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Importance of adequate sample preparation for the assessment of protein digestibility and quality

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ABSTRACT

Determination of the nutritional quality of dietary proteins requires *in vivo* or *in vitro* digestion experiments that evaluate the ileal amino acid digestibility. However, many studies assess protein quality using finely ground flours or isolates rather than the food in its commonly consumed form, which may lead to overestimation of bioaccessibility. This study utilizes the standardized INFOGEST Quant protocol to evaluate the impact of sample preparation on protein digestibility and quality of chia and quinoa seeds, popularly known for their high protein quality. Total nitrogen, amino groups and individual amino acids were analyzed to calculate digestibility and digestible indispensable amino acid ratios (DIAAR). Results demonstrated that proteins in whole chia seeds, the most prevalent form of consumption, are virtually indigestible (<4%), rendering their amino acids physiologically unavailable. Only upon grinding did chia's protein digestibility surge to 63.3%, significantly increasing its DIAAR values and transforming it into a viable amino acid source. In contrast, quinoa exhibited high total protein digestibility (>90%) upon both traditional processing (boiling) and grinding. These findings highlight that sample preparation is a determinant factor in protein quality assessment. To avoid overestimating the true nutritional value of plant-based "superfoods", digestibility assays must mimic real-world consumption practices.

1. Introduction

The nutritional quality of dietary proteins depends not only on their amino acid profile but also on their ability to meet human metabolic needs for individual amino acids and nitrogen. To assess this important quality metric, both digestibility and human daily requirements for individual amino acids must be considered (Adhikari et al., 2022; FAO, 2013). Consequently, the Food and Agriculture Organization (FAO) recommends using the digestible indispensable amino acid score (DIAAS) as the standard to describe protein nutritional quality, replacing the protein digestibility-corrected amino acid score (PDCAAS). Unlike PDCAAS, which uses fecal protein digestibility for calculations, DIAAS considers the ileal digestibility of individual amino acids, resulting in more accurate estimations (FAO, 2013). Given that proteins are complex sources of both amino acids and dietary nitrogen, quality metrics should preferably preserve the individuality of the amino acids (Adhikari et al., 2022).

Ideally, true ileal digestibility should be determined *in vivo* using humans, growing pigs, or growing rats, in descendant order of preference (FAO, 2013). This makes protein the only macronutrient that requires animal or human testing for quality assessment, a requirement that inherently raises ethical concerns. Furthermore, such *in vivo* trials are expensive, time-consuming, and difficult to implement for large sample sizes (Santos-Sánchez et al., 2024). A viable alternative is the use of *in vitro* simulations. The INFOGEST Quant method (Egger et al., 2026) is an *in vitro* approach based on the static INFOGEST 2.0 digestion protocol (Brodkorb et al., 2019) to precisely measure protein digestibility. This advanced protocol has been validated against *in vivo* data (human, pig, and rat; Sousa et al., 2023) and is currently undergoing international standardization (ISO/DIS/IDF, 2024).

Crucially, the INFOGEST and INFOGEST Quant protocols specify that food samples for protein nutritional quality assessment (digestion experiments) should be prepared to reflect their actual dietary consumption conditions (Brodkorb et al., 2019; Egger et al., 2026; Moughan & Lim, 2024). In fact, pre-digestion processing can significantly alter food composition and structure, thereby affecting amino acid digestibility (Craddock et al., 2021). The impact of thermal processing on protein structure and digestibility is well-documented (Gong et al.,

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2025; Martineu-Côte et al., 2023). However, the specific effect of mechanical grinding and protein isolation remains underexplored, and yet dietary proteins are most frequently evaluated using highly processed flours, protein isolates, or ground products (Han et al., 2019; L'Hocine et al., 2023; Martineau-Côté et al., 2023), rather than whole foods, to improve sample homogeneity and stability. With this, protein quality is then generalized to the food itself (for example, data on protein quality determined in pea protein isolate are extrapolated to peas in general). Some studies have already shown that grinding increases digestibility in foods like soybeans (Zahir et al., 2018, 2021), but the extent to which this truly affects protein digestibility and DIAAS values has yet been insufficiently studied. Understanding this is of utmost importance, though, as discrepancies in protein quality results among studies – arising from different sample handling procedures – prevent a reliable comparison with other *in vitro* or *in vivo* studies as well as the extrapolation to real consumption conditions.

The premise of this study was that preparing foods for digestibility measurements exactly as they are consumed constitutes a critical step in the experimental process for determining protein quality. Consequently, subjecting the food sample to fine grinding to enhance homogeneity, or to protein isolation to increase concentration, may substantially underestimate or overestimate its true protein quality, and it would not reflect the actual quality of the dietary proteins as they are typically consumed. In this regard, this study aimed to evaluate the true impact of food preparation on protein digestibility and DIAAS by comparing a sample preparation that reflects common dietary practices with an artificial preparation approach, such as fine grinding, using the INFOGEST Quant protocol. Chia and quinoa seeds were selected for the study because of their popularity as "superfoods", known for their high protein content and essential amino acids (S. Chen & Luo, 2024; Poshadri et al., 2024; Vilcacundo & Hernández-Ledesma, 2017). Quinoa is typically consumed cooked, while chia is frequently consumed as whole raw seeds, either hydrated (*e.g.*, as chia pudding) or non-hydrated (*e.g.*, as topping for salads and smoothies).

