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Dietary protein for human health

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Dietary protein for human health

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Editorial: Dietary protein for human health

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Editorial on the Research Topic
Dietary protein for human health

Amino acids found in food proteins are essential in the human diet not only for the maintenance of lean body mass and because of the involvement of amino acids in essential metabolic pathways, but also for modulating appetite and maintaining body weight, and optimal organ function, including muscle function. Optimal organ and muscle function underpin long term health.

Given projected world population growth, food protein demand, and the uncertainties in food production associated with global climate change and other drivers it is timely for an authoritative update on the subject of amino acids and protein in human nutrition.

It was in this context, and driven by the need for future world food and protein security coupled with environmental sustainability, that the international symposium "*Dietary Protein for Human Health*" organized by the Food and Agricultural Organization of the United Nations (FAO), the Riddet Institute, Massey University, Wageningen University and Research, and the International Atomic Energy Agency, was convened in Utrecht the Netherlands in September 2023. Themes covered at the Symposium included: protein nutrition and health; amino acid requirements; amino acid digestibility and availability; dietary protein quality including a review of the protein digestibility corrected amino acid score (PDCAAS) and digestible indispensable amino acid score (DIAAS) evaluation systems; the influence of protein quality on growth and development and on whole body protein metabolism; plant, animal and alternative proteins and their roles in sustainable nutrition; and future sustainable food protein production.

This Research Topic draws off the original research presented at the international symposium "*Dietary Protein for Human Health*" and the resultant collection of 25 scientific papers provides a comprehensive update of recent advances in the area.

The definition and quantification of protein and amino acid requirement values has long been contentious and uncertainty in this area still remains, with recent research pointing toward higher estimates of requirements. The ability of a food to deliver amino acids to meet a stated requirement has also been subject to intensive research over the years, though it has only been over the past decade that physiologically valid methods for determining amino acid digestibility and availability in humans have become generally available. The wider implications of amino acid uptake on growth and development

in children and on body metabolism and organ and muscle function in adults remain important subjects of ongoing research. All of these topics are covered in depth. Recently the effect of climate change on food production, and at the same time the effect of food production systems on climate change itself have become hot topics for research, accompanied by societal calls for changes in consumption patterns of foods. Such recommendations certainly have implications for environmental sustainability, but also implications for nutritional sustainability, food affordability, and cultural mores. The challenge of adequately feeding the future world population is complex and multifactorial, and this complexity is addressed in the present Research Topic.

The Research Topic follows a progression of themes. Review papers by Calvez et al. and Wolfe et al. set the scene by establishing the overall relevance of a study of protein metabolism, protein nutrition and dietary protein quality. Other authors (Layman, Deutz et al., Trommelen and Loon, Groenendijk et al., Deane et al., Manary et al., Mensink) hone in on the specific roles of protein and amino acids in body protein turnover and muscle metabolism as well as malnutrition and disease states. Paoletti et al. and Moughan et al. provide an update on the estimation of amino acid requirements, while Gaudichon and Moughan and Lim address recent developments in protein quality scoring patterns and systems of evaluation. Two contributions (Hodgkinson, Kashyap et al.) address the *in vivo* determination of amino acid digestibility in humans, and the paper of Stein discusses the need for animal models of *in vivo* amino acid digestibility and reviews the evidence for choice of the growing pig as a valid model for the adult human. To allow for a more routine determination of amino acid digestibility in foods a validated *in vitro* digestibility assay is urgently needed. Three papers (Singh, Krul et al., Santos-Sánchez et al.) interrogate this topic. Stanton and Sheffield et al. focus on animal vs. plant foods as supplies of protein, amino acids, and other nutrients, while the works of Burlingame et al., Fletcher et al., Chungchunlam and Moughan offer an holistic assessment of the different dimensions of food sustainability. The Research Topic is completed with a paper (Xipsiti) providing an FAO perspective on protein quality evaluation and the establishment of an international database of food amino acid digestibility, looking to move the area forward and secure greater accuracy of amino acid provision.

Overall, the Research Topic adds to knowledge in a critical area. It remains important that we have a solid scientific evidence base to support amino acid requirement values that will reflect optimal metabolic function and health. Equally we need accurate information on how different foods and novel protein sources differ in their ability to provide the body with dietary essential amino acids. This has never been more important than now, with a significant global challenge to properly feed a growing human population within acceptable environmental boundaries.

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Impacts of protein quantity and distribution on body composition

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The importance of meal distribution of dietary protein to optimize muscle mass and body remains unclear, and the findings are intertwined with age, physical activity, and the total quantity and quality of protein consumed. The concept of meal distribution evolved from multiple discoveries about regulating protein synthesis in skeletal muscle. The most significant was the discovery of the role of the branched-chain amino acid leucine as a metabolic signal to initiate a post-meal anabolic period of muscle protein synthesis (MPS) in older adults. Aging is often characterized by loss of muscle mass and function associated with a decline in protein synthesis. The age-related changes in protein synthesis and subsequent muscle atrophy were generally considered inevitable until the discovery of the unique role of leucine for the activation of the mTOR signal complex for the initiation of MPS. Clinical studies demonstrated that older adults (>60 years) require meals with at least 2.8 g of leucine (~30 g of protein) to stimulate MPS. This meal requirement for leucine is not observed in younger adults (<30 years), who produce a nearly linear response of MPS in proportion to the protein content of a meal. These findings suggest that while the efficiency of dietary protein to stimulate MPS declines with aging, the capacity for MPS to respond is maintained if a meal provides adequate protein. While the meal response of MPS to total protein and leucine is established, the long-term impact on muscle mass and body composition remains less clear, at least in part, because the rate of change in muscle mass with aging is small. Because direct diet studies for meal distribution during aging are impractical, research groups have applied meal distribution and the leucine threshold to protein-sparing concepts during acute catabolic conditions such as weight loss. These studies demonstrate enhanced MPS at the first meal after an overnight fast and net sparing of lean body mass during weight loss. While the anabolic benefits of increased protein at the first meal to stimulate MPS are clear, the benefits to long-term changes in muscle mass and body composition in aging adults remain speculative.

KEYWORDS

leucine, muscle mass, muscle protein synthesis, protein requirements, sarcopenia

Introduction

The meal distribution of dietary protein is thought to have a positive impact on body composition and skeletal muscle mass; however, outcomes are influenced by age, physical activity, and the quantity and quality of the protein consumed. In general, the total quantity of protein consumed each day appears to be the most important dietary factor affecting lean body mass (1). If quantity is high, the relative importance of quality and meal distribution is likely minimal. However, with an increasingly older population, epidemic health problems of

obesity and diabetes, and dietary guidelines shifting toward more plant-based diets, the combined impact of protein quantity, quality, and meal distribution may have increased importance to maintaining healthy skeletal muscles (1–3). This review provides a summary of the data supporting the hypothesis for meal distribution and addresses the limitations of current knowledge.

Recognizing metabolic roles of amino acids

In large part, the meal distribution hypothesis arises from the discovery of the role of the branched-chain amino acid leucine in the regulation of muscle protein synthesis (MPS). Post-meal changes in plasma and intracellular leucine concentrations serve as a unique meal-related signal for triggering MPS. While all amino acids have a fundamental role as substrates for protein synthesis, each amino acid, and certainly each of the 9 essential amino acids (EAA), has a metabolic role beyond the fundamental role as a building block for new proteins (4). Examples include tryptophan as a precursor to serotonin, methionine and cysteine as precursors to glutathione and taurine, threonine as a substrate for the production of mucin, lysine essential for the synthesis of carnitine, and leucine for the activation of mTORC1 for triggering MPS. For each of these metabolic roles, the pathway is driven by substrate availability and specifically the intracellular amino acid concentration.

Currently, the unique metabolic roles of each of the 9 EAA are often obscured by the use of the generic concept of dietary "protein." Protein represents a food source for the delivery of EAA. Protein is somewhat like a vitamin pill. There is no requirement for the pill, but there are requirements for each of the essential vitamins inside the pill. Similarly, protein is simply a food structure that delivers amino acids to the digestive tract. We recently suggested a new framework for evaluating the dietary impact of protein by shifting the focus to the individual nutrient requirements for each of the 9 EAA (5). This approach, called the "EAA-9 Equivalence," provides a transparent and additive framework for evaluating diet quality and optimizing personal nutrition.

Discoveries supporting meal distribution

There have been three critical discoveries that have modified our understanding of adult protein needs and led to new concepts about the importance of meal distribution. (1) The first discovery involved elucidating the role of the branched-chain amino acid leucine in regulating the meal response of MPS. The discovery of the regulatory role for leucine highlights the difference between the minimum protein required to provide amino acids as building blocks for new proteins versus an optimal protein intake for metabolic roles. (2) The second discovery was that aging results in a decreased response of MPS to a protein meal but that the age-related decline in efficiency could be overcome by increasing the EAA content of individual meals. (3) The third discovery was the finding that the post-meal anabolic response of MPS has a finite duration of 2 to 3 h, suggesting that a single large protein meal (i.e., dinner) might not be the optimal protein distribution for older adults.

Muscle protein synthesis responds to meal content of leucine in adults

In the 1970s, multiple investigators provided *in vitro* evidence that among all amino acids, leucine had a unique potential to stimulate MPS (6–8). Using isolated diaphragm muscle or the perfused hemi-corpus, these investigators demonstrated that leucine could stimulate protein synthesis in fasted rats, and the response was associated with increased activation of ribosomes (i.e., binding of ribosomes to mRNA), the cellular structures for assembling amino acids for creating new proteins.

Regulation of protein synthesis is complex, but on a macro-level, it can be viewed at two distinct stages: transcription and translation. Transcription reflects gene expression and long-term regulation of the capacity for protein synthesis by controlling the amounts of ribosomes, mRNA, tRNA, and enzymes, while translation reflects short-term regulations of protein synthesis primarily through regulation of proteins called initiation factors that control the activity or efficiency of the protein synthesis machinery (i.e., ribosomes, mRNA, and tRNA).

To test the specific effects of leucine on transcription versus translation, the research group at the University of Illinois conducted an experiment examining muscle protein synthesis with different lengths of food deprivation, including fed, 24-h fasted, and 72-h fasted treatment groups (9). The hypothesis was that leucine would have the greatest effects during short-term food restriction, reflecting regulation at the translation stage, while prolonged starvation would impact transcription and reduce the potential of leucine to stimulate MPS. Consistent with the hypothesis, leucine exhibited the greatest stimulation of MPS in the 24-h fasted animals with minimal to no effect after 72 h. These findings provided evidence that the anabolic effects of leucine were at the initiation stage of MPS and reflected metabolic regulations for recovery after a short-term catabolic period (i.e., in this case, food restriction). This aspect was an early indication that the composition of a meal could alter the rate of MPS.

Proof for the mechanism would wait for more than a decade to develop an antibody methodology for quantitative analysis of the proteins involved in initiation. In collaboration with colleagues at Penn State University, we demonstrated that MPS recovery after an acute catabolic period was regulated in large part by the eIF4 initiation complex (i.e., eIF4E and eIF4G), which is a key regulatory factor for the activation of mRNA and stimulation of MPS (10). Using exhausted exercise to generate an acute catabolic condition, we found that MPS was depressed by over 30% from the pre-exercise stage. Furthermore, this inhibition of MPS was produced by binding an inhibitory protein, binding protein 1 (BP1), to the eIF4E subunit of the eIF4 complex, creating an inactive complex. We showed that the BP1 binding could be reversed within an hour of feeding protein, allowing for eIF4E and eIF4G to bind together and creating the active eIF4 initiation complex

Abbreviations: BP1, inhibitory binding protein 1; BMI, body mass index; DEXA, dual-energy x-ray absorptivity; EAA, essential amino acids; eIF4 (eIF4E and 4G), eukaryotic initiation factor 4; LBM, lean body mass; mTORC1 (mTOR), mechanistic target of rapamycin complex 1; mRNA, messenger RNA; MPS, muscle protein synthesis; rpS6, S6 ribosomal protein; tRNA, transfer RNA.

