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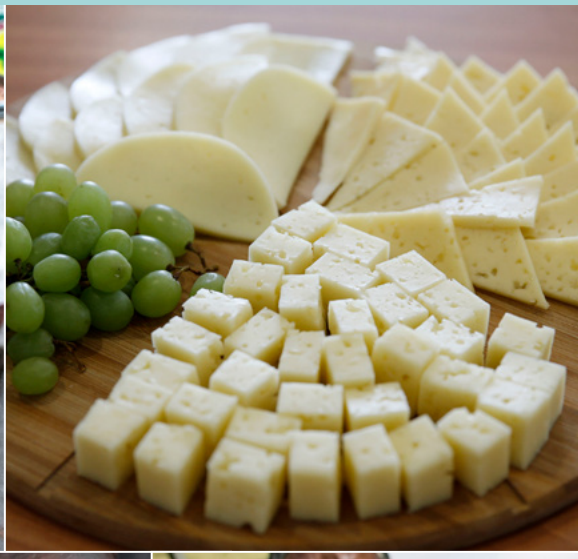
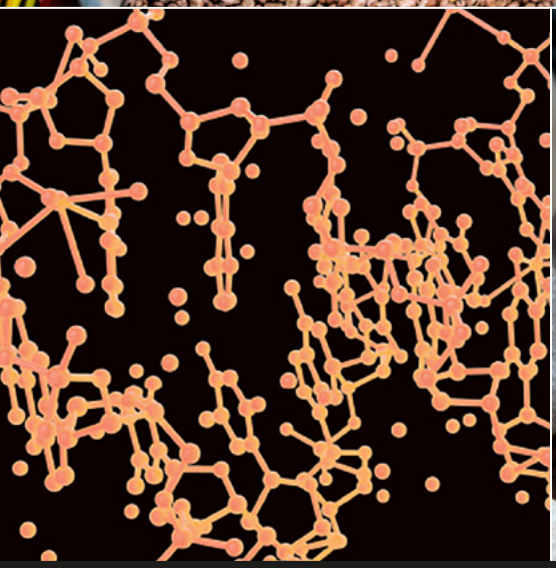
Development of a protein database and the way forward for reviewing protein requirements

Report of a joint FAO/IAEA technical meeting in Vienna, 10–13 October 2022

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Abbreviations

AA	amino acid
AAA	aromatic amino acids
APE	atom percent excess
BCAA	branched-chain amino acids
Cas	casein
CHO	carbohydrate
CO_2	carbon dioxide
COST	European Cooperation in Science and Technology
Dig_{ref}	digestibility of the standard protein
Dig_{test}	digestibility of the test protein
DIAAR	digestible indispensable amino acid ratio
DIAAS	digestible indispensable amino acid score
EAR	estimated average requirement
EED	environmntal enteric dysfunction
FAM	Food and Agriculture Microdata Catalogue
FAO	Food and Agriculture Organization of the United Nations
GWG	gestational weight gain
HIC	higher-income country
^2H_2O	deuterated water
H_2S	hydrogen sulphide
IAA	indispensable amino acid
IAAO	indicator amino acid oxidation
IAEA	International Atomic Energy Agency
INFOGEST	international network of excellence on the fate of food in the gastrointestinal tract
INFOODS	International Network of Food Data Systems
kcal	kilocalorie
kdn	kidney bean or black bean
$K^{15}NO_3$	potassium nitrate

LMICs	lower-middle-income countries
N	nitrogen
$^{15}HN_3{}^{15}NO_3$	ammonium nitrate
$(^{15}NH_4)_2SO_4$	ammonium sulfate
NPPU	net postprandial protein utilization
PAL	physical activity level
PDCAAS	protein digestibility-corrected amino acid score
ppi	pea protein isolate
PPU	postprandial protein utilization
PRI	population reference intake
RDA	recommended dietary allowance
SAA	sulphur amino acids
SDGs	Sustainable Development Goals
SOFI	State of Food Security and Nutrition in the World
spi	soy protein isolate
sun	sunflower protein isolate
TIM	TNO Gastro-Intestinal Model
tmp	total milk protein
UHT	ultra-high temperature
UNU	United Nations University
whbran	wheatbran, wheat, whole wheat/wheat bran
WHO	World Health Organization
wpi	whey protein isolate

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1. Introduction

For the first time, the Food and Nutrition Division (Formerly Nutrition and Food Systems Division) of the Food and Agriculture Organization of the United Nations (FAO) and the Division of Human Health of the International Atomic Energy Agency (IAEA) jointly organized a technical meeting held in hybrid format in Vienna from 10 to 13 October 2022. During the meeting, a framework for the development of a protein database and the way forward for reviewing protein requirements was discussed and agreed upon. The meeting was opened by the director of IAEA's Division of Human Health, the director of IAEA and the director of FAO's Food and Nutrition Division. It was also attended by a World Health Organization (WHO) representative and world experts in the areas of nutrition and protein quality assessment drawn from 16 countries. The meeting agreed on the need to create a database on protein quality with information on protein digestibility and amino acid absorption, to be jointly managed by FAO and IAEA, and defined a framework for establishing the database, including a set of criteria to be used for data inclusion. The meeting also recognized the dearth of data on protein quality from lower-middle-income countries (LMICs) and recommended that resources be mobilized to make this possible so that the database, when established, can include diverse data, including those on climate-smart foods such as those consumed in LMICs. To generate more protein quality data, the meeting identified stable isotope techniques as the best-suited tool to measure protein quality in humans accurately and in a minimally invasive way. An IAEA-coordinated research project bringing together multiple stakeholders to use stable isotope techniques to generate more data on protein quality was recommended. Further, the meeting reviewed and made suggestions on indispensable amino acid (IAA) reference values to be used in combination with amino acid (AA) absorption data to aid dialogue on individual protein requirements across age groups.

Opening remarks

The opening session was moderated by Ms Cornelia Loechl, Head of the Nutritional and Health-Related Environmental Studies Section of IAEA's Division of Human Health. The meeting was jointly opened by Ms May Abdel-Wahab, Director of IAEA's Division of Human Health, and Ms Lynnette Neufeld, Director of FAO's Food and Nutrition Division. Ms Abdel-Wahab noted the timeliness and significance of this unique multi-agency and cross-disciplinary convergence on a topic of great importance and observed that this was one step towards addressing the numerous factors that undermine our ability to combat malnutrition in all its forms by 2030, especially in light of a rapidly growing global population and more awareness of the environmental footprint of food consumption. She stated that this complexity has led to recommendations for a shift to more sustainable protein sources, especially with more protein of plant origin. But this comes with trade-offs related to how much of the protein consumed becomes available to the human body, she noted. Paradoxically, high atmospheric carbon dioxide emissions are linked to reduced nutrient concentrations and bioavailability in major food crops such as rice, wheat and barley, with protein, iron and zinc being greatly impacted. Pandemics and other emerging challenges such as rampant global inflation also continue to drastically limit our access to foods rich in high-quality protein. Ms Abdel-Wahab highlighted that in the course of time IAEA has joined hands with other players such as FAO in discussions and activities to generate much-needed data on protein quality. She emphasized that stable isotope techniques will continue to be central to our ability to provide an evidence base upon which efforts to ensure supply of adequate and high-quality protein to meet requirements across various ages and physiological states can be anchored. Moreover, all protein quality data, whether collected by isotopic techniques or other approaches, must be properly and sustainably curated and stored in secure databases. In closing, Ms Abdel- Wahab reminded the participants that their discussions on creating a framework for the protein quality database were very important.

Ms Neufeld further welcomed the participants to the technical meeting and noted the importance of two agencies coming together to co-organize a meeting of this significance. She emphasized that United Nations agencies have a unique role in achieving the Sustainable Development Goals (SDGs) and the aligned global nutrition targets by 2030. Referring to the 2022 State of Food Security and Nutrition in the

World (SOFI) report, she said that the world is moving backwards in its efforts to end hunger, food insecurity and malnutrition; hence, the meeting was of critical timely importance because providing an adequate, sustainable and nutritious supply of protein remains an increasing challenge. In this regard, plant-based proteins and novel protein sources such as insects have been suggested to have greater nutritional value, as well as less environmental impact, than "traditional" protein sources. Understanding the potential role of different protein sources by being able to assess protein quality is therefore also paramount in the light of changing food systems, Ms Neufeld continued.

Better nutrition is one of the four fundamental aspirations set out in FAO's strategic framework, alongside better production, a better environment and a better life. The right to adequate food and a transition towards healthy diets for national populations is at the core of better nutrition. In this regard, accurately defining the amount and quality of protein required to meet nutritional needs and appropriately describing the protein supplied by foods and diets is of critical importance. Ms Neufeld reminded the participants that FAO, alongside IAEA, WHO and others, has a long history spanning over 50 years in leading the work on establishing global nutrient requirements and coordinating discussions on accurately measuring protein quality in foods and diets.

Ms Neufeld said that since 2013, following FAO recommendations on better methods to assess protein quality, sufficient data have become available on ileal AA digestibility of foods and diets from various regions measured in different populations and different physiological states throughout the life cycle. FAO, with funding provided by the Government of Canada, recently initiated a project in collaboration with IAEA to inform future developments of a protein digestibility database to aid dialogue on the evaluation of protein quality and protein sufficiency in different populations. Ms Neufeld thanked the participants and reminded them of the importance of the task at hand.

Rationale for a protein database and purpose of the technical meeting

Accurately defining the protein amount and quality required to meet human nutritional needs and appropriately describing the protein supplied by foods and diets is critical for meeting global nutrition targets. Scientific advice on protein quality evaluation is also relevant for the development of Codex Alimentarius food standards and guidelines. Standardized data on food protein quality in humans has a potential to inform dialogue on recommendations for protein requirements for all age groups, especially in the first 3 years of life.

Evolution of expert discussions on protein quality

Many scientific developments related to protein quality have been achieved over the past decades. Amino acid metabolic availability, the protein digestibility-corrected amino acid score (PDCAAS), and the digestible indispensable amino acid score (DIAAS), which is based on the measurement of metabolic availability and the true specific oro-ileal digestibility of each individual AA, have been discussed in various expert consultations.[1, 2] In 2014, an FAO expert working group meeting held in Bengaluru, India, discussed the most appropriate methodologies for measuring protein digestibility and utilization in humans. Five research protocols currently in use or with potential for further development for studying true ileal AA digestibility in humans, pigs and rats were recommended for measuring the DIAAS, namely:

i. true ileal amino acid digestibility;

ii. the dual isotope tracer approach;

iii. indicator amino acid oxidation;

iv. postprandial protein utilization; and

v. net postprandial protein utilization.

The FAO expert working group meeting further recommended establishing a robust database of protein digestibility of foods commonly consumed worldwide, including those consumed in LMICs. Since then, data have progressively accumulated on ileal AA digestibility of foods, measured using different methods both in animal models (pigs and rats) and in humans, including populations from different regions and in different physiological states throughout the life course. A new, non-invasive indirect dual isotope tracer method was developed as part of the IAEA-supported Coordinated Research Project "Bioavailability of proteins from plant-based diets" (E4.30.31.) involving the labelling of the target food with deuterium and addition of a ^{13}C-labelled protein or crystalline AA mixture at the point of consumption. This method was used to evaluate individual AA digestibility of legume proteins in healthy adults in Brazil, India, Jamaica, Mexico, Morocco, Pakistan and Thailand and to

determine the protein quality of complementary foods used for young children in India.

Purpose of the technical meeting: To review and update evidence and related methods on protein requirements and protein quality assessment and to design a framework for development of a protein digestibility database to aid dialogue on the evaluation of protein quality and protein sufficiency in different populations.

Specific objectives

1. To critically review and evaluate rat, pig and human models and methods used to assess ileal AA digestibility and the data obtained with the models and methods related to food commodities currently available for human populations and animal models.

2. To review data obtained for human populations in different physiological or pathological states and environments throughout the life course on protein quality based on the DIAAS from protein sources including traditional foods, alternative protein sources and protein ingredients.

3. To propose a concrete framework for harnessing the knowledge into a future, fully accessible, robust database on protein digestibility of foods and diets from different regions of the world.

4. To discuss and propose mechanisms for utilizing information on protein digestion that occurs along the entirety of the gastrointestinal tract to inform dialogue on protein requirements throughout the life course.

5. To propose additional research that is needed, including validation of methods.

Report structure

This report is a synthesis of technical background presentations and discussions by participants during the technical meeting. It covers a global presentation of the concept of protein quality and requirements throughout the life course, touching on historical aspects of the recommendations set variously by FAO/WHO/United Nations University (UNU). It discusses the important elements needed to set protein requirements, such as IAA scores and IAA reference patterns and the associated methods. The report then narrows down on assessment of protein digestion and metabolic utilization and related assessment methods, including stable isotope tracer techniques. Lastly, the report summarizes the framework for a new protein quality database jointly managed by FAO and IAEA, which was agreed on by experts at the technical meeting.

2. Overview of protein in food and protein requirement issues

Proteins account for a significant part of animal and plant tissues and microorganisms and are the main nitrogenous constituents, with approximately 16 percent nitrogen (N) by weight.[3] The basic structural units of proteins are the AAs, which are characterized by a nitrogen-containing amine function, an acid carboxyl group and a specific lateral chain. Proteins are constituted by 20 AAs, which are linked together by peptide bonds.

2.1. Protein in foods: traditional, alternative and sustainable food protein sources

Proteins are a vital component of the diet. Dietary proteins provide nitrogen and AAs, and particularly the nine indispensable AAs (IAAs) that are not quantitatively synthesized in the human body and must be provided by the diet in adequate quantity and proportion. Nitrogen and AAs are required for the synthesis of protein and of other nitrogen-containing and AA-derived compounds that have various structural and biological functions in the body.[3]

For healthy adults, the daily estimated average requirement (EAR) for protein intake is 0.66 g/kg body weight, and the daily recommended dietary allowance (RDA) or population reference intake (PRI) is 0.83 g/kg body weight.[3, 4] Recommendations have also been provided for infants and children, and for women during pregnancy and lactation.[3, 5] The daily dietary protein intake in adult populations ranges approximately from 40 g/day to 100 g/day, which constitutes on average approximately 10 to 20 percent of daily energy intake.[3] However, protein consumption differs across the globe, particularly between higher-income countries (HICs) and LMICs.[6, 7] In most HICs, about 60 percent or more of dietary proteins are from animal source foods (meat, milk and dairy products, fish, eggs and other animal products), of which approximately 50 percent comes from meat and dairy.[8] In LMICs the amount of protein consumed is consistently lower than in HICs, especially for animal protein sources, which account for 15 to 30 percent of dietary proteins.[9-12] As global demand increases, a target for an environmentally sustainable protein supply aims towards a food systems shift to a ratio of about one-third animal-based and two-thirds plant-based proteins, in addition to having access to a diverse array of protein sources to increase the resilience of the protein supply.[8, 13]

Alternative and novel protein sources are currently being developed. A large majority of them are sourced from plants (such as by-products of legumes, cereals, seed crops, nuts and plant leaves)[9], while others are sourced from industrial co-streams such as brewery spent grains.[14] Yet other protein sources of increasing interest include microbial proteins (single-cell proteins or microbial biomass, and especially those that can grow on CO_2, H and O_2),[15] fungi (produced from their cellulose decomposition capabilities),[16] micro- and macro-algae (making use of light, seawater or minerals)[17] and insects (produced on substrates not directly applicable for feed or food application).[18] Lastly, cultured animal cells and recombinantly expressed proteins are also expected to become an integral component of the food industry within the coming years.[19] How sustainable and cost effective these novel sources can be, whether protein-rich products can be produced on a commercial scale using these substrates, and how much they ultimately contribute to reducing carbon footprint remain to be clarified.

In addition, to ensure the efficacy of novel proteins for widespread consumption, determining their safety through the appropriate regulatory framework is critical. The nutritional value of these protein sources and protein-rich products is subject to variability

and depends on their protein content, AA profile and digestibility. Although a dietary shift to increase consumption of plant proteins is a recognized strategy towards food system transformation,[9,20] it is well known that plant proteins can fall short in meeting human AA requirements and can have lower digestibility values than some of the commonly consumed animal-based protein sources.[21-23] An exception is soybean products, which appear to have a well-balanced protein source with good digestibility that is comparable to animal source protein.[24] It is therefore recommended for vegetarians and vegans to add more protein to their diet than those who consume animal source foods.[20] Considering current data, further research will be needed to determine whether a similar compensation factor will be applied to alternative protein sources (bacteria, fungi, micro-algae, etc.).