2. Materials and methods

2.1. Chemicals, samples, and sample preparation

Unless otherwise stated, all chemicals and enzymes were purchased from Merck (Darmstadt, Germany). All chemicals used for amino acid analysis and analysis of amino groups were of analytical and/or chromatography grade. The enzymes and bile salts used for *in vitro* digestion were pepsin from porcine gastric mucosa (activity in arbitrary units (AU): 2883 AU/mg), porcine bile extract (1.28 μmol/mg) and pancreatin from porcine pancreas (trypsin activity: 4.9 AU/mg). Enzyme activities and bile concentration were measured according to Egger et al. (2026). One AU for pepsin is defined as that which produces a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C, measured as TCA-soluble products; these units are often referred to "Sigma" or "Anson" pepsin units (Anson, 1938; Anson & Mirsky, 1932). One AU for trypsin is defined as that which hydrolyzes 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester per minute at 25°C and pH 8.1 (Hummel, 1959).

Chia (*Salvia hispanica*) and white quinoa (*Chenopodium quinoa*) seeds were purchased from a local supermarket in Bern, Switzerland. Raw chia was used unprocessed. To prepare the ground chia, whole raw seeds were roasted for 10–12 min at medium temperature (stove heating power 5/9); then, the chia was ground using an electric grinder (GM 200, Retsch, Haan, Germany), sieved to separate and repeat grinding of the coarse particles, and crushed with a mortar to ensure complete disruption of the seeds. Hydrated chia was prepared by soaking raw chia seeds in water at a 1:3 ratio (seeds:water, w:w) for 30 min. For the cooked quinoa, raw seeds were cooked in water (1:2 quinoa:water ratio, w:w) using a staggered heating profile: 3 min in boiling water (stove heating power 8/9), 5 min at medium temperature (3/9), and 10 min at low temperature (1/9). The seeds were left in the water for an additional 10 min without heat, cooled overnight at 4°C, and ground in an electric grinder (GM 200, Retsch, Haan, Germany). Lastly, raw quinoa seeds were ground in an electric grinder (GM 200, Retsch, Haan, Germany) to create the ground quinoa (quinoa flour).

All prepared samples were stored at −20°C until use. Fig. 1 shows the samples under different preparation conditions; raw quinoa is shown for reference only and was not subjected to digestibility assessment.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To evaluate the impact of sample preparation on protein integrity, the protein profiles of the treated samples (cooked and ground quinoa, hydrated and ground chia) were studied using SDS-PAGE. Approximately 100 mg of each sample was diluted in 200 μL Tris buffer (100 mmol/L, 1 g/100 mL sodium dodecyl sulfate (SDS), pH 7.4) and sonicated 5–7 times (2-s cycles at 60% power). Proteins were precipitated with 1 mL of pure acetone and isolated by centrifugation at 18,500 *g* for 10 min at 4°C (5427 R centrifuge, Eppendorf, Hamburg, Germany). After discarding the supernatants, the protein pellets were air-dried at room temperature and dissolved in 400 μL Tris buffer (100 mmol/L, 1 g/100 mL SDS, pH 7.4) with the aid of sonication (ultrasonic bath USC 600 TH, VWR, Dietikon, Switzerland). Following a second centrifugation under the same conditions, the supernatants were collected. The remaining pellets were re-extracted in 200 μL Tris buffer, centrifuged again, and the resulting supernatants were pooled with the first extraction.

The protein concentrations of the pooled extracts were measured using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). The extracts were mixed with SDS (1x final concentration), adjusted to 0.67 mg protein/μL with ultra-pure water, and denatured at 95°C for 5 min. A 15 μL aliquot of each extract and a pre-stained marker (BenchMark, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were loaded onto a 15% acrylamide SDS-PAGE gel. Following electrophoresis (PowerPac 3000, Bio-Rad, Hercules, CA, USA), the gels were stained with Coomassie Brilliant blue G 250, destained with an aqueous solution of ethanol (10 mL/100 mL) and ortho-phosphoric acid (2 g/100 mL) (Kang et al., 2002), and scanned using a calibrated densitometer (GS-900, Bio-Rad, Hercules, CA, USA).

2.3. Static in vitro digestion

The samples were subjected to static *in vitro* digestion following the INFOGEST Quant protocol by Egger et al. (2026). In advance to the experimental work, the procedures described in the cited protocol were followed to prepare all the simulated digestion fluids (oral, gastric and intestinal), determine the activity of the commercial enzymes and the concentration of the commercial bile extract, and perform the pH test of the individual foods (for pH adjustment during digestion). For *in vitro*

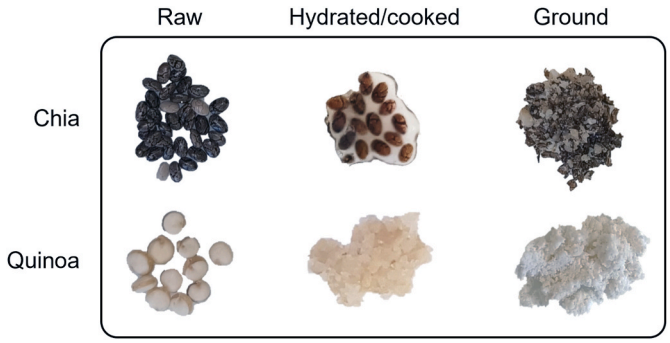


Fig. 1. Visual representation of chia and quinoa seeds before (raw) and after (hydrated (chia), cooked (quinoa) and ground (chia and quinoa)) sample preparation.