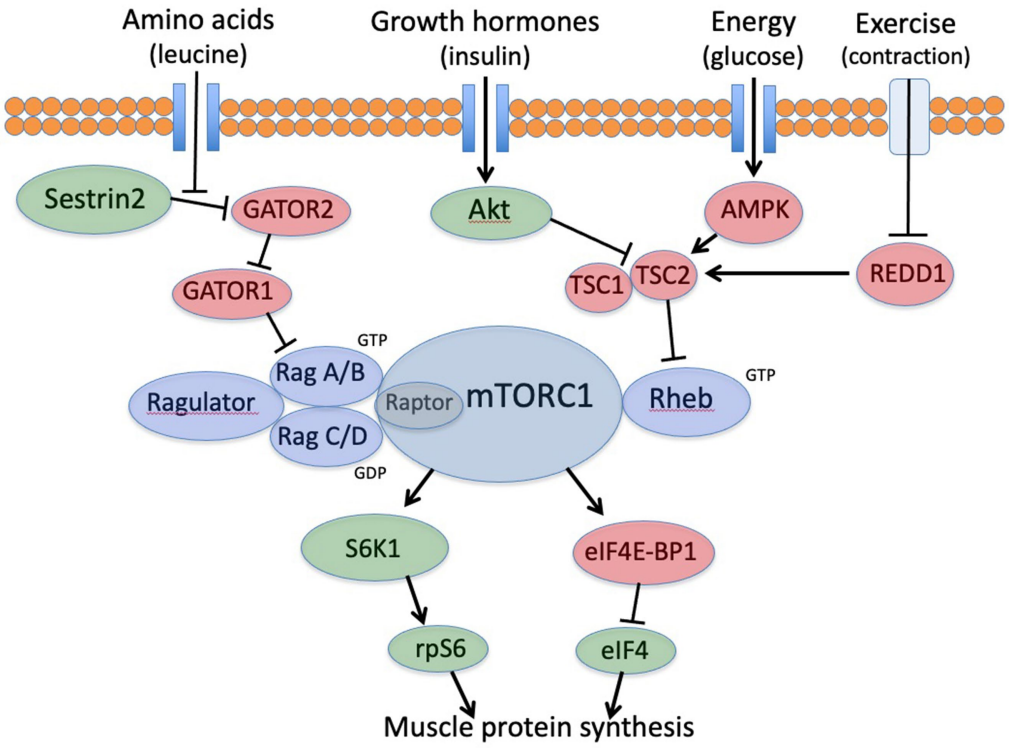

FIGURE 1
mTORC1 signaling cascade for translation initiation in skeletal muscle. mTORC1, mechanistic target of rapamycin; rpS6, ribosomal protein S6; S6K1, S6 kinase; eIF4-BP1, inhibitory binding protein complex; eIF4, active eIF4 initiation complex.

for the stimulation of MPS. Subsequently, we demonstrated that eIF4 activation was dependent on the cell concentration of leucine (11).

In the past 20 years, multiple laboratories have fully elucidated the leucine-mTORC1-eIF4 regulatory mechanism (Figure 1) (12, 13). The mTORC1 regulation is sensitive to multiple metabolic inputs, including amino acids (primarily leucine), hormones (primarily insulin), energy (regulated by AMPK), and resistance exercise (regulated via REDD1 and Sestrin 2) (14–16). When these inputs are optimally balanced, mTORC1 activates the downstream factors eIF4 and rpS6 (S6 ribosomal protein) to initiate MPS. These two regulatory factors serve to enhance MPS by selecting mRNAs to increase the capacity for MPS and to specifically increase the synthesis of myofibrillar proteins (17). It is important to note that the mTORC1 regulation in skeletal muscle differs from other tissues because it is sensitive to exercise (11, 18). Furthermore, the anabolic impact of insulin in skeletal muscle declines with aging while the importance of leucine increases (11, 18, 19). Other tissues remain sensitive to insulin with no known effects of exercise (19).

The efficiency of protein synthesis response to a meal declines with aging

The second discovery was that older adults require increased amounts of EAAs to stimulate MPS. Aging reduces metabolic efficiency. My first research project in graduate school was studying age-related changes in protein synthesis (20). We discovered that the fundamental mechanisms for protein synthesis involving ribosomes and mRNAs decreased in both capacity and efficiency with increasing age. The age-related decline in MPS reduces the capabilities for repair and remodeling of skeletal muscle and is considered a central cause of muscle atrophy and sarcopenia (21). However, the inevitability of these age-related changes began to be reevaluated during the late 1990s with the findings that infusion of EAA into older adults to produce hyperammonemia could produce a robust MPS response (22). This study demonstrated that with sufficient increases in plasma amino acid concentrations, the older adults retained a capacity similar to younger adults to stimulate MPS.

Subsequently, the research group in Galveston, TX, compared meal responses of MPS in young adults (~28 years old) versus older adults (~68 years old) (23). Both groups fasted overnight and were then provided an oral dose (i.e., breakfast meal) of 6.7 g of EAA created to mimic the composition of EAA in whey protein (~15 g of whey protein). Analyzing muscle biopsies, the young adults exhibited a significant increase in MPS, while the older adults exhibited no response from the oral dose of EAA. They repeated the experiment but enriched the EAA mixture with leucine from 1.7 g in the control group up to 2.8 g in the enriched group (24). The younger adults got no added benefit from the leucine enrichment, while the older adults exhibited a rate of MPS equivalent to the younger adults. These findings demonstrated that the age-related decline in MPS could be overcome by increasing the amount of leucine in the meal and suggested that MPS has an upper limit to a meal response.

The Galveston studies also highlight the important discovery that the meal effect of leucine observed in older adults is not present in younger adults. MPS in younger adults (and presumably children)

appears to respond in proportion to the amount of protein in a meal. Moore et al. (25) reported that in 22-year-old males, meals containing 5, 10, or 20 g of whey protein produced a nearly linear response in MPS in proportion to the protein in the meals. Assuming the whey protein used in the meals contained ~11% leucine, the meals provided approximately 0.55, 1.1, or 2.2 g of leucine, illustrating that the leucine effect on regulating MPS observed in older adults was not evident in the young adults. Churchward-Venne et al. (26) reported a similar proportional response of MPS with 27-year-old men consuming test meals of 15 g or 30 g of milk protein. Contrary to these findings, older adults generate no meal response to 1.7 g of leucine (equivalent to ~15 g of whey protein) but demonstrate a robust response to 2.8 g of leucine (equivalent to ~26 g of whey protein) (24). These findings led to the concept of a "meal threshold" requirement for leucine to produce an anabolic response in older adults (Figure 2). A meal threshold for dietary protein and specifically leucine represents a significant modification to dietary protein recommendations (2, 3, 27). These data provide support for the theory that both the amount of protein and the EAA composition of individual meals impact the anabolic response of skeletal muscle in older adults.

While a minimum meal threshold for leucine and total protein to stimulate MPS in older adults has been established, the maximum anabolic response to protein at a meal remains controversial (3, 28, 29). Studies have shown that the MPS response after a meal follows a logarithmic pattern trending toward a plateau with decreasing efficacy of higher protein meals (25, 26, 30, 31). Moore et al. (25) found linear increases in MPS response with meals from 5 g up to 20 g of protein with no significant increase from 20 g to 40 g. Similarly, Churchward-Venne et al. (26) reported a proportional response of MPS with protein meals providing 15–30 g but no detectable difference from 30 to 45 g. Consistent with these findings, other studies have shown that meals containing 70–90 g of protein produce similar rates of MPS as meals containing 30 or 40 g of protein (32, 33). While it seems logical that there is some cellular limit to the anabolic response to a protein meal, other investigators have argued that there is no upper limit to the anabolic response to ingested protein. These investigators suggest that understanding of the anabolic response is confounded in studies

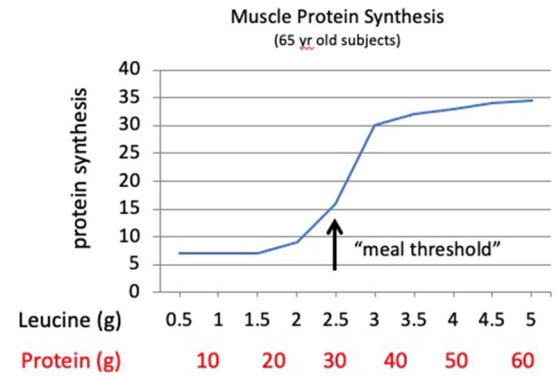

FIGURE 2
Theoretical response curve for muscle protein synthesis in older adults to increasing meal amounts of dietary protein or the amino acid leucine. Older adults demonstrate a "meal threshold" for leucine to stimulate the mTORC1 signal to initiate muscle protein synthesis. The protein amounts assume an average of ~8% leucine in meals with mixed protein sources.

of MPS because of a lack of measurement of protein breakdown (28) or because experimental designs lack sufficient duration of measurements to fully characterize the anabolic response (29). To fully characterize the optimal protein content of individual meals requires longer-term studies to establish changes in muscle mass.

An early demonstration of the impact of meal distribution was provided by the French group of Arnal et al. (34). They conducted a cross-over feeding experiment with 15 women with an average age of 68 years. The women consumed 64 g of protein daily throughout two 14-day trials. In one trial, the protein was distributed across four small relatively balanced meals (14, 20, 12, and 18 g/meal), called a spread pattern, while the other trial, known as a pulse pattern, the protein was distributed in three uneven meals (4, 51, and 20 g/meal) but with a single large meal. With the same daily intake of total protein, the pulse pattern generated higher rates of protein turnover and more positive nitrogen balance, resulting in greater fat-free mass after only 14 days. This study is consistent with a meal threshold hypothesis. Assuming that the leucine content of the meals created with a mixture of dietary proteins was ~8%, the spread pattern provided less than 1.7 g of leucine at any meal, while the pulse pattern grouped the dietary protein into a single meal providing more than 4.0 g of leucine. Similar benefits of a pulse meal pattern have been observed in hospitalized, bedrest elderly patients (35).

These findings are consistent with age-related changes in the metabolic roles of the EAA leucine. In young adults and children, leucine, along with growth hormones, contributes to the translational control of mTORC1 for MPS (18, 36), but the MPS response in young individuals appears to be proportional to the amount of protein in the meal (25). In older adults, leucine has a more specific role as a dietary signal, communicating to skeletal muscle that the meal contains adequate protein to support an MPS response (24). After a meal, activation of mTORC1 requires a twofold to threefold increase in plasma and intracellular leucine concentrations to stimulate MPS (Figures 1, 2). This metabolic role of leucine highlights the difference between the minimum dietary requirement for protein defined by the RDA versus an optimal metabolic need. The minimum leucine requirement defined by the Institute of Medicine is ~2.7 g/day for a 70-kg person (37), while the optimum amount of leucine to stimulate MPS is a minimum of 2.5 g/meal or approximately 7.5 g/day, nearly 3 times the minimum RDA (2, 3, 5).

The anabolic response of muscle protein synthesis has a finite duration after a meal

The third important finding that supports meal distribution was the elucidation of the duration of the MPS anabolic response to a meal. When leucine meets the required meal threshold for activating mTOR and the initiation factors, it triggers MPS. The duration of this anabolic response ranges from 2 to 2.5 h after the meal (38, 39). Using whey protein, which is rapidly digested, the leucine concentration in the blood rises rapidly, stimulating MPS, which peaks at 60–90 min after the meal and declines back to the fasted baseline by ~180 min. Understanding the meal duration led to the concept of oscillating anabolic and catabolic periods for muscle protein turnover. After a meal, there is an anabolic period

when MPS exceeds muscle protein breakdown, and then during post-absorptive times, there is a catabolic period when MPS declines, and protein breakdown exceeds synthesis. The catabolic period is most significant during the long overnight fast when skeletal muscle serves as a reservoir to provide amino acids to maintain essential protein turnover in vital organs.

A logical explanation for the decline in MPS after a meal would be the depletion of amino acids as they are incorporated into new protein structures. However, amino acids tend to remain elevated in the blood for 4 or 5 h or longer, depending on the amount and types of protein in the meal. However, more importantly, leucine and the regulatory proteins eIF4 and rpS6 remain elevated after MPS declines to baseline (39, 40). The limited duration response of MPS has been characterized as "muscle full" or a "refractory period" when MPS appears to be unresponsive to normal activation signals (38, 39). The underlying explanation remains speculative; however, the refractory period may be associated with declining levels of ATP to support the energy needed to maintain the elongation phase of protein synthesis (40).

The refractory period for MPS raises questions about second-meal responses. The importance of the leucine signal and the amount of protein in the first meal after an overnight fast to stimulate MPS are well-established. During catabolic periods, such as fasting or exhaustive exercise, the initiation factor eIF4 is inhibited by binding with BP1 (10, 13). This inhibition is reversed by the activation of mTORC1 and the downstream initiation proteins. While the MPS response to the first meal has been studied extensively, the MPS response to a second meal has not been studied. The findings that blood leucine and the regulatory proteins are still elevated 4 or 5 h after a first meal and after MPS returns to the fasted baseline (40, 41) suggest that the leucine threshold and eIF4 regulations may not be relevant at a second meal that occurs within 5 h after an initial stimulatory meal. Additional research is needed to characterize second meal responses and optimal dietary distribution of protein at mid-day meals.

Furthermore, the duration of the anabolic response to a meal has been recently questioned as an artifact of using rapidly digested proteins (29). These investigators suggest that consumption of 100 g of milk protein containing 80 g of slow-digesting casein can prolong the anabolic response to a meal up to at least 12 h.

Unanswered questions concerning meal duration and the oscillating pattern of protein turnover in skeletal muscle are as follows: (1) what causes MPS to decline after a meal, (2) is the observed decline an artifact of proteins selected and experimental design, and (3) is the meal response actually consistent across all meals. For example, is the first meal response after an overnight fast that inhibits translation initiation factors the same as the response to a mid-day meal when the initiation factors may still be fully active? To the best of my knowledge, there are no studies of anabolic response after a second meal (i.e., lunch), and there is some evidence that the response to protein meals late in the day is significantly lower than to the first meal (42).

In total, the available evidence from both mechanistic and clinical experiments supports that optimizing the meal response to dietary protein should be an important strategy for adults who struggle to maintain adequate protein intake and overall nutrient density while confronting declining energy needs (43). Currently, in the United States, most adults consume nearly 60% of their daily protein in a single large meal late in the day, while breakfast and the mid-day meal typically contain only 10–20 g of protein. This distribution of dietary protein fails to reach the meal threshold for leucine at either of the first two daily meals (32) and may ultimately lead to insufficient total daily protein (43).

