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Collaborative studies on in vitro digestibility of dietary proteins based on the INFOGEST Quant protocol: toward international standardization☆

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Abbreviations: CH, cheese; CP, chickpea; DDP, 3,3′-dithiodipropionic acid; DIAAR, digestible indispensable amino acid ratio; DIAAS, digestible indispensable amino acid score; FAO, Food and Agriculture Organization of the United Nations; IAA, indispensable amino acids; IDF, International Dairy Federation; ISO, International Organization of Standardization; IVD, in vitro digestion; NCF, nitrogen-to-protein conversion factor; OPA, *o*-phthalaldehyde; PDCAAS, protein digestibility-corrected amino acid score; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMP, skim milk powder; SPI, soy protein isolate; TAA, total amino acids; TN, total nitrogen; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet; WMP, whole milk powder; WPI, whey protein isolate; YOG, yogurt.

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ABSTRACT

Based on recommendations by the Food and Agriculture Organization, an international standardization was launched for a static in vitro protein digestibility method, recently published as the INFOGEST Quant protocol. Based on the validated INFOGEST 2.0 method, the INFOGEST Quant protocol allows quantification of total protein digestibility, individual amino acid digestibility, and digestible indispensable amino acid score (DIAAS) calculation. Comparability of protein digestibility obtained with the INFOGEST Quant method was confirmed using identical substrates in vitro and in vivo. Method standardization within the International Dairy Federation and the International Organization for Standardization was initiated, and precision data were established in two international collaborative studies by over 25 laboratories. The three applied analytical endpoints were comparable. Robust precision estimates for o-phthalaldehyde and total nitrogen showed repeatability and reproducibility limits of 10% and 15%, respectively. Moreover, the protein digestibility of fresh dairy foods was higher than in freeze-dried samples, highlighting matrix and processing effects.

1. Introduction

Proteins are elemental macronutrients with great nutritional relevance as sources of indispensable amino acids (IAA) and bioactive peptides (Loveday, 2019). Protein quality depends on both its composition in indispensable amino acids and the efficiency with which these become bioaccessible upon digestion (Bessada et al., 2019; Havenaar et al., 2016; Kashyap et al., 2023), i.e., their digestibility. The term"digestibility" refers to the proportion of bioavailable free amino acids and short peptides generated during digestion relative to the remaining undigested proteins and longer peptides (Stein et al., 2007). In 2013, the Food and Agriculture Organization (FAO) of the United Nations recommended the digestible indispensable amino acid score (DIAAS) as the preferred measure for protein quality (FAO, 2013), replacing the protein digestibility corrected amino acid score (PDCAAS) (FAO, 1991). DIAAS is defined by the lowest digestible indispensable amino acid ratio (DIAAR) of a protein source, which compares the apparently absorbed quantity of an indispensable amino acid to the human amino acid requirement based on true ileal digestibility (FAO, 2013). The limiting indispensable amino acid determines the DIAAS value of the protein source.

Ideally, DIAAS should be determined in humans via naso-ileal intubation (Moughan & Wolfe, 2019) or using methods with dual isotope labelling (Calderón de la Barca et al., 2021). However, due to ethical and practical constraints, animal models, such as ileum-fistulated growing pigs (Hodgkinson et al., 2022; Moughan et al., 2005) or rats (Moughan & Wolfe, 2019), are currently used. Animal studies, however, are costly and time-consuming, and they raise ethical concerns. Therefore, the FAO highlighted the need for validated and reproducible in vitro methods (FAO, 2013), particularly to support rapid growth in the production of alternative and novel foods.

A wide variety of in vitro digestion (IVD) protocols has been introduced over the past decades (Bohn et al., 2018; Kopf-Bolanz et al., 2012; Versantvoort et al., 2005) but they differ substantially in experimental conditions and often lack physiological relevance, which limits the comparability of results. To overcome these concerns, the COST Action INFOGEST network developed a harmonized static protocol for the in vitro simulation of gastrointestinal digestion that closely mimics human gastrointestinal conditions (Brodkorb et al., 2019; Minekus et al., 2014). This protocol was validated for its biological relevance by comparing the results of milk protein digestion with in vivo data from pigs (Egger et al., 2017) and humans (jejunal effluents; Sanchón et al., 2018). Building on the INFOGEST IVD model, an analytical workflow enabling the quantification of protein digestibility and DIAAS (INFOGEST Quant method) has recently been established (Egger et al., 2026; Sousa, et al., 2023b) and validated with in vivo data (Hodgkinson et al., 2022) using four foods (wheat bran cereal [All-Bran®], pigeon peas, black beans, and peanuts) and three isolated proteins (zein, whey protein isolate, and collagen) (Sousa, et al., 2023b).

A standardization process within the International Dairy Federation (IDF) and the International Organization for Standardization (ISO) has been initiated to advance this laboratory method toward a recognized international standard. In this vein, the present research paper describes two collaborative studies that were conducted to evaluate the reproducibility and repeatability of the in vitro protocol to determine protein digestibility, as required for the IDF/ISO standardization. These two collaborative studies were performed with lyophilized foods and protein isolates to ensure homogeneity and stability of samples and to facilitate shipment. However, freeze-dried substrates are not representative of

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real foods, since the processing severely changes the food structure, which may alter protein digestibility. Therefore, the method was further tested on 19 fresh dairy foods (milks, fermented milks, and cheeses) as an example of a real experiment (performed in the same lab by experienced technicians) using the same workflow (Sousa et al., 2023b). Performed in the context of IDF, the collaborative studies mainly focused on dairy products, including only two plant-based products. However, the method has also been successfully applied to other products, including meat- and plant-based burgers, soy products, and edible insects (Gómez-Marín et al., 2026; Hammer et al., 2023; Sousa et al., 2023), and interlaboratory comparisons including other substrates are ongoing.

2. Materials and methods

2.1. Collaborative study organization

Two international collaborative studies were conducted as illustrated in the graphical abstract. In Collaborative Study 1 (May to December 2021), 26 laboratories analyzed five freeze-dried dairy foods (skim milk powder [SMP], whole milk powder [WMP], whey protein isolate [WPI], cheese [CH, grated, freeze-dried], and yogurt [YOG, freeze-dried]). Collaborative Study 2 (February to October 2023) included 23 laboratories analyzing the same dairy products plus two plant-based products (soy protein isolate [SPI] and chickpeas [CP]; canned cooked chickpeas were shredded into 2–3 mm particles, and freeze-dried). All test materials were centrally prepared, homogenized, and freeze-dried (YOG, CH) at Agroscope (Bern, Switzerland), and distributed to all the participating collaborators in sealed bags, and their corresponding total nitrogen (TN) values (g/kg) determined with the Kjeldahl method (ISO-8968-3|IDF-20-3, 2004) were indicated on the bags. Although most of the participating laboratories were familiar with the static IVD protocol by Brodkorb et al. (2019), the additional analytical workflow for protein digestibility assessment developed by Sousa et al. (2023b) was previously introduced to them in a two-day hands-on workshop. The participating laboratories were asked to perform the experiments in triplicate following the draft ISO|IDF method (ISO/DIS-24167|IDF-261, 2024), selecting at least one of three analytical endpoints: primary amines with o-phthalaldehyde (OPA), TN with the Kjeldahl method, or total amino acids (TAA) with ultra-high-performance liquid chromatography (UHPLC). Besides the total digestibility for each substrate, the participating laboratories were asked to report the measured activity of the enzymes used for the IVD experiments and to provide Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for the qualitative assessment of the digesta. All results were returned to Agroscope for centralized data treatment and evaluation.

2.2. Samples for the collaborative studies

SMP, WMP, and WPI were obtained from Ingredia (Arras, France) and SPI from International Flavors & Fragrances (Solae® Supro®; New York, USA). Gruyère cheese and yogurt were produced at Agroscope (Bern, Switzerland) according to industrial standards and Swiss food legislation. Gruyère production also followed the procedure by Casey et al. (2004). Canned chickpeas were bought from a local store (Coop, Switzerland; bio chickpeas "Karma", boiled) (Table 1).

Cheese and chickpea samples were ground to 2–3 mm particle sizes using an electric mincer (GM 200, Retsch, Haan, Germany). All cheese, chickpea, and yogurt samples were freeze-dried (Alpha 1–4 LSCbasic and Lyocube, Martin Christ, Osterode am Harz, Germany). A protein-free cookie containing purified corn starch (Maizena), margarine, sucrose, cellulose, and baking powder (Egger et al., 2026; Moughan et al., 2005) was provided as blank control for digestion in parallel and to correct for amino acids originating from digestive enzymes.

Table 1
Substrates used in the two collaborative studies, including their total nitrogen (TN) content, the total protein content, and the corresponding sample weight subjected to in vitro digestion (IVD).