digestions, an amount of sample equivalent to 40 mg of protein (calculated from total nitrogen; Section 2.4) was digested each time. The first step in the digestion process was the oral phase, for which samples were completed to 1 g final mass with ultrapure water, and mixed with the simulated salivary fluid at pH 7.0 (1:1 ratio). Then, the oral bolus was mixed (1:1 ratio) with the simulated gastric fluid containing pepsin (2000 AU/mL final activity; the definition of AU can be found in Section 2.1), pH was adjusted to 3.0 with hydrochloric acid (2 mol/L), and the reaction was allowed for 120 min. Lastly, for the intestinal phase, the gastric chyme was mixed (1:1 ratio) with the simulated intestinal fluid containing bile (10 mmol/L final concentration) and pancreatin (100 AU/mL final trypsin activity; the definition of AU can be found in Section 2.1). pH was adjusted to 7.0 with sodium hydroxide (2 mol/L), and the reaction was allowed for 120 min. All digestion phases were performed at 37°C under continuous rotational agitation (SB3 rotator, Stuart, VWR Chemicals, Dietikon, Switzerland). For further details on the experimental procedure, refer to Egger et al. (2026).

To terminate digestion and separate the digestible from the undigestible fractions, 32 mL of ice-cold pure methanol (HiPerSolv CHROMANORM, VWR Chemicals, Dietikon, Switzerland) was added to the intestinal digesta, followed by incubation at −20°C for 1 h and centrifugation at 4000 *g* for 15 min at 4°C (Microfuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The supernatants (digestible fractions) were collected. The pellets (undigestible fractions) were twice washed with 5 mL methanol in water (800 mL/L), centrifuged (4000 *g*, 10 min, 4°C), and freeze-dried (Alpha 1-4 LD, Kuhner, Birsfelden, Switzerland) for approximately 60 h. The whole pellets were individually transferred to 10 mL glass vials for analysis.

Tube weights were recorded throughout the digestion process to calculate digestibility, using the supplementary material by Egger et al. (2026). Each sample was digested in eight replicates: four for total nitrogen determination (Section 2.4) and four for amino acid and amino group analysis (Sections 2.5, 2.6 and 2.7). Replicates were digested in four separate sets, with each set including a protein-free cookie (Egger et al., 2026; Moughan et al., 2005; Sousa et al., 2023) as a blank to correct for enzyme-derived nitrogen, amino acids, and amino groups.

2.4. Analysis of total nitrogen in foods and digested samples

The total nitrogen content in the undigested foods, supernatants, and pellets was determined to assess protein digestibility using the Kjeldahl method according to the ISO 5983-1:2005 and ISO 5983-2:2009 (ISO, 2005; ISO, 2009) normalized procedure. Before performing the Kjeldahl method, the digested supernatants were concentrated to approximately a half of the original volume using a centrifugal vacuum concentrator (CentriVap, Labconco, Kansas City, MO, USA), whereas pellets were dissolved in 2 mL sulfuric acid (96 mL/100 mL) and 2 mL hydrogen peroxide (35 mL/100 mL), and vortexed for up to 20 min until solubilization. Then, 0.5 g of undigested foods or approximately 2 g of concentrated supernatants or dissolved pellets were transferred to Kjeldahl digestion tubes and mixed with three catalyst tables (95.4 g/100 g potassium sulfate, 1.8 g/100 g copper sulfate pentahydrate, and 2.8 g/100 g titanium dioxide), 20 mL sulfuric acid (96 mL/100 mL), and 200 μL of silicone anti-foaming agent. The solutions were incubated for 2 h at 420°C and cooled down at room temperature. Total nitrogen was measured using a Kjeltec 8400 nitrogen analyzer with an 8460 autosampler (FOSS, Hillerød, Denmark).

2.5. Hydrolysis of proteins and peptides in food and digesta

Acid hydrolysis was used to release the individual amino acids in the foods, supernatants, and pellets. For the hydrolysis of the foods, approximately 30 mg of the samples were weighed into 10 mL glass vials. For the digestible fractions, 220 μL of supernatants were dried in 2 mL glass vials using a centrifugal vacuum concentrator (CentriVap, Labconco, Kansas City, MO, USA). The freeze-dried pellets were transferred entirely to 10 mL glass vials (the ratio of the transferred pellet to the total pellet was monitored and considered for digestibility calculations). The samples were mixed with 22% (v/v) ultra-pure water, 12% (v/v) DPP (1 g/mL in water), 12% (v/v) HCl (0.2 mol/L), 4% (v/v) norvaline (internal standard), and 50% (v/v) concentrated HCl (37 mL/100 mL). To prevent oxidation, the headspace of the 10 mL vials was flushed with N_2. A cystine solution (200 μmol/L final concentration) was separately hydrolyzed as an external standard. Hydrolysis was performed at 110°C for 15 h (digesta) and 24 h (foods) (Egger et al., 2026).

An additional basic hydrolysis was performed specifically to determine the tryptophan content of the foods. Approximately 50 mg of food was mixed with 170 mg starch, 200 μL 1-methyl-tryptophan (internal standard), 1750 μL ultrapure water, and 2 mL NaOH (8.4 mol/L) in Teflon 30 mL tubes. An external tryptophan standard (40 μg/mL final concentration) was also prepared. After incubation at 110°C for 20 h, the samples were immediately neutralized with 5 mL phosphate buffer (0.2 mol/L, pH 7) according to ISO 13904:2016 (ISO, 2016). As digestibility calculations are based on the amino acid ratio between supernatant and supernatant plus pellet, tryptophan was determined only after acid hydrolysis in the digested samples (Egger et al., 2026).