We tested the distribution theory for impact on MPS. Using a cross-over design with 15 adult women (~37 years old), the women consumed 90 g of protein from mixed food sources (i.e., leucine content ~8%) for 7 days in either an unbalanced or balanced meal pattern (44). In the unbalanced trial, the protein was distributed as 10, 20, and 60 g at breakfast, lunch, and dinner, respectively, similar to consumption patterns in the United States. In the balanced trial, the women received 30 g of protein at each meal, designed to provide at least 2.5 g of leucine at each meal. After the first day and the seventh day, 24-h net protein synthesis was measured in skeletal muscles. While the women consumed the same total protein each day, the balanced meal distribution produced greater net 24-h MPS than the unbalanced distribution.

Evidence that protein distribution at meals impacts body composition

While the application of the meal threshold hypothesis has been tested by redistributing protein from dinner to the first meal to enhance MPS (44), the long-term effects on body composition and muscle mass remain unclear. There are studies reporting the benefits of meal distribution of protein for body composition and muscle mass (44–46), while other studies fail to find significant effects (47). The inconsistency of the findings may, at least in part, be explained by considering the likely magnitude of body composition changes during short-term studies, which are likely within the detection limits considering variations among subjects and current body composition methods.

Meal distribution of dietary protein impacts body composition in animals

To test the meal distribution hypothesis and estimate the magnitude of the body composition effects, we designed a meal distribution study with adult rats (48). Rats were trained to consume meals similar to the meal pattern used in our human MPS study (44), with their daily ration partitioned at meals providing 4, 4, and 6 g of food. One group of rats received protein in a balanced pattern with 16% of energy (%En) from protein at each meal, while the other group received an unbalanced distribution of 8%En, 8%En, and 27%En, respectively. The total daily diets for both groups were exactly the same for calories, protein, carbohydrates, fat, and fiber. The only difference was the distribution of the protein and carbohydrates. The design was built around both protein and leucine distributions. Previous studies (28) identified the meal threshold for leucine with this age and size of adult rats as 55–60 mg. With the balanced distribution, the meals provided 74, 74, and 111 mg of leucine, and in the unbalanced distribution, the meals provided 38, 38, and 184 mg. With the balanced distribution, all three meals provided sufficient leucine to activate MPS, but with the unbalanced distribution,

only the last meal exceeded the leucine threshold for activation of MPS.

After 2 and 11 weeks, MPS was determined after the first meal, and eIF4, rpS6, and MPS were found to be 30 to 45% higher in the animals consuming the higher leucine meal. Body composition was measured by DEXA at 11 weeks. Surprisingly, there were no significant differences in fat mass or fat-free mass between the groups, suggesting the meal distribution had no effects. However, direct dissection of tissues revealed that the hindlimb muscle mass was ~10% larger in the animals with the balanced distribution, while the liver was ~10% larger in animals receiving the unbalanced distribution with the large dinner meal (48). These findings are consistent with the leucine threshold hypothesis for MPS and also demonstrate that whole-body DEXA measurements do not differentiate small, tissue-specific changes in lean body mass.

Meal distribution of protein impacts body composition during weight loss

Recognizing that meal distribution likely has a small impact on muscle mass and is likely secondary to protein quantity and quality, definitive proof for benefits related to aging and sarcopenia that are characterized by changes of only 5–8% per decade will be difficult to obtain. An alternative approach is to apply meal distribution concepts during weight loss when body weight is changing more rapidly, and lean body mass can account for up to 50% of the total weight lost.

We applied the leucine threshold and meal concepts to a series of weight-loss studies (49, 50). These studies modified both the quantity and the meal distribution while protein quality remained similar across treatment groups. In each of the studies, the diet design was the same, and the daily energy restriction was approximately 500 kcal from their pre-study diet. Participants were randomly assigned to either a high carbohydrate, low protein diet (55%En carbohydrates, 30%En fat, 15%En protein; 0.8 g protein/kg body weight) with meals providing 10, 15, and 45 g of protein, respectively, or to a reduced carbohydrate, higher protein diet (40%En carbohydrates, 30%En fat, 30%En protein; 1.6 g/kg) with protein distributed as 35, 35, and 50 g. While higher protein at the first meal has been shown to enhance appetite regulation (satiety) and thermogenesis, the hypothesis for these studies was that increasing protein at the first meal would enhance MPS, minimizing loss of lean body mass and resulting in greater loss of body fat.

In the 12-month diet study, 130 overweight men and women (BMI ~33; age ~ 45 years) were randomly assigned to either the low-protein or high-protein diet groups (49). The average weight loss at 12 months was 24% greater in the higher protein group with significantly greater loss of body fat (5.3 kg vs. 7.3 kg, in low- and high-protein groups, respectively). Loss of lean body mass (LBM) was similar (2.7 kg vs. 2.6 kg, respectively); however, the net change in body composition was significantly different, with LBM accounting for 34% of the weight loss in the low-protein group and 26% in the higher protein group.

Similarly, in a weight loss study conducted with community-dwelling older adults (~70 years old), participants who voluntarily shifted daily protein intake from dinner to earlier meals lost more total weight and more body fat without changing total daily protein intake (51). The researchers concluded that "a more even pattern of protein intake was associated with a greater decline in BMI and abdominal fat".

In a second study utilizing the same diet protocol, we evaluated the additive and synergistic effects of dietary protein and resistance exercise on body composition changes during weight loss (50). Utilizing a 2 × 2 design, 48 women (BMI ~33; age ~ 46 years) were randomly assigned to one of four treatment groups: low protein, low protein with exercise, higher protein, and higher protein with exercise. Similar to the previous study, the dinner meals were similar across all groups. The primary diet differences were increased protein and reduced carbohydrates at the first two meals in the higher protein groups. After 16 weeks, the higher protein (diet only) group lost 12% more body weight, 18% more body fat, and 25% less lean body mass compared to the low protein group. Consistent with the previous study, 35% of the weight lost for the low protein group was fat-free mass, and 25% for the higher protein group.

The exercise treatment consisted of 5 days/week of walking for 30 min and 2 days/week of resistance exercise (49). After 16 weeks, the higher protein + exercise group lost 46% more body weight, 60% more body fat, and 40% less fat-free mass compared with the low protein + exercise group. This study demonstrated the synergistic effects of dietary protein and exercise to improve body composition during energy restriction for weight loss. Furthermore, the addition of 16 weeks of exercise to the low protein treatment group resulted in the loss of an additional 0.5 kg of body fat compared with the low protein group without exercise, while the addition of exercise to the higher protein group resulted in the loss of an additional 2.9 kg of body fat compared to the diet group without exercise. To the best of our knowledge, this was the first study to demonstrate the interactive effect of dietary protein and exercise on improving body composition in adult women during weight loss.

While these weight loss studies appear to demonstrate the benefits of increased protein at the first meal, the studies do not differentiate effects due to increasing daily quantity versus meal distribution. However, the studies build on the discoveries that increasing dietary protein at the first meal stimulates MPS and increases net MPS for the day. The assumption inherent to this design was that adding 50 g of additional protein to a dinner meal that already contained ~50 g of protein would have a minimal additive effect on net daily MPS (25, 26, 32) or muscle mass (45).

Population survey support for meal distribution of protein

Population studies, in general, have not focused on meal distribution of protein, and the quality of information on meal-specific protein distribution is limited in most food surveys. The NHANES data reveal that higher daily protein intake is inversely correlated with BMI and waist circumference (52), and the findings appear to be associated with increased protein at breakfast (46, 47). Again, meal distribution is often intertwined with total protein intake. Studies using NHANES data show that adults consuming 2 or 3 meals with at least 25 g of protein at each meal are more likely

to meet the minimum RDA for protein (43) and maintain greater muscle mass (44, 46). Kim et al. (47) reported adults who consume a greater percentage of their total daily protein at breakfast maintained greater muscle mass and grip strength than individuals consuming a high percentage at the dinner meal. These same investigators also conducted an intervention study and found that supplementing 30 g of protein at the breakfast meal with older adults produced greater muscle mass than supplementing 30 g of protein at the dinner meal. Overall, while the number of studies is limited, population-based surveys appear to support the merit of multiple protein meals per day, with increased protein at the breakfast meal providing additional value.

Summary and conclusion

In summary, the direct effects of meal distribution of dietary protein on muscle mass in older adults are difficult to assess. Changes in mass occur slowly and are likely small in magnitude, and methods for directly measuring muscle mass are limited. There is a general assumption that short-term measurements of MPS provide a biomarker for anabolic changes in muscle mass; however, changes in MPS are of much greater magnitude than changes in muscle mass (53). Still, there are some fundamental metabolic responses that support meal distribution. The first is the discovery of the meal threshold for leucine to trigger MPS and the related discovery of the duration of the post-meal anabolic response. Triggering the mTOR signal complex to initiate MPS requires approximately 3.0 g of leucine, which is equivalent to a meal containing approximately 30–35 g of high-quality protein, and once activated, MPS will remain elevated for approximately 2.5 h. Adding more protein to a meal does not increase the magnitude or duration of the anabolic period (25, 26). The logical extension of these findings is that adding protein to a low-protein meal would be more beneficial than adding protein to an existing meal already containing maximum protein for MPS effects. Furthermore, there is a general belief that MPS is most responsive at the first meal after an overnight fasting period. Essentially, every study of MPS in either humans or animals has been done at the first meal, maximizing the recovery of translation initiation factors inhibited during the overnight fast. If MPS measured at the first meal is not a relevant biomarker for anabolic changes in muscle mass, then the significance of studies measuring MPS after this first meal must be re-evaluated.

Furthermore, evidence accumulates that protein quantity and meal distribution are interrelated in protecting adult muscle mass. The first priority is achieving a single meal with adequate protein and leucine to stimulate MPS (26). If the daily protein intake is limited to the RDA of 0.8 g/day (~60 g/day), the daily protein intake needs to be aggregated into at least one meal with >35 g of protein. Evenly distributing the low protein intake across multiple meals with <20 g of protein minimizes MPS responses and the benefits to skeletal muscle. However, if protein intake is higher (~1.6 g/kg; 120 g/day), adding additional protein to large dinner meals that may already provide >50 g of protein is likely inefficient for muscle benefits. Research demonstrates that adding protein to the first meal enhances MPS and produces benefits to muscle mass and body composition (46–51). The application of these findings and the meal distribution hypothesis to long-term muscle health, such as aging and sarcopenia, remains difficult to prove and awaits additional research.

Recommendations

Based on the weight of available evidence, we believe that older adults benefit from daily protein intakes above the RDA ranging from 1.2 to 1.6 g/kg (27). Furthermore, the evidence supporting the anabolic response at the first meal is robust, and we strongly recommend increasing protein intake at breakfast to at least 30 g of high-quality protein (2, 3). The optimal distribution of dietary protein across all meals requires additional research and an integrated understanding of the interrelationships of dietary protein quantity, quality, and meal distribution with age and physical activity.

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Conflict of interest

DL participates on speaker's bureaus for the National Dairy Council (NDC) and the National Cattlemen's Beef Association (NCBA); he is a nutrition consultant to NCBA; and he serves on Scientific Advisory Boards for The Nutrient Institute, Institute for the Advancement of Food and Nutrition Science (IAFNS), and Herbalife.

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Compartmental analysis: a new approach to estimate protein breakdown and meal response in health and critical illness

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Purpose of review: This study aimed to discuss the use of the pulse stable isotope tracer approach to study changes in metabolism in healthy individuals and critically ill patients.

Recent findings and conclusion: We found that in the postabsorptive state and healthy condition, intracellular protein breakdown and net intracellular protein breakdown, when calculated using the pulse tracer approach, are about double what has previously been reported using the more traditional primed-constant and continuous stable isotope approaches (600 versus 300 grams of protein/day). In critically ill patients, protein breakdown is even higher and calculated to be approximately 900 grams of protein/day, using the pulse tracer approach. Based on these data, we hypothesize that reducing protein breakdown in the postabsorptive state is key when trying to improve the condition of critically ill patients. Moreover, we also used the pulse tracer approach during feeding to better estimate the intracellular metabolic response to feeding. Our first observation is that endogenous protein breakdown does not seem to be reduced during feeding. We also have shown that when consuming a meal with a certain amount of protein, the biological value of that protein meal can be calculated with the pulse tracer approach. In conclusion, using the pulse stable isotope tracer approach to study protein kinetics in the postabsorptive state and during feeding expands our understanding of how dietary proteins can affect human protein metabolism. The intracellular protein synthesis stimulatory effect of a meal is an important factor to consider when calculating the exact protein requirements and needs, particularly in critical illness.

KEYWORDS

amino acids, critically ill, ICU, stable isotopes, nutrition

Introduction

Meeting the enhanced needs of critically ill patients through optimal protein intake has been studied for many years. The current protein intake requirements (1) are based on the requirements established for healthy humans, to which a multiplication factor is added. However, protein turnover is highly upregulated in critically ill patients (2–4), suggesting an increased availability of amino acids as more amino acids are released intracellularly and into the circulation from protein breakdown (PB). Therefore, a higher disposal of those amino

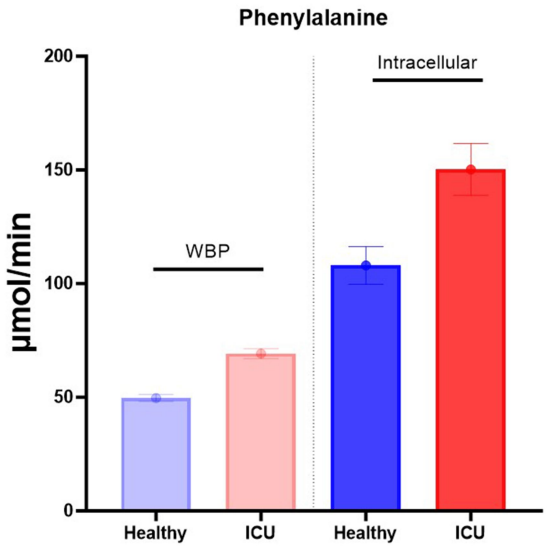

FIGURE 1
Intracellular phenylalanine production is much higher than WBP. Data are from Deutz et al. (4).