Sample	TN	Total protein (TN x NCF)	Sample weight for IVD
	(g/kg)	(g/kg)	(mg)
Skim milk powder (SMP)	49.7	317	126
Whole milk powder (WMP)	38.9	248	161
Gruyère (CH), freeze-dried	66.7	426	94.0
Whey protein isolate (WPI)	133	849	47.1
Yogurt (YOG), freeze-dried	50.7	323	124
Enzyme blank	0	0	1000
Soy protein isolate (SPI)	137	855	47.1
Chickpea (CP), (freeze-dried)	26.8	168	239

The enzyme blank refers to a protein-free cookie. The total protein content was calculated with a nitrogen-to-protein conversion factor (NCF) of 6.38 for dairy products (as required by IDF) and 6.25 for non-dairy substrates (SPI and chickpea).

2.3. In vitro digestions performed by the participating laboratories

The participating laboratories of the collaborative study were requested to follow the IVD method as described in detail in the draft ISO|IDF method (ISO/DIS-24167|IDF-261, 2024), which builds on the static INFOGEST 2.0 protocol (Brodkorb et al., 2019) and the protein digestibility method by Sousa et al. (2023b). No further specifications were given concerning the mixing during the gastric and intestinal steps of digestion, and these parameters were not systematically recorded. Prior to digestion, the participating laboratories analyzed the enzyme activities of pepsin, trypsin in solubilized pancreatin, and bile salt concentration according to the draft ISO/IDF method (which corresponds to Sousa et al., 2023b).

All substrates were normalized to 40 mg protein using a nitrogen-to-protein conversion factor (NCF) of 6.38 for dairy products and 6.25 for plant products. This protein load, which corresponds to the amount of protein contained in 1 g of cow milk, has been validated against in vivo data using substrates from animal and plant origin, and different matrix complexities (Sousa et al., 2023b). Normalization to 40 mg of protein ensures an equal protein-to-enzyme ratio across experiments. The samples were not reconstituted to their original moisture content. Instead, freeze-dried samples were to be reconstituted in water to a final mass of 1 g and mixed with 1 mL simulated salivary fluid (SSF; pH 7.0), following food-to-digestive-fluid ratios described in the original INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). For SPI and CP, the participating laboratories were asked to report whether salivary amylase (75 U/mL of digesta) was included in the oral phase.

2.4. In vitro digestions and analytical methods performed at Agroscope

To demonstrate the applicability of the method to real foods, the protein digestibility of 19 fresh dairy products (without freeze-drying) was analyzed in triplicate, using all three analytical endpoints. In the next sections (2.4.1–2.4.9), details are provided on the experimental conditions and analyses applied for the dairy products and the collaborative studies conducted at Agroscope.

2.4.1. Chemicals and reagents

All chemicals and enzymes were purchased from Merck (Zug, Switzerland). The simulated digestive fluids were prepared according to the draft ISO/IDF method (ISO/DIS-24167|IDF-261, 2024), which was based on INFOGEST 2.0 (Brodkorb et al., 2019).

2.4.2. Fresh dairy foods

Fresh dairy products included skimmed cow's milk (homogenized, ultra-high-temperature treated), pasteurized cow's milk (whole, homogenized), goat's milk (3% fat, homogenized, high-temperature, and

pasteurized), sheep's milk (whole, homogenized, and pasteurized), fresh yogurt, skyr, kefir, quark, and ricotta, cottage, feta (sheep and goat's milk), Ziger (whey protein cheese), Gruyère, Parmigiano Reggiano, Cheddar, Brie, Camembert, Tilsiter, and Manchego (sheep's milk) cheeses. All products except for Manchego cheese were purchased from a local supermarket (Coop, Bern, Switzerland). As Manchego cheese showed lower digestibility values than the other cheeses (see Section 3), five Manchego cheeses were analyzed to confirm the results: two were purchased in Switzerland (Coop, Bern) and three were purchased in Spain (Mercadona and Alcampo, Vitoria-Gasteiz). Liquid foods were homogenized by gently inverting the sample tubes 10 times (avoiding foam formation); semi-solid samples were homogenized by gently mixing with a spatula; cheeses were ground using an electric mincer (GM 200, Retsch) to a final particle size of 2–3 mm. These homogenization procedures were selected to minimize changes to the physical structure of the different foods in order to be consistent with the real form of consumption. The rinds of Manchego, Parmigiano Reggiano, and Tilsiter cheeses were removed before grinding, whereas those of Brie and Camembert cheeses were kept in place, as these cheeses are typically consumed with their rinds. The substrates were normalized to 40 mg of protein using an NCF of 6.38 and adjusted with ultrapure water to reach a final mass of 1 g for digestion.

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

For the qualitative profiling of proteins in the fresh dairy foods, 1 g of skyr, quark, and all cheeses (ricotta, cottage, feta, Brie, Camembert, Ziger, Gruyère, Parmigiano Reggiano, Cheddar, Manchego, and Tilsiter) were homogenized in 25 mL ultrapure water with a bead mill (Bead Ruptor 24, Omni International, Kennesaw, GA, USA). The cheese extracts and the liquid foods (cow's milk, sheep's milk, goat's milk, yogurt, and kefir) were diluted with a sample buffer (Tris-HCl 350 mmol/L, pH = 6.8, sodium dodecyl sulfate 10%, dithiothreitol 100 mmol/L, glycerol 50%) to a final protein concentration of 0.33 μg/μL. Proteins were denatured at 95 °C for 10 min, and 15 (cheese extracts) or 10 μL (liquid foods) of each sample were loaded before separation by SDS-PAGE (15% polyacrylamide). A molecular weight marker (Benchmark™, Invitrogen) was run along with the samples, and the gels were stained with colloidal Coomassie Blue, as described previously (Kang et al., 2002).

2.4.4. In vitro digestions performed at Agroscope for dairy products and the collaborative studies

All IVDs performed at Agroscope (collaborative studies and fresh dairy products) were carried out using the same draft ISO/IDF method (ISO/DIS-24167|IDF-261, 2024) as all other participating laboratories of the collaborative studies, ensuring full comparability across datasets. Briefly, all substrates were normalized to 40 mg protein, adjusted to 1 g final mass, and mixed with 1 mL simulated salivary fluid (pH 7.0). For SPI and CP (and for their corresponding cookie control), salivary amylase (75 U/mL of digesta) was included. Thereafter, the samples were incubated for 2 min at 37 °C (incubation chamber). Then, 2 mL of simulated gastric juice (pH 3.0) containing pepsin (2000 U/mL of digesta) was added, and the mixture was incubated for 120 min at 37 °C. Finally, 4 mL of simulated intestinal fluid (pH 7.0) containing pancreatin (100 U trypsin activity/mL of total digesta) and bile (10 mmol/L of bile salts in total digesta) were added and incubated for 120 min at 37 °C. Digestion was performed under constant gentle mixing (16 rpm) on a rotating wheel (Stuart™ SB3 rotator; Bibby Scientific, Stone, United Kingdom) or on other slow mixing devices.

At the end of the intestinal phase, all digestions were immediately stopped by the addition of pure ice-cold methanol to a final concentration of 80% (*v*/v). Precipitation of the undigestible fraction was aided by incubation of the samples at −20 °C for at least 1 h and subsequent centrifugation (2000 ×*g* at 4 °C for 15 min). The supernatants were collected in new tubes avoiding the interface, and the pellets were washed twice with an aqueous methanol solution (80%, *v*/v) followed by centrifugation (2000 ×*g* at 4 °C for 5 min). The washed pellets (whole pellets obtained from digestion) were dried in a freeze-drier (Alpha 1–4 LSCbasic and Lyocube, Martin Christ). All digestions were conducted in triplicate on three separate sets/days.

2.4.5. Analysis of total nitrogen in foods and digesta

TN in undigested foods and digesta (pellets and supernatants) was quantified using the Kjeldahl method (ISO-8968-3|IDF-20-3, 2004). Representative aliquots of foods and supernatants were analyzed directly. The results for foods and supernatants were recorded as TN concentration (g/kg). For the pellets, the whole pellet was first separated into two fractions, which were analyzed separately to obtain two measurements and to ensure a backup sample in case one aliquot failed during the analysis. The samples were solubilized in 2 mL of H_2SO_4 (96%, *v*/v), followed by vigorous mixing for 1 min, subsequent addition of 2 mL of H_2O_2, and mixing again until completely solubilized. The TN content of both fractions was then summed to obtain the total TN amount per pellet (mg), representing the undigested protein fraction.

2.4.6. Acid hydrolysis for amino group (OPA) and/or amino acid (TAA) quantification in foods and digesta

Undigested foods and digesta (pellets and supernatants) were subjected to acid hydrolysis prior to determination of total amino groups and total amino acids, following the procedure by Jaudzems et al. (2019). Because acid hydrolysis partially degrades tryptophan, alkaline hydrolysis was used for accurate quantification of tryptophan in undigested foods (section 2.4.7). Tryptophan in digesta was quantified from the acid hydrolysates together with the other amino acids. Although absolute tryptophan recovery is also reduced during the optimized acid hydrolysis time, its digestibility can be calculated from the relative amounts determined in the soluble and insoluble digesta fractions. Briefly, 220 μL of individual supernatants were dried in glass vials in a centrifugal concentrator (CentriVap, Labconco, Kansas City, MO, USA) and resuspended in 220 μL ultrapure water, 120 μL 3,3′-dithiodipropionic acid (DDP; 0.1%, *w*/*v*, in NaOH 0.2 mol/L), 120 μL HCl (0.2 mol/L), 40 μL L-norvaline (10 mmol/L), and 500 μL HCl (37%, *v*/v).