2.6. Analysis of individual amino acids

Following acid hydrolysis, 40 μL aliquots were neutralized with 40 μL NaOH (6 mol/L) and 80 μL HCl (0.1 mol/L). The samples were filtered through 96-well cellulose filter plates (SuperPlate, Thermo Fisher Scientific, Waltham, MA, USA) at 370 *g* for 5 min at 15°C. A 10 μL volume of each filtrate was derivatized with 70 μL AccQ-Tag borate buffer and 20 μL AccQ-Tag Ultra reagent (Waters, Milford, MA, USA) and incubated at 55°C for 10 min at 1000 rpm using a Thermomixer (ThermoMixer C, Eppendorf, Hamburg, Germany). Individual amino acids were quantified using ultra-high-performance liquid chromatography (UHPLC) coupled to an ultraviolet detector (Vanquish Flex, Thermo Fisher Scientific, Waltham, MA, USA) and an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm; Waters, Milford, MA, USA). Separation was done using a gradient flow at 0.4 mL/min for 32 min at 50°C with a 2 μL injection volume and detection at 260 nm. The mobile phases were (A) AccQ-Tag eluent A (Waters, Milford, MA, USA) in water (15 mL/100 mL), and (B) acetonitrile containing 2 mL/100 mL formic acid (ISO/IDF, 2022). Samples were run in gradient elution as follows (min/%B): 0.00/0.1, 5.50/0.1, 15.22/9.1, 20.47/21.2, 21.26/59.6, 21.29/90.0, 22.84/90.0, 26.00/0.1, 32.00/0.1. Hydrolyzed cysteine and a mixture of individual amino acids were injected repeatedly in each sequence and used as external standards.

For basic hydrolysis, samples were neutralized with HCl (6 mol/L) and phosphate buffer (0.2 mol/L, pH 7), and filtered through a cellulose filter plate (SuperPlate, Thermo Fisher Scientific, Waltham, MA, USA) at 164 *g* for 5 min at 15°C. Filtrates were injected into the same UHPLC system coupled to a fluorescence detector (excitation: 285 nm/emission: 340 nm) utilizing identical column and separation conditions as for individual amino acids.

2.7. Analysis of amino groups

The concentration of amino groups ($R\text{-}NH_2$) released into the digestible (supernatant) and undigestible (pellet) fractions was analyzed using *o*-phthalaldehyde (OPA), as described by Kopf-Bolanz et al. (2012). Digesta (supernatants and pellets) after acid hydrolysis were diluted with 0.5 mol/L perchloric acid (1:5 (v/v) for supernatants; 1:10 (v/v) for pellets) and centrifuged at 13,000 *g* for 1 min (5427 R centrifuge, Eppendorf, Hamburg, Germany). An OPA working solution was prepared by mixing 10 mL boric acid (0.1 mol/L in water, pH 8.5), 2 mL SDS (100 g/L in water), 0.4 mL OPA (40 g/L in ethanol), 0.4 mL 2-mercapto-ethansulfonic acid sodium salt (200 g/L in water), 1 mL Tergitol 15S9 (100 g/L in water) and 6.2 mL ultrapure water. The samples (8 μL) were derivatized with 232 μL OPA working solution and incubated for

10 min at 30°C in the dark. Absorbance was measured at 340 nm (Spectramax iD3, Molecular Devices, San José, CA, USA). Glutamic acid (0.4–8.0 mmol/L) diluted in perchloric acid (0.5 mol/L) served as the standard for the calibration curve, and perchloric acid (0.5 mol/L) as the blank.

2.8. Digestibility calculations

Protein digestibility was calculated according to Egger et al. (2026) using total nitrogen (Section 2.4), individual amino acids (Section 2.6), and primary amino groups (Section 2.7). Digestibility was determined by dividing the nitrogen, amino acid or amino group content in the supernatant by the content recovered in both the supernatant and pellet fractions. Total protein digestibility based on individual amino acids was calculated with the same equation by summing all the individual amino acids in the supernatant/pellet. True digestible indispensable amino acid ratio (DIAAR) values were calculated from the content of individual amino acids in the food, corrected by their individual digestibility, and relative to the FAO (2013) recommended requirements for children (6 months to 3 years) and adults (older than 3 years). The DIAAS corresponds to the lowest DIAAR value within a food, representing its limiting amino acid.