TABLE 1 Recalculation of postabsorptive protein breakdown as grams of protein/day in humans.

	Healthy	ICU	ICU minus healthy
Non-compartmental PB (WBP)	292 [286, 305]	411 [398, 424]	115 [100, 131]
Non-compartmental net PB	44 [42, 47]	31 [29, 33]	−13 [−17, −10]
Intracellular PB	642 [592, 691]	892 [825, 960]	251 [167, 334]
Intracellular net PB	87 [82, 92]	60 [56, 65]	−26 [−33, −20]

Data are grams of protein/day [mean (95% CI)] protein breakdown (PB), obtained from recalculation of data, described previously (4). We used phenylalanine and tyrosine decay curve parameters to calculate the non-compartmental PB (comparable to the rate of appearance in plasma), intracellular production, and net protein breakdown, using the conversion of phenylalanine to tyrosine. We assumed that 4% of protein is phenylalanine.

acids will become available for protein synthesis and other disposal routes. In steady-state conditions, the production of amino acids released from PB is in balance with the disposal of those amino acids.

Stable isotope tracer methodology is often used to measure amino acid kinetics (5). The basic principle is that the dilution measurement of the infused stable isotope amino acid makes it possible to calculate the endogenous substrate production and disposal, and when infusing stable isotopes of essential amino acids, PB can be estimated (6). Protein turnover in healthy individuals and during a variety of disease states, including critical illness, has predominantly been calculated using traditional methods such as primed-constant and continuous infusion of combinations of stable isotope amino acids such as leucine, phenylalanine, and tyrosine (7, 8). This approach requires intravenous infusion of stable amino acid tracers using a calibrated pump and accurate priming of the tracer pool to instantly obtain a tracer steady state, which is not always easy (4).

Recently, we reported a novel pulse tracer approach that enables the calculation of the intracellular production of amino acids (2–4, 9, 10). When using this approach, a pulse of stable isotopes is administered intravenously in a small volume and within 10 s. Measuring the decay of the isotope enrichments in plasma makes it possible to calculate simultaneously both the whole-body production (WBP) rate of these amino acids [is equal to the non-compartmental rate of appearance (Ra) as calculated by the primed-constant and continuous infusion model (7, 8)], and the intracellular production rate (3, 4) from the compartmental analysis. We have used this approach to compare the WBP and intracellular production of amino acids to better understand the balance between protein degradation rate and dietary protein intake (Figure 1).

Protein turnover measurement in the post-absorptive state

As critically ill patients can have a wide range of changes in plasma amino acid concentrations (2, 11), correct priming of the pool to obtain instant tracer and tracer product steady state can be very difficult (12). To overcome this, we developed a pulse amino acid tracer approach (9), which does not need an infusion pump or knowledge of the pool sizes, and requires much smaller amounts of tracers than the traditional primed-constant and continuous infusion method. The pulse tracer approach is therefore an easy-to-use method for critically ill patients to study in depth their whole-body amino acid kinetics. Amino acid production can be assessed by calculating the area under the curve (AUC) of the tracer-tracee ratio decay (10) in the measured time period or by fitting the decay with a 2 exponential functions (3).

By measuring the phenylalanine production in healthy and critically ill patients (Figure 1), we can calculate the turnover of protein per gram protein/day/subject (Table 1 and Supplementary Figure S1) (4). As previously reported by others (13), ~300 grams of protein are broken down per day in a healthy individual in the postabsorptive state (4) as measured by the primed-constant and continuous stable isotope infusion protocol. If food only increases protein synthesis (see later), a dietary intake of approximately 75 grams of balanced protein would enhance protein synthesis to 375 grams, which is an ~25% increase.

The leucine/KIC approach (reciprocal model) (6) is often used to better estimate the total PB by assuming that the plasma KIC enrichment represents better the whole-body intracellular enrichment of leucine than the plasma leucine enrichment, as the conversion of leucine to KIC mainly takes place in muscle (14). PB measured with the KIC plasma enrichment is 1.3 times higher than when using the leucine plasma enrichment (15). In contrast, using the pulse approach, PB appears to be 2.6 times higher when calculated with the intracellular appearance of leucine than when calculated with Ra (4). Therefore, using plasma KIC enrichment to better estimate PB is not sufficient to correctly estimate intracellular PB. One of the reasons for this difference could be that it is based on the assumption that the conversion of leucine to KIC takes place in all organs at the same rate. Therefore, although this approach seems better than using plasma

Abbreviations: PB, Protein breakdown; WBP, Whole-body production, comparable to the rate of appearance.

enrichment of leucine (6), it does not seem to correctly estimate intracellular PB.

As net PB depends on the careful measurement of oxidation when using the leucine/KIC approach, we and others used the combined phenylalanine/tyrosine approach, which only needs plasma enrichment measurements. The pulse approach with compartmental analysis and the phenylalanine/tyrosine approach, in our opinion, have the advantage of only requiring plasma enrichment measurements to calculate intracellular PB.

However, calculating PB from intracellular production (Table 1) leads to approximately 650 grams/day of PB in the postabsorptive state. In this case, a dietary intake of approximately 75 grams of protein/day would only lead to an 11% increase in protein synthesis. Consequently, if we calculate the net protein loss, which is the difference between PB and synthesis, a net loss of 87 grams of protein/day will take place in a healthy individual when there is no food intake. The net loss would be less if protein synthesis was lower in the absence of protein intake. However, during 12 h fasting (16), PB and oxidation of leucine are not increased, while after 3 days of fasting, protein oxidation is increased by 13% and PB by 30% (17). We therefore conclude that it is likely that the PB rate is not reduced by 24 h of fasting, and thus that our calculations of net loss are probably a good estimation.

In critically ill patients (Table 1), both non-compartmental PB and intracellular PB are increased by approximately 40% as compared to the healthy state. When using the intracellular PB measurement, protein degradation is approximately 900 grams/day and net protein loss is approximately 60 grams/day, which clearly shows that the turnover of protein is substantially increased in relation to the net loss. However, the net loss in critically ill patients is only 6.6% of total PB.

The calculations of net protein synthesis and breakdown are also affected by intracellular appearance. The calculation of phenylalanine hydroxylation as a proxy for oxidation is the enrichment ratio between plasma phenylalanine and the phenylalanine product tyrosine multiplied by the appearance of tyrosine. The conversion of phenylalanine to tyrosine occurs intracellularly, and thus the ratio estimates the correct ratio for both Ra and intracellular appearance calculations. However, the Ra of tyrosine underestimates the intracellular appearance of tyrosine, and therefore, the calculation of net protein synthesis/breakdown is higher than when the Ra of tyrosine is used.

One remarkable observation is that net PB in critically ill patients is not increased but decreased in the postabsorptive state (Table 1 (2–4)). We believe that we should try to interpret this observation physiologically. We hypothesize that reducing net protein loss in ICU patients could be a protective mechanism to reduce protein loss during disease. We also observed the same phenomenon in patients with chronic illnesses or at a higher age (18). Further research is needed to provide a more mechanistic explanation.

TABLE 2 Estimated net lean mass loss in grams of protein/day in humans when no food is provided.

Healthy	577 gram [544, 611]
Critically Ill	402 gram [373, 431]
ICU minus healthy	−175 gram [−220, −131]

Data are gram lean mass/day loss, calculated from intracellular net protein breakdown, assuming lean mass contains 15% protein.

According to the calculations of the reduction of net PB in critically ill patients and thus of loss of lean mass, using the compartmental calculations (Table 2), critically ill patients will still lose approximately 400 grams of lean tissue/day (0.8%/day when total lean mass is approximately 50 kg). Others have found that loss of muscle mass, which is approximately 50% of total lean mass (19) in critically ill patients in the ICU is approximately 1%/day (20).

So how can protein loss be attenuated in healthy subjects in the postabsorptive state? We calculated (Table 1) that net protein loss in healthy subjects is approximately 87 grams, indicating that at least 87 grams of dietary amino acids are needed for a healthy subject to become anabolic. When protein is ingested, other factors such as digestion play a leading role in reduced protein efficiency, which may partly explain the higher protein intake advised (21). Reduced digestibility of dietary proteins likely becomes even more important in critically ill patients.

What are the clinical implications of these observations (2–4)? If PB is much higher in critically ill patients than previously thought, the protein synthesis rate will also increase. The energy costs of protein synthesis are approximately 1.3 kcal/gram protein (22), suggesting that these energy costs of critically ill patients are approximately 900 × 1.3 kcal = 1,170 kcal, 65% of the total 1,800 kcal REE we measured in critically ill patients (2). In addition, we suggested that not so much the amount of protein intake needs to be increased in critically ill patients, but that particularly the upregulated PB needs to be reduced (4). Therefore, we hypothesize that critically ill patients need dietary components that can reduce PB. We recently showed a reduction in PB when providing HMB to critically ill patients (23). However, additional research is needed on whether certain dietary amino acids and/or proteins are also able to reduce PB, as we previously showed for arginine in the critically ill (24).

Protein breakdown and synthesis during feeding

During feeding, there is an increase in amino acids released into the circulation, due to enhanced digestion and absorption of the meal-derived amino acids and from amino acids that become available from intracellular PB. The increased appearance of amino acids in the circulation and intracellularly will stimulate the disposal of amino acids (mainly for protein synthesis) and result in an increased intracellular concentration that could reduce PB. Several studies, including our own, have observed a reduced endogenous PB when using the primed-constant and continuous tracer infusion model (7, 8).

One important complicating factor could be the splanchnic extraction of amino acids that could affect the dilution of plasma enrichment. Endogenous PB is the rate of appearance (Ra), corrected for the amount of tracee entering the whole-body pool from nutrition. So we need to establish how much of the meal-derived amino acids are absorbed in the gut. If we assume that absorption is 100% for free dietary amino acids, the amount of nutrition entering the body pool in the mucosa cell needs to be subtracted from the Ra (calculated from the primed-continuous or pulse approach) or the intracellular appearance (from the pulse approach) to estimate endogenous PB.

So how does splanchnic extraction of meal-derived amino acids (e.g., phenylalanine) play a role in calculating endogenous PB? The

basic assumption is that when using the Ra, a correction needs to be made for the Ra with the rate of meal-derived phenylalanine, appearing in the hepatic vein (thus post-splanchnic). The misunderstanding with this approach could be that the Ra only represents the PB of non-splanchnic organs and that subtracting the post-splanchnic appearance corrects the Ra on a whole-body level. In actuality, the Ra includes PB in all organs, and thus the absorbed meal-derived amino acids into the mucosa cell should be subtracted to calculate endogenous PB.

So what is the role of the splanchnic extraction measurement with the continuous stable isotope tracer infusion approach during feeding? In our opinion, this calculation is not needed at all. Only the estimation of how many amino acids from food are absorbed should be sufficient (6) to calculate endogenous PB. Using the pulse tracer approach, the same arguments will hold. There is no need to estimate splanchnic extraction to estimate endogenous PB. Therefore, the calculation that was used for many years, Ra = protein synthesis + oxidation = protein breakdown + intake from food remains valid (7, 25, 26). Using the pulse approach, Ra is replaced by the intracellular appearance.

However, it remained unclear whether the proteins in the meal were indeed able to reduce PB. We recently performed a pilot study on 11 human subjects to examine whether intracellular PB (using compartmental analysis) can also be measured in the prandial state (27, 28). For that purpose, we developed a protocol in which nutrition was provided every 20 min as sips containing a mixture of free amino acids, representing the composition of whey protein. Nutrition needs to be given as sips to obtain a steady state influx of dietary amino acids, and we previously observed that using a sip protocol gives comparable information on net protein synthesis and other measures, with some caveats (8). After the steady state was obtained, as verified by adding stable isotopes of amino acids to the sips and measuring the plasma enrichment and concentration of these amino acids, we administered the pulse of stable isotopes as previously conducted in the postabsorptive condition.

We subsequently compared the turnover of phenylalanine (as a measure of PB) obtained by non-compartmental and intracellular (compartmental) analyses in the prandial state (Figure 2, upper panel). Non-compartmental protein breakdown (WBP), measured during feeding (WBPfed), was approximately 50 µmol/min using this approach. When subtracting the WBP when no food was given (WBPfed—fasted), the difference in WBP was approximately 20 µmol/min. As the amount of phenylalanine given enterally was greater than the increase in WBPfed—fasted, the difference became negative (delta endoWBP). This means that there is a reduction in endogenous protein breakdown (endoWBP) when calculating the effect of feeding. A consistent reduction of PB during feeding has previously been observed by us (7, 8) and others (29–31) when WBP was measured using the primed-constant and continuous infusion models.

However, as indicated above, the non-compartmental protein breakdown calculation (WBP) underestimates the true intracellular PB, suggesting that our calculation of the intracellular PB provides a better reflection of the true PB. When the same calculations were performed using the intracellular PB approach (Figure 2, lower panel), the amount of phenylalanine given enterally as sips was very well matched with the difference between the intracellular appearance fed and fasted. Therefore, no reduction in PB was observed anymore. Therefore, we believe that feeding does not reduce endogenous PB and that these findings in the past might have likely been caused by the stable isotope tracer model used.

