparison to cooked chicken breast (Hammer et al., 2023). Bars without common letters differ ($P < 0.05$).

In vitro DIAAR values for tofu of the cultivar Galice were lower compared to tofu from other cultivars ($P < 0.05$), except for SAA, AAA, and tryptophan (Fig. 4b). For all AA except leucine and tryptophan, *in vitro* DIAAR values were higher in tofu of the cultivar Amandine compared to tofu of the cultivar Protéix ($P < 0.001$). *In vitro* DIAAR values of cooked soybeans of the cultivar Amandine were generally lower than soybeans from other cultivars, while values for the soybeans of cultivars Amandine and Protéix did not differ for most AA (Suppl. Fig. 5). *In vitro* DIAAR values for different soymilk products were not statistically different across cultivars (Suppl. Fig. 6).

In vitro DIAAS values of products from all cultivars are presented in Fig. 5. Cooked soybeans had *in vitro* DIAAS values below 75%, while the values for soymilk and tofu products ranged from 75% to 99%. Soymilk and tofu did not differ significantly in *in vitro* DIAAS but were both higher than cooked soybeans ($P < 0.001$). In comparison to cooked chicken breast, all soy foods had significantly lower values ($P < 0.001$). Soybean cultivar type was not significantly different in DIAAS when compared across all soy foods.

The presented DIAAR and DIAAS results were calculated by considering the in vitro digestibility of individual AA (Suppl. Table 3), assessed by TAA analysis (Suppl. Table 4a), and as an approximation of *in vitro* DIAAR "proxy *in vitro* DIAAR" can be calculated based on total protein *in vitro* digestibility by TAA analysis instead of *in vitro* digestibility of individual AA (Suppl. Table 4b). Additionally, proxy *in vitro* DIAAR values were calculated based on R-NH_2 analysis, which does not allow for the assessment of digestibility of individual AA (Suppl. Table 4c). Proxy *in vitro* DIAAS was generally higher than *in vitro* DIAAS, although the protein quality classification of < 75% (no claim), 75–99% (good quality), or ≥ 100% (excellent quality) was not affected. Proxy *in vitro* DIAAR by TAA and R-NH_2 analyses resulted in similar values and identified the same limiting AA (Suppl. Table 4b, Suppl. Table 4c), but did not correspond to limiting AA of *in vitro* DIAAS calculation (Suppl. Table 4a).

4. Discussion

4.1. Protein quality

Food protein sources can be categorized by DIAAS value into no claim (DIAAS < 75), good (DIAAS = 75–99), and excellent (DIAAS ≥ 100) protein quality (Fao, 2013). By this definition, cooked soybeans of all Swiss soybean cultivars are of low ("no claim") quality, which could be increased by producing soymilk and tofu products, resulting in "good" protein sources, based on our *in vitro* results and considering IAA requirements for preschool children aged 6 months to 3 years. These *in vitro* DIAAS values are comparable to *in vivo* DIAAS values for true ileal digestibility (6 months to 3 years) assessed in mini pigs reported by Reynaud et al. (2021) of 99 (Lys) and 83 (SAA) for commercially obtained soymilk and tofu, respectively. It is important to note that a direct comparison of our products to the commercial products studied by Reynaud et al. (2021) is not possible due to non-identical soybean cultivars, climatic conditions, and potentially different processing steps to produce soymilk and tofu, all of which could modify the AA profile of products as well as their digestibility. To date, no *in vivo* DIAAS for cooked whole soybeans has been reported; however, Han, Moughan, Li, and Pang (2020) found similarly low values (6 months to 3 years) ranging from 53 to 77 for six cooked pulses in growing pigs.

Available *in vivo* data reveal that processed soy products seem to be among the highest-quality plant-based protein sources: Soy protein