2.2. Protein requirements throughout the life course

Proteins are a major active component of all cells and have essential structural and functional roles for optimal human metabolism and physiology.[25, 26] Protein turnover (the process of endogenous synthesis and degradation) is tightly regulated in the human body in order to maintain proteostasis.[27] In healthy adults, about 250 g of protein is broken down into AAs and then re-synthesized daily.[28] Into this cycle, there is a daily addition of dietary AAs, and a daily loss of AAs through oxidation and subsequent nitrogen excretion. Roughly, this loss is about a quarter of the daily turnover and is replaced by the daily intake. Intake and loss should be in balance with each other to ensure that there is no net loss of body protein. Protein turnover is not distributed equally across all tissue and cells, and some are more active in this process as a response to internal and external stimuli.[25, 27] There is a rapid turnover in visceral tissues and a slow turnover in muscle. For example, the liver and intestine account for about 50 percent of the body protein turnover, but only about 8 percent in terms of the lean body mass. Skeletal muscle accounts for half the lean body mass but only 25 percent of total protein turnover. Protein turnover therefore depends on body composition and pathophysiological conditions (e.g. resting, fasting, exercise and ageing). There is also an energy cost to this activity, and with some assumptions, the cost is equivalent to about 1 kcal/g of protein turnover. Thus, in healthy adults, protein turnover has been estimated to account for about 20 percent of the basal metabolic rate.[29, 30] It is important to emphasize that there is no relation between protein turnover and daily intake requirement. In fact, protein turnover is approximately four times greater than the average daily dietary intake, but it is dependent on body composition.[31] Additionally, while there is a relationship between the amount of protein intake and the amount lost through oxidation, there will always be a minimal, or obligatory, protein loss from the body even if protein intake is zero. This relation between protein intake and loss is an important part of experimental design in studies that measure protein (or AA) oxidation, in which subjects should be adapted for a sufficient period to their experimental protein intake before oxidation is measured.

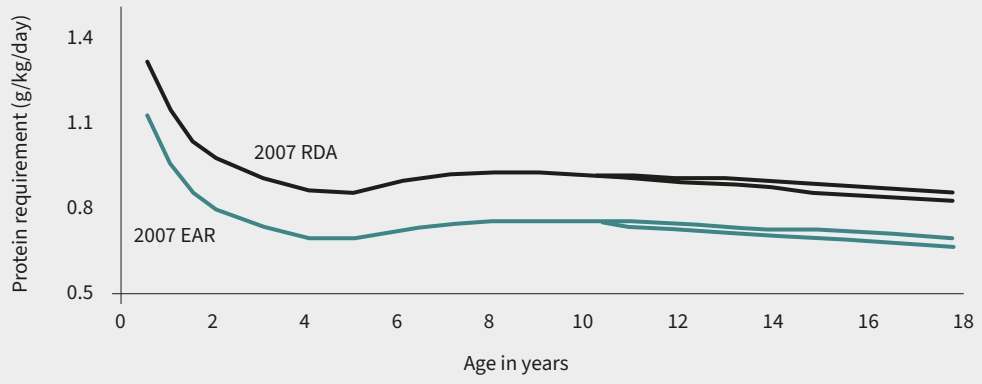
The requirement of protein is based on the relationship between protein intake, measured in terms of total N, and net N balance, measured as the difference between intake and losses (urinary, faecal and integumental), under otherwise normal conditions, including energy balance. It is critical to remember that energy intake influences protein breakdown (as stated above) and would spare the requirement for protein. While N is measured in this relationship, protein and N are used interchangeably, with the implicit assumption that 1 g of N is equivalent to 6.25 g of protein.[32, 33] The N balance to N intake relation will show a positive slope, increasing

from negative values when N (protein) intake is lower than the requirement to a zero-balance and subsequent plateau when the intake is at, or exceeds, the requirement. In effect, the oxidation will match the requirement, as stated above, and this is an important part of adaptation to habitual intakes, particularly for nutrients that cannot be easily stored in the body. Thus, the protein intake at which the N balance is zero will represent the protein requirement. With respect to the statement of the protein (or IAA) requirement, two important statistical constructs are used.[3] This is because the requirement must be proposed for populations, and the requirement will never be the same in all individuals in any population. The first construct is the EAR, which is the mean (or median) value for the distribution of requirements in a population and is used to estimate the adequacy of protein intake in a population. The second construct is the RDA, which is the intake at which an individual would have a very low risk (<2.5 percent) of having a deficient intake. While appropriate for individuals, the RDA should never be used to estimate deficiency in a population because it would grossly overestimate the risk of deficiency in that population.

In 2007, the FAO/WHO/UNU consultation report[3] determined values for protein requirements based on N balance experiments conducted around the world at different N intakes and the factorial method (Table 1). For adults, the zero intercept (EAR) for daily protein requirement was determined to be 0.66 g/kg (105 mg N/kg) and the RDA (or PRI) was 0.83 g/kg (132 mg N/kg). It is worth noting that these data were extracted from 19 primary N balance studies with 235 observations, and no plateau state (of zero N balance) was achieved as protein (N) intake increased.[34] Considering that in adults a higher protein intake does not lead to protein accretion, it is difficult to reconcile these data with optimal nutritional status. Subsequently, an attempt was made to re-evaluate these data, with the addition of nine further N balance studies but using a breakpoint (protein requirement) that would define a zero-balance (rather than a zero intercept) derived from a bi-phase linear regression analysis.[35] This breakpoint gave a higher value for the daily N requirement, at 0.91 g/kg (143 mg N/kg), but the N balance at the breakpoint was still positive (at ~10 mg N/kg), which is still difficult to reconcile with adult physiology. At present, the WHO/FAO/UNU zero-intercept daily EAR value of 0.66 g protein/kg (or 105 mg N/kg) is still used to determine the protein requirement globally.

It is difficult to conduct similar N balance experiments in children to determine their protein requirement; in addition, growth and physical activity are also important factors to take into consideration. The protein requirements of infants and children are therefore computed indirectly, by the factorial method.[36, 37] In this calculation, the protein needs added for growth are based on actual protein accretion values (measured by

Figure 1. Protein requirements of children and young people (aged 18 years and below)



Note: The black line is the "safe" requirement, or RDA. The green line is the population requirement, or EAR. The lines diverge at 10 years of age into two, for boys (upper line) and girls (lower line).

Source: Adapted from: **WHO/FAO/UNU**. 2007. *Protein and Amino Acid Requirements in Human Nutrition. Report of a joint WHO/FAO/UNU Expert Consultation.* World Health Organisation Technical Report Series 935. Geneva, WHO.

Table 1. Protein requirement and protein to energy ratio for different age groups

Age group	Protein requirement [i] g/kg/d	Energy requirement kcal/kg/d	P/E ratio (requirement)	P/E ratio after adjusting for PDCAAS [vi]
Pre-school children [ii]				
1–5 years	0.94	81	4.6	5.9
Schoolchildren [ii]				
6–10 years	0.91	71	5.1 [iii]	6.6
Adolescents [ii]				
11–18 years (boys)	0.88	60	5.8 [iv]	7.4
11–18 years (girls)	0.86	55	6.3 [v]	8.1
Adults				
Men (sedentary)	0.83	39	8.5	10.3
Women (sedentary)	0.83	36	9.2	11.2
Men (moderate activity)	0.83	46	7.2	8.7
Women (moderate activity)	0.83	42	7.9	9.6

Note: i: Safe requirement of high-quality protein.
ii: Assuming moderately active children.
iii: If sedentary (PAL of 1.4), then P/E ratio increases to 5.9.
iv: If sedentary (PAL of 1.4), then P/E ratio increases to 6.7.
v: If sedentary (PAL of 1.4), then P/E ratio increases to 7.1.
vi: P/E ratio of the requirement adjusted for the PDCAAS value of the dietary protein in a standard Indian low-cost vegetarian diet. In this case, using an Indian balanced diet protein based on a cereal/pulse/milk mix, with a PDCAAS of 77 percent for children up to 10 years of age, 78 percent for children up to 18 years of age and 82.5 percent for adults.
P/E ratio = protein energy ratio; these values refer to the requirement
PAL = physical activity level

Source: Adapted from: **WHO/FAO/UNU**. 2007. *Protein and Amino Acid Requirements in Human Nutrition. Report of a joint WHO/FAO/UNU Expert Consultation.* World Health Organisation Technical Report Series 935. Geneva, WHO.

whole body potassium counting in children of different age groups), corrected for efficiency of utilization and for maintenance (as in adults). These protein requirement values, measured directly by the zero intercept of the N balance to N intake relation for adults and calculated by the additive factorial approach for children, are shown in Figure 1 and Table 1.

Protein requirements during pregnancy have also been assessed by the factorial method as the additional daily requirement for foetal growth and expansion of maternal tissue. When new data on protein deposition during pregnancy were considered, along with a relatively inefficient rate of protein deposition, it was estimated that a woman gaining 12 kg of gestational weight gain would require an extra protein intake (at safe levels) of 0.6 g/d, 8.1 g/d and 27.0 g/d during the first, second and third trimesters, respectively.[3, 38] Protein requirements were also determined using the indicator amino acid oxidation (IAAO) method, resulting in values of ~79 g/d (~16 weeks of gestation) and 108 g/d (~36 weeks of gestation).[39, 40] According to the authors, these higher values account for the changing needs in protein requirement throughout the stages of pregnancy and are ~14 to 18 percent of total energy from protein, which are within the Acceptable Macronutrient Distribution Range.[39]

Although it is recognized that a decline in skeletal muscle mass and muscle strength is a physiological characteristic of ageing, protein requirements for older people remain unchanged. Several studies have suggested that a higher protein intake would benefit older individuals to compensate for a reduced protein absorption capacity and to stimulate muscle protein synthesis.[41] However, N balance studies do not provide sufficient evidence to justify a change in requirements.[34] Likewise, protein requirements derived using the IAAO method are in line with EAR values following biphasic linear regression analysis on existing N balance data.[35, 41]

2.3. IAA requirements throughout the life course

Dietary proteins should supply the nine IAAs in proper proportions and in adequate quantity. The other 11 AAs present in the dietary proteins, though required for protein synthesis, are not considered nutritionally indispensable because the body can synthesize them from other carbon and nitrogen sources. Table 2 gives the requirements for IAAs. There is no evidence to suggest that these IAA requirements are different in older people, or during pregnancy and lactation.

Table 2. WHO/FAO/UNU (2007) indispensable amino acid requirements (mg/kg/d) in adults and children. 1985 FAO/WHO/UNU values for adults are given for comparison

Amino acid	Adults All ages including older people and pregnancy		Children (years)				
			0.5	1–2	3–10	11–14	15–18
	1985	2007	2007				
Histidine	8-12	10	22	15	12	12	11
Isoleucine	10	20	36	27	23	22	21
Leucine	14	39	73	54	44	44	42
Lysine	12	30	64	45	35	35	33
SAAs	13	15	31	22	18	17	16
Threonine	7	15	34	23	18	18	17
AAAs	14	25	59	40	30	30	28
Tryptophan	3.5	4	9.5	6.4	4.8	4.8	4.5
Valine	10	26	49	36	29	29	28
Total (rounded off)	94	184	378	268	214	212	201

Note: To calculate protein score for each amino acid, divide its requirement by the daily EAR of the protein requirement (0.66 g/kg). SAAs, sulphur amino acids; AAAs, aromatic amino acids.

Source: Adapted from: **WHO/FAO/UNU**. 2007. *Protein and Amino Acid Requirements in Human Nutrition. Report of a joint WHO/FAO/UNU Expert Consultation.* World Health Organisation Technical Report Series 935. Geneva, WHO.

A theoretical paradigm for assessing IAA requirements was based on estimates of the intake of AAs necessary to balance the minimum obligatory losses of AAs (when protein intake was zero), as predicted from the composition of mixed body proteins. There is always a loss of protein from the body, even under zero-protein intake conditions. If one could make an estimate of the amount of protein that was lost, and then assume that IAAs contributing to this loss occur in proportion to their concentrations in body mixed proteins, then one has an estimate of the minimum intake required to balance these losses. However, an estimate of the efficiency with which AA are used is also needed; this was assumed to be about 70 percent.

The use of N balance to measure IAA requirements was considered error-prone because unmeasured integumental losses could be a major confounder. An alternative experimental approach to measuring IAA requirements used AA (or carbon) balance as the criterion of adequacy. This method is based on measuring AA oxidation over a whole day by tracer techniques to provide more accurate estimates of irreversible IAA losses that need to be balanced by intake. This is best done for those IAAs in which the kinetic measure of oxidation is well established and validated because this is, in turn, dependent on the measurement of the enrichment of the precursor pool from which AAs are subject to oxidization. This

is the intracellular AA pool, which is inaccessible, and for which validated proxy measurements from the extracellular fluid, or blood, are needed. These validated proxies (such as plasma alpha-keto-isocaproic-acid as a proxy for intracellular leucine) exist, so leucine oxidation can be thought to be accurately measured, and leucine balance therefore serves as an accurate alternative to the N balance method. Different time-based approaches (fasting vs fed state) have been used in this method, but the best was a 24-hour measurement method including both fasted and fed states that used the quantification of leucine or phenylalanine oxidation as an indicator of the balance of AAs in different diets, with limiting amounts of the test AAs under consideration. In these measurements, an important consideration was that the subjects were adequately adapted to the experimental level of AA intake (seven days, validated against 21 days, with similar results). Based on these stable isotopically measured balances, the 2007 WHO/FAO/UNU report presented revised IAA requirement figures from the 1985 values.[3] They showed a two- to three-fold increase and make protein quality an important issue.

For children, it is difficult to perform such intensive and demanding experimentation to assess IAA requirements. In children, a factorial method was used, as was detailed above for protein requirements, with the additional consideration that the maintenance protein requirement had the same IAA requirement pattern as in adults, but the growth protein accretion had an AA pattern of mixed body tissue protein.

2.4. Scoring methods and the IAA reference pattern

The quality of dietary protein sources has been directly assessed by measuring utilization and retention of dietary N and AAs in the body, but this approach is difficult because of the complexity of the physiological and metabolic processes of protein digestion and absorption, and metabolic utilization of AAs.[3, 42] Alternatively, the quality of dietary protein is defined by the ability to meet age-specific nitrogen and IAA requirements for growth, maintenance and specific physiological states.[3] The three limiting factors for protein quality from foods and diets, i.e. their capacity to meet nitrogen and IAA nutritional needs, include the total protein content, IAA content and profile of these proteins, and the metabolic availability of the dietary protein-derived AAs.[3] Accordingly, protein quality has been assessed by the widely accepted chemical scoring approach, which compares the IAA pattern of a protein with reference age-specific IAA requirement patterns and correction for protein or IAA digestibility. The IAA reference pattern is calculated for each AA by dividing its requirement by the daily protein requirement (i.e. 0.66 g/kg for adults) (Table 3). The amino acid score for lysine, based on the 1985 and 2007 IAA requirement pattern, is described in Table 4.

Table 3. Reference indispensable amino acid (IAA) profile calculated from the requirements for protein and for each of the nine IAAs

Mean protein requirement (g/kg/d)		IAA Reference profile (mg/g protein)									
		His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val	Total IAAs
Adult	0.66	15	30	59	45	22	38	23	6	39	277.0
1–2.9 years	0.86	18	31	63	52	26	46	27	7.4	42	312.4

Note: His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; SAA, sulphur amino acids; AAA, aromatic amino acids; Thr, threonine; Trp, tryptophan; Val, valine.

Source: Adapted from: **Shivakumar, N., Jackson, A.A., Courtney-Martin, G., Elango, R., Ghosh, S., Hodgkinson, S., Xipsiti, M., *et al.*** 2020. Protein quality assessment of follow-up formula for young children and ready-to-use therapeutic foods: recommendations by the FAO Expert Working Group in 2017. *The Journal of Nutrition,* 150(2): 195-201.