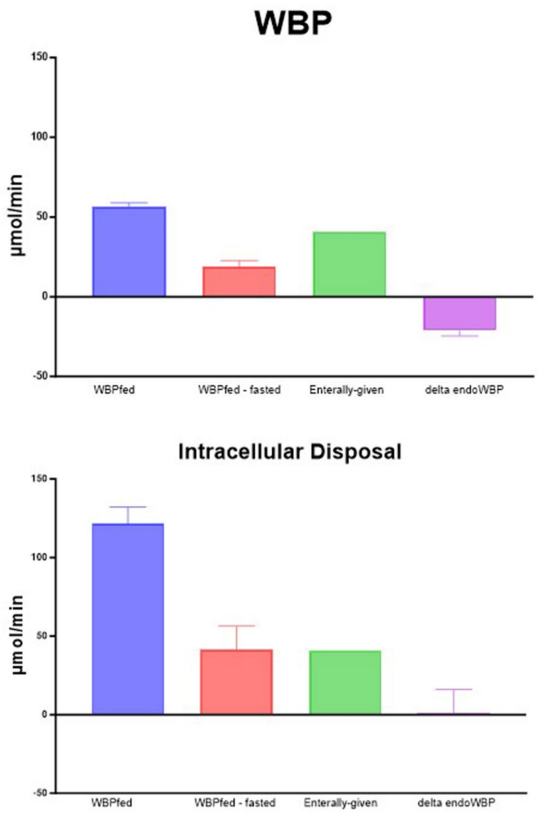

FIGURE 2
Phenylalanine production. WBPfed is the non-compartmental production during feeding. WBPfed—fasted is when the WBP in the post-absorptive condition is subtracted from the WBP during feeding. Enteral-given is the amount of phenylalanine given with the food. Delta EndoWBP is enterally given and subtracted from WBPfed—fasted, representing the change in the endogenous protein breakdown.

Perspectives and limitations

Our approach of combining sip feeding with the isotope pulse method can be used to measure the intracellular appearance of amino acids from any food protein. The intracellular appearance depends on how many amino acids are left after digestion and absorption of the food protein, the appearance of the plasma pool, and other factors that could have reduced the intracellular appearance and availability. Our pilot study shows that when using a mixture of free dietary amino acids and assuming that the digestion and absorption of free amino acids are not limited, the intracellular appearance of amino acids matches the amount consumed. However, a limitation of our observation is that we still do not know the exact digestion and absorption rates of amino acids.

Furthermore, when consuming a meal with a certain amount of protein, the biological value of that protein in principle can be calculated with our new approach, but this needs more validation studies. The same is true for complex meals with different types of

proteins. Protein synthesis stimulatory effects of a meal seem to be an important factor in calculating the exact protein requirements and needs.

Therefore, we propose to check our approach in critically ill patients during feeding to establish which factors affect the anabolic capabilities of certain dietary amino acid mixtures or proteins as it might guide nutritional approaches in the critically ill.

Conclusion

We have provided a new view on protein metabolism in the postabsorptive and fed state when using the pulse stable isotope tracer approach. We have concluded that the estimation of protein turnover with the primed-constant and continuous infusion protocol is too low and healthy human PB is more in the range of 600 grams of protein/day.

We also presented new data that show that during feeding, endogenous PB is likely not reduced by food, but that feeding only stimulates protein synthesis.

Author contributions

ND: Writing – original draft, Writing – review & editing. ME: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1388969/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Graphic representation of the data as shown in Table 1.

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Quantification and interpretation of postprandial whole-body protein metabolism using stable isotope methodology: a narrative review

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Stable isotopes are routinely applied to determine the impact of factors such as aging, disease, exercise, and feeding on whole-body protein metabolism. The most common approaches to quantify whole-body protein synthesis, breakdown, and oxidation rates and net protein balance are based on the quantification of plasma amino acid kinetics. In the postabsorptive state, plasma amino acid kinetics can easily be assessed using a constant infusion of one or more stable isotope labeled amino acid tracers. In the postprandial state, there is an exogenous, dietary protein-derived amino acid flux that needs to be accounted for. To accurately quantify both endogenous as well as exogenous (protein-derived) amino acid release in the circulation, the continuous tracer infusion method should be accompanied by the ingestion of intrinsically labeled protein. However, the production of labeled protein is too expensive and labor intensive for use in more routine research studies. Alternative approaches have either assumed that 100% of exogenous amino acids are released in the circulation or applied an estimated percentage based on protein digestibility. However, such estimations can introduce large artifacts in the assessment of whole-body protein metabolism. The preferred estimation approach is based on the extrapolation of intrinsically labeled protein-derived plasma bioavailability data obtained in a similar experimental design setting. Here, we provide reference data on exogenous plasma amino acid release that can be applied to allow a more accurate routine assessment of postprandial protein metabolism. More work in this area is needed to provide a more extensive reference data set.

KEYWORDS

absorption, anabolism, protein requirements, protein quality, RDA, splanchnic extraction, indicator amino acid oxidation

1 Introduction

All living tissues are in a constant state of protein turnover, regulated by the balance between protein synthesis and breakdown rates. This turnover provides tissues with plasticity, e.g., by replacing damaged protein or protein remodeling in response to stress. Furthermore, tissue can hypertrophy or atrophy, based on a prolonged net positive or negative protein balance, respectively. In a fasted state, protein balance is negative, resulting in a net loss of protein mass (catabolism). An influx of exogenous amino acids is required for protein balance

to become positive (anabolism) and offset fasted losses. Dietary protein intake is essential to maintain lean body mass, with the current recommended daily allowance (RDA) estimated at 0.8 $g \cdot kg^{-1} \cdot day^{-1}$ (1). However, it is generally believed that the RDA is insufficient to attenuate lean body mass loss during conditions such as energy restriction or aging (2, 3). Moreover, protein intakes exceeding the recommended daily allowance may further stimulate anabolism and elicit benefits such as improving the adaptive response to exercise, improving immune function, and accelerating wound healing (4–8). Therefore, there is much interest in the determination of the optimal dietary protein intake to maximize health and function and how this is modulated by factors such as protein quality, protein timing, and/or protein distribution. However, there is much debate on the methodology to accurately assess protein requirements and the impact of protein quality on post-prandial protein handling. Despite known limitations, recommendations for protein requirements and quality are currently primarily based on nitrogen balance, the Indicator Amino Acid Oxidation (IAAO) method, and Digestible Indispensable Amino Acid Score (DIAAS) (1, 9). Theoretically, the accurate assessment of whole-body protein metabolism would provide an ideal method to not only assess protein requirements and protein quality, but also provide insight in the underling metabolic rates (protein synthesis, breakdown, oxidation, and net balance). Whole-body protein metabolism can be quantified using stable isotope methodology (10, 11). By applying a constant amino acid tracer infusion and taking frequent blood samples, the assessment of postabsorptive whole-body protein metabolism is relatively simple. In contrast, the assessment of postprandial whole-body protein metabolism is more challenging when exogenous protein-derived plasma amino acid bioavailability (hereafter referred to as "exogenous plasma amino acid bioavailability") needs to be taken into account. Here we discuss (1) the plasma amino acid kinetics model to determine whole-body protein metabolism in the postabsorptive and postprandial state, (2) the impact of exogenous plasma amino acid bioavailability in the amino acid kinetics model, and (3) the various approaches available to determine and/or estimate exogenous plasma amino acid bioavailability and subsequently postprandial protein metabolism.

2 The plasma amino acid kinetics model

Whole-body protein metabolism can be assessed based on plasma amino acid kinetics, i.e., the rates at which amino acids are released into and taken up from the circulation (10, 11). In the fasted state (Figure 1A), amino acid release into the circulation originates solely from tissue protein breakdown (endogenous protein-derived plasma amino acid rate of appearance). Thus, the total amino acid rate of appearance, the endogenous rate of appearance, and whole-body protein breakdown rate are all equal in the fasted steady state. The rate at which amino acids disappear from the circulation represents the rate of amino acid uptake into tissues. Amino acids taken up by tissues are assumed to be either incorporated into proteins (protein synthesis) or oxidized. Amino acid oxidation can be measured by the irreversible hydroxylation of phenylalanine to tyrosine (12) or by the production of $^{13}CO_2$ in expired air (13). Subsequently, protein synthesis rate can be calculated by subtracting the rate of amino acid oxidation from the rate of disappearance. Finally, protein balance can be assessed by subtracting protein breakdown from protein synthesis. The calculations to assess plasma amino acid kinetics and whole-body protein metabolism in a fasted (and fed) state have been described in detail before (11).

In the fed state (Figure 1B), the assessment of whole-body protein kinetics is more challenging because amino acids not only appear into the circulation from protein breakdown (endogenous protein-derived plasma amino acid appearance), but also from the ingested protein (exogenous plasma amino acid appearance). This exogenous plasma amino acid rate of appearance needs to be quantified and accounted for (subtracted from the total plasma amino acid appearance rate) to calculate postprandial protein breakdown rates and, consequently, net protein balance. The exogenous plasma amino acid rate of appearance cannot be directly assessed with the amino acid stable isotope approach used to assess the total plasma amino acid kinetics. Therefore, the tracer methodology needs to be extended to directly assess the exogenous plasma amino acid bioavailability or alternatively the exogenous plasma amino acid bioavailability needs to be estimated.

3 Exogenous plasma amino acid bioavailability

Plasma amino acid concentrations are often used as a proxy for exogenous plasma amino acid bioavailability (Figure 2A), as it does not require the application of (more) amino acid tracers. However, plasma amino acid concentrations are not only impacted by exogenous plasma amino acid release, but also by endogenous amino acid release into the circulation (tissue protein breakdown) and the rate at which amino acids are taken up by tissues. Therefore, plasma amino acid concentrations cannot quantify exogenous plasma amino acid bioavailability. However, changes in plasma amino acid concentrations over time can provide some insight in the time course of exogenous plasma amino acid release, which is important for the interpretation of postprandial protein metabolism as will be discussed later. Following the ingestion of protein, plasma amino acid concentrations will rise and subsequently return to baseline. A complete return to baseline concentrations suggests that the ingested protein has been fully digested, absorbed, and released into the circulation (maximal exogenous plasma amino acid bioavailability has been reached). However, the experimental baseline sample may not always be representative of basal conditions. For example, many studies investigate the impact of protein ingestion directly following exercise when plasma amino acid concentrations are elevated due to exercise-induced catabolism (12). Therefore, the time point at which plasma amino acid concentrations in a postexercise feeding treatment do no longer differ from a placebo treatment would give a better indication of when maximal exogenous plasma amino acid bioavailability has been reached. Ideally, exogenous plasma amino acid bioavailability is assessed directly using tracer methodology. Following protein ingestion, exogenous plasma amino acid rate of appearance becomes positive and will eventually return to its baseline of zero, indicating maximal exogenous plasma amino acid bioavailability has been reached (Figure 2B). The area under the curve of the exogenous rate of appearance represents exogenous plasma amino acid bioavailability in absolute amounts (g). This can be divided by the ingested amount of protein to express it in a relative amount (percentage of the ingested

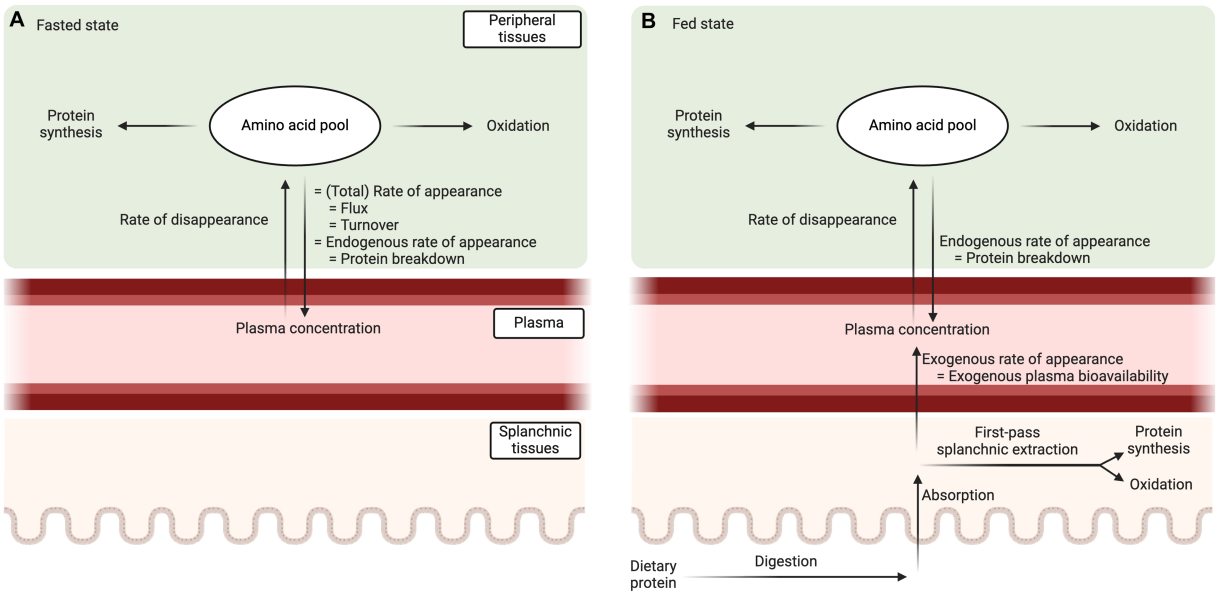

FIGURE 1
Schematic representation of the plasma amino acid kinetics model in fasted **(A)** and fed state **(B)**. In the fed state, the total amino acid rate of appearance into the circulation consists out of an endogenous (tissue protein breakdown) and exogenous (dietary protein-derived plasma amino acid availability) component. Exogenous plasma amino acid bioavailability needs to be assessed or estimated to allow the calculation of whole-body protein breakdown rates.

protein). A plateau in the cumulative timeline of exogenous plasma amino acid bioavailability represents maximal exogenous protein/AA bioavailability (Figure 2C).