For pellets and foods, the whole digesta pellet (transferred into a glass vial) or ~ 10–30 mg of undigested food were dissolved in 880 μL ultrapure water, 480 μL DDP (0.1%, *w*/*v*, in NaOH 0.2 mol/L), 480 μL HCl (0.2 mol/L), 160 μL L-norvaline (10 mmol/L), and 2 mL HCl (37%, *v*/v). All samples were placed in an oven at 110 °C for 15 h (digesta) or 24 h (foods).

2.4.7. Alkaline hydrolysis for tryptophan quantification

Tryptophan in the undigested foods was quantified after alkaline hydrolysis according to ISO 13904:2016 (ISO-13904, 2016). Around 50 mg of food sample (or 150 mg for chickpeas and 500 mg for liquid milk) were mixed with 170 mg starch, 200 μL 1-methyltryptophan internal standard (320 μg/mL), and 2 mL NaOH (8.4 mol/L). The solution was filled to 4 mL with ultrapure water and placed in an oven at 110 °C for 20 h. The samples were then cooled down for approximately 10 min and neutralized with 5 mL phosphate buffer (0.2 mol/L, pH 7.0). An L-tryptophan standard (1.6 mg/mL) was hydrolyzed in parallel.

2.4.8. Quantification of total amino groups

Total amino groups (R-NH_2) in the supernatant and pellets after acid hydrolysis (Section 2.4.6) were quantified using the OPA method. Supernatants and pellets were diluted 5 and 10 times, respectively, with perchloric acid (0.5 mol/L) and derivatized with OPA in the presence of 2-mercapto-ethansulfonic acid. Total amino groups were determined using ultraviolet (UV)/visible spectrophotometry at 340 nm and based on a glutamic acid standard curve (Egger et al., 2026; Kopf-Bolanz et al., 2012).

2.4.9. Quantification of individual amino acids and tryptophan

The amino acid profile of undigested foods and digesta (pellets and

supernatants) was determined based on the AOAC International method 2018.06 corresponding to ISO-4214|IDF-254:2022 (ISO-4214|IDF-254, 2022) for infant formula (Jaudzems et al., 2019) and a documented procedure (Waters, 2007). Briefly, 40 µL acid hydrolysate (Section 2.4.6) was neutralized with 40 µL NaOH (6 mol/L) and 80 µL HCl (0.1 mol/L). The samples were transferred to cellulose filter plates (Eppendorf, Schönenbuch, Switzerland) and centrifuged for 5 min at 370 x*g* and 15 °C. Then,10 µL filtered samples were derivatized with 20 µL AccQ-Tag Ultra reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate in acetonitrile, saturated solution), and 70 µL borate buffer (5%, *w/v*); the reaction was allowed for 10 min at 55 °C in agitation (1000 rpm; ThermoMixer C, Eppendorf). Individual amino acids were analyzed using a Vanquish UHPLC system (Thermo Fisher Scientific, Reinach, Switzerland) equipped with a UV detector. Chromatographic separation was carried out on an Acquity UHPLC BEH C18 column (2.1 × 150 mm, 1.7 µm; Waters, Baden, Switzerland) using the following conditions: flow rate 0.4 mL/min, injection volume 2 µL, column temperature 50 °C, and UV detection at 260 nm.

For the analysis of tryptophan in the undigested material, 100 µL of alkaline hydrolysates (Section 2.4.7) were neutralized with 50 µL ice-cold HCl (6 mol/L) and 2.35 mL phosphate buffer (0.2 mol/L, pH 7.0). Then, 200 µL of the neutralized samples were placed in a cellulose filter plate (Eppendorf) and centrifuged for 5 min at 370 x*g* and 15 °C. Quantification was performed with a Vanquish UHPLC system (Thermo Fisher Scientific) coupled to a fluorescence detector and equipped with an Acquity UHPLC BEH C18 column (2.1 × 150 mm, 1.7 µm; Waters), according to ISO 13904:2016 (ISO-13904, 2016). Analytical conditions were 0.4 mL/min flow rate, 2 µL injection volume, 50 °C column temperature, and 285/340 nm excitation/emission wavelengths.

2.5. Calculation of protein digestibility, DIAAR, DIAAS, and proxy-DIAAS

Calculation of total protein digestibility (%), individual amino acid digestibility (%), DIAAR, DIAAS, and proxy-DIAAS based on quantification of TN, amino groups (OPA), and amino acids (TAA) were done according to the draft ISO/IDF method (ISO/DIS-24167|IDF-261, 2024) based on (Sousa et al., 2023b). As stipulated by the FAO for regulatory purposes, the amino acid requirement reference pattern values for children (6 months to 3 years) (FAO, 2013) were considered for the calculation of DIAAS and proxy-DIAAS. For the calculation of DIAAS and DIAAR, an NCF of 6.25 was used for all foods, as recommended by the FAO, irrespective of the NCF used for protein normalization in the digestion experiments.

The digestibility of total protein and individual amino acids was calculated based on Eq. (1). The calculation of DIAAS requires the digestibility coefficient (DC) of IAAs, calculated from their individual digestibility as in Eq. (2). The amount of digestible IAA per gram of food protein (DIAA) was calculated using Eq. (3). DIAAR for each IAA was finally calculated using the reference values for children given by FAO (FAO, 2013) as in Eq. (4). If only total protein digestibility is available, an approximation of DIAAR/DIAAS (proxy-DIAAR/proxy-DIAAS) can be calculated by replacing the digestibility of IAA with the total digestibility obtained with TN, OPA, or TAA in Eq. 2 and applying the same DC for all IAA in Eq. 3 (Sousa et al., 2023b).

$$\text{Digestibility } [\%] = \frac{F_S - C_S}{(F_s - C_S) + \max(0; F_P - C_P)} \times 100 \quad (1)$$

F_S and C_S refer to the supernatants of food and cookie (enzyme blank), respectively, and F_P and C_P refer to the pellets of the same fractions.

$$\text{Digestibility coefficient (DC)} = \frac{\text{digestibility IAA}}{100} \quad (2)$$

$$\text{Digestible IAA (DIAA)} = \frac{\text{IAA in food [mg]}}{\text{Food protein [g]}} \times \text{DC} \quad (3)$$

$$\text{DIAAR} = \frac{\text{DIAA}_{\text{measured}}}{\text{DIAA}_{\text{reference}}} \times 100 \quad (4)$$

2.6. Data treatment and statistical evaluation

The statistical test for inclusion of data from both collaborative studies was a robust two-sided Wilcoxon test programmed in R language (wilcox.test(substrate ~ RT, alternative="two.sided", data = all)) using RStudio (version 2024.02.29; Posit PBC, Boston, MA, USA). To test the equivalence of the three methods for analyzing total protein digestibility, a one-way analysis of the variance (ANOVA) test followed by Tukey's post hoc and Welch's ANOVA test were done in SPSS (version 31.0.1.0; IBM, Armonk, NY, USA) with all the data included in the reproducibility and repeatability tests. Individual foods were tested separately. Median digestibility results (categorized by food) were used as the dependent variables, while the method (OPA, TN, and TAA) was the independent variable; outliers were removed from the dataset for this analysis. Homoscedasticity of the data was assumed (Levene's test non-significant), but the data did not always follow normal distribution (Shapiro-Wilk test significant for most substrates). Significance was considered when $p < 0.05$.

In the collaborative studies, the general mean (assigned value) for total digestibility using OPA and TN was calculated for each substrate by calculating the median value over the medians from each individual laboratory, and the uncertainty was calculated from the median of the standard deviations of each individual laboratory. Exclusion criteria were applied independently to each substrate for (i) laboratories with technical problems; (ii) results with low trueness (> 20% difference to assigned value (general mean, calculated as described above); and (iii) high variability (> 20% standard deviation), as detailed in Table 2. Two iteration rounds of exclusion and recalculation of the general mean (assigned value) and standard deviation were performed. Based on the cleaned datasets, repeatability (*r*) and reproducibility (*R*) were calculated using robust statistics (rlmer; Koller, 2016) for OPA and a linear mixed model in R (Fox et al., 2024) for TN analyses, respectively. Only four labs reported TAA results, so repeatability and reproducibility could not be calculated for this analytical method.