Table 4. Amino acid score for lysine based on 1985 and 2007 IAA requirement pattern

Protein source	Lysine content mg/g protein	FAO/WHO/UNU 1985 lysine score (16 mg/g protein)	FAO/WHO/UNU 2007 lysine score (45 mg/g protein)
Wheat	27	>100	60
Rice	35	>100	78
Sorghum	24	>100	53
Millet	22	>100	50
Nuts/seeds	35	>100	77
Vegetables	43	>100	96
Legumes	73	>100	>100
Animal protein	82	>100	>100

Source: Adapted from: **WHO/FAO/UNU**. 2007. P*rotein and Amino Acid Requirements in Human Nutrition. Report of a joint WHO/FAO/UNU Expert Consultation.* World Health Organisation Technical Report Series 935. Geneva, WHO.

To account for protein and AA digestibility, protein quality is assessed through the two simple indexes, the PDCAAS and the DIAAS.[1-3, 42, 44-49] The PDCAAS, which corrects the chemical IAA score by a single faecal nitrogen digestibility value, has been subject to criticism.[1-3, 44, 50, 51] Indeed, faecal digestibility is not always a good proxy of AA digestibility, especially for low digestible proteins, possibly because of the contribution of colonic microbes to nitrogen transactions through the fermentation of undigested protein entering the colon. Moreover, when the PDCAAS is positioned for single protein ingredients, if the value is above 1.0, it is truncated to a maximum value of 1.0. To overcome these concerns, the DIAAS corrects the chemical score of each individual IAA for its true specific oro-ileal digestibility.[1, 2, 51] Unlike the PDCAAS, for a single protein source the DIAAS is not truncated and can thus indicate the potential of a high-quality protein to complement low-quality protein in mixed diets.[1, 2, 51] For the PDCAAS, complementary proteins are best identified by using the untruncated AA scores. This is essentially the process advocated by FAO in the 1991 report, in which the final PDCAAS of mixed dishes is calculated using the independent values for true faecal protein digestibility and the AA scores for the IAAs rather than the PDCAAS values.[44] For both the PDCAAS and the DIAAS approaches, any values for final mixed diets above 1.0 are truncated to 1.0. To overcome these concerns, the DIAAS corrects the chemical score of each individual IAA for its true specific oro-ileal digestibility.[1, 2, 51] Unlike the PDCAAS, for a single protein source the DIAAS is not truncated and can thus indicate the potential of a high-quality protein to complement low-quality protein in mixed diets, while for the final mixed diets a DIAAS above 100 percent is truncated to 100 percent.[1, 2] A critical aspect of the chemical scoring approach is measurement of protein and IAA digestibility to correct the chemical score. The two methods differ in the fact that the PDCAAS corrects the chemical score by single protein nitrogen faecal digestibility, while the DIAAS corrects it by true ileal digestibility of each individual IAA. This question of AA metabolic availability has been discussed during recent expert consultations.[1-3, 5, 43, 51]

3. Protein digestion in the intestine and digestibility issues

The metabolic availability of the dietary protein-derived AAs is related to the digestion of protein in the intestine and the subsequent absorption of AAs so that they are made available to the organism.[52, 53]

3.1. Overview of protein digestion and metabolic utilization

Protein digestion is a complex process, whose purpose is the progressive cleavage of protein into smaller fragments constituted by AAs, dipeptides and tripeptides so that they can be absorbed. The cleavage of proteins in the gastrointestinal tract involves a coordinated series of sequential processes by which proteins are progressively hydrolysed by proteolytic enzymes, leading to the release of AAs and small peptides that are absorbed and transferred as free AAs into the bloodstream. This intricate and coordinated system of digestion ensures that, under normal conditions, 50 to 99 percent of ingested protein is cleaved in the intestinal lumen and the AAs are absorbed and made available to the organism to support metabolic needs.

After food ingestion, dietary proteins, accounting for 40 g to 100 g daily in adults, are subjected to digestion in the gastrointestinal tract. A part of protein entering the gastrointestinal tract daily is also derived from endogenous sources, including salivary, gastric, biliary, pancreatic and intestinal secretions accounting for approximately 20 g to 30 g and desquamated villus epithelial cells and mucous proteins accounting for an additional 30 g; a smaller amount (2–4 g) is derived from plasma proteins leaking into the lumen. Exogenous dietary proteins derived from the food consumed and endogenous proteins are mixed in the intestinal lumen, and the protein load requiring digestion within the gastrointestinal tract is approximately 100 g to 150 g daily. Protein digestion starts with chewing to mechanically increase the surface area. After swallowing, digestion of proteins in the stomach involves the action of pepsins with a broad proteolytic specificity, splitting peptide bonds mostly involving phenylalanyl, tyrosyl and leucyl residues.[54] The presence of gastric juices leads to protein denaturation, creating a more open protein structure which allows digestive enzymes to perform their specific function with more ease. Gastric emptying determines the rate at which the ingested protein is delivered in the duodenum, where segmentation contractions further facilitate luminal hydrolysis by pancreatic proteolytic enzymes such as trypsin, chymotrypsin, carboxypeptidase A and B, and intestinal brush border enzymes.[55, 56] The pancreatic endopeptidases trypsin, chymotrypsin and elastase primarily split peptide bonds located within the protein molecules, resulting in the production of short-chain polypeptides. These are further hydrolysed by the exopeptidase's carboxypeptidase A and B, acting, respectively, on aromatic/aliphatic C terminals and basic C terminal residues to remove single AAs. These pancreatic peptidases cannot hydrolyse peptide bonds with proline at the C-terminus.

The product of the coordinated intraluminal digestion of proteins by the endopeptidases and exopeptidases is a mixture of neutral and basic AAs (30 percent) and peptides with chains varying in length from two to six AAs (70 percent). They are then subjected to cleavage by peptidases at the level of the intestinal brush border, leading to the release of free AAs, dipeptides and tripeptides, taken up across the intestinal mucosa and absorbed through a variety of transporters.[57, 58] Di- and tripeptides can cross the brush border membrane by a peptide transport system with broad specificity (Pept-1) that is able to transport dibasic as well as diacid peptides and peptides consisting of up to three AA residues.[59, 60] Although some diffusion of free AAs does occur, they are mostly absorbed by active transport systems.[61-64] Unlike peptides, which are absorbed equally well in both the proximal and distal small intestine, free AAs are absorbed more rapidly in the duodenum and jejunum. In contrast

to the single peptide transport system, there are multiple transport mechanisms for free AAs at both the luminal and the basolateral membrane of the enterocyte.[61-64]

Absorption as di- and tripeptides is a major mechanism for absorption of protein-derived AAs from the human intestinal lumen and is considered a more efficient form of AA absorption than that of single free AAs. Di- and tripeptides are further hydrolysed by peptidases within the cytoplasm of enterocytes into free AAs, which are excreted through AA transporters into portal circulation. Thus, most products of protein digestion that reach the portal circulation are single free AAs. The absorbed AAs are released into the portal and then the systemic circulation, where they are taken up by peripheral tissues.[65] However, following absorption, a substantial part of the ingested AAs undergo first-pass splanchnic extraction, i.e. AA uptake and disposal in intestinal and hepatic tissues.[53, 66] For instance, ornithine and citrulline, which are not present in proteins, can be synthesized in enterocytes from several AAs present in proteins and play a role in the inter-organ metabolism. In addition, the enterocytes use several AAs (glutamine, glutamate and aspartate) as fuels in the context of a high energy requirement for cell renewal in the epithelial layer and for nutrient absorption.[67-69] AAs are also actively metabolized by the liver, but with an important difference between AAs: branched-chain AAs are less subjected to hepatic metabolism and are released in higher quantity in the peripheral circulation.

The fraction of ingested protein that is not digested and absorbed from the small intestinal lumen reaches the large intestine, where AAs are not quantitatively absorbed but metabolized by the microbiota.[70-84] In the first steps of protein catabolism by the intestinal bacteria, these compounds are hydrolysed by extracellular proteases and peptidases into free AAs and peptides. The catabolic fate of AAs is transamination or deamination by the gut microflora, mostly leading to ammonia and to the corresponding keto acids or saturated fatty acids. AAs and primary amines can be deaminated by the same processes, and urea recycled to the intestinal lumen is also hydrolysed into carbon dioxide and ammonia. The ammonia generated through deamination can be utilized as a nitrogen source, absorbed or excreted. Several free AAs released from proteins in the large intestine are precursors for short-chain fatty acid synthesis (mainly acetate, propionate and butyrate), organic acids (mainly formate, lactate and succinate), ethanol and gases (mainly H_2 and CO_2, with some H_2S). The branched-chain fatty acids (isobutyrate, 2-methylbutyrate and isovalerate) are derived from the branched-chain AAs valine, isoleucine and leucine. AAs can also be metabolized via decarboxylation, leading to the production of amines and polyamines. Tyrosine gives rise to 4 ethylophenol, phenol and p cresol, whereas tryptophan results in the production of indole, skatole and kynurenine. Sulphur-containing AAs yield sulphide that can be utilized by colonocytes or directly incorporated in de *novo*-synthesized AAs.

In the large intestine, bacteria can synthesize de novo some, if not all, of the 20 AAs required for protein biosynthesis.[92] However, both the recycling towards the host of these locally synthesized AAs (suggesting that the exchange of AAs between the microbiota and the host could take place in both directions) and the quantitative contribution of this recycling to the systemic pool of AAs of the host remain unclear.[85-92]

3.2. Apparent versus true digestibility

As a proxy for measuring the dietary intake that is made available as AAs to the organism after digestion and absorption, the disappearance of dietary AAs is usually determined in different parts of the gastrointestinal tract.[93] This is performed by measuring the proportion of ingested nitrogen or AAs that are not absorbed in the intestine and recovered in faeces (faecal digestibility), at the terminal ileum (ileal digestibility) or in the caecum (in rats) (caecal digestibility, as a proxy of ileal digestibility).[52, 94-96]

A limitation for oro-faecal and ileal balance approaches is that digesta in the faeces or terminal ileum contain AAs from both exogenous origin (i.e. undigested and/or unabsorbed dietary protein) and endogenous origin (e.g. gut AA losses from digestive enzymes and other proteins secreted into the intestinal lumen, and desquamated epithelial cells).[97-99] In pigs, measurement of endogenous AAs secreted into the small intestine showed that approximately 75 percent of the endogenous AAs are reabsorbed by the end of the small intestine, at the distal ileum, and 25 percent of the endogenous AAs are not reabsorbed and enter the large intestine.[100] Therefore, failing to correct for endogenous losses leads to the underestimation of the actual dietary AA digestibility.[101-103]

In the traditional assessment of digestibility, when oro-faecal or oro-ileal disappearance is not corrected for endogenous losses, the terms *apparent faecal* or *ileal digestibility* are used.[52, 53, 99, 104] *Standardized* and *true digestibility* discriminates between exogenous N and AAs provided from foods and endogenous N and

AA losses. Gut endogenous N and AA losses can be distinguished into basal and specific losses.[95, 104] Basal losses represent the minimal losses which are not impacted by foods, and specific losses are losses above basal losses that result from dietary composition. For example, foods rich in dietary fibre and antinutritional factors enhance digestive enzyme secretion and epithelial cell turnover and consequently increase specific losses of endogenous N and AAs.[105, 106] When oro-faecal or oro-ileal disappearance is corrected for basal faecal or ileal endogenous losses, measured by feeding a protein-free diet, for example, the terms *standardized* or true faecal or ileal digestibility are used. When faecal or ileal digestibility is corrected for total faecal or ileal endogenous losses (i.e. both basal and specific losses) by differentiating between endogenous and exogenous losses using intrinsically labelled markers when the test food is fed, the term *real digestibility* is used.[42, 95, 107-111]

Several methods have been proposed to measure gut endogenous N and AA losses. If a protein-free meal is administered, the N and AA recovered at the ileal or faecal level are only from endogenous origin, and this method can be used in humans and in animal models, particularly in pigs.[99, 112-116] The results are reproducible but with an important dispersion of the values, and the method usually tends to underestimate endogenous losses, leading to an overestimation of digestibility values.[117-119] Another method is to provide a meal with a hydrolysed protein such as casein; after collection, intestinal samples are filtered to remove small molecular weight peptides and free AAs from dietary hydrolysed casein origin while retained higher molecular weight polypeptides (greater than 10 kDa), mainly from endogenous origin, are quantified.[120] When this method is used, the intestinal endogenous flux of AAs was somewhat different in rats adapted to different diets with different protein sources.[119] This method provides higher intestinal luminal endogenous AA fluxes than the protein-free method, but some uncertainties originate from the endogenous and dietary origin of the peptides removed or retained by filtration.[120] Guanidination of lysine to homoarginine in the meal by addition of O-methylisourea has also been used for assessing endogenous AA fluxes.[121, 122] As homoarginine is naturally absent in foods, its apparent digestibility corresponds to the true digestibility of lysine, and after calculation of endogenous losses of lysine, the endogenous losses and the true digestibility of other AAs are derived from the ratio of homoarginine to other AAs in the guanidinated meal and in the ileal contents.[121, 123] Unless the protein under study is labelled, it should be studied without other protein sources in the diet.

Lastly, intestinal endogenous AA fluxes have been directly measured by the isotope labelling of either endogenous AAs or dietary proteins. The labelling of endogenous AAs is performed by infusing ^{15}N- or ^{13}C-leucine in humans and pigs.[97, 124-127] Close values of true ileal digestibility of rapeseed protein of 73.5 and 73.7 percent were obtained in pigs with this method and with a protein-free meal, respectively.[127, 128] Alternatively, labelling of exogenous dietary protein N and AAs with stable isotopes such as ^{15}N, ^{13}C and ^{2}H is used to discriminate between dietary and endogenous N and AAs and to assess the ileal digestibility and metabolic utilization of dietary protein-derived N and AAs.[42, 110, 129-138]

3.3. Faecal versus ileal digestibility

Digestibility measurements can be made at the faecal level[139] with the inaccuracy that this entails, or at the ileal level with greater accuracy. True ileal AA digestibility in the pig has been shown to accurately predict body protein accretion, thus validating this assay.[93] However, gaining access to a human's intestinal ileal contents can be invasive and cumbersome.[140, 141] As described above, dietary AA absorption occurs in the small intestine, and there is currently no evidence to suggest that intact AA can be absorbed in the large intestine in relevant amounts. Therefore, from a practical perspective, intestinal AA absorption is completed by the end of the small intestine, and the fraction that enters the large intestine is degraded by the microbiota with the release of ammonia and AA-derived metabolites.

The easiest way to measure digestibility is by faecal measurement, in which N and AA losses that have not been absorbed in the small intestine are measured in the faeces. This oro-faecal balance method is the simplest, oldest and most basic method of measuring protein and AA digestibility by assessing the disappearance of ingested AA between oral intake and faecal excretion, in which AA intake represents the AA content of ingested foods and AA faecal excretion represents the AA content of faecal material. Although non-invasive, a major limitation of the oro-faecal approach is the substantial hindgut microbial modification and metabolism in the large intestine of the undigested dietary protein exiting the terminal ileum, which can strongly differ from the AA composition of faecal material.[142] The large intestine contains large numbers of microbes, which will ferment any dietary protein present, and in the pig 80 percent of the AAs in the faeces have been shown to be of microbial origin. Net microbial AA degradation

occurs in the large intestine, and the disappearance of AAs in the large intestine does not necessarily reflect AA absorption by the host organism. Intestinal bacteria can transform AAs into nitrogen-containing metabolites such as polyamines, indoles or ammonia, part of which can be absorbed.[143] These changes lead to overestimation of faecal digestibility, which ranges, according to study models, from 2 to 15 percent.[52] This overestimation is considered tolerable for protein nitrogen digestibility but is more problematic for the digestibility of individual AAs. To avoid interference from microbial metabolism in the large intestine, the disappearance of ingested AAs is preferentially determined at the terminal ileum, i.e. at the end of the small intestine (oro-ileal disappearance). While there is bacterial activity in the small intestine, it is considered minimal compared with colonic activity.[72]

Thus, to determine AA digestibility, digesta must be collected from the terminal ileum, giving ileal digestibility.[144] Although complicated, the collection of ileal effluents is feasible in humans using naso-ileal probes[133, 145-147] and in ileostomy patients.[111] Whereas digesta can be collected from the terminal ileum in humans, the methods are not routine, and an animal model is often used. In the pig model, intestinal cannulas are used.[123, 148, 149] In rats, measurement of ileal digestibility is feasible but difficult, as it is not possible to insert intestinal cannulas, and the volume of digesta taken after euthanasia is relatively small. In addition, as a meal-eating omnivore, the pig is preferred over the rat, which is a naturally selective nocturnal feeder. The description of the methods for evaluating oro-ileal digestibility in the different models is presented in part 4.