4 Impact of protein bioavailability on the assessment of postprandial protein metabolism

Insight in the timeline of exogenous plasma amino acid bioavailability is required to properly evaluate whole-body protein metabolic rates in the experimental context. As meals are typically consumed as a (single) bolus, this introduces a non-steady state and results in a time-dependent variation in whole-body protein metabolic rates. To characterize a more complete postprandial response to a meal, the assessment period should at least match the time required to achieve maximal exogenous plasma amino acid bioavailability (Figure 3A, dotted line b). But when the assessment period is longer than the time point at which maximal exogenous protein derived plasma amino acid bioavailability is reached, this introduces a postabsorptive period within the assessment (Figure 3A, dotted line c). Consequently, this will lower the average protein synthesis rates during the "assumed postprandial" assessment period. The impact of an experimental period that is too short to reach maximal exogenous plasma amino acid bioavailability (Figure 3A, dotted line a) will depend on the pattern of protein-derived amino acid release into the circulation (fast vs. slow). For example, most protein-derived amino acids are released in the initial hours following the ingestion of a more rapidly digestible protein (14, 15). When the assessment period is short and matching this peak amino acid availability, this would overestimate average whole-body protein synthesis rates during the complete postprandial period. Conversely, the exogenous plasma amino acid rate of appearance following the ingestion of a more slowly digestible protein or a large whole-foods mixed meal may not peak until several hours into the post-prandial period (14–16). When the assessment period would end before the peak exogenous plasma amino acid availability, it may result in a gross underestimation of the average whole-body protein synthesis rates during the complete postprandial period and total whole-body protein synthetic response to the meal. In support, we have recently demonstrated that the ingestion of a large amount of protein (100 g milk protein) results in a much larger and more prolonged (>12 h) protein synthetic response than was previously assumed based on shorter experiments (12). Thus, the expected time course of exogenous plasma amino acid bioavailability is a crucial consideration in study design and the interpretation of data (Figure 3B).

While the time course of exogenous plasma amino acid bioavailability only impacts the interpretation of whole-body protein synthesis and amino acid oxidation rates, exogenous plasma amino acid bioavailability needs to be quantified to for the calculation of whole-body protein breakdown rates (11). Specifically, whole-body protein breakdown is calculated by:

$$Protein\ breakdown = Total_{Ra} - Exo_{Ra} \quad (1)$$

$Total_{Ra}$ and Exo_{Ra} represents the total and exogenous plasma amino acid rate of appearance, respectively. Assessment of the exogenous amino acid rate of appearance allows the time course of whole-body protein breakdown rates to be determined. When only a single estimated value for exogenous plasma amino acid bioavailability is available, only an average whole-body protein breakdown rate during the entire assessment period can be calculated. This does not impact the validity, but time-course data can provide additional

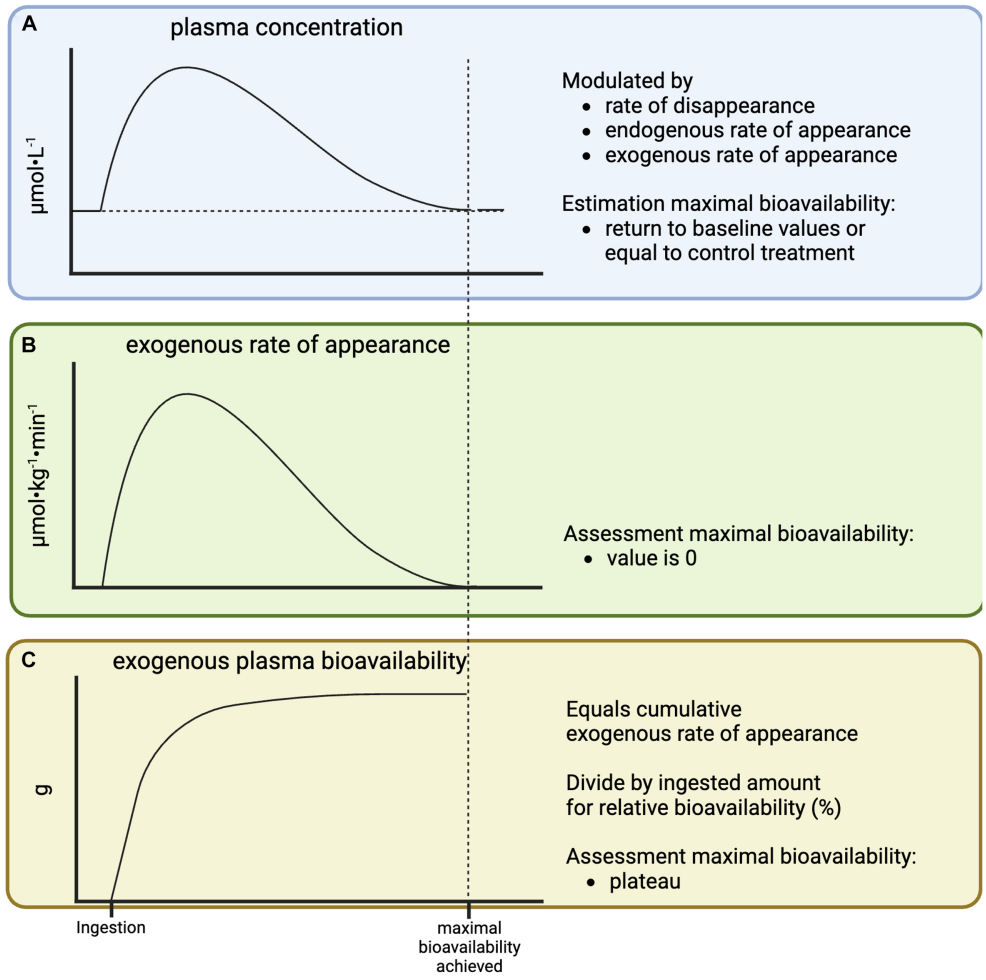

FIGURE 2
Schematic representation of plasma amino acid concentration **(A)**, exogenous rate of plasma amino acid appearance **(B)**, and exogenous plasma amino acid bioavailability **(C)** in response to the ingestion of a single bolus of protein.

valuable insights, such as, whether the effects are short-lived, increase over time, or correspond with other variables such as insulin levels. As can be deduced from the formula, any inaccuracy in the assessment or estimation of plasma bioavailability directly translates in inaccurate whole-body protein breakdown rates (Figure 4A). However, there are various plasma amino acid kinetic models routinely applied that differ greatly in their estimations of exogenous plasma amino acid bioavailability.

5 Plasma amino acid kinetics models to assess postprandial whole-body protein metabolism

5.1 100% bioavailability model

Initial work on whole-body protein metabolism developed a simplified plasma amino acid kinetics model that did not account for the bioavailability of amino acid released from the ingested protein (17):

$$Q = PS + OX = PB + ING \quad (2)$$

Q represents whole-body flux/turnover (or total rate of amino acid appearance as used in contemporary models). PS represents protein synthesis, OX represents oxidation (catabolism), PB represents protein breakdown, and ING represents protein ingestion. Note that despite older terminology for the elements, formula 2 can be rearranged to construct formula 1, with the exception that formula 2 does not account for the plasma amino acid bioavailability of the ingested protein. Therefore, all ingested protein is assumed to appear into the circulation, which generally is a substantial overestimation and results in incorrect assessment of whole-body protein breakdown. To illustrate, we applied the 100% bioavailability model to our data set of our recent work in which 100 g of protein was ingested (12) (Figure 4B). This allows a direct comparison of plasma amino acids kinetics and whole-body protein metabolism as assessed/estimated by various models based on the same raw data. This resulted in negative values for protein breakdown rates in the 100% bioavailability model, which is physiologically impossible. In contrast, protein breakdown rates were only reduced by ~5%, assessed using the gold standard labeled protein method (methodology discussed in

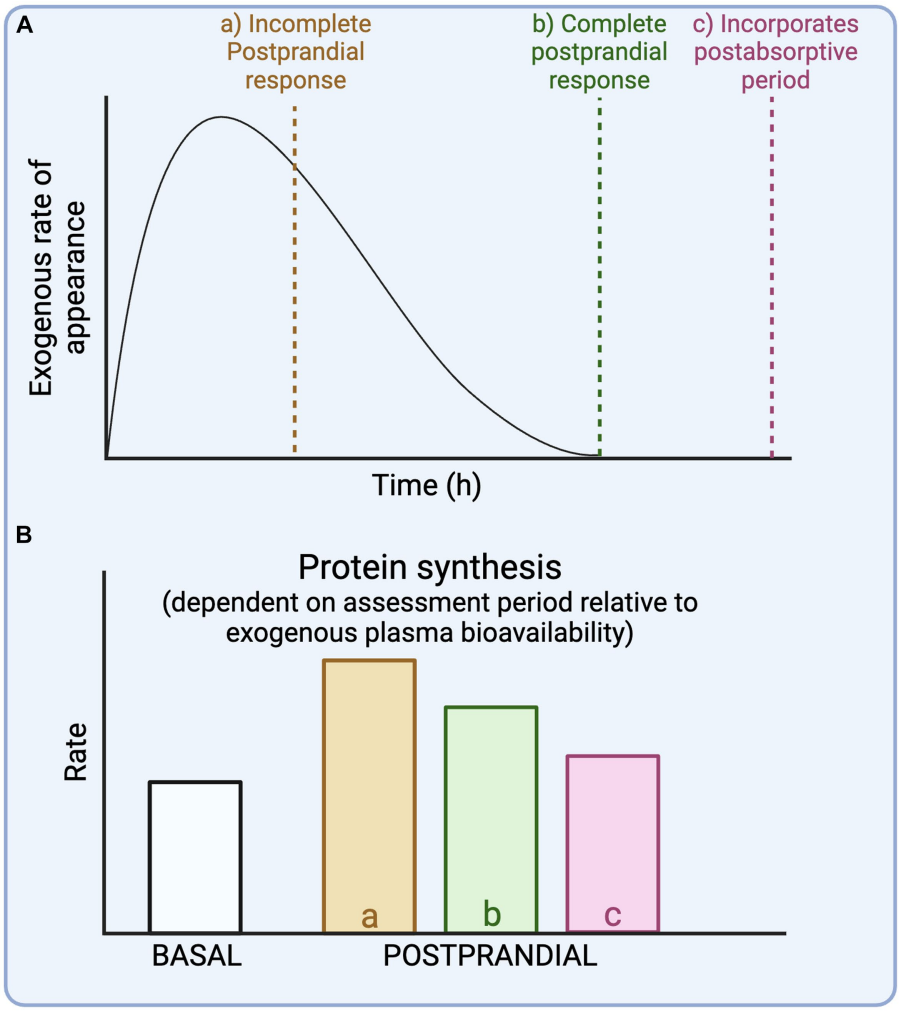

FIGURE 3
Schematic representation of different experimental durations relative to exogenous plasma amino acid bioavailability **(A)** and their impact on postprandial whole-body protein synthesis rates **(B)**.

section 5.3). In general, protein ingestion has only a modest impact on protein breakdown, with reductions between 5 and 25% in whole-body protein breakdown rates observed following protein ingestion as assessed with the labeled protein method (18–20). The magnitude of error in the full bioavailability model is largest during short experimental methods where the overestimation of exogenous plasma amino acid bioavailability is greatest. As the model ignores the true exogenous plasma amino acid bioavailability, it is fundamentally flawed. Therefore, the full bioavailability model should be considered outdated, as there are alternative approaches that do not require additional measurements but estimate exogenous plasma amino acid bioavailability to improve accuracy of the model.