Total in vitro protein digestibility results from the freeze-dried dairy products (collaborative studies) were compared with those from the fresh dairy foods. In vitro data from the collaborative studies and from the analysis of the 19 dairy foods were also compared with in vivo data available in the literature. These comparisons were conducted through statistical analysis in SPSS (version 31.0.1.0; IBM) using a generalized linear model and considering gamma distribution of the data, food type and digestion method (in vitro/in vivo) or food preparation (fresh/dry) as independent variables, and digestibility as the dependent variable. The threshold for significance was set at $p < 0.05$. A comparison of freeze-dried versus fresh dairy products was done only with data from equivalent food types. For the in vitro versus in vivo comparisons, the foods analyzed in vitro were categorized into four groups: dairy powders (SMP, WPI, and WMP), liquid milk (skim milk, pasteurized whole milk, yogurt, skyr, and kefir), cheese (quark, ricotta, cottage, feta, Ziger, Brie, Camembert, Gruyère, Parmigiano Reggiano, Cheddar, Tilsiter, and Manchego) and plant proteins (SPI and wet chickpea).

3. Results

3.1. Collaborative studies

3.1.1. Enzymatic activity among laboratories and SDS-PAGE results

Most, but not all, participating laboratories reported their source reference and activity of pepsin and pancreatin (trypsin), as well as bile

Table 2
Precision data for total protein digestibility results obtained with the analysis of primary amines (OPA; using the o-phthalaldehyde method), total nitrogen (TN; using the Kjeldahl method), and total individual amino acids (TAA; by UHPLC).

	SMP	WMP	Cheese	WPI	Yogurt	SPI	Chickpea
OPA							
Total results received	25	25	25	25	25	18	18
Excluded: technical issues (number of labs)	3	3	3	3	4	0	0
Excluded: trueness, variability (number of labs)	4, 2	5, 3	3, 4	2, 2	4, 3	1, 2	3, 2
N	16	14	15	18	14	15	13
Digestibility [%]	92.93	80.71	81.06	99.4	87.43	100	96.67
SD	6.35	10.01	7.16	3.21	8.82	2.91	6.06
SEM	1.6	2.7	1.8	0.8	2.4	0.8	1.7
Robust linear mixed model							
s_L	5.4	6.1	9.5	0	7.3	3.1	7.4
s_r	4.7	9	8.1	4.1	9.4	2.8	7.9
s_R	7.1	10.9	12.5	4.1	11.9	4.2	10.8
r	13.4	25.4	23	11.7	26.5	8	22.3
R	20.2	30.8	35.5	11.7	33.7	11.7	30.5
TN							
Total results received	14	15	15	15	15	6	6
Excluded: technical issues (number of labs, single value)	1, 2	1, 3	1, 2	1, 2	1, 2		
Excluded: trueness, variability (number of labs)	1, 1	1, 1	0,1	1,1	0, 1	1, 0	1, 0
N	9	9	11	10	11	5	5
Digestibility [%]	90.7	78.1	83.3	100.0	82.3	90.0	88.8
SD	4.0	4.1	8.2	0.2	8.6	6.2	6.9
SEM	1.3	1.3	2.5	0.1	2.7	2.8	3.1
Robust linear mixed model							
s_L	5.8	5.6	5.3	5.0	0.0	4.2	14.8
s_r	3.9	12.0	8.0	0.9	12.9	5.1	7.5
s_R	7.0	13.3	9.6	5.0	12.9	6.6	16.6
r	10.8	33.7	22.3	2.6	36.0	14.2	20.9
R	19.5	37.2	26.8	14.1	36.0	18.4	46.5
TAA							
Total results received	4	4	4	4	4	3	2
N	4	4	4	4	4	3	2
Digestibility [%]	91.4	82.81	84.54	92.3	85.81	95	86.2
SD	2.2	4.3	3.6	2.4	4.5	2.7	4.2
SEM	1.1	2.1	1.8	1.2	2.2	1.3	2.4

Statistical terms – N: number of labs included in the evaluation; digestibility: assigned value, general mean; SD: standard deviation of the median; SEM: standard error of the median; s_L: between laboratory variance; s_r: standard deviation of the repeatability; s_R: standard deviation of the reproducibility; r: repeatability; R: reproducibility; % are given in absolute values. *Sample names* – SMP: skim milk powder; WMP: whole milk powder; WPI: whey protein isolate; SPI: soy protein isolate. Cheese, yogurt, and chickpea samples were freeze-dried.

salt concentration. However, only a few reported amylase activity (only for Collaborative Study 2) (Supplementary Material, Tables S1 and S2). All participating laboratories used porcine pepsin, but this was obtained from different sources and showed highly variable activities (393–5605 U/mg pepsin powder). Most participating laboratories used the same pancreatin product (Sigma P7545), whose trypsin activity ranged between 1.7 and 7 U/mg powder. Bile acids were mainly of bovine (Sigma B8381, B3883) or porcine (Sigma B8631) origin, although some participating laboratories used bile salts (Sigma, B3301, B8756) instead, and concentrations ranged from 1 to 6 mmol/mg powder. For the second study, only five laboratories used salivary amylase (Sigma, A1031), with activities ranging from 79 to 123 U/mg powder. Low reported pepsin or trypsin activity could not be linked to a higher variability or lower trueness of protein digestibility results. However, enzyme activity data were not available for all participants, and different pepsin and pancreatin products were used in different combinations, precluding a robust correlation analysis. Due to the low number of laboratories that added amylase, no separate statistical evaluation was performed.

Protein patterns analyzed by SDS-PAGE (Supplementary Material, Fig. S1 shows an example of a gel run with cheese and yogurt pellets after digestion) showed that pellets were extensively hydrolyzed after digestion, with bands most probably originating from digestive enzymes, since they differed from the casein control (lane 2) or from the corresponding undigested controls (lanes 3 [cheese] and 7 [yogurt]). As expected, no intact proteins were visible in the supernatants (data not shown).

3.2. Total digestibility, repeatability, reproducibility, and equivalence of the three analytical endpoints

The two rounds of collaborative studies were performed with the same freeze-dried dairy products, using the same method for IVD, and by mostly the same laboratories. Statistical testing did not reveal significant differences between the two rounds ($p > 0.2$ for all the substrates); therefore, the results from both collaborative studies were included in the evaluation. Moreover, no statistically significant differences in total digestibility were found among the three different analytical methods for the tested substrates (OPA, TN, and TAA), as shown in Fig. 1. This was further confirmed by ANOVA followed by Tukey's post-hoc ($p > 0.05$) and Welch's test ($p > 0.2$) (Supplementary Material, Table S3).

For each substrate, the assigned value (general mean), number of excluded laboratories, and reason for exclusion, as well as the calculated repeatability (r) and reproducibility (R) values, are listed in Table 2. The repeatability and reproducibility standard deviations (s_r and s_R, respectively) were within acceptable ranges for both endpoints. The total protein digestibility results using the TAA method are presented in Table 2. Unfortunately, the data using this method were provided by only four laboratories, which was not sufficient to calculate repeatability and reproducibility. Nevertheless, the individual performance of the four participating laboratories is presented in Supplementary Material,

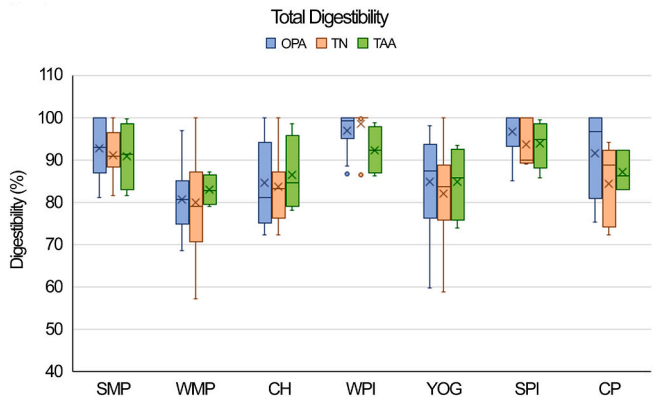

Fig. 1. Total protein digestibility analyzed by the participating laboratories using the three analytical endpoints (total amino groups [OPA], total nitrogen [TN], and total amino acids [TAA]) for the seven substrates of the collaborative study: skim milk powder (SMP), whole milk powder (WMP), cheese (CH), whey protein isolate (WPI), yogurt (YOG), soy protein isolate (SPI), and chickpea (CP). Box plots show median values (lines), mean values (x), minimum and maximum values (error bars), and outliers (dots). For OPA and TN, general means were calculated using robust statistics (section 2.6). TAA results represent the average of the four datasets reported by participating laboratories.

Fig. S2.

3.3. Performance of individual laboratories applying the digestibility method

The performance of the individual participating laboratories was evaluated for the OPA and TN results only (OPA, Fig. 2; TN, Supplementary Material, Fig. S3; outliers included). For this, the results of each participating laboratory were compared with the assigned value for total protein digestibility calculated for each of the substrates (Table 2), while the method's reproducibility standard deviation (s_R) was set as the standard deviation of the collaborative study (σ_{PT}). For all substrates, most laboratories reported average digestibility values close to the assigned value (green lines in the figures) and within the upper and lower limits of $\pm 2\sigma_{PT}$ (orange dotted lines) or $-3\sigma_{PT}$ (red dotted lines), which show the calculated acceptable range for each substrate.