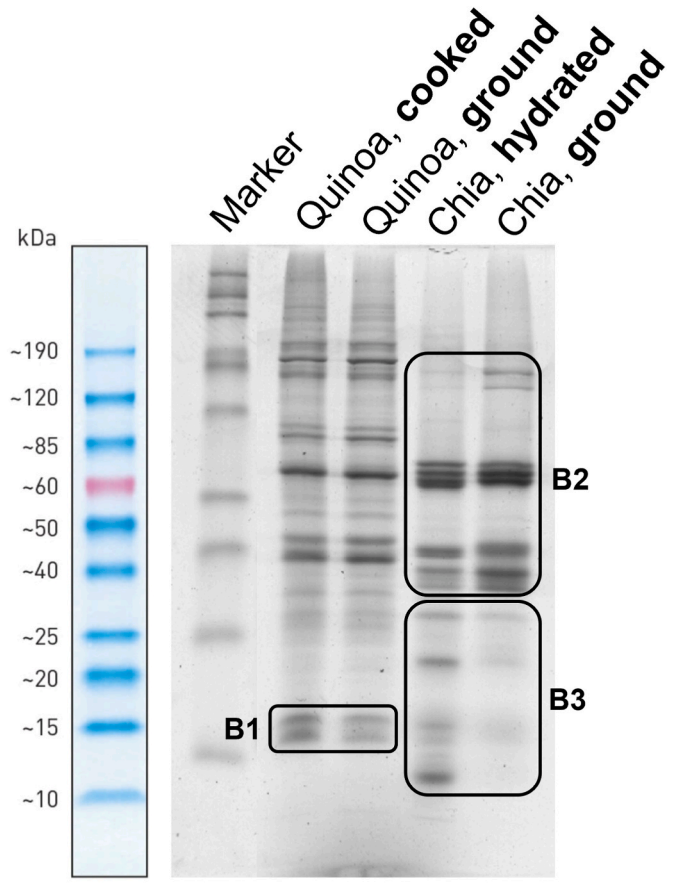


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel showing the protein profile of cooked quinoa, ground quinoa, hydrated chia and ground chia, based on apparent molecular weight (kDa). Black rectangles highlight the protein bands that exhibit changes in relative density between the unmechanized (cooked/hydrated) and ground samples.

3. Results and discussion

3.1. Protein size

Structural changes in quinoa and chia proteins induced purely by sample processing were investigated using SDS-PAGE (Fig. 2). The protein profile of quinoa seeds remained largely unchanged between cooked and ground samples, with only a minor increase in band density observed around 10 kDa when the seeds were cooked (Fig. 2, B1). The slight intensification of these specific, smaller proteins upon thermal treatment may be due to heat-induced denaturation and aggregation (Gong et al., 2025).

By contrast, for chia seeds, the density of protein bands above 15–20 kDa (Fig. 2, B2) slightly increased after grinding, whereas the density of lower molecular weight bands (Fig. 2, B3) decreased. This shift is likely the result of differential extraction efficiencies. As the SDS-PAGE samples were normalized to a standard protein concentration prior to loading, the visible bands reflected the relative composition of the extracted protein pool. According to the scientific literature, the mucilage extracted during the hydration of whole chia seeds contains between 2.6 g/100 g and 29.3 g/100 g of protein on a dry matter basis (depending on the extraction and analytical conditions) (Capitani et al., 2013; Segura-Campos et al., 2014; Timilsena et al., 2015). These are probably small, highly soluble proteins that easily diffuse into the surrounding mucilage layer, making them readily accessible to the extraction buffer. Conversely, the main storage proteins remain trapped inside the protected seed. Grinding completely disrupts this physical barrier, allowing the extraction buffer full access to the entire internal seed matrix. This enables the comprehensive solubilization of all proteins, including the larger structural and storage proteins, which naturally shifts the visible protein profile toward higher molecular weights.

3.2. Protein digestibility

The total protein digestibility and digestibility of the individual amino acids of the foods were determined following *in vitro* digestion. Total digestibility was assessed based on three analytical methods (total amino acids, primary amino groups, and total nitrogen), all of which yielded consistent results (Fig. 3; Supplementary Material Table S1). The digestibility of raw and hydrated chia seeds was extremely low, remaining below 4% across all analyses. Given the inherent 4–8% method variability associated with the protocol (validated by Sousa et al., 2023), these values indicate that raw chia seeds are virtually indigestible. Notably, soaking for 30 min (hydration) provided no improvement to the protein digestibility of the seeds, while it was improved to 28.7% in pigs by a prolonged overnight soaking (Hodgkinson et al., 2025). However, when raw chia seeds were finely ground prior to digestion, their total protein digestibility surged to 63.3% (average of the three analytical methods; Fig. 3). Correspondingly, the digestibility of individual amino acids in ground chia averaged above 57.0%, with the exception of tryptophan (39.2 ± 25.6%) (Fig. 4; Supplementary Material Table S1). This demonstrates the critical role of the food matrix and its physical disruption.

Chia seeds are surrounded by a coat of various layers that provide structural support and mechanical reinforcement, whereas the cell walls are rich in indigestible fibers (Capitani et al., 2013). Therefore, intact seed shells restrict digestive enzymes from reaching intracellular proteins, while fine grinding physically shatters this robust barrier. This exposes the internal matrix, facilitating protein–enzyme interactions (Holland et al., 2020) and artificially inflating digestibility compared to its typical consumption form. When the seeds are hydrated for different lengths of time, intracellular proteins get probably more accessible by digestive enzymes that when the seeds are consumed raw, although the proteins secreted to the hydrogel-like structure might be more or less protected by the mucilage polysaccharide, which is mostly undigestible (Chen et al., 2024). Therefore, while chia possesses a highly interesting