The differences between ileal and faecal digestibility show that faecal digestibility is more often overestimated than ileal digestibility, ranging between 5.0 and 9.0 percent in rats, 8.3 and 15 percent in pigs, and 2.0 and 5.9 percent in humans.[52, 96] The ileo-faecal difference in nitrogen (2–9 percent) and AA digestibility (0.4–15 percent) have been reported in monogastric animals (including humans) for highly digestible proteins, and these differences were reported to be as high as 20 percent in rats for less digestible plant proteins. The observation of these large differences could be due to microbial fermentation of dietary fibre and undigested AAs during colonic transit.[96, 104, 118, 139, 150] Fermentable dietary fibre has been shown to increase colonic N retention by increasing microbial biomass. Indeed, dietary fermentable fibre has been used clinically to reduce circulating ammonium by locking it into the microbiome.[151] In humans, the difference was not significant for total nitrogen and for some AAs, but on average the overestimation was 2.4 percent for all AAs except lysine, alanine, isoleucine and methionine, and varied from 0.9 percent for leucine to 15 percent for glycine[139] (Table 5). In growing rats, true faecal digestibility compared with true ileal digestibility was not significantly different for legumes (pea, kidney bean and pea concentrate) but was overestimated by 15 percent for a cereal-based breakfast with an ileal digestibility of 67 percent, and by only 3 percent for a whey isolate with an ileal digestibility of 99 percent.[119] Therefore, ileal and faecal digestibility are not well correlated for the pig (r^2 = 0.67) or rat (r^2 = 0.27) model, and a correction factor is therefore difficult to implement.[96]

Table 5. Ileal and faecal digestibility in adult humans receiving a meat/cereal/dairy-based diet

	Ileal	Faecal	Difference
Glycine	0.72	0.87*	0.15
Serine	0.87	0.92*	0.05
Methionine	0.93	0.83*	0.10
Tryptophan	0.77	0.83*	0.06

Note: * Highly significant ($p < 0.001$).

Source: Adapted from: **Rowan, A.M., Moughan, P.J., Wilson, M.N., Maher, K. and Tasman-Jones, C.** 1994. Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71(1): 29-42.

3.4. Nitrogen versus individual AA digestibility

To determine the amount of digestible AAs, N digestibility or individual AA digestibility values can be used. A concern in the assessment of dietary protein and AA digestibility is the uncertainty associated with assuming overall protein (N) digestibility as a proxy for individual AA digestibility. A modest variation in ileal digestibility of IAAs has been reported in humans, ranging from 89 percent (threonine) to 95 percent (lysine) with an N digestibility of 94 percent in soy protein isolate.[108-110, 150] In contrast, considerable differences were observed with less digestible whole plant protein sources such as pea cultivars, in which ileal digestibility of IAAs varied from 75 percent (tryptophan) to 89 percent (methionine) with an N digestibility of 76 percent in pigs.[125] These results suggested that there was a need to measure the digestibility of each IAA to evaluate the overall quality of dietary protein. It was thus recognized that true ileal digestibility of each individual AA is theoretically better than faecal digestibility and single protein N digestibility.[1, 2, 5, 43, 51]

An additional aspect that is important for protein digestibility is that of available (reactive) lysine. Food processing can damage lysine, leading to the formation of nutritionally unavailable analogues. However, when AA content of food or digesta is analysed using strong acids, a number of these unavailable analogues revert to lysine with conventional AA analysis methods, leading to an overestimation of the available lysine content. Therefore, it is important to determine available or "reactive" lysine and true ileal digestibility of reactive lysine, especially in processed foods, considering that lysine is often one of the first-limiting AAs. This can be done using the method described by Moughan and Rutherfurd.[152]

For determining protein quality, the recommendation is to determine true individual AA digestibility at the ileal level.

3.5. Food factors impacting digestibility

Food processing technologies have evolved to meet the growing global demands, leading to more complex food formulations that could impact protein digestibility.[13, 153] Many products such as soybeans are processed in several ways. Protein and AA digestibility is usually higher when protein purity is increased. Comparison of standardized ileal digestibility of several oilseeds in pigs showed that the digestibility of rapeseed was improved by 5.6 percent when the food consisted of a complete meal (17 percent protein) instead of seeds (10.5 percent protein), while no difference was observed between sunflower seeds and meal.[128] Similarly, a difference of 3.2 percent was observed in pig for the digestibility of a rapeseed isolate compared with a rapeseed meal.[108, 154] These differences can be explained by the greater accessibility of proteins to digestive enzymes for a protein ingredient than for a matrix with complex molecular interactions. Plant cell walls and the availability of indigestible polysaccharides typically make proteins and other nutrients more resistant to digestion and therefore less digestible.[155-157] The published review on *in vivo* digestibility data of soybean, soybean meal, soy protein concentrates and other soy derived products show great variation,[153] and it is currently unknown whether this is caused by the soy variety, the production region, postharvest handling or the processing method. Additional key considerations related to sources of variability in both the AA composition and digestibility assessments are environmental (e.g. soil fertility, temperature and moisture), genetic (e.g. varietal selection) and processing factors (e.g. thermal treatment, milling and protein isolation), along with their interactions. The presence of these factors and interactions between them can lead to changes in the ratio of storage proteins, influence the resulting AA content and impact the availability of anti-nutritive factors that affect digestibility.

The availability of multiple processing methods has resulted in an increase in the number of processing steps (such as fermentation, shear cell technology and 3D printing) and food ingredients (also because of clean label strategies) in composed products and an overall diversification of the food matrix. However, processing methods (cooking, heating and mechanical treatment) may impact protein digestibility and concentrations of available IAAs in several ways.[158-162] Generally, dry heating may increase protein aggregation and reduce digestibility, while wet heating may increase digestibility as a result of protein denaturation. High-temperature or long cooking durations can modify the chemical structure of AAs, resulting in decreased accessibility of proteins to digestive enzymes and impaired digestibility.[163, 164] A well-observed example of the effect of high temperatures on protein degradation is the Maillard reaction, whereby the conjugation of AAs (usually lysine) with reducing sugars leads to their glycation and a reduction in metabolic availability.[165-168] In humans, post-prandial lysine absorption was reduced following intake of milk powder in which 20 or 50 percent of lysine

was glycated, in contrast with a 3 percent lysine glycation.[165] Likewise, the digestibility of milk proteins in rats was affected by a spray-type treatment at high temperatures (250 °C inlet temperature), while short treatments at high temperatures did not decrease digestibility (UHT, 140 °C, 5 s).[169] Heat treatment of skimmed milk powder has also been shown to decrease lysine digestibility by 14 percent in young rats and 22 percent in older rats.[170]

The processing of most animal source proteins increases the overall protein digestibility.[158, 171] However, the duration, method and temperature of the cooking process may decrease digestibility, highlighting the need to assess individual AA digestibility as well as overall protein digestibility. In particular, in the pig model it was observed that steamed ground beef (72 °C) and roasted topside steak (160 °C) resulted in a reduction of the limiting IAAs (leucine and valine, respectively).[171, 172] Furthermore, long-duration, high-temperature cooking of beef decreases protein digestibility in rats, with a digestibility for raw meat of 97.5 percent, reduced by 3 percent through cooking in boiling water for 3 h and by 0.6 percent by cooking in the oven at 180 °C. for 40 min.[173] The same results have been observed in humans, with a decrease in ileal N digestibility of 4 percent between raw cooked and fully cooked meat.[133, 168]

Some antinutritional factors such as trypsin inhibitors, phytates and polyphenols that are naturally present in plant foods can also influence digestibility by interacting with proteins.[108, 174, 175] Trypsin inhibitors are found in several plant foods. Large quantities of trypsin inhibitors are found in soybeans (between 20.3 mg/g and 122.6 mg/g of protein). With the presence of these inhibitors, trypsin activity is reduced across soybeans and other plant foods, thereby affecting the digestion of proteins and AAs.[174, 176, 177] For example, a 623 mg dose of trypsin inhibitor in a meal decreased protein digestibility by 11.5 percent in rats.[178] These inhibitors are commonly deactivated by heat treatment.[174, 176] A known anti-nutrient, phytic acid, is found in several plant foods (grains, legumes, nuts and oilseeds); for instance, sunflower meal contains 27 g/kg of phytic acid.[179, 180] Phytic acid binds monovalent and divalent cations (e.g. potassium, sodium and magnesium) through chelation, leading to phytate, which can alter the bioavailability of proteins or decrease the activity of digestive enzymes, either through cofactor chelation or by direct or indirect interaction with dietary proteins.[179] These phytates are relatively temperature-resistant but can be neutralized by phytases or by prolonged soaking.[181] In pigs, the addition of phytase to a meal in a complex diet increased the apparent ileal digestibility of nitrogen by between 1.6 and 12 percent according to a dose-response effect.[182] Polyphenols are a large group of compounds with antioxidant effects, including flavonoids and tannins, and are also found in many plant products.[183-185] They are known to bind to digestive enzymes and protein, decreasing overall digestibility. Addition of *Vicia faba* extract leading to 2 percent tannin in the feed decreased the apparent ileal nitrogen and AA digestibility of casein from 91 percent to 60 percent in rats.[186, 187] In short, the reduced digestibility of plant proteins has been ascribed to reduced bio-accessibility because of plant cell walls, the presence of antinutritional molecules such as anti-proteases and the presence of substances such as tannins (polyphenols) that may bind food proteins or digestive enzymes. The latter two mechanisms can affect other components in mixed meals. As conventional methodology has necessarily estimated protein digestibility of single foodstuffs, such interactions have not been considered. Humans tend to consume mixed meals that may contain proteins from several sources. Newer digestibility technology applying stable isotope tracers can quantify the potential interactions of plant and animal proteins.

4. Protein and IAA ileal digestibility assessed by different models and methods

Protein and AA digestibility are currently assessed by various methods in humans, pigs and rats and using *in vitro* models. The oro-ileal balance method traditionally assesses nitrogen and AA disappearance in the intestine, and more recent methods measure the systemic availability of dietary AAs.[1, 2, 5, 43, 51]

4.1. Intrinsic stable isotope labelling of dietary protein

Several methods for measuring dietary protein nitrogen and AA digestibility require stable isotope labelling of dietary protein to distinguish it from other protein sources, to discriminate between dietary and endogenous nitrogen and AAs, and to assess their transfer and metabolic fate in various body pools and excretion pathways. Indeed, stable isotope-labelled dietary protein can be used to track the digestive and metabolic fate of the labelled compound, such as nitrogen and AAs. It is thus possible to assess true oro-ileal digestibility of dietary protein and AAs using ^{15}N, ^{2}H or ^{13}C labelled dietary proteins, and it is also possible to assess metabolic bioavailability expressed as net postprandial protein utilization using ^{15}N- or ^{13}C labelled dietary proteins through the recovery of the tracer as ^{15}N urea or $^{13}CO_2$, respectively.[42, 144] Several methods for ^{15}N-, ^{2}H- and ^{13}C labelling of protein have been proposed.[144]

Intrinsic and uniform labelling with ^{15}N can be carried out in plants using ^{15}N enriched nitrogen fertilizers such as ammonium nitrate ($^{15}NH_3{}^{15}NO_3$) and potassium nitrate ($K^{15}NO_3$) during plant growth.[145, 147, 188] It is also possible to intrinsically and uniformly label with ^{15}N animal proteins, especially proteins from ruminants, because the bacteria contained in the rumen can easily convert inorganic nitrogen into AAs, thus allowing the incorporation of ^{15}N enriched AAs into the proteins of the animal.[189] This method has been used to label cow meat as well as cow and goat milk proteins by supplying ammonium sulphate ($(^{15}NH_4)_2SO_4$) orally or directly infused in the rumen of the animal.[133, 136, 146, 190] Animal proteins not derived from ruminants have also been labelled with ^{15}N. For instance, egg white protein has been labelled by giving laying hens access to ^{15}N leucine in their meal. Certain reactions such as transamination, an equilibration process, will incorporate label into IAAs at specific positions (the N and H atoms at C2). However, the reaction varies greatly between IAAs. Transaminases are absent in lysine and threonine and most active for the branched-chain AAs such as leucine. The transamination correction factor determined in the dual tracer method shows how individual IAAs have differing transamination activities.[111]

Intrinsic and uniform labelling with ^{2}H can be carried out in plants using deuterated water ($^{2}H_2O$) during plant growth.[129, 191] In animals, egg and meat from hens were intrinsically and uniformly labelled with ^{2}H by providing the 20 ^{2}H labelled AAs to the feed; goat milk was intrinsically and uniformly labelled with ^{2}H by providing a mixture of ^{2}H labelled maize and ^{2}H labelled cowpea to the feed.[131, 132] Intrinsically and uniformly doubly labelled $^{15}N/^{2}H$ protein was produced by giving $(^{15}NH_4)_2SO_4$ and $^{2}H_2O$ orally to a goat to obtain $^{15}N/^{2}H$ labelled goat milk protein, or providing $^{15}NH_3{}^{15}NO_3$ and $^{2}H_2O$ during plant growth to obtain $^{15}N/^{2}H$ labelled sunflower protein.[136]

Protein labelled with ^{13}C can also be used. For example, ^{13}C labelled cow's milk proteins or hen's egg proteins can be obtained by infusing a single AA or a mixture of AAs labelled with ^{13}C phenylalanine or leucine.[111, 137, 138] Intrinsically and uniformly doubly labelled ^{15}N, ^{13}C hens egg protein is created by feeding mixtures of 20 $^{15}N/^{13}C$ labelled AAs.[130, 192] Plant proteins labelled with ^{13}C are obtained when cultivated in an atmosphere enriched in $^{13}CO_2$, which enriches the organisms uniformly, but this method is extremely expensive and technically very challenging as the plants must be held in a sealed atmosphere, in which leakage of this expensive tracer can occur. Heterotrophic organisms such as mycoprotein and insect larvae can be easily labelled with ^{13}C glucose added in their substrate.

Working at modest enrichment levels, use of either ^{15}N or ^{2}H is feasible when plants are grown in containers in a glasshouse or hydroponic system or an area protected from rainfall and insects, with simple precautions to reduce evaporative loss. As the desired enrichment is low because experiments are planned for analysis by isotope ratio mass spectrometry, dilution of added tracer by soil water and loss by evaporation are acceptable. Depending on the protein source, the cost of the isotopic tracers and the method of supply, ^{15}N or ^{2}H labelling may be easier and more economically viable.

4.2. Ileal digestibility – oro-ileal balance methods in humans

The direct determination of true ileal nitrogen or AA digestibility requires the collection of ileal digesta. In humans, digesta can be collected using naso-ileal intubation methods or through a surgically exteriorized ileum in ileostomized patients.[5, 95, 193]

For collection of ileal digesta, healthy humans are equipped with a naso-ileal tube.[144] For this purpose, a radio-opaque tube is introduced into the nose and migrates to the terminal ileum, and its location is confirmed through radiography and pH measurements. The tube is made of three lumens, one to infuse a non-absorbable marker, one to collect digesta and one to inflate a ballast balloon to help the peristaltic movement of the intestinal tube. The non-absorbable marker is PEG 4000, which is infused at a known concentration and at a slow rate (the "slow marker" method), allowing the total flow rate in the ileum to be determined. Once the ileal contents are collected, the volume is noted, a liquid aliquot is frozen for further determination of PEG 4000 by the turbidimetric method, and the remaining sample is freeze-dried. Dry matter is measured, and analytical work is performed to quantify N, ^{15}N, ^{2}Hor $^{13}CO_2$ and AA concentrations and ^{15}N, ^{2}H and $^{13}CO_2$ AAs, along with other analytes if required. The use of naso-ileal tubes is a promising approach for collecting ileal samples, but there are several limitations: namely, the invasiveness of the method, the interindividual variability in tube migration and the minimal tolerance among subjects.[194]

A less invasive approach for direct access to ileal content in humans is to recruit ileostomized patients.[109] If individuals do have a permanent ileostomy, then collection of digesta is typically deemed as non-invasive because collection is considered part of the routine ostomy system. In addition, it is much more convenient to have ileostomates participate in a crossover design. However, there are several limitations to collecting digesta from an external ostomy system in humans. One is that very few people have a permanent ileostomy. The individuals who do may also have other pre-existing conditions that prevent participation in a human trial. Thus, to date, only a few studies involve ileal digesta collection through subjects with a permanent ileostomy. Additionally, as ileostomies are performed following severe digestive pathologies (i.e. colon cancer and Crohn's disease), the validity of this model compared with healthy subjects has been questioned in relation to morphological and microbial changes in the terminal ileum of ileostomy patients, which tend to be closer to colonic characteristics.[94, 112] In some studies, nitrogen recovered in the ileal effluent of ileostomized patients was higher than in subjects with an intact colon.[149, 195]

Data on true ileal digestibility of proteins, and to a lesser extent AAs, were obtained for various animal and plant proteins in different meal conditions in healthy volunteers equipped with intestinal tubes using protein intrinsically labelled with ^{15}N and in ileostomates (Table 6).