Furthermore, the relative difference to the assigned value was calculated for each participating laboratory and substrate (OPA, Fig. 3; TN, Supplementary Material, Fig. S4). Here, $\pm\ 2\sigma_{PT}$ (red lines in the figures) was used as the acceptance threshold, corresponding to the relative standard deviation of the reproducibility limit (s_R) of the method. These results confirmed that most laboratories performed within the acceptance criteria, with three to five laboratories reporting considerably lower values for certain samples with lower homogeneity, such as yogurt, cheese, and chickpea.

3.4. Digestibility and DIAAS of dairy foods

The scans of the SDS-PAGE performed with the fresh dairy products (Fig. 4A) present the molecular weight distribution of their native proteins. As expected, cheeses exhibited more protein bands than milks, which is attributed to the partial proteolysis that occurs during cheesemaking and ripening. However, the protein profiles suggest that all samples were in good condition before digestion.

Overall, proteins in dairy foods were highly digestible, with total protein digestibility (average of the three endpoints) exceeding 96% in all fresh products except goat's milk (94%) and Manchego cheese (93%) (Fig. 4B). Fermented dairy foods tended to show slightly higher average digestibility than liquid milks. This difference is probably a consequence of lower and more variable digestibility values in liquid milk samples when measured with TN (Supplementary Material, Fig. S5). Similarly, values slightly decreased when hard cheeses were studied using the TAA method, whereas all three analytical endpoints performed similarly in the other fermented foods.

The average digestibility of cow's milk was slightly higher than that of sheep's milk, although the variability of the results (represented by the error bars in Fig. 4B) suggests no real differences. There was also no apparent difference between skim and pasteurized cow's milk. Conversely, goat's milk presented lower total protein digestibility. On the other hand, Manchego cheese displayed the lowest protein digestibility among cheeses, which was confirmed by analyzing four additional cheese samples from different farms. The total protein digestibility of the five Manchego cheeses ranged between 87% and 95% with the analysis of TAA, and between 87% and 100% using OPA and TN (Supplementary Material, Fig. S6).

The DIAAS values based on the reference patterns for young children (as recommended by FAO, 2013) exceeded 100% for all dairy products, underscoring their excellent protein quality (Table 3). Among Manchego cheeses, however, some variation was observed: three of the five samples exhibited DIAAS values below 100% (86.7, 90.6, and 96.4%), consistently attributable to tryptophan as the limiting amino acid. In these samples, tryptophan digestibility was markedly reduced (51–57%), and it was the only amino acid for which the DIAAR fell below 100% (Table 3).

3.5. Digestibility of freeze-dried versus fresh dairy products and comparability with in vivo digestibility

Total protein digestibility of the freeze-dried dairy foods (SMP, WMP, cheese, and yogurt) was significantly lower ($p < 0.001$) than that of the corresponding fresh products (skim milk, whole milk, cheese, and yogurt) (Fig. 5A). Considering all four food types together, freeze-drying resulted in an average reduction of 13.8% in total protein digestibility (84.8% versus 98.6%), emphasizing that drying can alter protein accessibility and/or matrix properties.

No statistically significant differences were observed between the in vitro (one-laboratory study) and in vivo digestibility values obtained from the literature ($p = 0.817$) (Fig. 5B), indicating that the in vitro method provides physiologically relevant estimates of dairy protein digestion.

4. Discussion

4.1. International standardization process of the in vitro protein digestibility method based on the static INFOGEST protocol

The IDF/ISO standardization process for the in vitro protein digestibility method was initiated in 2023, following its first validation against in vivo digestibility using seven substrates (Sousa et al., 2023b). Since then, the method has been applied to a wider range of substrates, including meat and plant-based burgers (Sousa et al., 2023a), soy products (Hammer et al., 2024, 2025), and edible insects (Hammer et al., 2023), with each study confirming its agreement with in vivo measurements. Although human or pig ileal digestibility analysis remains the gold standard for the assessment of protein digestibility and DIAAS (FAO, 2013), a validated and standardized in vitro alternative that closely simulates physiological digestion offers major practical and ethical advantages.

The total digestibility values obtained with the three different analytical endpoints OPA, TN, and TAA in the collaborative studies presented herein were not statistically different. However, so far, this conclusion is based on the seven substrates of this study and the seven substrates previously reported by Sousa et al. (2023b). Therefore, more results comparing the three methods are needed to confirm the full equivalence of the three methods.

For IDF/ISO standardization of any method, its robustness must be demonstrated. This is why the repeatability and reproducibility of the in

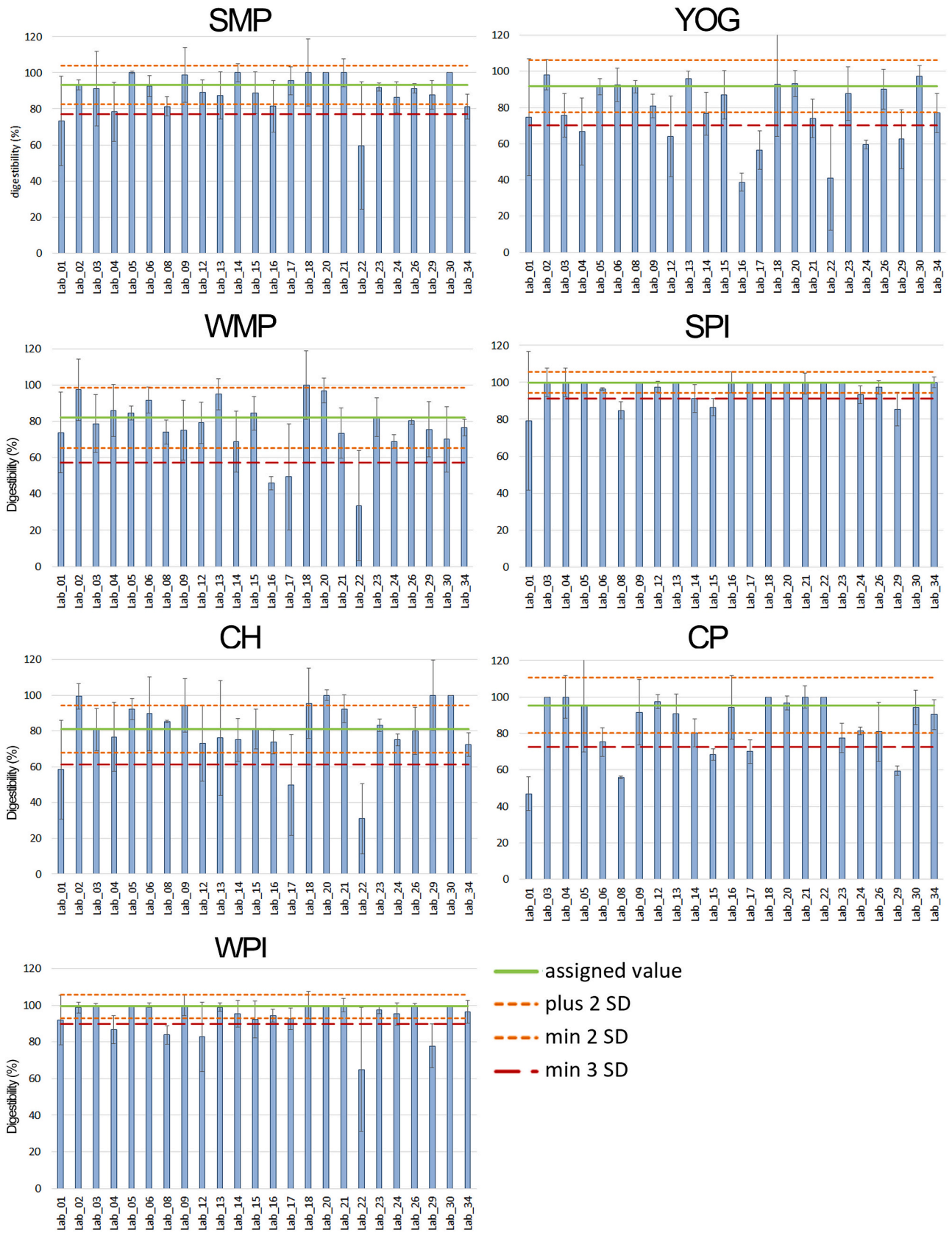

Fig. 2. Average total protein digestibility (%, with standard deviation where replicates were available) measured by the o-phthalaldehyde method (OPA) for each participating laboratory, combining data from both collaborative studies. The green lines represent the assigned value, orange dotted lines show ±2 standard deviations (SD), and the red dotted line indicates −3 SD for each substrate (Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