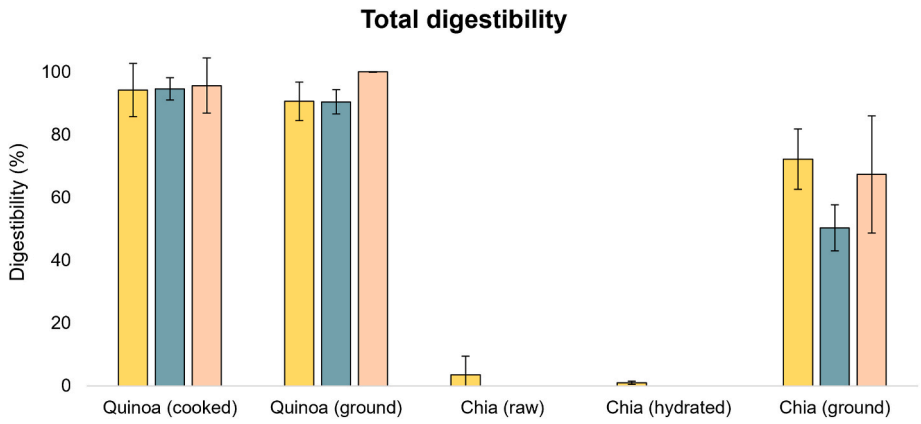


Fig. 3. Total protein digestibility of quinoa (cooked and ground) and chia (raw, hydrated, and ground) samples, expressed as the percentage (%) of digested protein; error bars represent standard deviations. Digestibility was evaluated based on total amino acids (▫), total amino groups (▪) and total nitrogen (▫).

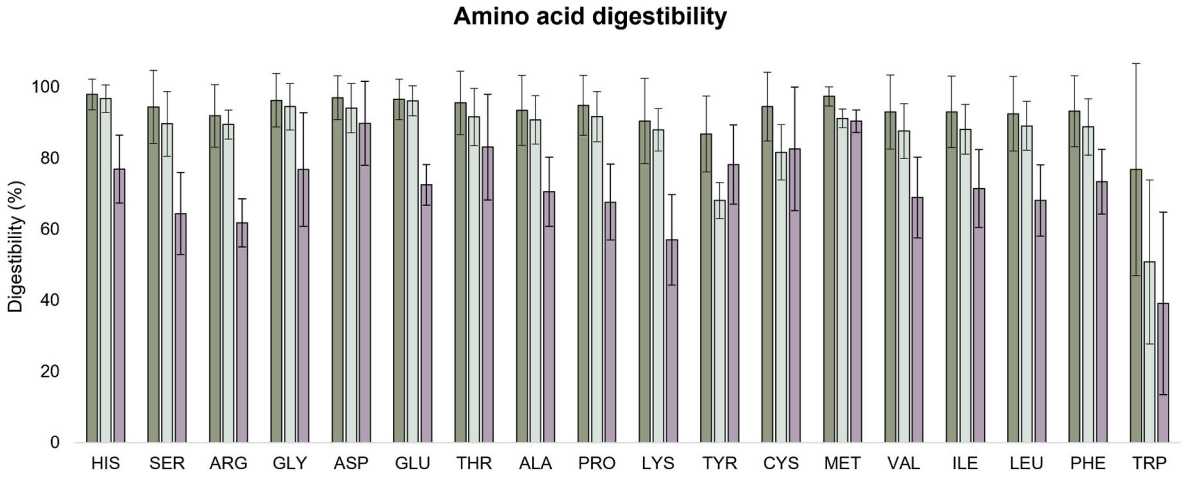


Fig. 4. Digestibility of individual amino acids in cooked quinoa (▪), ground quinoa (▫) and ground chia (▪) samples, expressed as the percentage (%) of digested amino acid moles; error bars represent standard deviations. Individual amino acid digestibility for raw and hydrated chia is not shown, as total digestibility was < 4%. ALA: alanine; ARG: arginine; ASP: aspartic acid; CYS: cysteine; GLU: glutamic acid; GLY: glycine; HIS: histidine; ILE: isoleucine; LEU: leucine; LYS: lysine; MET: methionine; PHE: phenylalanine; PRO: proline; SER: serine; THR: threonine; TRP: tryptophan; TYR: tyrosine; VAL: valine.

profile of essential amino acids (Table 1; S. Chen & Luo, 2024) and is widely marketed as a protein-rich "superfood", the physical form in which it is consumed dictates whether these amino acids can be absorbed at all. If chia seeds are merely allowed to swell or shortly hydrate in liquid, arguably their most prevalent method of consumption, this potentially excellent protein source remains fundamentally unutilized by the digestive system. Therefore, to truly justify chia's reputation as a nutritional superfood in terms of protein provision, the seeds must be appropriately ground prior to ingestion.

By contrast, the total digestibility of quinoa exceeded 90% across all analytical methods, with no differences observed between cooked and raw ground samples. Accordingly, the digestibility of individual amino acids in quinoa remained high, exhibiting only slight decreases in tyrosine, cysteine, and methionine upon grinding (Fig. 4). In this case, cooking already achieves a profound structural breakdown in quinoa without the need for intense mechanical grinding. Thermal processing depolymerizes and solubilizes dietary fiber (López et al., 2024; Marconi et al., 2000; Zia-ur-Rehman et al., 2003), increasing cellular permeability and porosity (Kohli & Singha, 2024). Concurrently, the abundant starch in quinoa undergoes massive swelling and gelatinization during boiling, disrupting the seed architecture from the inside out. In this context, in both cooked and ground quinoa, the cell wall was damaged, and the proteins were exposed to digestive enzymes, resulting in high total protein digestibility. The results for cooked quinoa are in good agreement with *in vivo* digestibility of individual amino acids (between 80 and 97%; Hodgkinson et al., 2025).