The data presented in Table 6 indicate that excellent digestibility (95–100 percent) has been obtained for milk, total milk protein isolates and casein. Good digestibility (90–94 percent) has been observed for bovine meat (two cooking processes), eggs, whey, pea flour, pea isolate (two independent studies), soy isolate, lupin flour, flaxseed isolate and gluten (cooked in French biscuits). Moderate digestibility (80–90 percent) has been found for sunflower isolate (cooked in biscuits) and rapeseed (two independent studies, one with a raw isolate and one with a concentrate cooked in a biscuit). One protein isolate, zein, displayed a low digestibility of 60 percent. The mean AA digestibility was close to that of protein (Table 6). However, there were variations in AA digestibility across foods, with IAAs having the highest digestibility in some foods and the lowest digestibility in others. A study in ileostomates[198] assessed the ileal AA digestibility of zein and whey protein using the same meal conditions and comparable procedures (an unlabelled protein source and a protein-free diet to assess endogenous losses) as in the ileal tube study,[204] and the values across both study designs were very similar. However, ileal protein digestibility values of soy, whey and casein protein isolate were overestimated in ileostomized patients in comparison with healthy volunteers with intestinal tubes when indirect comparisons were made.[113, 136, 197, 202] In contrast to the ileal tube studies, most of the studies using participants with a permanent ileostomy were carried out without any isotope labelling, so differences in protocols may also account for the differences

Table 6. Ileal protein and mean AA digestibility (in percentage) in humans equipped with an ileal tube and with ileostomies

Protein source	Protein type	Food matrix	Ileal tube		Ileostomates	
			Protein digestibility	Mean AA digestibility	Protein digestibility	Mean AA digestibility
Milk	Total milk protein	Skimmed milk	95.5 ± 1 n = 5			
	Total milk protein	Beverage, with or without carbohydrate or fat	94.6 ± 1.2 n = 25	95.3 ± 1.8 n = 7		
	Casein	Puree	94 ± 1.7 n = 8		101.9 ± 0.70 n = 8*	
	Casein	Beverage	94.1 ± 1.4 n = 10			
	Hydrolysed casein	Beverage	92.2 ± 1.3 n = 11			
	Whey	Beverage	91.2 ± 6.9 n = 8*	92.3 ± 2.3 n = 8*	98.3 ± 0.8 n = 8*	92 n = 8*
Egg	White	Raw (liquid)			51.3 ± 21.9 n = 5	
	White	Cooked (solid)			90.9 ± 1.8 n = 5	
Bovine Meat	Raw	Steak	90.1 ± 5.9 n = 8			
	Fully cooked	Steak	94.1 ± 1.9 n = 8			
	Fried beef				94 n = 5*	
Pea	Flour	Beverage	89.4 ± 1.1 n = 7			
	Isolate	Beverage	89.9 ± 4 n = 9			
	Globulin	Beverage	94 ± 2.5 n = 7			
	Isolate	Puree	92 ± 2.7 n = 8			
Soy	Isolate	Beverage with or without carbohydrate	91.4 ± 4.4 n = 21	91 ± 7 n = 6	99.5 ± 0.8 n = 8*	
Lupin	Flour	Beverage	91 ± 3 n = 7			
Gluten	Isolate	French biscuits	90.3 ± 4.3 n = 9			
Rapeseed	Isolate	Beverage	84 ± 8.8 n = 7			
	Concentrate	Biscuits	80.7 ± 6.5 n = 10	83.9 ± 7.3 n = 10		
Sunflower	Isolate	Biscuits	86.8 ± 4.4 n = 12	86.5 ± 5.1 n = 12		
Flaxseed	Isolate	Biscuits	92.8 ± 2 n = 9	93.8 ± 3.1 n = 9		
Zein	Isolate	Beverage	60.2 ± 12.7 n = 8*	63 ± 4.6 n = 8*		60 n = 8*

Note: *Without any intrinsic labelling of protein with isotope.

Sources: See source notes: 107-111, 133, 135, 145-147, 193 and 196-203.

between studies. In conclusion, when related food sources and methods are compared, studies measuring protein and AA digestibility in ileostomates seem to report values that are similar to values reported in studies with healthy volunteers who have undergone an intestinal tube procedure. However, there are limited data on ileostomates for further comparison across models, largely because of the scarcity of people with permanent ileostomies. Although insertion of the intestinal tube at the ileum has quite a few drawbacks (it is invasive, cumbersome and expensive), it does have the advantage of being robust and having a good interstudy repeatability, and it can generate values that are very consistent with those obtained in other models.

4.3. Ileal digestibility – oro-ileal balance methods in pigs

The pig may be used as a model to determine the ileal digestibility of AAs in human foods. The pig has several advantages because its physiology and anatomy are similar to those of humans, its diet can be similar to that of humans, and, unlike rats, it does not perform coprophagia.[139, 205-207] Additionally, IAA requirements in pigs are only 1.2 times higher, on average, than human IAA requirements.[208] However, pigs require space for housing, and experiments using the pig model are generally quite expensive.[208] Direct measurements of ileal protein and AA digestibility based on their ileal disappearance can be precisely performed in pigs equipped with an ileal T cannula.[205, 209-212] Ileal cannulas can be easily installed in the distal ileum of pigs, allowing the collection of digesta from the end of the small intestine. Pigs tolerate the procedure well and values for AA digestibility obtained in pigs are repeatable over time.

Despite some differences between models, the results obtained in human studies are usually confirmed in pig studies. Particularly, it has been shown in pigs, as in humans, that digestibility of proteins of animal origin is usually higher than that of plant proteins, and that the food matrix and treatment influence protein and AA ileal digestibility.[172, 175, 205, 213-220]

Depending on the AA considered, ileal digestibility measured in ileostomized pigs and humans was not significantly different or slightly higher, with a mean deviation of 1.9 percent and differences ranging from 0.1 percent for serine to 8.1 percent for cysteine.[139] Recently, ileal AA digestibility measured in cannulated pigs and ileostomized patients was compared across a range of foods.[198] Pig and human ileal AA digestibility values were significantly correlated with a linear regression equation derived for the true ileal AA digestibility for the overall mean of all IAAs of $y = 1.001x - 0.008$, very close to $y = x$ (y = human, x = pig). This suggests that true ileal AA digestibility values determined in the growing pig may be directly used for predicting digestibility in adult humans. In another study, true ileal digestibility values were measured in pigs with a T cannula and in humans with a naso-ileal tube; somewhat higher digestibility values were observed in the pig model than in humans for nitrogen and for AA, with a difference of about 3 percent for nitrogen and a difference ranging between 0.2 and 6 percent for AAs.[108]

Ileal AA digestibility and DIAAS values have been determined in many human foods using the pig as a model, and it has been demonstrated that the pig model can be used to detect low digestibility of lysine caused by heat damage.[221] It has also been demonstrated that values for ileal digestibility of AAs obtained in individual ingredients can be used to calculate the DIAAS values in a mixed meal that is typically eaten by humans.[222] Ileal AA digestibility determined in the pig model and DIAAS values of more than 200 foods have already been published. Developing digestibility data using the DIAAS, however, requires accurate analysis of the amino acid score, which assumes that the amino acid analysis of food proteins is trivial, which it is not. Potential interactions between components of a human mixed meal require further study. A plant protein with some residual anti-protease activity could potentially reduce the digestibility of an animal protein source.

4.4. Ileal digestibility – oro-ileal balance methods in rats

Direct measurements of ileal protein and AA digestibility based on ileal disappearance can also be performed in rats, but, in contrast to humans and pigs, there is no standardized method for continuously collecting intestinal digesta because rats cannot be equipped with an ileal or caecal cannula.[205, 209-212] The mouse model has also been proposed, although its use for assessing protein and AA digestibility has been very limited.[223, 224] Rats can serve as a model of ileal AA digestibility1 because their upper digestive tract is comparable to that of humans. Anatomically, rats are distinguishable from humans primarily by the presence of a caecum developed between the small intestine and the colon, leading to the term oro-caecal balance.[225] The main advantages of rats are that they are readily available, easily housed and economically viable in terms of price, space and care. Thus, the rat model can be used to test multiple protein sources, different processing or

cooking methods and various physiological conditions in a limited amount of time. However, rats and humans have different digestive behaviours, so the test protein given to rats should be adapted accordingly. Rats are small animals, which limits the quantity of sample collection. Since it is not possible to perform intestinal cannulation in a rat model, euthanasia of rats is required to collect digestive content.

Currently, two protocols for evaluating AA digestibility in rats are available. The first method follows the ingestion of repeated meals containing a non-absorbable marker and the collection of a single ileal sample after euthanasia of the animal. Rats are submitted to a specific feeding protocol, including nine hourly meals, in which the test protein and non-absorbable marker are given.[103, 226] The rats are euthanized 5 to 7 hours after the first meal intake; their ileal digesta is collected (the last 20 cm of the small intestine) and their contents (e.g. AAs and non-absorbable marker) are determined. Endogenous AA losses are usually estimated with a group fed a protein-free meal. The main advantage of this protocol is that true ileal amino acid digestibility is directly evaluated. However, the feeding protocol is burdensome, and the use of a non-absorbable marker can lead to uncertainties.[227, 228] Additionally, only a small quantity of digesta (~150 mg of dry matter) is collected in the ileum, which does not allow for the use of labelled protein and isotopic analyses unless samples are pooled. The use of a protein-free meal may potentially underestimate endogenous AA losses, which results in an underestimation of AA digestibility values.[113, 229]

Another method for evaluating AA digestibility in rats is one that quantitatively assesses dietary AA content in the caecum of rats after a limited post-digestion period (usually 6 hours).[136, 169, 197] This novel method involves several assumptions that still require extensive validation. One assumption is that 6 hours after meal intake, the digestion of protein is nearly complete; thus, the dietary AA losses are predominately in the caecum, and fermentation time is limited. In this case, caecal digestibility is used as a surrogate of ileal digestibility. For this protocol, isotopic analyses are possible (e.g. ^{15}N labelled proteins can be used) because of the large quantity of digestive content available in the caecum for collection (500–600 mg of dry matter). Six hours after meal intake, the entire digestive tract is removed to collect the content of every segment (stomach, proximal small intestine, ileum, caecum and colon). Dietary N recovery is quantitatively determined in the digestive tract to verify the assumption that it is mainly in the caecum. Then, caecal dietary AA losses are analysed to estimate true caecal AA digestibility.

In addition to the determination of the digestive fate of dietary protein, the use of labelled protein makes it possible to study the postprandial utilization of dietary N (deamination losses and incorporation of dietary N in splanchnic and periphery organs). There is no use of non-absorbable marker in this method because dietary AA losses are quantitatively evaluated. However, it is highly possible that the caecal microbiota influences the dietary AA content, which may lead to some uncertainties in the digestibility values. Caecal microbial fermentation within the 6-hour period can be highly significant and affect the results, especially when proteins with a lower digestibility are evaluated. Under- or overestimation of digestibility may also occur if high quantities of dietary AAs are still in the small intestine or are already in the colon, undergoing fermentation, 6 hours after meal intake. These errors are limited for highly digestible protein sources. In addition, it was also proposed to use the dual isotope method approach to assess caecal AA absorption in rats using a test protein that is intrinsically labelled with one isotope (^{15}N, ^{2}H) and a reference protein labelled with another isotope (^{13}C).[230]

Comparison of the mean true AA digestibility values obtained with these two protocols shows that the differences are small for highly digestible proteins. If labelled protein sources are tested, the caecal digestibility protocol is preferred. For instance, mean ileal AA digestibility of whey protein concentrate was 98.0 ± 0.7 percent,[103] while mean caecal AA digestibility was 97.5 ± 0.2 percent.[136] Similarly, mean ileal AA digestibility of casein was 96.2 ± 1.2 percent,[226] while mean caecal amino acid digestibility was 95.8 ± 0.4 percent.[231] Indirect comparisons of mean true ileal or caecal AA digestibility obtained in rats and true ileal digestibility obtained in humans with the oro-ileal balance method are available in the literature (Table 7). A very good correlation exists between rats and humans (R^2 = 0.9, Figure 2), and differences are especially low (<1 percent) for highly digestible protein sources. However, larger differences (up to 10 percent) between rats and humans are observed for moderate to low digestibility proteins.

Several controlled studies have compared true ileal digestibility in growing rats and pigs.[93] Generally, a high level of agreement is found, but for more poorly digested proteins (especially those with high amounts of antinutritional factors) poorer agreement has been observed. The comparison between rats and pigs for several sources of protein shows that the overall ileal digestibility of AA was similar between the two models (96.0 percent for the pig and 96.1 percent for the rat), but with large disparities. For example, 9 of

Table 7. Mean true ileal amino acid digestibility determined in human volunteers and rats

Protein source	Human	Rat
Soy protein isolate	97.0 ± 0.8 [i]	97.9 ± 0.7 [iv]
Whey protein concentrate	98.0 ± 0.7 [i]	96.9 ± 0.8 [iv]
Casein	95.8 ± 0.4 [iii]	96.8 ± 0.4 [vi]
Total milk protein	96.0 ± 1.2 [iii]	95.3 ± 0.7 [iv]
Pea protein isolate	94.6 ± 1.4 [iii]	93.6 ± 1.0 [v]
Whole wheat/wheat bread	93.3 ± 2.0 [i]	92.0 ± 3.0 [iv]
Sunflower protein isolate	96.3 ± 0.2 [iii]	86.5 ± 1.5 [vi]
Kidney bean/black beans	77.0 ± 0.9 [i]	80.0 ± 4.0 [iv]
Wheat bran	74.0 ± 0.6 [i]	70.0 ± 8.0 [iv]
Zein	53.1 ± 3.4 [ii]	63.0 ± 4.6 [iv]

Notes: [i] Evaluated in ileostomized volunteers with protein-free meal to determine endogenous losses.
[ii] Evaluated in healthy volunteers using a naso-ileal tube and with a protein-free meal to determine endogenous losses.
[iii] Evaluated in healthy volunteers using a naso-ileal tube and with ^{15}N labelled dietary protein.
[iv] Ileal amino acid digestibility determined in rats with a protein-free meal to determine endogenous losses.
[v] Caecal amino acid digestibility determined in rats with a protein-free meal to determine endogenous losses.
[vi] Caecal amino acid digestibility determined in rats with ^{15}N labelled dietary protein.