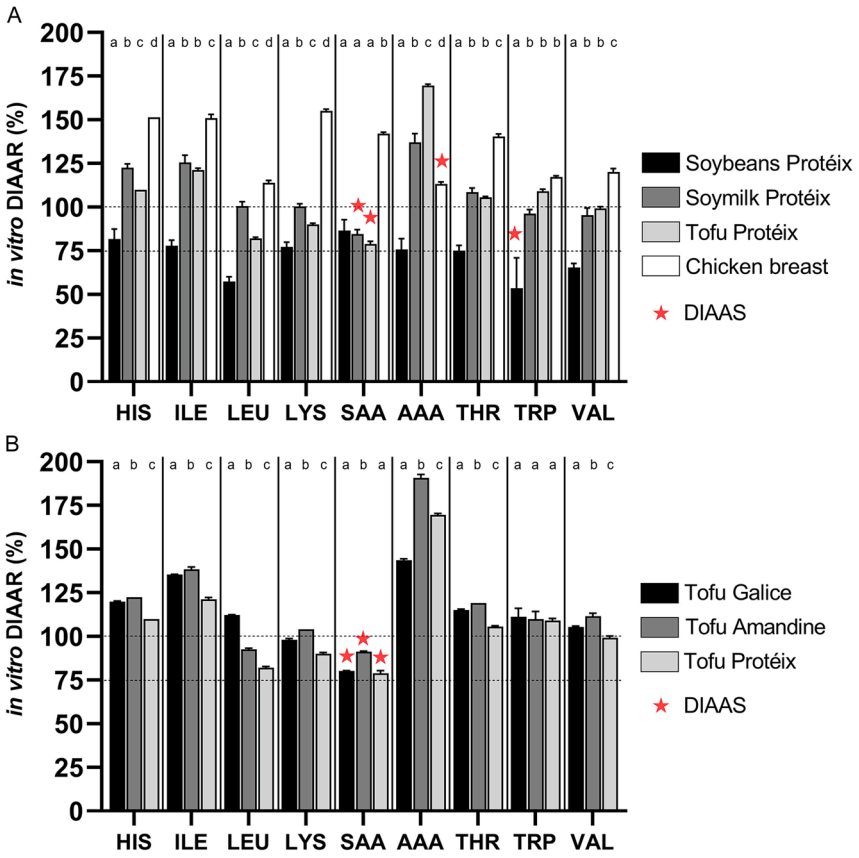


Fig. 4. (A) Comparison of *in vitro* DIAAR of cooked soybeans, soymilk, and tofu from the soybean cultivar Protéix with cooked chicken breast (Hammer et al., 2023). (B) Comparison of *in vitro* DIAAR of tofu from the Galice, Amandine, and Protéix soybean cultivars. Calculations were done using the recommended amino acid pattern for children aged 6 months to 3 years (Fao, 2013), *In vitro* DIAAS, being the lowest of the DIAAR, are highlighted with a red asterisk for each soy food. Differences in *in vitro* DIAAR values of the same IAA were compared; bars without common letters differ ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

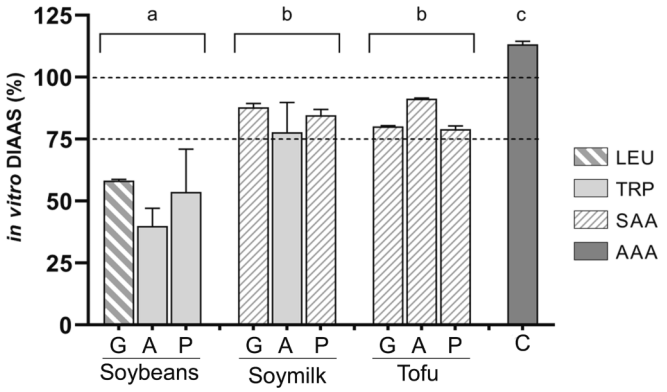


Fig. 5. *In vitro* DIAAS of soy foods from all soybean cultivars (Galice, G; Amandine, A; Protéix, P) are compared to cooked chicken breast (C) (Hammer et al., 2023). Patterns and colors of bars indicate limiting amino acids for the soy food. Grouped bars without common letters differ ($P < 0.05$). Recommended amino acid patterns for children aged 6 months to 3 years were used for the calculations. The dotted line divides DIAAS values into the following categories: <75 (low); 75–99 (good); and ≥100 (excellent).

isolate and soy meal (Mathai, Liu, & Stein, 2017) reportedly have relatively high DIAAS values (6 months to 3 years) of 84 (SAA) and 89 (SAA), respectively, which are higher than other legumes, such as pea protein concentrate (Mathai et al., 2017), peas, and chickpeas (Han et al., 2020), as well as cereals, such as wheat, maize, and oats (Pahm, Liu, & Stein, 2014). While plant-based proteins can be of "good" protein quality based on the food and processing steps applied, animal-based protein sources generally fall into the category of "excellent" protein quality, with reported *in vivo* DIAAS values for beef (Bailey, Mathai, Berg, & Stein, 2020), pork (Bailey et al., 2020) and dairy (Mathai et al., 2017) exceeding 100 (6 months to 3 years).