3.3. *In vitro digestible indispensable amino acid ratio (DIAAR)*

The profound impact of sample processing on protein digestibility was directly reflected in the DIAAR values (Fig. 5; Supplementary Material Table S2), which refer to the ratio of a digestible indispensable amino acid in reference to its recommended daily intakes (FAO, 2013). Describing the DIAAR values is crucial for understanding true protein quality, with the lowest DIAAR value representing the DIAAS (the limiting amino acid) (Moughan & Lim, 2024). For quinoa, mechanical grinding resulted in only minor shifts across most DIAAR values compared to the cooked sample. Interestingly, however, due to a lower tryptophan digestibility in ground quinoa, the DIAAR value of tryptophan for children and adults decreased by 35% in the ground compared to the cooked quinoa. This specific difference shifted the limiting amino acid from leucine (in cooked quinoa) to tryptophan (in ground quinoa). Thus, even when overall digestibility remains high, introducing artificial mechanical processing can still alter the perceived availability of specific sensitive amino acids. By contrast, and in accordance with the observations above, the implications of the sample preparation were massive for chia seeds (Fig. 5). Given that the proteins in the whole raw or hydrated chia were virtually indigestible in our study and only

Table 1
Results of the total nitrogen and protein content, and amino acid composition of the raw, ground quinoa and chia seeds analyzed in this study, and the age-related Recommended Scoring Patterns (RSP) defined by FAO (2013).

	Quinoa	Chia	RSP	
			Children	Adults
Total nitrogen (mg/g food)	16.9	37.4		
Protein (mg/g food)	106	234		
Amino acids (mg/g protein)				
Essential				
AAA[a]	67.7	84.3	52	41
HIS	26.7	27.5	20	16
ILE	37.6	37.4	32	30
LEU	59.1	63.7	66	61
LYS	53.3	45.9	57	48
SAA[b]	34.4	45.6	27	23
THR	34.6	35.3	31	25
TRP	10.5	12.4	8.5	6.6
VAL	44.2	47.1	43	40
Non-essential				
ALA	41.7	49.1		
ARG	78.9	103		
ASP	79.3	87.3		
GLU	132	176		
GLY	48.3	44.8		
PRO	33.1	35.5		
SER	41.6	52.2		

[a] AAA: aromatic amino acids (phenylalanine and tyrosine).
[b] SAA: sulfur-containing amino acids (methionine and cysteine). The "children" group corresponds to 6 months to 3 years; "adults" group corresponds to older children, adolescents and adults. ALA: alanine; ARG: arginine; ASP: aspartic acid; GLU: glutamic acid; GLY: glycine; HIS: histidine; ILE: isoleucine; LEU: leucine; LYS: lysine; PHE: phenylalanine; PRO: proline; SER: serine; THR: threonine; TRP: tryptophan; TYR: tyrosine; VAL: valine. The protein content was calculated from total nitrogen using a nitrogen-to-protein conversion factor of 6.25.

digestible to 28.7% in other *in vivo* studies (Hodgkinson et al., 2025), their functional DIAAR values were effectively zero or 40%, respectively. Therefore, in chia seeds' popularly consumed forms (raw or hydrated), their proteins cannot be considered a source of high-quality dietary protein. Upon fine grinding, their DIAAR values surged to 46–147% and 55–173% for children and adults, respectively. Thus, simply milling the seeds transformed chia from a non-source of digestible amino acids into a moderate or even excellent source of essential amino acids. Specifically, histidine, sulfur amino acids (cysteine and methionine) and aromatic amino acids (phenylalanine and tyrosine) were highly available upon digestion of ground chia. Yet, even in a ground state, several DIAAR remained far below the 100% threshold required to be considered a good source of essential amino acids (FAO, 2013). In particular, lysine, leucine and tryptophan presented very low DIAAR values, which could be explained by the high human dietary requirements of lysine and leucine (FAO, 2013) and the low digestibility of tryptophan in the foods studied here.

These data reinforce the central premise of this study: evaluating foods in a finely ground, experimentally manageable state when they are predominantly consumed whole leads to a severe overestimation of their real nutritional quality. This is minimized, however, when the common consumption form involves intense processing (*e.g.*, cooking). Despite the scarce literature on the topic, these results align with previous work showing an increase in protein digestibility when the particle size of the meal was reduced (Lee et al., 2025). With this, the present study demonstrates that sample preparation is a critical determinant in the INFOGEST Quant method, and the specific ways in which foods are typically consumed must be strictly mirrored when designing digestion experiments.

The principle of mirroring real-world consumption must also be strictly applied to highly processed ingredients, such as pure protein powders or isolates. In human *in vivo* trials, such refined ingredients are rarely consumed in complete isolation; instead, they are typically ingested as part of a broader meal or mixed into a complex food matrix. Conversely, a common *in vitro* practice is to test them purely on their own. As these powders are extremely finely ground and entirely devoid of any protective structural matrix, their *in vitro* digestibility is inherently maximized, leading to a predictable overestimation of their physiological value. When digested alongside other macronutrients, however, matrix interactions often alter and potentially reduce the digestibility of these otherwise highly accessible proteins. Therefore, to evaluate protein powders realistically and prevent this artificial overestimation, they must be tested within a representative food matrix, preferably the same as consumed in real life. The most standardized *in vitro* approach involves co-digesting an isolated protein with a protein-free cookie, as explicitly outlined in the INFOGEST Quant protocol (Egger et al., 2026), although further investigation of the protein isolate–matrix interactions remains necessary.