Sources: See source notes: 107, 110, 113, 135, 136, 197, 198, 201, 226, 231, 233 and 234.

the 16 AAs showed higher values in the pig model, with differences that ranged from 0.1 percent for threonine to 3.5 percent for histidine. Other AA values were higher in the rat model, ranging from 0.2 percent for alanine to 10.6 percent for cysteine. Additionally, there was a significant effect of the species on 7 of the 16 AAs.[232]

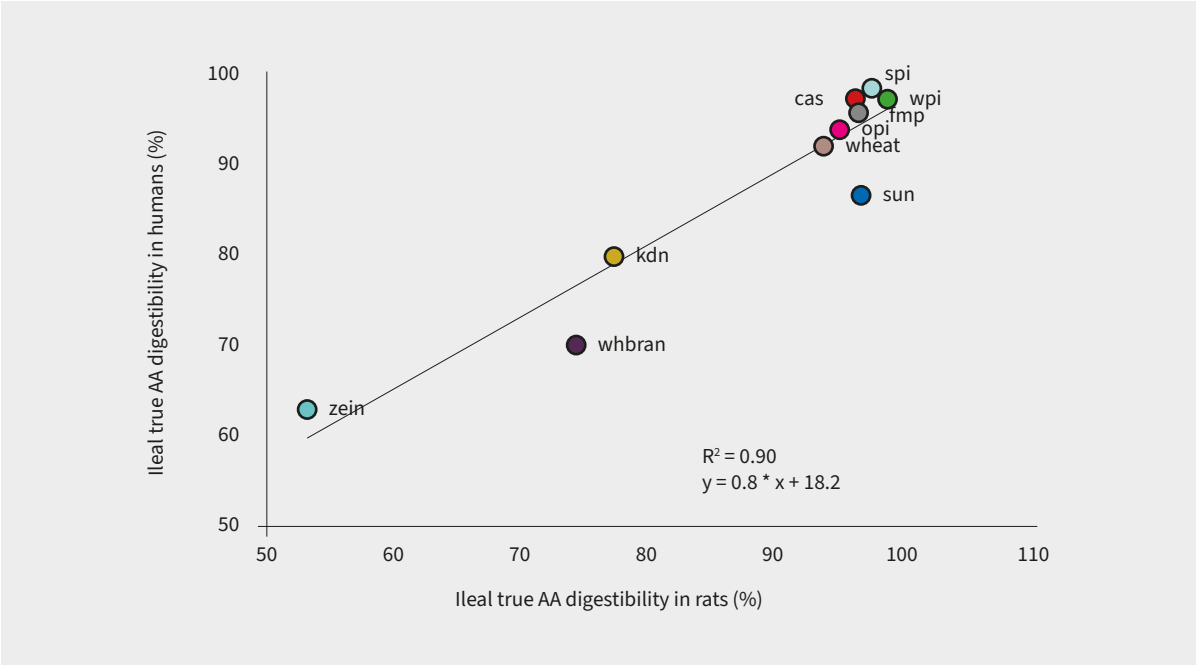
In conclusion, ileal or caecal true AA digestibility can be easily and rapidly determined in the rat model. This model also has the advantage of being more economical than other animal models of protein digestibility. Indirect comparisons of data in humans and rats are promising and show good correlations. Further studies are required with direct comparisons, especially for medium to low digestibility protein sources.

4.5. True digestibility – the dual isotope method in humans

The dual isotope tracer method is a minimally invasive approach based on blood sampling and using two stable isotope-labelled markers simultaneously fed in a plateau feeding protocol, the test protein intrinsically being labelled with one isotope (^{15}N, ^{2}H) and a standard marker labelled with another isotope (^{2}H, ^{13}C) of known nitrogen and AA digestibility constituted by either a single AA, a mixture of AAs or an intrinsically labelled protein.[2, 235, 236] A dual isotope test was developed with ^{15}N spirulina and ^{2}H phenylalanine to estimate the capacity of the gut to digest protein-bound phenylalanine from spirulina in a pathological context. No correction for transamination was made as this was not required in a clinical test in which a relative isotope ratio was calculated between the protein tested and a standard marker that was differently isotopically labelled. This method was introduced to assess simultaneously the digestibility of all dietary protein IAA using ^{2}H labelling of different test proteins and a ^{13}C labelled standard protein of ^{13}C labelled spirulina whole cells.[2, 235, 236]

The method involves simultaneous ingestion of two intrinsically stable isotopically labelled proteins, one a test protein ($^{2}H/^{15}N$) and the other a standard protein (^{13}C) of known digestibility, in a plateau feeding protocol. The (primed) plateau feeding protocol, in which frequent meals are administered, was adopted in the dual isotope tracer technique to reduce the blood sampling burden on the subject. Plateau feeding helps to achieve a steady state that requires fewer blood samples to define than a non-steady state curve. It is also important because two protein sources can vary in their digestion and absorption kinetics, which could lead to differences in their plasma appearance: when free labelled AAs or a protein with fast digestion and

Figure 2. Mean true ileal amino acid digestibility values determined in human volunteers



Note: Adapted from Table 7. Mean true ileal amino acid digestibility values determined in human volunteers and rats for ten protein sources obtained in independent studies. cas, casein; kdn, kidney bean or black bean; ppi, pea protein isolate; spi, soy protein isolate; sun, sunflower protein isolate; tmp, total milk protein; whbran, wheatbran; wheat, whole wheat/wheat bran; wpi, whey protein isolate.

Sources: See source notes: 107, 110, 113, 135, 136, 197, 198, 201, 226, 231, 233 and 234.

absorption are used as a standard and administered as a bolus, their plasma AA appearance shifts to the left of the test protein. The principle is to compare the ratio between the enrichment in the two isotopes ($^{15}N/^{2}H$ or $^{2}H/^{13}C$) for each IAA analysed in the meal and at plateau in the plasma.[235, 236] The digestibility of each IAA in the test protein is derived from the known digestibility of each IAA in the standard, corrected by the change in this ratio between the meal and the plasma. The ratio of enrichment of labelled AAs of the test protein at the plateau with respect to their enrichment in the meal is compared with a similar ratio of the standard protein obtained simultaneously. This "ratio of ratios", when corrected for true ileal AA digestibility of the standard protein (Dig_{ref}), provides a measure of true ileal AA digestibility of the test protein (Dig_{test}).

Dig_{test} = [Plasma ^{2}H IAA (**APE**)/Meal ^{2}H IAA (**APE**)] / [Plasma ^{13}C IAA (**APE**)/Meal ^{13}C IAA (**APE**)] *100 * Dig_{ref}/100

APE, atom percent excess.

As ^{2}H is less subject to transamination exchanges in the liver and other body tissues, it is usually preferred to ^{15}N for the labelling of the test protein, and because ^{13}C does not exchange, it is preferred to ^{15}N for labelling the standard component.[236, 237] The method assumes a similar metabolic fate and the absence of differentiations in the absorption of differently labelled AAs from test and standard protein, and that they enter a common pool after digestion and absorption.[238, 239] Because of the capacity of intestinal proteases, digestibility estimates probably do not vary drastically with different meal patterns, although a positive effect on low digestible protein cannot be excluded.[240, 241] Using the plateau feeding protocol leads to a steady isotopic plasma enrichment, which avoids different rates of absorption of AAs from test and standard components in the test meal that could influence the rate of AA metabolism.[242]

The standard used can either be an intrinsically labelled (bound) protein of known digestibility or a labelled free AA mixture, which is assumed (by definition) to have 100 percent digestibility. The standard component must meet several criteria, including the preferability of high ^{13}C enrichment, allowing its use at tracer dose, and being highly digestible and acceptable in food. In the study by Devi and colleagues,[122] the spirulina whole cell

standard protein contributed just 3 percent of the meal protein. A single highly U ^{13}C enriched AA, a mixture of highly U ^{13}C enriched AAs or intrinsically and uniformly highly U ^{13}C enriched whole cells of the cyanobacterium spirulina have been used as a standard.[116, 135, 235, 236] In either case, the standard protein chosen should be labelled at positions which are not involved in isotope exchanges. For instance, if only the methyl group of methionine is labelled, then it is lost during the transmethylation reaction and hence would lead to overestimation of test protein methionine digestibility.

The standard protein should also have high digestibility and be commercially available and easily producible at a reasonable cost. The stable isotopic label that is preferred and generally not involved in isotopic exchanges is ^{13}C in the carbon backbone; therefore, ^{13}C labelled protein or free AAs are most suitable as a standard. Animal source proteins such as milk, casein, whey, egg and meat are known to have high digestibility and could be used as a standard protein. However, the amount of free labelled AA mixture required for production of these proteins in quantities required for the digestibility studies is high and processing them for long-term storage is cumbersome. A commercially available cyanobacterial protein, U ^{13}C spirulina (Cambridge Isotope Laboratories) has been previously used as a standard protein in a dual isotope tracer study, with a mean true ileal IAA digestibility of 85.6 percent. (This was determined against a ^{2}H labelled free AA mix.) The digestibility of IAA from U ^{13}C spirulina, determined in humans by plasma sampling using the dual tracer method, and as a standard a mixture of ^{2}H or ^{15}N labelled free AAs, indicates an average digestibility of the IAAs from spirulina of 85 to 88 percent, ranging from 80 to 90 percent for lysine to 80 to 95 percent for phenylalanine.[129, 243] These values are consistent with the values obtained in rats by the oro-caecal balance method.[134] However, AA digestibility of spirulina protein is moderate (<90 percent) and involves interstudy variability. U-^{13}C-free AA mixture can be used as a standard, with the advantage of high digestibility and low interindividual variability.[116, 135, 244] However, it has been argued that a protein versus protein comparison for the test and standard could be preferred because the rate of protein-derived AA absorption, metabolism and utilization can be different from, and is usually slower than, that of free AAs.[245, 246] Also, it would be easier to ascribe a test protein with low measured IAA appearance as being due to low digestibility, while it would be more difficult to differentiate between low digestibility and low digestive function when crystalline AAs are used as a standard.

This method has been used to assess the digestibility of IAAs of a range of different plant and animal food proteins.[129, 132, 191, 236, 243, 247-249] These proteins have been measured in young children and adults, as well as in scenarios to define the effect of food processing or the food matrix (Table 8).

Interestingly, AA digestibility of some legume protein did not differ when measured with the use of either U ^{13}C spirulina protein or U ^{13}C free AAs as the reference in healthy individuals.[247] A U ^{13}C free AA mixture has been used as a standard when the digestion might be affected in situations such as cystic fibrosis and environmental enteropathy.[235, 250, 251] The range of individual AA digestibility values reported for IAAs by the dual isotope tracer technique are higher for plant protein than for animal protein (although their median digestibility is lower). For instance, for mung bean protein the true IAA digestibility ranged from 42.5 ± 1.2 percent for threonine to 75.8 ± 2.6 percent for isoleucine; for goat milk protein it ranged from 89.9 ± 1.2 percent for threonine to 97.9 percent ± 1.8 for methionine.[129, 131] Therefore, the difference between the highest and lowest true ileal IAA digestibility for mung bean protein was 33 percent for isoleucine and threonine, whereas for goat milk protein the difference was 8 percent for methionine and threonine. This result is expected because various antinutritional factors present in plant foods affect the digestibility of each IAA to a different extent. A similar effect of antinutritional factors on true IAA digestibility was observed when black tea was co-ingested with egg protein[243] and illustrates that, in mixed meals, antinutritional factors derived from plants could reduce the digestibility of animal source protein.

The dual stable isotope method involves some assumptions.[93] First, differently labelled AA from the test and standard protein have similar absorption kinetics. Second, after their digestion and absorption, differently labelled AA from the test and standard protein undergo a similar and equivalent first-pass splanchnic extraction and metabolism before entering the common body pool as sampled, so the ratio of enrichments of test to reference protein AAs cancels out this metabolism and extraction. These assumptions appear to be reasonable because the isotopic effects for absorption and metabolism are not conclusively known but are likely to be very small, and when at plateau the isotopic ratio will reflect amino acid appearance from the different components. Therefore, the measure can be assumed to be a true estimate of the test protein's digestion and absorption. The dual tracer method could potentially be made non-invasive by sampling IAA appearance in urine. However, this development has not been pursued aggressively because a slower

Table 8. True ileal digestibility of a variety of food proteins in different age groups

Test protein	Standard protein	Age group (years)	N	Methionine	Phenylalanine	Threonine	Lysine	Leucine	Isoleucine	Valine	Mean IAA	Tryptophan
Desi chickpea	U-^{13}C-spirulina	18–45	1	59	61	54	44	69	69	64	57	-
Kabouli chickpea			6	72	81	73	60	80	81	76	75	-
Kabouli chickpea extruded		6–11	12	82	106	83	79	92	91	90	88	-
Mung bean		18–45	6	52	73	43	63	68	76	68	63	-
Mung bean		1.5–2	4	54	77	62	65	68	63	68	65	67
Dehulled mung bean		18–45	6	64	75	55	63	76	83	80	71	72
Yellow pea			6	56	81	67	62	79	80	77	72	-
Yellow pea extruded		6–11	13	80	106	81	75	94	91	90	86	-
Rice		1.5–2	4	80	84	73	78	79	81	75	79	-
Finger millet			4	60	70	67	75	67	75	65	68	-
Egg white		18–45	6	80	93	89	89	88	84	82	86	-
Whole egg			6	86	96	96	89	88	85	87	89	-
Whole egg		1.5–2	4	86	90	90	94	86	85	81	87	-
Faba bean		18–45	5	-	61	44	63	66	63	69	-	-
Chicken skeletal muscle			6	93	94	94	96	89	89	90	92	-
Pinto bean			6	79	71	68	73	83	81	84	77	-
Milk	^{13}C-AA		7	98	93	90	93	95	97	92	94	94
Spirulina	^{2}H-AA	18–45	6	84	95	83	78	86	84	87	85	79

Sources: See source notes: 129, 132, 191, 236, 243 and 247-249.

urinary appearance is likely to obscure any kinetic differences between labelled IAAs from different sources to which blood samples are more sensitive. The dual tracer method is expensive and requires access to the facilities of an experienced nutrition centre. Moreover, it will not be feasible to measure every possible protein source by this method. Thus, there is a role for a modest screening method that has the capacity to screen hundreds of proteins, including different plant varieties and processing and preparation methods, while the digestibility of tens of important proteins is measured *in vivo* by the dual tracer method.

In conclusion, the dual isotope tracer method, as currently used, is pragmatic for use in different populations. However, it does have many assumptions: the post digestive transactions of AAs from two differently labelled proteins are similar, and one protein does not influence the digestion of the other (test or standard) protein. Although the technique needs validation against other methods which measure digestibility at the end of the ileum, the true ileal IAA digestibility values of different proteins measured by the dual isotope tracer technique are comparable to those obtained by other methods. The technique is also sensitive to changes in food matrices and processing. Nevertheless, it has great potential as a minimally invasive technique for measuring true ileal IAA digestibility of a wide variety of proteins to inform protein quality not only in different populations but also in populations with different physiological states, especially vulnerable populations.

4.6. Metabolic bioavailability – the IAAO method in humans

The indicator amino acid oxidation approach (IAAO) measures metabolic availability through the oxidation of an indicator IAA with an increasing level of the IAA under test. The IAAO method thus compares the response of the indicator IAA in subjects consuming graded intakes of a test protein relative to the response obtained in the same subjects consuming similar intakes of a reference protein. The subjects' response to the intakes of the test and reference proteins are presented as two different slopes, with the ratio of the test to the reference slope representing the relative metabolic availability/bioavailability of the test protein. Hence, the IAAO method is considered a slope ratio method for assessing AA metabolic availability.[252, 253]

The IAAO method is based on the physiological premise that when one AA is limiting in the diet, all other AAs are in relative excess and must be oxidized. This produces a linear response in indicator IAA oxidation, so comparison of the slope of test IAAO vs reference IAAO produces the relative metabolic availability/bioavailability of the test limiting AA and is used to measure the protein quality of a test food. In the method combining the IAAO and slope ratio methods, the oxidation of a ^{13}C labelled indicator IAA (often [1 ^{13}C]phenylalanine) given orally is used as a proxy for the ability of an unlabelled dietary IAA to contribute to protein synthesis; the higher the oxidation of the indicator IAA, the lower the protein synthesis.[254] This is achieved by comparing the oxidation response slopes to graded intakes of the selected test IAA from a test protein at sub-requirement levels of the labelled indicator IAA ([1 ^{13}C]phenylalanine) with that of a reference crystalline IAA mixture.[144, 253]

Changes in the oxidation of L [1^{13}C]-phenylalanine to $^{13}CO_2$ is used as the outcome variable (y) when the response to changes in intake of a limiting IAA in a food protein is assessed in comparison with that obtained using a crystalline AA. The oxidation of the indicator IAA is measured in breath as $^{13}CO_2$. Since changes in indicator oxidation reflect whole body bioavailability, the term *metabolic availability* is used to reflect the effect of digestion, absorption and utilization of the test AA. The IAAO slope ratio method has been validated in pigs.[253] It has been argued that a highly digestible protein such as casein and hydrolysed casein would be a better reference than a crystalline AA mixture because of their effect on gut protein metabolism, gut endogenous AA secretions, AA absorption and body protein synthesis.[245, 246, 255] However, while casein is suitable for use in animals such as pigs, it is not a practical reference protein in humans with a 4 to 5 times lower AA requirement. Therefore, crystalline AA is a more practical reference in human subjects because it allows the provision of lower intakes of the amino acid under study.