We recently reported insect-based foods, such as mealworm larvae and crickets, to possess good to excellent protein quality when assessed with the same methodology as in the current investigation. Consistent with current results, insect protein quality varied depending on the food preparation methods and food processing (Hammer et al., 2023). The slightly superior protein quality of insects compared to plant-based foods such as soy must be related to the environmental footprint required for their production.

Although the protein quality of plant-based sources is usually lower than that of animal-based foods, combining suitable food proteins can result in a mixed meal with enhanced protein quality, which was demonstrated with the combination of a plant-based burger with a burger bun in diets for gilts in a study by Fanelli et al. (2022). Considering that our *in vitro* DIAAR values for soymilk and tofu products are low for SAA and tryptophan, combining them with foods providing additional SAA and tryptophan, such as legumes, nuts, seeds, or small portions of animal-based protein sources (Fao, 1970), may result in a balanced meal, able to provide adequate amounts of IAA to meet dietary requirements. Soymilk and tofu products, being abundant in lysine, would furthermore be a suitable complementary food to lysine-deficient cereal grains (Cervantes-Pahm, Liu, & Stein, 2014).

4.2. Protein hydrolysis and processing

At the end of digestion, protein hydrolysis was evaluated by analyzing proteins and peptides in the soy foods, and by quantifying the proportion of dietary protein being bioavailable as free amino acids and small peptides. As no complete proteins of any of the soy foods were detected after completion of the IVD, all food proteins are assumed to have been hydrolyzed to some extent. For some soy proteins, kinetics of peptide release during IVD was illustrated by peptide patterns, suggesting that similar proteins are generally less digestible in cooked soybeans than in soymilk and tofu products. This observation is in line with the low total protein *in vitro* digestibility of around 50–60% observed for cooked soybeans, which is comparable to the reported standardized ileal digestibility of crude protein of 72.3 ± 1.3 determined for roasted full-fat soya beans in diets fed to growing pigs (Kaewtapee et al., 2018). Differences between *in vitro* results and the study by Kaewtapee et al. (2018) might be due to the soybean cultivar and growing conditions, but primarily expected to be the processing steps applied, which included exposure to 110–115 °C during roasting and, though not mentioned, most likely a grinding step before mixing soybeans in feed, which can render proteins more accessible to digestive enzymes. Legumes may have variable digestibility because of the presence of several antinutritional components that can negatively affect protein and AA digestibility, namely, lectins (soybean agglutinins), trypsin and chymotrypsin inhibitors, phytic acid and its salt phytate, and polyphenols (tannins) (Gilani et al., 2005; Liener, 1994). Moreover, soybean cell walls have a complex structure composed of structural proteins and nondigestible polysaccharides (pectin, hemicellulose, and cellulose) (Zahir, Fogliano, & Capuano, 2020). Perkins et al. has reported differences in dietary fiber content of roasted soybeans, soymilk, and tofu to be 4.6, 1.1, and 0.1 g per 100 g edible portion (Perkins, 1995). The intact physical structure and rigid plant cell walls can build a physical barrier and limit the bioaccessibility and subsequent protein digestibility of plant proteins (Drulyte & Orlien, 2019; Holland & Edwards, 2020). Our low *in vitro* digestibility results for cooked soybeans therefore suggest that the soaking and cooking steps did not have a major effect on cell wall integrity.

Soymilk and tofu products are significantly more digestible than cooked soybeans. Our *in vitro* results are comparable to the *in vivo* study by Reynaud et al. (2021), who found the true ileal digestibility of total AA to be 92.3 ± 3.0 and 95.0 ± 2.3 for commercially obtained soymilk and tofu, respectively, when fed to mini pigs. As all soy foods contain similar relative protein compositions and comparable quantities of antinutritional components, the strongly enhanced digestibility of soymilk and tofu products compared with cooked soybeans is most likely due to the processing steps affecting the physical food structure and plant cell wall integrity. Soymilk production includes wet grinding of overnight-soaked soybeans, followed by boiling and filtration to remove fiber-rich okara (Riaz, 2005; Wilson, 1995). The wet grinding can modify the plant tissue matrix by separating or rupturing cells and can change the porosity of the cell walls (Holland & Edwards, 2020) so that lipids and protein bodies can be released from the food matrix (Riaz, 2005). During filtration, the insoluble fraction is removed, creating a protein-rich oil-in-water emulsion with soy proteins that can be more easily reached by digestive enzymes during digestion than if they remained in the tightly packed soybean. Tofu is produced by the coagulation of proteins and oil in the heated soymilk, removal of soy whey, and pressing of the curds (Wilson, 1995). The slightly enhanced digestibility of tofu compared to soymilk products might be due to the heat-induced denaturation step, which unfolds proteins and consequently exposes the hydrophobic regions of soy proteins, facilitating gelation by hydrophobic interactions after the addition of coagulant (Peng, Ren, & Guo, 2016).