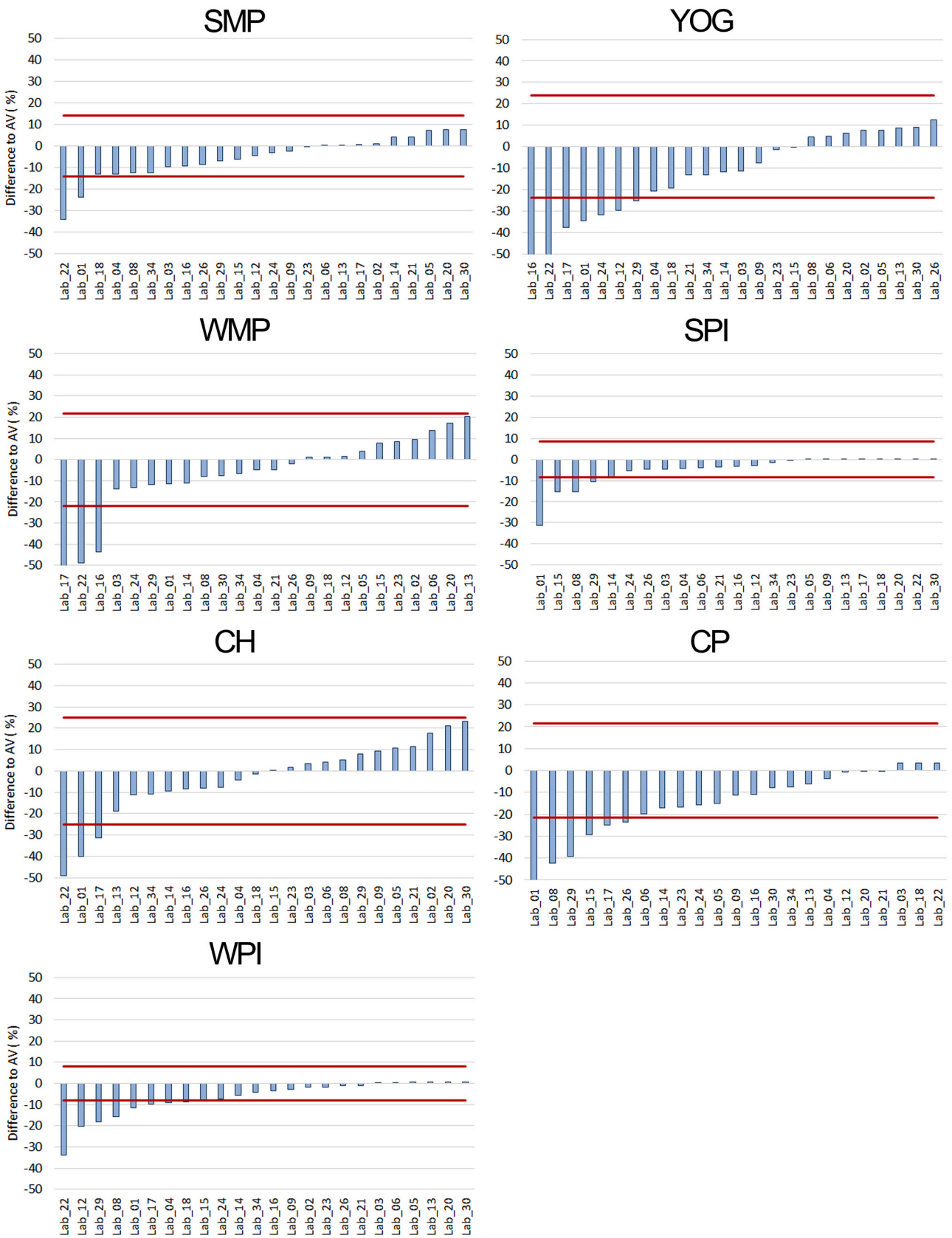

Fig. 3. Difference (+/−) to the assigned value (digestibility, Table 2) for the OPA-based digestibility results for each substrate and each participating laboratory. The red lines represent $\pm 2^{*}\sigma_{PT}$ corresponding to the reproducibility standard deviation (s_R) of the method (Table 2). Outliers are included. SMP: skim milk powder; YOG: yogurt; WMP: whole milk powder; SPI: soy protein isolate; CH: cheese; CP: chickpea; WPI: whey protein isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

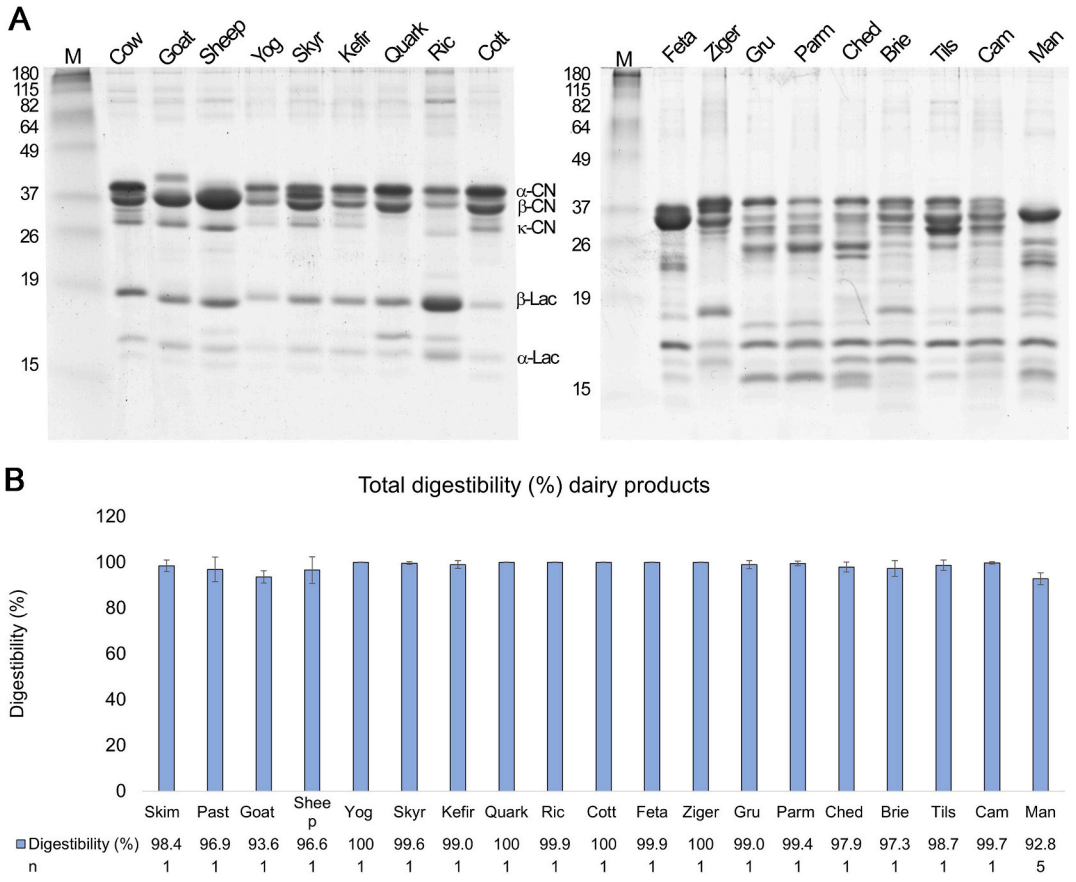

Fig. 4. A) Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the fresh dairy foods before digestion. B) Total protein digestibility of the dairy foods, expressed as the average (%) and standard deviation (error bars) of the three analytical endpoints (total amino groups [OPA], total nitrogen [TN], and total amino acids [TAA]), each method performed in triplicate. Past: pasteurized cow milk; Yog: yogurt; Ric: ricotta cheese; Cott: cottage cheese; Gru: Gruyère cheese; Parm: Parmigiano Reggiano cheese; Ched: Cheddar cheese; Tils: Tilsiter cheese; Cam: Camembert cheese; Man: Manchego cheese.

vitro protein digestibility method were assessed through two collaborative studies conducted within the INFOGEST network. The two studies used freeze-dried dairy and plant-based products (5 dairy and 2 plant-based) to ensure homogeneity of the test materials and facilitate shipment around the globe, recognizing that these substrates do not fully represent real consumption conditions. To address this, 19 fresh dairy foods were additionally analyzed and compared with their freeze-dried counterparts.

4.2. *Calculation of assigned values and performances of individual laboratories*

According to ISO 5725-1, accuracy data should preferably be generated by laboratories that already have high expertise in performing the method (ISO-5725-1, 2023). In these collaborative studies, although the participants were familiar with the static INFOGEST 2.0 digestion protocol, many were implementing the analytical workflow for quantification of digestibility for the first time. Moreover, in some cases, the experiments might not have been performed strictly under repeatability conditions (e.g., short time between replicates, identical operator, or the same calibration of the equipment). Another source of variability is that although the protein content was standardized at 40 mg for all digestions, the enzyme preparations differed in source and activity among laboratories. Although the participating laboratories predominantly used the same pancreatin source, the reported trypsin activity fluctuated between 1.7 and 7 U/mg, most probably due to batch differences. As established by the INFOGEST network, rigorous standardization of enzyme activity is vital for ensuring study reproducibility (Brodkorb et al., 2019; Egger et al., 2026).