It should be noted that the model with assumption of full exogenous amino acid bioavailability is still applied with some frequency, most commonly when applying the Indicator Amino Acid Oxidation (IAAO) method (11, 21). While the full exogenous plasma amino acid bioavailability model was designed for study designs in which amino acid tracer infusions were applied, the IAAO method typically applies only the ingestion of an amino acid tracer. However, not all ingested amino acids (tracers) appear into the circulation as the exogenous amino acid bioavailability in the circulation never reaches 100% [maximal exogenous plasma amino acid bioavailability is ~80% (18)]. Therefore, this approach has the inherent limitations of the 100% bioavailability method, but also violates the model assumption of 100% plasma bioavailability of the tracer (as the model was developed for intravenous tracer infusion). In support, plasma amino acid kinetics differ substantially in the IAAO model when comparing an intravenous vs. oral tracer approach (22). Nevertheless, the intake level that results in a breakpoint in indicator amino acid oxidation (assumed to represent the protein or essential amino acid requirement) is consistent between the intravenous and oral tracer method. It has been suggested that the oral amino acid tracer approach can still be applied to evaluate changes in (oral tracer-derived) whole-body protein metabolism (21). However, this approach has not been validated against gold-standard dual tracer feeding-infusion methods and, therefore, should be considered exploratory. In conclusion, IAAO-derived plasma amino acid kinetics rates are likely not accurate for either the oral or infusion method and should not be reported as secondary outcomes. Both models give consistent estimates for the indicator amino acid oxidation breakpoint, which suggests that they

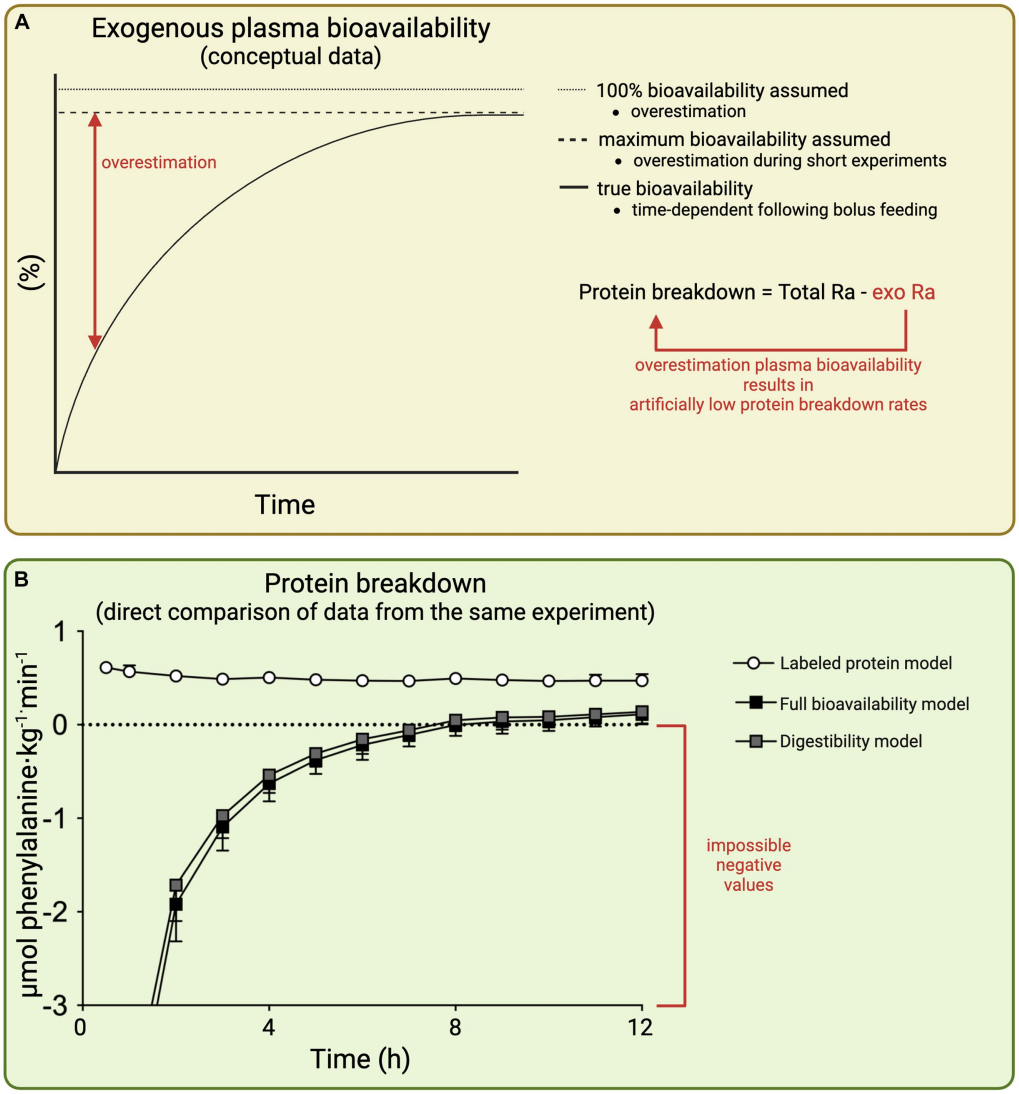

FIGURE 4
Schematic representation of the impact of inaccurate estimation of exogenous plasma amino acid bioavailability on whole-body protein breakdown rates **(A)** and whole-body protein breakdown rates as calculated based on different plasma amino acid kinetic models following the ingestion of 100 g protein; all three models calculated from the same raw data from Trommelen et al. (12) (9) **(B)**. Total Ra: total rate of both endogenous plus exogenous amino acids appearing into the circulation. Exo Ra, rate of exogenous amino acids appearing into the circulation.

may be valid for the assessment protein and essential amino acid requirements.

5.2 The digestibility model estimates exogenous plasma amino acid bioavailability

The 100% bioavailability model can be improved by correcting the amount of ingested protein for estimated exogenous plasma amino acid bioavailability. Exogenous plasma amino acid bioavailability (Formula 3) represents the ingested protein-derived amino acids that are absorbed in the gut, subsequently escape first pass splanchnic extraction, and are released into the systemic circulation (23, 24):

$$BIO_{estimated} = ING * digestibility * SPE_{estimated} \quad (3)$$

$BIO_{estimated}$ represents the estimated exogenous plasma amino acid bioavailability, digestibility represents the true ileal digestibility of the ingested protein, and $SPE_{estimated}$ represents the estimated first pass splanchnic extraction. By dividing $BIO_{estimated}$ by the duration of the assessment period, it is converted to the (estimated) average exogenous rate of plasma amino acid appearance. The latter can be used in formula 1 to calculate the average protein breakdown rate over the assessment period. While true ileal protein digestibility has long been challenging to assess in humans due to the requirement of invasive techniques, there are data from animal (especially pig) models that seem to correspond well with data derived from human *in vivo* models (25). Moreover, the development of the minimally invasive dual tracer digestibility techniques has allowed more human data to be collected in recent years (26, 27). As digestibility represents the exogenous protein-derived amino acids that may be absorbed in the gut, an additional correction for first-pass splanchnic extraction

needs to be applied to estimate subsequent exogenous plasma amino acid bioavailability. This first-pass splanchnic extraction can be estimated based on the postprandial increase in whole-body amino acid oxidation (postprandial – postabsorptive rates) (28, 29).

An advantage of the digestibility method is that there are considerable amounts of data available for the digestibility of most proteins. Therefore, the digestibility approach can be applied in most experiments. The main drawback is that the method relies on multiple assumptions and extrapolations that have the potential to introduce errors. Digestibility data are typically obtained during steady state conditions which do not reflect the bolus feeding approach used in stable isotope studies to reflect the response to ingesting a normal meal. The digestibility obtained during steady state conditions represents the maximal protein digestibility of the protein source when given sufficient time. Therefore, maximal digestibility data should not be extrapolated to a bolus feeding study of relatively short duration with insufficient time to allow maximal digestibility or maximal exogenous plasma amino acid bioavailability to be reached. For example, milk protein may have a 95% digestibility given sufficient time (25), but clearly not all protein is digested, absorbed, and released into the circulation within the first hour after the ingestion of a large milk protein bolus (12). Thus, extrapolating the maximal digestibility data to short experimental duration results in overestimation of exogenous plasma amino acid bioavailability and consequently results in artificially low protein breakdown rates (Figure 4). Other limitations of the model are the assumptions that are made regarding first-pass splanchnic extraction. It needs to be assumed that splanchnic tissues are in net balance (28), although there is some indication that net balance may be negative in the postprandial state (30). In addition, the postprandial increase in whole-body amino acid oxidation is assumed to reflect first-pass oxidation. However, oxidation is assessed by the application of a continuous amino acid tracer infusion directly into the circulation. Therefore, any tracer-derived oxidation in the model cannot be the result of first-pass splanchnic extraction. While the digestibility model has substantial limitations, the method is conceptually superior to approaches that do not account for exogenous plasma amino acid bioavailability.

5.3 Intrinsically labeled protein to assess exogenous plasma amino acid bioavailability

Exogenous plasma amino acid bioavailability can be assessed by combining the application of an amino acid tracer constant infusion with the ingestion of a different stable isotope of the same amino acid (e.g., L-[$^{2}H_{5}$]-phenylalanine and L-[1-^{13}C]-phenylalanine, respectively). The ingested amino acid tracer should reflect the properties of the amino acids it traces which requires them to be in the same matrix. The ingestion of a free amino acid tracer can be applied to assess the exogenous amino acid bioavailability following the ingestion of a free amino acid mixture, but not following the ingestion of a protein source. In a real-life setting, exogenous amino acids are typically consumed in the form of dietary proteins. Therefore, the assessment of postprandial whole-body protein metabolism following the ingestion of intact dietary proteins is of particular relevance. This requires the dietary protein to be intrinsically labeled, i.e., the amino acid tracer should be incorporated into the protein matrix. The intrinsic labeling of dietary protein can be achieved in multiple ways, such as feeding or infusing amino acid tracers to, for example, insects (31), chickens (32), or cows (33). The intrinsic labeling of plant proteins is also possible and has been applied to assess protein digestibility (27), but not yet for exogenous plasma amino acid bioavailability. Exogenous plasma bioavailability is calculated as follows:

$$Exo_{Ra} = total_{Ra} * \frac{E_{plasma}}{E_{pro}} \quad (4)$$

$$Exogenous\ plasma\ AA\ bioavailbility_{(t)} = area\ under\ the\ curve\ of\ Exo_{Ra} \quad (5)$$

Exogenous plasma AA bioavailability$_{(t)}$ represents the cumulative amount of dietary protein-derived amino acids that have been released in the circulation at a specific time point (Formula 5). E_{plasma} represents the enrichment of the labeled protein-derived tracer in the circulation. E_{pro} represents the enrichment of the labeled protein before ingestion. The combination of an amino acid tracer infusion with the ingestion of intrinsically labeled protein is the preferred method to quantify postprandial protein metabolism, as it is the only method to directly quantify exogenous plasma amino acid bioavailability. The drawback of this method is that the production of intrinsically labeled protein is expensive and labor intensive to apply. Therefore, there is a need for alternative approaches that can provide a more routine evaluation of postprandial whole-body protein metabolism.

It should be noted that the accuracy of the intrinsically labeled protein to assess exogenous plasma amino acid bioavailability has been questioned (28, 34). It was suggested that the enrichment of the labeled protein-derived tracer gets diluted across the splanchnic bed, which would result in an underestimation of exogenous plasma amino acid bioavailability. While the enrichment of the labeled-protein derived tracer gets diluted following ingestion, this has no impact on the assessment of exogenous plasma amino acid bioavailability or E_{pro} as used in formula 4. E_{pro} represents the enrichment of the labeled protein before ingestion, which also can be defined as the enrichment of the exogenous amino acids or the tracee. By definition, the enrichments of the exogenous tracee are not diluted by any endogenous flux. The enrichment of the exogenous tracee is used in the formula to calculate the plasma appearance rate back from tracer to tracee. For example, an E_{pro} of 50% MPE indicates that for every tracer appearing in the circulation (calculated by total$_{Ra}$ * E_{plasma}), and equal amount of exogenous tracee appears into the circulation. Therefore, the exogenous rate of appearance is two times (equals dividing by 50% MPE) the exogenous rate of appearance of the tracer. Figure 5 demonstrates that E_{pro} is not diluted throughout the splanchnic bed and that calculation of the exogenous rate of appearance is accurate using the intrinsically labeled protein model. The model can be challenged by modifying variables like the protein intake dose, the labeled protein enrichment, any of the metabolic rates, and/or incorporating additional factors such as digestibility, additional endogenous rate of appearance, additional rates of disappearance, or a net splanchnic extraction/release, but the model remains valid under all these challenges. Therefore, the intrinsically labeled protein method can