4.3. Differences across Swiss soybean cultivars

The three soybean cultivars selected for this study were different in yield (Galice), in improved taste (Amandine), and increased protein content (Protéix). However, although, the three varieties were different in protein composition (Amandine: lack of lipoxygenase) and content (Protéix: higher total protein), only minor differences in *in vitro* digestibility and *in vitro* DIAAR values were observed. The latter can be explained by the higher protein content in Protéix that was due to more dispensable AA, which do not result in higher protein quality. In contrast to the insignificant differences between varieties, major differences were observed among the product types, with increasing *in vitro* digestibility from soybeans, to soymilk, and tofu, as a consequence of the food processing steps the products underwent. Therefore, when appropriate processing and food preparation steps are applied, the differences between varieties could come into play.

4.4. Soy foods as mineral source

While the total iron content in soybean is substantial, it is important to consider that plant sources contain non-heme iron, which is less bioavailable than heme iron from meat (Hallberg, Rossander, & Skånberg, 1987). Moreover, the absorption of non-heme iron is susceptible to inhibitory components found in soybeans, such as phytate (Hallberg et al., 1987), polyphenols (Gillooly et al., 1983), and inorganic calcium (Monsen & Cook, 1976). Although soybeans contain high amounts of zinc, the presence of phytate will most likely strongly inhibit its absorption, considering the high molar ratios of PA to zinc (Zhang, Stockmann, Ng, & Ajlouni, 2022). It is therefore likely that soy foods are an inferior source of iron and zinc compared to meat, as they lack heme iron, contain polyphenols, and have a high proportion of phytic acid.

4.5. Strengths and limitations

The strengths of the study are, first, the application of the widely recognized and validated INFOGEST static IVD protocol (Brodkorb et al., 2019) with a subsequent *in vitro* DIAAS analytical workflow (Sousa et al., 2022), and second, the assessment of the characterization and protein quality comparison of traditionally consumed soy foods produced from Swiss soybean cultivars that are currently available on the Swiss market. However, more *in vitro* and *in vivo* comparison studies are required to further validate the applied *in vitro* model.

Our key findings are as follows:

(1) Total protein *in vitro* digestibility is strongly increased across the traditional food production value chain, being low for cooked soybeans (<65%), high for soymilk (>80%), and very high for tofu products (>90%).
(2) Protein quality by *in vitro* DIAAS is low for cooked soybeans (DIAAS < 60), while soymilk (DIAAS = 78–88) and tofu products (DIAAS = 79–91) have similar protein quality, which is considerably better than that of cooked soybeans ($P < 0.001$).
(3) Limiting AA for cooked soybeans is either leucine or tryptophan, and sulfur-containing AA (SAA) is a limiting AA for all soymilk and tofu products, except soymilk of the Amandine cultivar, where it is tryptophan.
(4) A higher total protein content does not necessarily lead to a higher DIAAS (e.g. Protéix cultivar).

5. Conclusions

In conclusion, soymilk and tofu products produced from Swiss soybean cultivars were found to be good protein sources for anyone older than six months and would therefore be suitable plant-based alternatives to animal-based foods. The processing steps to produce soymilk and tofu significantly enhance the protein quality of soybeans by increasing the *in*

vitro digestibility of individual AA. Differences in fiber content might be an important reason for the increase in digestibility between unprocessed and processed soy foods. Thus, further research regarding the optimal preparation of soybeans to reduce the negative effects of the plant tissue matrix, as well as processing steps to reduce mineral absorption inhibiting components, such as phytic acid and polyphenols, would help increase the nutritional value of soy foods. Future characterization and protein quality assessment can assist continued breeding programs in the development of suitable soybean crops for human consumption.

CRediT authorship contribution statement

Laila Hammer: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Diego Moretti:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Claude-Alain Bétrix:** Methodology, Investigation. **Pabiraa Kandiah:** Formal analysis. **Agostino Pellegri:** Formal analysis. **Lychou Abbühl-Eng:** Formal analysis. **Reto Portmann:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Lotti Egger:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.113947.

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