Despite these challenges, it is very promising that such a multistep method that includes several enzyme activity determinations could achieve an average repeatability limit (r) of 19% and an average reproducibility limit (R) of 26.6% after the exclusion of outliers (Table 2). These values are well in line with those of simpler enzymatic methods, such as methods for alkaline phosphatase activity in dairy products (e.g. r = 12%, R = 26%) (Milk and milk products – Determination of alkaline phosphatase activity – Part 2: Fluorometric method for cheese, 2024; ISO-22160|IDF-209, 2007). The average reproducibility standard deviation (s_R) for all tested substrates for the methods OPA and TN achieved 9%. In the future ISO/IDF method, it is therefore recommended that triplicates may be accepted if they differ in digestibility by <10%, whereas larger variation would necessitate a fourth replicate. Fully establishing the method's precision requires further collaborative trials that extend the scope to other substrates and incorporate amino acid digestibility analyses.

Laboratory performance was assessed in two complementary ways: (1) comparing each laboratory's mean and standard deviation for each substrate with the statistically calculated assigned value (OPA, Fig. 2; TN, Supplementary Material, Fig. S3) and (2) evaluating the deviation of each laboratory's mean digestibility from the assigned value (OPA, Fig. 3; TN, Supplementary Material, Fig. S4). Both approaches showed that digestibility results of pure protein powders (WPI and SPI) were less variable (s_R < 7%, Table 2), whereas freeze-dried yogurt, cheese, and chickpea showed higher dispersion (10% < s_R < 17%, Table 2). Freeze-dried milk powders (SMP and WMP) exhibited intermediate behavior (7% < s_R < 13%, Table 2). The higher variability could be caused by differences in matrix complexity and product homogeneity.

Because the precision estimates were calculated after application of

Table 3
Digestible indispensable amino acid ratios (DIAAR) for children (6 months to 3 years) of whole dairy foods, expressed as percentages (standard deviations of triplicate analyses are given in parentheses).

	HIS	ILE	LEU	LYS	SAA	AAA	THR	TRP	VAL
Skim milk	129	170	145	143	134	192	141	**123**	151
	(0)	(0)	(0)	(0)	(2)	(0)	(0)	(0)	(0)
Pasteurized milk	129	173	144	145	141	195	143	**124**	151
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(2)	(0)
Goat's milk	120	147	141	139	133	165	156	**110**	159
	(3)	(21)	(6)	(3)	(20)	(11)	(13)	(30)	(14)
Sheep's milk	**129**	164	146	147	143	186	140	135	159
	(0)	(0)	(0)	(0)	(2)	(0)	(0)	(0)	(0)
Yogurt	**121**	158	133	134	131	180	132	125	139
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(9)	(0)
Skyr	150	192	160	166	153	217	160	**136**	169
	(0)	(0)	(0)	(0)	(2)	(0)	(0)	(0)	(0)
Kefir	**125**	165	137	139	133	183	136	131	143
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(5)	(0)
Quark	145	175	156	158	150	209	**141**	147	158
	(0)	(0)	(0)	(0)	(2)	(0)	(0)	(0)	(0)
Ricotta	**144**	195	185	181	226	192	170	212	151
	(0)	(0)	(0)	(0)	(5)	(2)	(0)	(0)	(0)
Cottage	177	192	159	162	**143**	238	156	158	173
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Feta	155	166	151	148	**130**	217	131	167	164
	(0)	(0)	(0)	(0)	(1)	(3)	(0)	(0)	(0)
Ziger	**113**	147	126	123	118	175	114	168	133
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Gruyère	162	159	149	145	120	220	**117**	183	160
	(0)	(5)	(0)	(0)	(3)	(3)	(0)	(22)	(0)
Parmigiano Reggiano	163	183	165	152	126	216	**124**	154	168
	(0)	(4)	(0)	(0)	(1)	(3)	(0)	(0)	(0)
Cheddar	159	155	145	149	**121**	203	123	132	155
	(0)	(14)	(2)	(0)	(8)	(9)	(2)	(21)	(7)
Brie	161	148	160	165	138	222	140	**118**	165
	(0)	(6)	(3)	(0)	(2)	(3)	(6)	(4)	(6)
Tilsiter	164	161	168	166	**134**	235	138	151	176
	(0)	(2)	(0)	(0)	(0)	(2)	(0)	(4)	(2)
Camembert	127	148	117	117	**103**	175	117	169	135
	(0)	(1)	(0)	(0)	(1)	(1)	(0)	(0)	(0)
Manchego	156	138	155	160	125	207	125	**110**	161
	(6)	(14)	(13)	(9)	(8)	(16)	(10)	(27)	(14)
Manchego 1	147	116	132	145	111	179	110	**90.6**	138
	(1)	(0)	(1)	(0)	(2)	(2)	(2)	(11.3)	(1)
Manchego 2	157	135	156	167	130	210	123	**96.4**	159
	(0)	(2)	(1)	(1)	(1)	(1)	(1)	(9.2)	(1)
Manchego 3	156	141	156	159	**124**	208	126	128	165
	(0)	(3)	(3)	(0)	(1)	(2)	(4)	(8)	(4)
Manchego 4	158	153	167	162	**131**	217	137	149	176
	(0)	(2)	(1)	(0)	(1)	(1)	(0)	(22)	(2)
Manchego 5	164	144	162	167	130	219	127	**86.7**	167
	(0)	(2)	(1)	(0)	(2)	(0)	(0)	(7.9)	(1)

DIAAR values were calculated using digestibility coefficients determined through the analysis of individual amino acids (TAA), and a nitrogen-to-protein conversion factor (NCF) of 6.25 as recommended by the Food and Agriculture Organization (FAO) of the United Nations (FAO, 2013). Using a dairy-specific NCF of 6.38 would reduce DIAAR values by ~2% (data not shown). Bold values indicate digestible indispensable amino acid scores (DIAAS).

the predefined exclusion criteria, they represent method performance after data quality control. To provide transparency on the variability of the complete interlaboratory dataset, Figs. 2 and 3 display results from all participating laboratories, including datasets excluded from the calculation of assigned values and precision parameters.

4.3. Protein quality of dairy foods

Even though isolated dairy proteins and milk powders are among the most studied ingredients in terms of protein digestibility (Herreman et al., 2020; Komatsu et al., 2023; Mathai et al., 2017), relatively little data is available for whole dairy foods. It is well known that processing can significantly affect the nutritional quality of proteins by altering their native physicochemical structure or by modifying the activity of endogenous antinutritional factors (Martineau-Côté et al., 2023; Wada & Lönnerdal, 2014). Indeed, the results of the experiments presented herein demonstrate that digestibility differs substantially between fresh products and their freeze-dried forms (Fig. 5A). Freeze-drying is known to induce protein denaturation due to multiple factors such as formation of ice crystals and dehydration stress. This change in protein structure increases hydrophobicity and induces aggregation (Roy & Gupta, 2004; Zhou et al., 2027), which can in turn negatively affect digestibility by hiding the binding sites of proteins and limiting accessibility of the digestion enzymes to them (Lee et al., 2024). Therefore, the protein quality of whole dairy foods (milk, cheese, and other fermented forms) should be assessed as they are consumed rather than extrapolated from freeze-dried powders or isolated proteins.

The present results confirm the high protein quality of dairy foods, which generally showed a favorable amino acid pattern coupled with a high total protein digestibility above 94%, resulting in DIAAS values (for children) above 100%. This is in accordance with in vivo data (Hodgkinson et al., 2025; Mathai et al., 2017) and earlier work showing near-complete intestinal hydrolysis of dairy proteins (Fang et al., 2016; Ménard et al., 2018; Nguyen et al., 2020; Žolnere et al., 2019),