4. Conclusions

This study confirms that food matrix structures and processing methods are primary determinants of protein digestibility. Therefore, sample preparation is a critical step in digestion assays and must replicate human consumption practices, while deviating from these real-world conditions profoundly compromises the validity of the digestibility results. This principle is equally critical when reproducing *in vivo* digestion tests *in vitro*, as deviations in sample preparation between the two models can profoundly compromise the comparability, validity, and practical relevance of the results.

Furthermore, this study highlights that a food's native amino acid profile alone is an insufficient indicator of its actual nutritional value. As demonstrated by our findings in chia seeds, an excellent amino acid profile does not guarantee nutritional value, since their essential amino acids remain largely unabsorbable when consumed in their popular whole (raw or hydrated) forms. To benefit from chia's potential as a protein source, the robust physical barrier of the seed coat must be mechanically disrupted by fine grinding. By contrast, standard thermal preparation methods, such as cooking, effectively disrupt the food matrix in quinoa and maximize digestibility without the need for mechanical processing, rendering cooked quinoa a superior and readily available protein source in practice.

CRediT authorship contribution statement

Ana Blanco-Doval: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Cédric Brügger:** Writing – review & editing, Validation, Formal analysis, Data curation. **Reto Portmann:** Writing – review & editing, Validation, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Lotti Egger:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI use

The authors did not use generative AI and AI-assisted technologies in the manuscript preparation process.

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Declaration of competing interest

The authors declare that they have no known competing financial

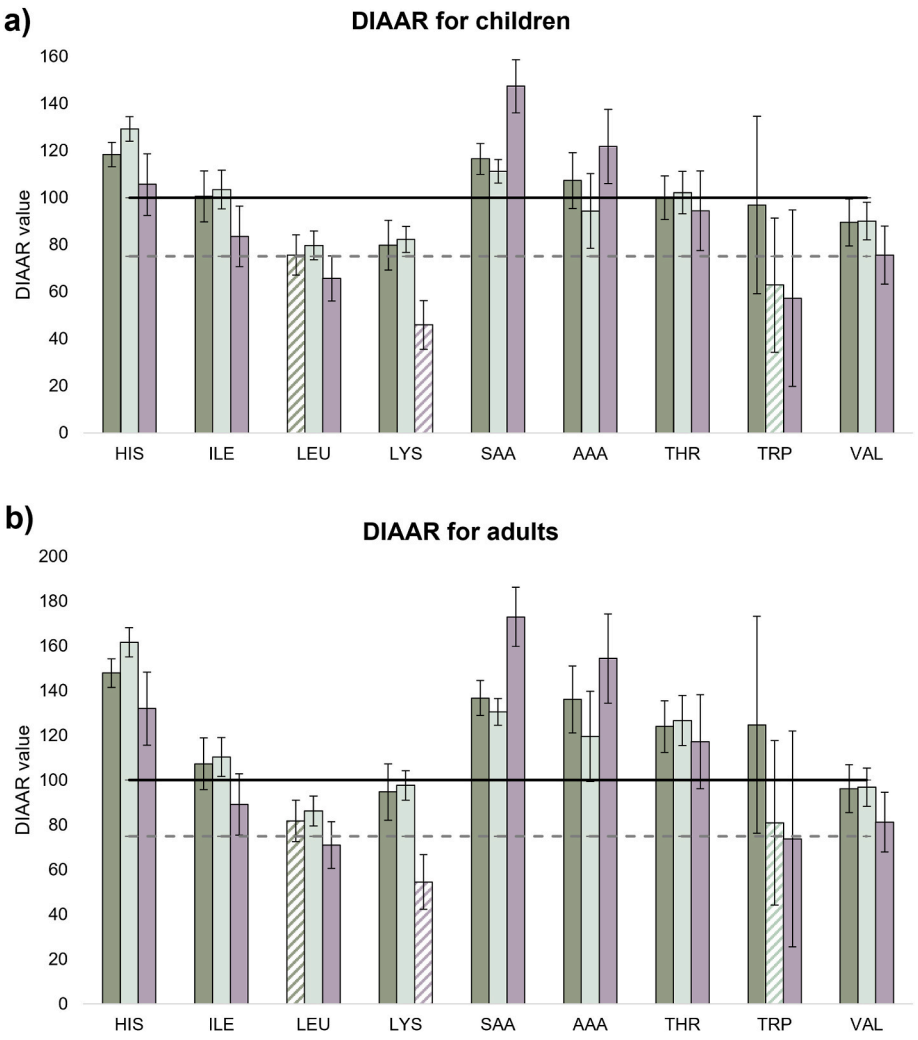


Fig. 5. Digestible indispensable amino acid ratios (DIAAR) calculated for cooked quinoa (■), ground quinoa (□) and ground chia (■) samples, based on requirements for a) children, and b) adults given by FAO (2013); error bars represent standard deviations. DIAAR for raw and hydrated chia is not shown, as total digestibility was < 4%. The diagonally striped bars indicate the DIAAS, i.*e.* the amino acid showing the lowest DIAAR, for each food. The dotted and continuous black lines represent, respectively, the threshold to consider the food a good (DIAAR: 75) or an excellent (DIAAR: 100) source of the corresponding essential amino acid according to FAO (2013). AAA: aromatic amino acids (phenylalanine and tyrosine); HIS: histidine; ILE: isoleucine; LEU: leucine; LYS: lysine; SAA: sulfur-containing amino acids (cysteine and methionine); THR: threonine; TRP: tryptophan; VAL: valine.

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.lwt.2026.120131.

Data availability

Data will be made available on request.

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