A key condition in the IAAO slope ratio method is that the indicator IAA oxidation must be linear in its response to changes in test IAA intakes, which then requires all tested IAA intakes to be below their requirement. To account for variability in the requirement, subjects are provided with test IAA intakes well below (<60 percent) the estimated average IAA requirement.[2] Keeping intakes below 60 percent of the EAR ensures the response if the indicator oxidation is linear at each intake of the test and reference proteins. Therefore, when metabolic availability is determined using the IAAO method, the requirement of the test amino acid must be known.[144, 256] The low-test IAA content in the experimental meals (which is usually <60 percent of the test IAA requirement) with a relative surplus of

other AAs (~120 percent of the requirement) could lead to an AA imbalance with reduced efficiency of protein synthesis and increased oxidation of other AAs, including the indicator IAA.[257-259] Although AA imbalances are more of a concern in protein-deficient diets and in metabolic availability studies, diets should be planned to be not limiting in total protein. This is fulfilled in IAAO metabolic availability studies by providing daily total protein at 1.0 g/kg.

The main advantage of the of the IAAO slope ratio method is that it is non-invasive because it measures $^{13}CO_2$ in breath. In addition, it is analytically simple and does not require intrinsically labelled test proteins because only the indicator IAA is labelled. It measures bioavailability, which means it can capture the effect of processing that can damage AAs such as lysine. Also, measures of bioavailability instead of digestibility can be used to test the effect of protein complementation of two plant protein sources in the same meal. The major disadvantages of the IAAO slope ratio method are that it is expensive because it requires many clinical experimental sessions and that only one IAA can be studied at a time. Strict control of non-protein intake and physical activity are also required to minimize underlying variations in the ^{13}C abundance of the large, exhaled CO_2 term not derived from protein. However, because in most cases the limiting AA determines protein quality, the method can provide important data, particularly in vulnerable populations (e.g. children) who cannot be studied with more invasive methods.

Using the IAAO slope ratio method, the protein quality of various plant foods has been studied in humans by assessing the metabolic availability of the limiting AA lysine or methionine in rice,[260, 261] corn,[262] sorghum,[263] millet,[264] lentils[265] and chickpea.[266] Additionally, the metabolic activity of total sulphur AA[267] in casein and in soy protein isolate have been evaluated (Table 9).

Table 9. Metabolic availability of limiting amino acids across various foods

Food	Limiting amino acid	Metabolic availability (%)
Casein	Sulphur amino acids	87
Soy protein isolate	Sulphur amino acids	72
Rice	Lysine Methionine	97 100
Corn	Lysine Tryptophan	71 80
Sorghum	Lysine	94
Millet	Lysine	97
Lentils	Lysine Methionine	80 69
Chickpea	Methionine	63

Sources: See source notes: 260–267.

Comparable values of IAA metabolic availability have been found in various studies, suggesting a good repeatability.[261, 262, 268] Furthermore, the IAAO method is sensitive to detecting the reduction in bioavailability caused by food processing. When proteins are extensively processed or heat-treated, which leads to them being modified and made unavailable by the Maillard reaction, racemization and cross-linked protein aggregation,[174, 269, 270] the assay has shown a considerable decrease in the metabolic availability of lysine.[263, 266, 271] A limitation of the method lies in the relatively high interindividual variability, with a coefficient of variation of 15 to 52 percent as measured from the standard error of the slopes in these estimates of IAA metabolic availability.[261, 262, 266, 271] This might be improved by a longer duration adaptation (~7 d) to the test level of IAA intake.[272, 273] Studies have demonstrated that a prior adaptation to the test IAA has no effect on the turnover rates of protein and hence does not influence the IAA requirements and the metabolic availability estimates.[256, 274] However, the variability of ^{13}C recovery was approximately double

with shorter adaptation periods (8 h and 3 d) than with 7 d of adaptation, at lower daily intakes of lysine (5 mg/kg and 20 mg/kg).[271] A longer adaptation to test IAA intakes might therefore reduce the interindividual variability. The method has not been comprehensively validated in controlled studies in humans.

4.7. *In vitro* digestibility

In vivo methods are greatly required to directly assess the effectiveness of protein sources for growth or other physiological functions. However, the need to develop complementary *in vitro* methods is recognized because of ethical and economic constraints. *In vitro* digestion models that mimic the gastrointestinal tract have been proposed as an alternative to *in vivo* experiments, although there are various limitations to mirroring the complexity of the digestive tract. Wherever possible, research and food authorities should reduce animal experiments, and *in vitro* methods are animal-free (except for the currently used enzymes) and therefore increase the sustainability of the food system. The two types of *in vitro* models are static and dynamic ones. These methods analyse the processes of protein digestion and their transformation into peptides and AAs.[275-278] Analytical techniques such as size exclusion chromatography can be used to estimate the proportion of small peptides potentially available for uptake, and, combined with determination of total dissolved protein, the percentage of small peptides and free AAs per total protein appears as a physiologically relevant estimate of protein and AA digestibility.[279]

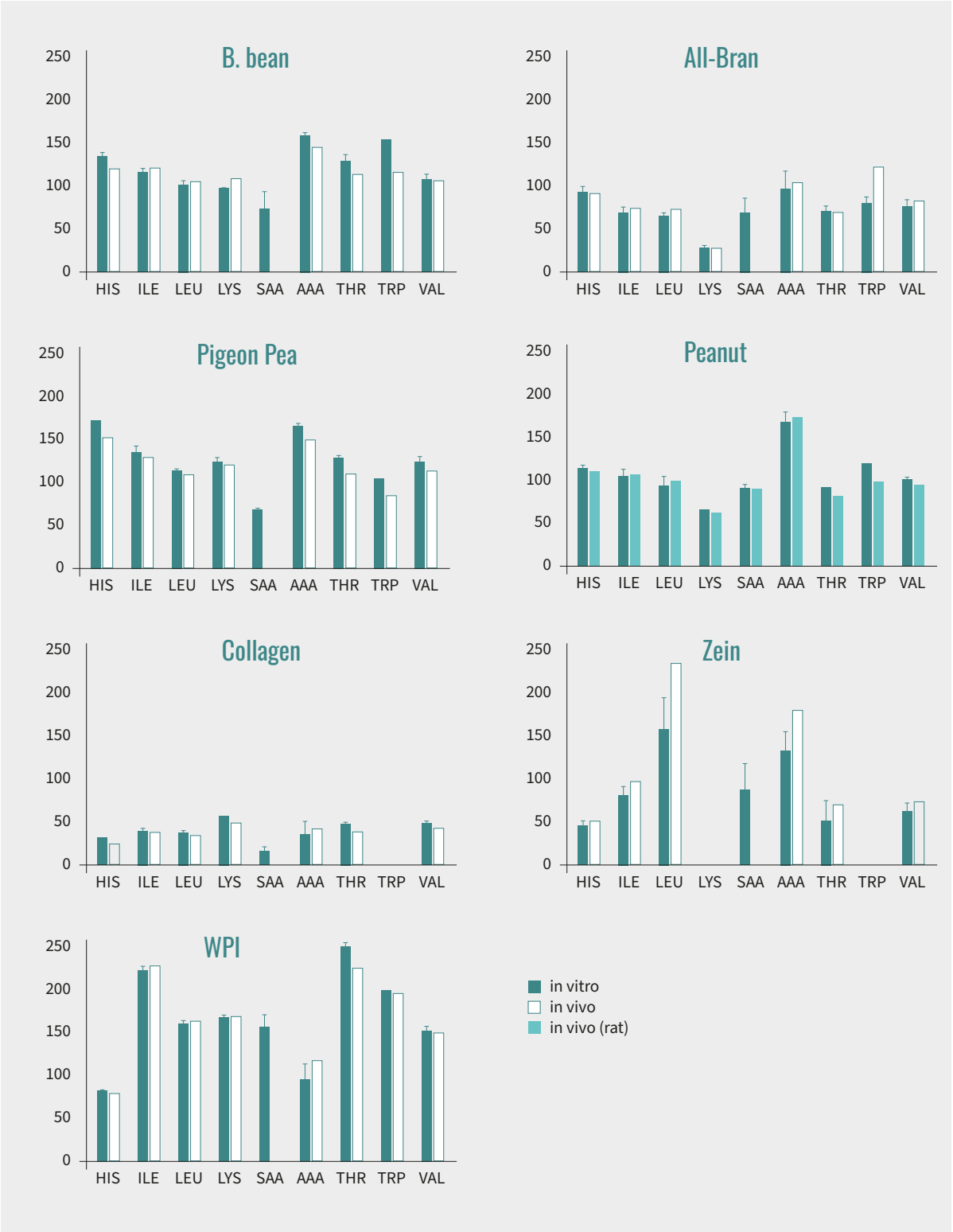
In the static models, meals are successively incubated for given times in flasks representing different steps of *in vivo* digestion and containing the corresponding digestive enzymes at the appropriate pH to mimic the oral phase, the gastric phase and the intestinal phase of the digestion in the intestinal lumen.[280, 281] Until recently, there were many static *in vitro* digestion models using different digestive enzymes, ionic strengths, pH, digestion times, etc. In the framework of the COST Action international network of excellence on the fate of food in the gastrointestinal tract (INFOGEST), an international consensus on a set of digestion parameters for a static *in vitro* simulation of adult digestion was reached.[282, 283] This INFOGEST protocol standardizes the experimental conditions in terms of enzymes, concentration, pH and incubation time. Using this protocol, food samples are subjected to sequential oral, gastric and intestinal digestion, and conditions such as electrolytes, enzymes, bile, dilution, pH and time of digestion are based on available physiological data. After optimization, this method has been applied to seven selected samples (whey protein isolate, zein, collagen, black bean, pigeon pea, All-Bran® cereal, and peanut), and the total protein digestibility and digestibility of individual AAs agreed with the *in vivo* data[103,198] for the same substrates with a mean difference of 1.2 percent. The *in vitro* digestible indispensable amino acid ratio (DIAAR) also correlated with the *in vivo* DIAAR obtained from true ileal digestibility values, with a mean difference of 0.1 percent (Figure 3).[284]

Although the obtained results are promising, more *in vivo*/*in vitro* comparative studies are needed using foods from different sources (plant, animal and other novel sources), different food matrices, and foods subjected to different technological processes. Such *in vitro* protocols should be developed in parallel to *in vivo* experiments, using the same food products and analytical methods to validate the results.

Static digestions are fast, simple, inexpensive and replicable but cannot reproduce all the conditions of digestion *in vivo*, including mechanical peristaltic and contraction processes.[280] The dynamic models are more complex, involving successive interconnected compartments to best reproduce *in vivo* digestion with mechanical forces used to reproduce peristalsis and contractions of the stomach and the small intestine.[280] The simpler dynamic models such as the Dynamic Gastric Model and the Human Gastric Simulator include only one compartment that only simulates digestion in the stomach.[285, 286] Other models such as the TIM system are more complex, with different compartments mimicking the entire digestive system.[287-289] Dynamic models of digestion are closer to digestive physiological conditions, allowing a better comparison with *in vivo* conditions,[280] although their complexity might hamper the quantitative determination of nitrogen required to evaluate protein digestibility.

In vitro methods for assessing protein digestibility are easier, faster and less inexpensive than *in vivo* methods but can lead to oversimplification of *in vivo* digestion processes and usually do not provide absolute digestibility values.[290, 291] One main difficulty is transcribing the complexity of the digestion processes *in vivo*.[291] In contrast, *in vitro* models provide relative values of digestibility and are mainly used to compare the digestibility of different protein sources and food, and to evaluate the influence on digestibility of food treatments and processes. Static and dynamic *in vitro* digestions of protein to peptides and AAs were compared with *in vivo* digestion models.[292-296] In a dynamic model with two steps, the gastric phase and the intestinal phase, a difference of 6.6 percent was

Figure 3. Comparison between *in vivo* and *in vitro* methods for AA digestibility



Note: *In vitro* digestible indispensable amino acid ratio values compared with *in vivo* AA digestibility-based data from pigs and humans (average values) for black bean, collagen, pigeon pea, zein, All-Bran® wheat cereal and whey protein isolate (WPI), and from rats for peanut.

Sources: Adapted from: **Hodgkinson, S.M., Stroebinger, N., Van Der Wielen, N., Mensink, M., Montoya, C., Hendriks, W.H., de Vries, S., *et al.*** 2022. Comparison of true ileal amino acid digestibility between adult humans and growing pigs. *The Journal of Nutrition,* 152(7): 1635-1646; **Rutherfurd, S.M., Fanning, A.C., Miller, B.J. and Moughan, P.J.** 2015. Protein digestibility-corrected amino acid scores and digestible indispensable amino acid scores differentially describe protein quality in growing male rats. *The Journal of Nutrition,* 145(2): 372-379; and **Sousa, R., Recio, I., Heimo, D., Dubois, S., Moughan, P.J., Hodgkinson, S.M., Portmann, R., *et al.*** 2023. *In vitro* digestibility of dietary proteins and in vitro DIAAS analytical workflow based on the INFOGEST static protocol and its validation with *in vivo* data. *Food Chemistry,* 404: 134720.

observed for the digestibility of nitrogen and differences ranging from 8 to 15 percent for AAs when measured *in vitro* or *in vivo* in pigs.[297] The digestion of skim milk powder according to the INFOGEST protocol leads to the same proportion of free AAs recovered in the intestinal phase *in vitro* as that recovered in the pig intestine.[282, 292] Moreover, a good correlation has been shown between the true digestibility obtained *in vivo* and the nitrogen digestibility obtained *in vitro*.[298] The main challenges in the development of *in vitro* methods to measure protein digestibility are related to the simulation of the complex enzymatic system found along the upper gastrointestinal tract, the definition of the absorbable and non-absorbable fraction, the differentiation of the nitrogen added in the form of enzymes, and the use of standardized conditions. Among the available devices (static, semi-dynamic or fully dynamic), static *in vitro* digestion methods are simple and cost effective and offer good inter-laboratory reproducibility if performed under standardized conditions.

5. Principle for the development of a database on protein and amino acid digestibility

5.1. Initial considerations

The importance of meeting protein requirements by supplying sufficient available AAs in the diet is recognized. With pressure arising from climate change, concerns to reduce the proportion of dietary protein derived from animal source foods have been raised, and the protein quality of plant proteins has come to the forefront. FAO recommends the use of the DIAAS to describe protein quality, which is a combination of the AA score, true ileal AA digestibility and the IAA reference pattern. Given the multitude of factors that can influence protein quality, it is critical for a new database to include such information as the protein source, proximate analysis values, AA composition and nitrogen and AA digestibility coefficients established by both *in vivo* and *in vitro* assays, in addition to the corresponding statistical analysis (min., max. and range). Processing conditions for all proteins should be clearly presented within the database. Furthermore, the database should be a living document and align with national food compositional databases.

5.2. Identification of a reference method and validation issues of other methods

Protein quality, from a nutritional standpoint, represents the product of the AA composition of the food or ingredient in question and the digestibility/availability of the constituent AAs. Protein quality estimates, including the PDCAAS and eventually the DIAAS, are used by regulatory bodies to substantiate protein content claims and for public health policy, including international food security programmes and national dietary assessments.

The measurement of protein quality has many technical considerations, including analytical issues, choice of species, measurement of digestibility vs availability, and whether the measures are performed *in vivo* vs *in vitro*. As stated in FAO reports, oro-ileal digestibility should be determined, in order of preference, in humans, then in pigs, and alternatively in rats.[1, 2, 5, 43, 51] Moreover, *in vitro* methods are also progressively being developed. These methods contribute to the accumulation of values for true ileal protein and AA digestibility for human food sources. Human *in vitro* models are being developed, or one can use human *in vivo* studies. For human *in vivo* studies, a diverse array of methods are available, and they are often based on oro-ileal digestibility or make use of labelled proteins, or both. However, these methods also have their advantages, drawbacks and limitations, depending on the models studied (Table 10). Labelling may be difficult and costly, and low volumes of the protein make it hard to perform food processing experiments and subsequent digestibility experiments on the same batch.