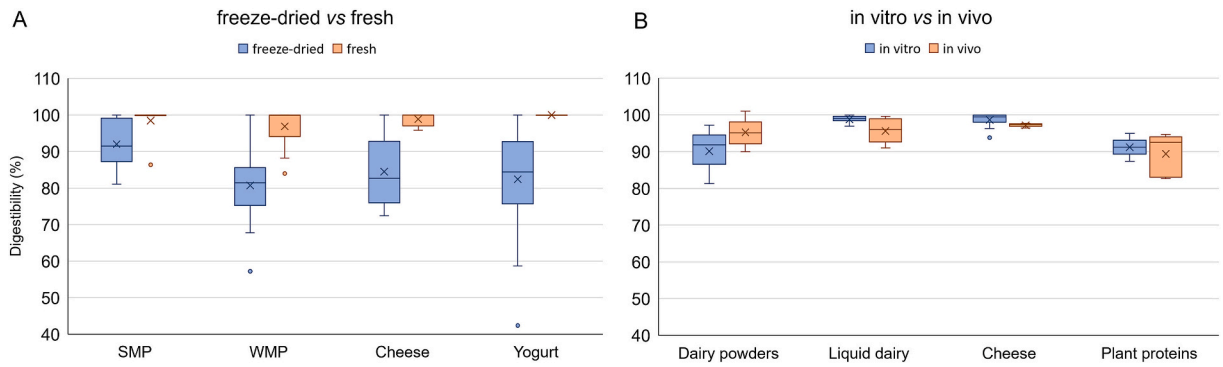

Fig. 5. Comparison of total protein digestibility between: A) freeze-dried dairy products used in the collaborative study (skim milk powder [SMP], whole milk powder [WMP], freeze-dried cheese (Gruyère), and freeze-dried yogurt) and their corresponding fresh products, * indicates a significant difference of $p < 0.001$; and B) in vitro results from this study (average of the three analytical endpoints: OPA, TN and TAA) and in vivo digestibility data from literature. In panel B, the protein sources are grouped as dairy powders (SMP, WMP and whey protein isolate [WPI]), liquid dairy (yogurt, skyr, kefir), cheese (quark, ricotta, cottage, feta, Ziger, Brie, Camembert, Gruyère, Parmigiano Reggiano, Cheddar, Tilsiter and Manchego), and plant proteins (soy protein isolate [SPI] and wet chickpea). No in vivo data were available for the remaining foods included in the in vitro study. In vivo data sources: (Cai et al., 2025; Calvez et al., 2021; Hodgkinson et al., 2025; Mathai et al., 2017; Rutherfurd et al., 2015) (B). Box plots show median values (lines), mean values (x), minimum and maximum values (error), and outliers (dots).

irrespective of differences in the proteolysis rate during the gastric phase (Fang et al., 2016; Nguyen et al., 2020). This stands in contrast to the quality of many plant proteins, which often have one or more limiting IAA coupled to a lower digestibility and present DIAAS values below 100% and sometimes much lower, such as wheat proteins (limited in lysine) with a DIAAS of 24–48% (Herreman et al., 2020; Komatsu et al., 2023; Mathai et al., 2017), or zein with reported DIAAS values near 1% (Cai et al., 2025; Komatsu et al., 2023).

Goat's milk showed lower total protein digestibility than other milk types, consistent with previous observations comparing goat and cow cheeses (Asensio-Grau et al., 2019), presumably due to a lower affinity of gastric pepsin for goat's milk caseins (Asensio-Grau et al., 2019). By contrast, no differences were observed between ultra-high-temperature (skimmed milk) and pasteurized cow's milk, in agreement with earlier studies (Aalaei et al., 2021; Cai et al., 2025; Wada & Lönnerdal, 2014).

Among the cheeses, Manchego exhibited the lowest protein digestibility, which could be caused by the observed slightly lower digestibility of sheep's milk. However, this may also relate to its shorter ripening time (likely less than 4–6 months, according to the Spanish legislation; BOE, 2006) compared to the other hard cheeses studied (Cheddar, Gruyère, and Parmigiano Reggiano; aged for at least 8 months according to the labeling). Longer ripening of Parmigiano Reggiano cheese, which prolongs bacteria-induced proteolysis, has been associated with enhanced proteolysis during digestion (Cattivelli et al., 2024). Since the exact ripening times of the Manchego cheese samples were not provided by the manufacturers, their effect on protein digestibility should be confirmed in future studies. The cause of the low digestibility of tryptophan in Manchego cheeses is also unclear. Tryptophan is particularly susceptible to chemical degradation, and matrix-dependent differences in its stability or analytical recovery during digestion and hydrolysis may have contributed to the observed values. Chemical modifications during cheese manufacture and ripening, including oxidation or Maillard-type reactions, as well as reactions promoted by transition metals during digestion, may further reduce recoverable tryptophan (Bellmaine et al., 2020). Because neither tryptophan degradation products nor the mineral composition of the cheeses were determined, these possible mechanisms remain speculative and should be investigated in targeted studies.

The digestibility and DIAAS data presented here expand the knowledge base on whole dairy foods and provide relevant inputs for future food composition and protein quality databases. However, this study assessed only one sample *per* dairy product type (except for Manchego cheese), so the results cannot be generalized to all market products. In fact, the five Manchego cheese samples highlight the considerable variability in protein digestibility within a single food category. Hence, these data should be considered only as references. Moreover, the DIAAR values in Table 3 were calculated using an NCF of 6.25 as recommended by FAO (2013), ensuring comparability with in vivo data. However, using a dairy-specific NCF of 6.38 would reduce DIAAR values by ~2% (data not shown).

4.4. Comparison with in vivo data

The total protein digestibility results were further compared with the available in vivo data (Cai et al., 2025; Calvez et al., 2021; Hodgkinson et al., 2025; Mathai et al., 2017; Rutherfurd et al., 2015; Fig. 5). Such studies are scarce due to ethical constraints and because true in vivo–in vitro comparison is only meaningful when foods are prepared under comparable conditions (for example, it is imprecise to compare a study that used raw and freeze-dried chickpeas with a study that used cooked and mashed chickpeas). In total, nine foods were used for the comparison in the presented comparative studies: SMP, WPI, SPI, liquid cow's milk (skimmed plus pasteurized milks), yogurt (fresh), ricotta cheese, feta cheese, Cheddar cheese, and cooked/mashed chickpeas. For comparison, only those studies that reported on ileal digestibility of foods that were as close as possible to those used in the present research were selected. However, the foods were not exactly the same between the in vivo and in vitro studies, and changes in processing, physical structure, etc. could exist. Due to these limitations in available in vivo data, various foods from the same category have been grouped. Therefore, this is just an overview of the results obtained using both techniques, and it is not intended to serve as a genuine comparison for the validation of the in vitro method (as it was done by Sousa et al., 2023b). The boxplot in Fig. 5B shows that the in vitro method validated here aligns closely with in vivo digestibility, supporting its physiological relevance. Moreover, the mean in vitro digestibility for all analyzed dairy products was 96.2%, closely aligning with the 95% digestibility observed in vivo (Hodgkinson et al., 2025). Nonetheless, the limited number of datasets, including products over the entire span of digestibility, precludes stronger statistical conclusions at this stage.

4.5. Scope and limitations of the protocol

This work provides precision data for total protein digestibility using OPA and TN analysis following the IVD according to the draft method ISO/DIS 24167|IDF 261 entitled: Milk and milk products – in vitro digestion protocol for the determination of protein digestibility and in vitro digestible indispensable amino acid score (DIAAS). This was

achieved in two international collaborative studies using seven protein substrates. However, the full validation of the TAA method for digesta, which is needed for DIAAR and DIAAS calculations, remains ongoing, as the required 15 h of acid hydrolysis must still be assessed across laboratories. Importantly, the demonstrated equivalence of the three analytical endpoints (OPA, TN, and TAA) for total protein digestibility suggests that future validation efforts for DIAAR and DIAAS may no longer need to include the full IVD step. Instead, validation could focus solely on the 15 h acid hydrolysis procedure of the supernatants and pellets. This would substantially simplify the remaining validation requirements while ensuring methodological robustness.

Moreover, the method was validated using foods with very different structure and complexity (WPI, zein, collagen, black beans, pigeon peas, All-Bran®, and peanuts; Sousa et al., 2023b), and since then, it has been applied to many products beyond dairy. However, digestion of more substrates, especially plant-based sources, is needed to fully understand the method's applicability and limitations. Its applicability to more delicate food preparations such as artificial emulsions also remains underexplored. Therefore, although the method has been shown to be equivalent to in vivo digestion in many types of foods, these gaps will direct future research that will broaden the range of foods studied.

5. Conclusion

Two international collaborative studies, involving over 20 laboratories across 16 countries, established the repeatability and reproducibility data required for the ISO/IDF standardization of the INFOGEST-based in vitro protein digestibility method (ISO/DIS-24167|IDF-261, 2024). For two analytical endpoints (OPA and TN), robust precision data were obtained, yielding an average reproducibility standard deviation (s_R) of 9%, which in practice means that analytical replicates should not differ by more than 10%. Moreover, all three analytical endpoints (OPA, TN, and TAA) produced equivalent total protein digestibility results for the substrates and laboratories tested. However, given that only four participating laboratories used the TAA method (ISO-4214|IDF-254), the analysis of individual amino acids in digesta requires further validation, which is already ongoing.

In addition to the precision data for the new method (ISO/DIS-24167|IDF-261, 2024) on in vitro protein digestibility, this study provides new in vitro digestibility and DIAAS data for a wide range of fresh dairy products, demonstrating their consistently high protein quality and contributing valuable information for future protein quality databases.

Together, these findings pave the way for an IDF/ISO-standardized method that enables a harmonized, reproducible, and physiologically meaningful assessment of protein digestibility and quality across the global food system.

CRediT authorship contribution statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.120598.

Data availability

Data will be made available on request.

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