5.3. The criteria to be used

Protein (nitrogen) and AA digestibility is measured in humans, in animal models or using *in vitro* models:

- Results obtained in animal models or using *in vitro* models should be validated in comparison with results obtained in humans. Correction factors can be determined and may be suitable for obtaining values for humans from data obtained in validated animal models or using *in vitro* models.

- Data from all methods (human, animal and *in vitro*) will be included.

Table 10. The strategies for studying protein/AA digestibility

Model	Costs	Through-put	Compara-tive data available	Can be adapted for specific populations	Provides info on individuals	Accepted by food authorities	Requires food-grade products	Additional remarks
in vitro static	+	High/ medium	-/+	-/+	No	Needs validation	No	No ethical concern
in vitro dynamic	++	Medium/ low	-/+	-/+	No	Needs validation	No	
Oro-ileal rat-based	++	Low	++	-/+	No	Yes	No	Ethical concern
Oro-ileal pig-based	+++	Low	+++++	-/+	No	Yes	No	
Oro-ileal in humans (nasal tubing)	+++++	Low	Reference method	-/+	Yes	Yes	Yes	Highly invasive
Oro-ileal in humans (ileosto-mates)	++++	Low	+	No	Yes	Yes	Yes	Not invasive
Dual isotope in humans	+++++	Low	-/+	-/+	Yes	Yes	Yes	Partially invasive
IAAO in humans	++++	Low	-/+	Yes	Yes	Yes	Yes	Not invasive

Source: Authors' own elaboration.

- Only the direct measurement of oro-ileal disappearance of the dietary component provides absolute values of protein (nitrogen) and AA digestibility.

- The contribution of the intestinal endogenous fraction of protein (nitrogen) and AA must be considered to provide values of true digestibility.

- For protein (nitrogen) digestibility, oro-faecal measurement can be a proxy of oro-ileal disappearance, but oro-ileal disappearance should be preferred. For AA digestibility only oro-ileal disappearance should be measured. Faecal digestibility measures are needed to describe overall body N transactions.

- Other *in vivo* methods are indirect and do not provide absolute values. Oro-ileal disappearance is used as the absolute reference for other relative methods. Protein (nitrogen) and AA digestibility are determined relative to reference values of either standard protein or free AAs. The choice of standard needs validation.

Ethical questions must be considered:

- Only *in vitro* methods are completely non-invasive. But the origin of digestive extracts should be considered.

- Methods using the collection of intestinal effluents in human are very invasive and cannot be used routinely.

- Methods using the collection of intestinal effluents in animals are also very invasive and need to be justified. Animal welfare needs to be considered.

- Methods using blood sampling are partially invasive and need to be justified. The number of blood samples and the volume of collected blood are limiting factors.

5.4. Initial agreements regarding the database

Experts at the technical meeting agreed that the objective of the database would be to develop, populate and maintain a fully accessible, robust database on true digestibility of protein and individual amino acids in foods consumed by humans, and to provide up-to-date information on the protein quality from food sources, according to the appropriate scoring method.

There was a discussion surrounding which agency would be best suited to house the database. Some rationale was provided as to why FAO may be best suited to do so.

- Most of the protein or IAA digestibility data are derived from animal or *in vitro* models; fewer data have been obtained using stable isotope methods, which are the main techniques supported by IAEA.

- As the data itself represents protein and IAA digestibility in foods, the FAO food composition database would be an ideal example to draw from when the protein database is designed.

- The data will have widespread use and will potentially be more visible to the larger nutrition community if housed at FAO rather than at IAEA.

No clear statement was made as to whether IAEA or FAO should house the database. Based on the rationale provided above, FAO would be an ideal place to host the database. However, it was confirmed that it will a joint FAO/IAEA database.

Users would have access free of charge to a comprehensive, robust database on ileal digestibility of protein and individual amino acids in foods that are part of human diets, together with information on the protein quality of foods and mixed meals. Data on any food that is part of human diets will be included, covering plant and animal foods and novel protein sources, with a conscious effort to include foods from LMICs, underutilized foods and climate-resilient crops. Various processing and food preparation methods and postharvest storage conditions will be covered, as well as proteins in mixed meals and in complementary foods for young children.

Intended users of the database would be research institutions, governments and industry. Users with various levels of skill and background knowledge will be able to use the data to calculate the protein quality of individual foods and mixtures of foods. The data will allow public health professionals to provide guidance on translating requirements into foods consumed, based on the dietary patterns of individuals or population sub-groups. It could be used to assess complementarity protein sources such as combining different foods that complement one another to provide the IAAs as part of a mixed diet or combining such foods in food products as complementary foods. Finally, it could be used to assess how poorly digestible proteins can be supplemented with limiting amino acids in order to improve the quality of some traditional plant-based diets. Following the eventual regulatory adoption of the DIAAS by governments, the data can be used by food regulatory agencies to evaluate food health and nutrition claims by industry.

The technical meeting participants agreed on the need to establish a scientific advisory and management group consisting of at least two members of FAO and IAEA staff – at least one from each organization – who would work with three to five external experts. Some of the steps required would be the following:

- To develop consent forms for data sharing and data use, working with FAO legal experts.

- To develop a template for data owners to submit data in a format that is compatible with the database.

- To provide guidance to data owners on preparing the data, for example, using a standardized system for classifying and describing food, which will allow the data to be used across domains (food composition, food consumption, food safety, etc.).

- To publish calls for data that may be submitted to the database.

- To populate the database with peer-reviewed published data and unpublished microdata from these sources to allow meta-analyses to be carried out.

However, the first hurdle would be to obtain sufficient funds to create the database and ensure it can run for several years.

6. Concluding remarks and future directions

Protein quality is the ability of a protein food source to provide the right amount of N and a balance of IAAs, which is critical for human health, especially in the most vulnerable periods of rapid growth and in old age. The methods for measuring protein quality over the years (e.g. measuring nitrogen balance in faecal matter after digestion) are inaccurate and are known to underestimate protein requirements. In 2014, FAO made a call for a new way to measure protein quality that focuses not on the whole protein but on the absorption of individual IAAs. Several methods, including isotopic techniques, were recommended for use in assessing true IAA absorption in human and animal models. Data on true AA digestion and absorption have been accumulating since the call by FAO. An urgent need was identified to have a repository for this unique set of data while continuing to collect more information.

For the first time in October 2022, agencies of the United Nations (FAO, IAEA and WHO) and nutrition and health experts drawn from academia, research and government institutions met at the IAEA headquarters in Vienna, and some attended virtually, to discuss how available protein quality data based on the DIAAS may be collated and secured in a global database for posterity. After deliberations, the technical meeting set beacons for the envisaged database. The main objective of the database will be to provide a fully accessible, robust database on amino acid digestibility of foods and diets. It will include available data from proteins that are part of the human diet (including alternative protein sources) and data from peer-reviewed publications, plus individual data from those studies, even if unpublished; and it will use FAO recommended protein and AA scoring patterns outlined in WHO/ FAO/ UNU 2007 for adults, infants and young children.

The main outputs and functions of the database will be to act as a repository to facilitate information and data sharing. It will include a calculator for the DIAAS (with a working example showing how to carry out the calculations). It was discussed that FAO may be best suited to host the joint FAO/IAEA database. The database will be available for access by both experts and non-experts, encompassing research/technical experts, food manufacturers, public health professionals and policymakers. Some users might also be interested in individual data to allow for meta-analysis, etc.

Data will be included in the database based on a set of agreed criteria and following existing guidelines (such as for the FAM/FAO INFOODS database, which can be adapted). Published data from peer-reviewed publications and raw data from published sources will be included. (In the case of unpublished/raw data, separate selection criteria will be established to account for variability.) Additionally, data will be classified based on the method/model of collection: human > pig > rat > *in vitro* models. Information to be included will be food classification/description of food groups (an example proposed was the FoodEx2 classification system), processing aspects and seasonal variation. In consideration of legal aspects, copyright of data will stay with the data owners and FAO/IAEA will be redistributing data through platform users. Users will be obliged to acknowledge the data owners. There will be an advisory on data use for commercial purposes. To ensure quality, an international database expert group will review published work as a first validation step for consideration for inclusion.

In closing, the technical meeting recognized emerging concerns on how to sustainably feed the world's population. The world is moving backwards in its efforts to end hunger, food insecurity and malnutrition. A shift to more sustainable protein sources has been suggested because they have greater nutritional value and less environmental impact than "traditional" protein sources. However, this comes with trade-offs related to protein quality. In this case, more data on protein quality are needed, especially in LMICs. While there are many methods for generating protein quality data, *in vitro* methods, if optimized, will hold the future for a rapid generation of information needed to inform protein nutrition. To make this a reality, there is need to establish an *in vitro* protocol. (The INFOGEST protocol can be used as an existing protocol with the possibility of adapting to the egg protocol.) Criteria for expected variability and reproducibility in comparison with *in vivo* methods will be needed.

Additionally, the following considerations were discussed:

- The criteria to be used to validate *in vitro* data should be included in the database.

- It is considered a validation when studies are performed with the same substrate and using the same analytical method.

- Five percent variability was discussed as an acceptable variability between *in vivo* and *in vitro* data – calculated as the average difference across variation in IAAs, tryptophan and cysteine rather than the average difference across all AAs.

- Static, semi-dynamic and dynamic models can be used to simulate gastrointestinal digestion, but the simplicity of static models allows for a better inter-laboratory reproducibility in the quantitative determination of amino acid digestibility.

- Inter-nutrient influences should be considered.

7. Recommendations for the future

1. To generate protein quality data from various foods and diets in LMICs.

2. To generate digestibility data on climate-resilient crops if we are to pioneer the consumption of sustainable protein sources in diets.

3. To create protocols that include the assessment of protein requirements and digestibility data for vulnerable groups such as infants (<12 months) and older people.

4. To try to identify functional/health indicators that affect protein and AA digestibility in humans.

5. As a practical steps forward, to identify and stimulate the accrual of funds to support research, data generation and human/technical resources.

6. To consider proposing protocols that include novel protein sources.

7. To assess the effect of dietary constituents and changes to foods on protein digestibility (such as antinutritional factors, high-fibre diets and food processing).

8. To recommend implementing in papers an accurate and complete description of a food source, as well as the processing and preparation methods, using a harmonized method (e.g. FoodEx2) so that future data can be easily added to the database.

Notes

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ANNEX
Opening remarks

Ms Abdel-Wahab welcomed participants to IAEA and to the technical meeting on the Development of a Protein Database and the Way Forward for Reviewing Protein Requirements, saying that IAEA was greatly honoured to co-organize the meeting together with our sister agency FAO and with the participation of WHO. This multi-agency and cross-disciplinary convergence on a topic of such great importance is not only significant but long overdue, given the many factors that undermine our ability to combat malnutrition in all its forms by 2030. The world population is projected to hit 10 billion by the year 2050, and with this statistic comes the dire need to feed an ever-growing population with foods containing high-quality protein to ensure an adequate child growth and nutritional status of the population. We know that the debate on protein is not complete unless it considers the impact on the environment, especially in relation to global warming. In this regard, a shift to more sustainable protein sources, especially with more protein of plant origin, is recommended. But this comes with trade-offs related to how much of the protein consumed becomes available to the human body. Paradoxically, high atmospheric carbon dioxide emissions are linked to reduced nutrient concentration and bioavailability in major food crops and have a great impact on protein, iron and zinc. Pandemics and other emerging challenges, such as rampant global inflation, also continue to drastically limit our access to foods rich in high-quality protein. Not only has it become more difficult to acquire foods with optimal protein quality, but there is also a distinct lack of readily available tools and technologies for accurately measuring and collating data on the quality of protein across diets.

To address these issues, IAEA has been at the forefront, alongside FAO and others, in discussions and activities to generate much-needed data on protein quality.

For example:

- In 2014, IAEA was part of discussions in Bangalore, India, in which recommendations on methods for assessing true ileal protein digestion shifted to the use of stable isotope tracers to complement the protein quality method proposed by FAO, the DIAAS.

- In 2020, IAEA hosted a technical meeting dedicated to understanding how changing food systems influence our diets and health, considering adverse events such as climate change and the COVID-19 pandemic. Central to the discussions were comments on the role nuclear techniques could play under these circumstances.

- The report from this technical meeting in 2020, now published in *Frontiers in Climate Change*, called for accurate tools for assessing the complexity of interactions between food systems, climate change and diet quality using a “soil to fork to human health outcome” approach.

- Through an IAEA-supported coordinated research project from 2014 to 2020, seven LMICs applied a novel dual stable isotope tracer technique to generate unique data on true ileal absorption of IAA from plant-based diets consumed in Asia, Africa and Latin America.

- A new IAEA-supported regional project in Asia, with over ten participating countries, will generate further data on protein quality in the region.

- A new IAEA-supported coordinated research project (2020–2027), with the participation of seven LMICs from Asia, Africa and Latin America, is evaluating how environmental factors contributing to chronic gut inflammation or environmental enteric dysfunction influence amino acid absorption.

From these key discussions and activities, we see that stable isotope techniques will continue to be central to our ability to provide an evidence base upon which efforts to ensure supply of adequate and high-quality protein to meet requirements across various ages and physiological states can be anchored. Moreover, all protein quality data collected by isotopic techniques and other approaches must be properly and sustainably curated and stored in secure databases.

In this regard, the discussions this week on creating a framework for the protein quality database are extremely important. Each participant in the technical meeting has a special role to play in achieving this objective.

Ms Neufeld said:

Dear colleagues, ladies and gentlemen,

I would like to join Ms May Abdel-Wahab in welcoming you to this technical meeting on the Development of a Protein Database and the Way Forward for Reviewing Protein Requirements, and I would like to thank our colleagues in IAEA for hosting this important meeting.

FAO is honoured to co-organize this meeting with our sister agency IAEA, with the participation of WHO. As UN agencies, we have a unique role in achieving the Sustainable Development Goals (SDGs) and aligned global nutrition targets by 2030. However, according to this year's State of Food Security and Nutrition in the World (SOFI) report, the world is moving backward in its efforts to end hunger, food insecurity and malnutrition, so this meeting is of critical timely importance because providing an adequate, sustainable and nutritious supply of protein remains an increasing challenge. In this regard, plant-based proteins and novel protein sources such as insects have been suggested to have greater nutritional value, as well as less environmental impact, than "traditional" protein sources. Understanding the potential role of different protein sources by being able to assess protein quality is therefore also paramount in light of changing food systems.

Better nutrition is one of the four fundamental aspirations set out in FAO's strategic framework, alongside better production, a better environment and a better life. The right to adequate food and a transition towards healthy diets for national populations is at the core of better nutrition. In this regard, accurately defining the amount and quality of protein required to meet nutritional needs and appropriately describing the protein supplied by foods and diets is of critical importance.

FAO, alongside IAEA, WHO and others, has a long history spanning over 50 years in leading the work on establishing global nutrient requirements and coordinating discussions on accurately measuring protein quality in foods and diets. These include the FAO 2013 Expert Consultation on Dietary Protein Quality Evaluation in Human Nutrition, which was a prelude to the FAO expert working group meeting in 2014 that specifically discussed the most appropriate methodologies for measuring protein digestibility and utilization in humans.[1, 2] One of the main recommendations from this meeting was the need to establish a robust database of protein digestibility of foods commonly consumed worldwide, including those consumed in low-income countries, along with recommendations to advance research and data collection.

Since then, sufficient data have become available on ileal AA digestibility of foods and diets from various regions measured in different populations and different physiological states throughout the life cycle. FAO, with funding provided by the Government of Canada, has recently initiated a project in collaboration with IAEA to inform the future development of a protein digestibility database to aid dialogue on the evaluation of protein quality and protein sufficiency in different populations. Therefore, your discussions this week on creating a framework for the protein quality database are extremely important.

Thank you all for the hard work and dedication that has gone into furthering this area of work. I wish you fruitful discussions for today and for the coming sessions and I am looking forward to the outcomes.

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