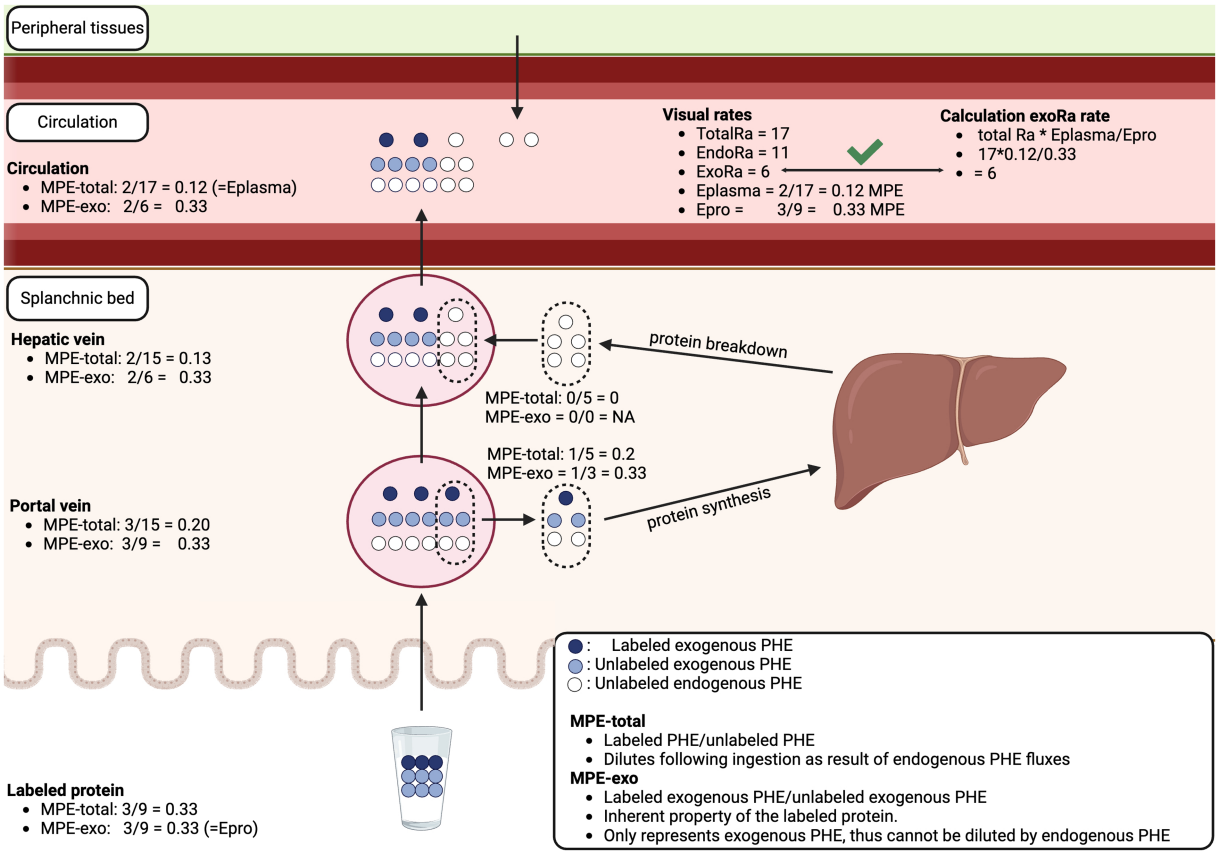

FIGURE 5
Schematic overview of exogenous protein-derived amino acid release into the circulation as assessed with intrinsically labeled protein model. The enrichment of the exogenous amino acid is an inherent property of the labeled protein and cannot be impacted by endogenous fluxes. The validity of the calculation of exogenous rate of plasma amino acid appearance (ExoRa) can be visually confirmed. The model can be extended to include additional amino acid kinetics or other physiological changes while remaining valid. MPE, Mole percent excess; PHE, Phenylalanine; TotalRa, Total rate of plasma amino acid appearance; EndoRa, Endogenous rate of plasma amino acid appearance; Eplasma, Enrichment of the exogenous protein-derived amino acid tracer in the circulation; Epro, Enrichment of the exogenous amino acid tracer in the dietary protein.

be used to accurately quantify exogenous plasma amino acid bioavailability.

5.4 Estimation of exogenous plasma amino acid bioavailability based on published literature

When exogenous plasma amino acid bioavailability cannot be directly assessed by using intrinsically labeled protein, the most accurate estimation would be based on reference data from such dual tracer isotope-feeding models that have been performed under similar experimental context. This is conceptually the same approach as the digestibility method but requires less assumptions. The main limitation of this method is that there are not that many reference data available. Only for bovine milk protein, there are substantial data obtained during various experimental conditions (e.g., different doses, protein fractions, nutrient co-ingestion, age, different assessment periods, exercise, and sleep). There are few data on exogenous plasma amino acid bioavailability following the ingestion of most other protein sources, and plant-based protein sources in particular. This is further complicated by the fact that exogenous plasma amino acid bioavailability data are not always (completely) reported. For example, even when the labeled protein approach is applied, exogenous rates of plasma amino acid appearance are not always calculated and/or reported. Moreover, studies have only recently started to report data on the cumulative exogenous plasma amino acid bioavailability. To address this, we have compiled our previous data (12, 18–20, 31, 35–51) and provide data on cumulative exogenous plasma amino acid bioavailability expressed as a percentage of the ingested protein in Table 1. These data allow estimation of the average exogenous rate of plasma amino acid appearance which can be used in formula 1, similar as discussed for the digestibility approach (example provided below). There is a need to establish a database on exogenous plasma amino acid bioavailability of the most common protein sources in *in vivo* in various populations and experimental conditions.

6 Discussion

A variety of methods have been applied to assess postprandial whole-body protein metabolism based on the plasma amino acid kinetics model. The accuracy of these methods differs greatly, based on their capacity to accurately assess exogenous plasma amino acid

TABLE 1 Overview of exogenous plasma amino acid bioavailability data at different time points following bolus protein during various experimental settings and designs.

Study	Type	Dose (g)	Age (years)	Exercise	Other	Cumulative plasma bioavailability (%)					
						3 h	4 h	5 h	6 h	8 h	12 h
(18)	AA	30	23 ± 3			63	70	73	76		
	Milk					36	46	53	59		
(45)	Whey	10	73 ± 5			53	56				
		20				53	58				
		35				48	54				
(39)	Whey	25	62 ± 5		low PRO	53	56	58			
					high PRO	49	52	54			
(49)	Whey	24	22 ± 3		immob	54	58				
(43)	Casein	35	23 ± 3			24	30	35	40		
			64 ± 4			24	30	36	40		
(46)	Casein	20	21 ± 2	-		33	41	46	49		
			21 ± 3	REX		29	37	43	47		
			75 ± 4	-		36	42	46	50		
			73 ± 3	REX		32	39	43	47		
(38)	Casein	20	21 ± 3		-	47	56	61			
			20 ± 2		CHO	41	55	64			
			74 ± 3		-						
			76 ± 4		CHO	38	45	51			
						33	46	54			
(40)	Casein	20	65 ± 7		-	36	43	47			
					FAT	34	42	48			
(50)	Casein	25	71 ± 6		-	30	36	41			
					serum	27	36	42			
(41)	Casein	20	23 ± 3		-	34	42	48			
			21 ± 2		insulin	36	49	61			
			68 ± 4		-						
					insulin	27	34	40			
			68 ± 2		-	26	34	40			
(47, 48)	Casein	30	23 ± 4	-	sleep	26	34	40	46	54	
				REX	sleep	27	35	41	47	54	
				REX	sleep+leu	28	37	44	51	58	
(42, 44)	Casein	20	72 ± 5	REX	sleep	37	46	52	57	61	
		20			sleep+leu	28	37	45	51	58	
		40			sleep	20	28	35	41	51	
		40			sleep	23	31	38	45	52	
(51)	Casein	20	24 ± 3		-	40	49				
					immob	42	47				
(19)	Milk	15	27 ± 4	END	CHO	62	68	71	74		
		30				64	69	72	74		
		45				55	63	67	69		
(12)	Milk	25	26 ± 6	REX		42	51	56	58	62	66
		100				21	26	31	36	44	53

(Continued)

TABLE 1 (Continued)

Study	Type	Dose (g)	Age (years)	Exercise	Other	Cumulative plasma bioavailability (%)					
						3 h	4 h	5 h	6 h	8 h	12 h
(20)	Milk	15	66 ± 6	REX	-	59	67	72	75		
		30			-	41	51	58	63		
		45			-	41	49	55	59		
		15			leu	52	61	67	70		
(36)	Milk	20	21 ± 2	REX	CWI + CHO	62	68	71			
(37)	Milk	20	23 ± 3	REX	HWI ± CHO	66	71	73			
(35)	Milk	30	22 ± 3	REX		51	60	65			
	Beef*					39	40	58			
(31)	Worm*Milk	30	23 ± 3	REX		58	68	73			
						58	70	77			

Age: mean ± SD. *provided in a whole foods matrix (cooked beef and ground mealworms, respectively). All data based on phenylalanine. AA, Free amino acids; Casein, Micellar casein; Whey, Whey protein; Milk, Milk protein concentrate; REX, Resistance exercise; END, Endurance exercise; low PRO, Following lower protein diet; high PRO, Following higher protein diet; CHO, Carbohydrate co-ingestion; FAT, Fat co-ingestion; insulin, Insulin infusion; sleep, Overnight sleep; leu, Leucine co-ingestion; immob, Leg immobilization; CWI, Cold-water immersion; HWI, Hot-water immersion.

bioavailability. We introduced a novel exogenous plasma amino acid model based on intrinsically labeled protein-derived reference data that are more accurate than data used in previous estimation approaches.

Extension of amino acid tracer infusion with ingestion of intrinsically labeled protein is the preferred approach to assess exogenous plasma amino acid bioavailability and postprandial whole-body metabolism. When this is not feasible, exogenous plasma amino acid bioavailability can be estimated based on reference data obtained with labeled protein during similar experimental conditions. For example, if an experiment is conducted with the ingestion of 25 g of milk protein in healthy, young adults following resistance exercise with a 6-h post-prandial assessment period, it can be estimated that ~58% of the ingested protein appear will have appeared in the circulation at the end of the assessment period (Table 1). In case of a phenylalanine tracer infusion, the exogenous plasma amino acid bioavailability expressed as percentage of ingested protein would be multiplied with phenylalanine content of milk protein (~6.3 mmol/25 g) and divided by 6-h to calculate the average exogenous rate of plasma amino acid appearance rates. The latter can be used to calculate the average whole-body protein breakdown rate over the full 6-h period. In this example, the estimate would be highly accurate based on appropriate reference data, with only inter-individual differences in protein digestion, amino absorption, and splanchnic extraction not being accounted for. Additional error would be introduced if the experimental conditions differ more from the reference data (e.g., no exact match of dose, population, co-intervention, etc.). It should be carefully considered if there are suitable reference data for extrapolation, to what extent extrapolation errors may impact conclusions, and a brief rationale should be provided in the methods and/or limitations sections. When no suitable exogenous plasma amino acid bioavailability reference data are available (for nearly all non-dairy proteins), the digestibility approach could be applied. Digestibility of many dietary protein source are available (25, 27). However, digestibility scores typically represent the maximal value that would be obtained if the ingested protein is given sufficient time to be absorbed. Therefore, digestibility scores should only be extrapolated to bolus feeding if there is an indication that maximal exogenous plasma amino acid bioavailability can be achieved within the assessed post-prandial period. In the digestibility approach, this could be estimated by a return of plasma amino acid concentrations to basal or control conditions (Figure 2). However, even if the assumption of maximal digestibility has been met, exogenous plasma amino acid availability or whole-body protein breakdown estimated by the digestibility method still differ from those obtained with intrinsically labeled protein as the gold standard (Figure 4B). Therefore, there is an urgent need to obtain more labeled protein-derived exogenous plasma amino acid bioavailability data for various protein sources (e.g., various plant proteins and whole-foods protein sources) This will allow the more routine assessment of postprandial whole-body protein metabolism based on (just) an amino acid tracer infusion to be more accurate when compared to the digestibility or 100% bioavailability methods.

It should be noted that all plasma amino acid kinetics have some limitations. It is assumed that amino acids taken up from the circulation are either incorporated into tissue protein (protein synthesis) or catabolized (amino acid oxidation). However, there can also be a (transient) expansion of tissue-free amino acid pool after bolus feeding. At least in muscle tissue (often referred to as the largest protein pool in the body), such expansion has returned to baseline in <4 h for phenylalanine, but not for the branched-chain amino acids (12). Another limitation is that the metabolic outcomes are assessed based on the kinetics and of single amino acid tracer and extrapolated to all amino acids and/or protein. A final limitation is that the plasma amino acid kinetics model only accounts for fluxes into and out of the circulation. For example, intracellular amino acid (re)cycling may occur. Therefore, actual protein breakdown and protein synthesis rates are likely higher than observed based on plasma amino acid kinetics, although it should not impact protein balance. Another example of a protein flux that is not observed is the loss of endogenous amino acids into the gastrointestinal tract with no reabsorption, which may be far from negligible (52). Therefore, the plasma amino acid kinetics model will need to evolve further to become more complete and accurate. Furthermore, it should be questioned what inferences can be made from the assessment of whole-body protein metabolism. Whole-body protein metabolism reflects the cumulative protein metabolism of all

individual organs and tissues. The contribution of an individual organ to whole-body protein metabolism is determined by its individual tissue protein mass and protein metabolic rate. When an increase in whole-body protein synthesis is observed, it cannot be inferred which tissues have contributed to this effect and by what magnitude. Generally, the anabolic response to feeding on a muscle and whole-body level tend to correspond (12, 38, 40). Whether such a relationship exists for other organs remains unclear, as most of these tissues cannot be routinely sampled. Resistance exercise can stimulate muscle protein synthesis rate without a large impact on whole-body protein synthesis rates (42, 47). While muscle tissue represents the largest protein pool, muscle tissue protein synthesis rates are much lower than tissue protein synthesis rates of most organs (11). Therefore, insights on tissue specific and whole-body protein metabolism should be taken into account when evaluating the impact of interventions, populations and/or conditions on protein metabolism.

7 Conclusion

The application of the labeled protein model can accurately assess exogenous plasma amino acid bioavailability and, as such, postprandial protein metabolism. However, the cost of producing and applying intrinsically labeled protein limits widespread application. Exogenous plasma amino acid bioavailability can be estimated based on reference data obtained with labeled protein during similar experimental conditions. If no appropriate plasma bioavailability data are available, the digestibility approach can be applied. Application of these models requires an understanding of their underlying assumptions and the consequences when violating them. There is an urgent need for more exogenous plasma amino acid bioavailability data on common dietary protein sources in various experimental contexts to facilitate the accurate assessment and/or estimation of postprandial whole-body protein metabolism.

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Discussion on protein recommendations for supporting muscle and bone health in older adults: a mini review

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Muscle and bone tissues are interconnected, and both rely on an adequate protein intake. Recommendations for protein intake for older adults specifically vary across countries. The purpose of this narrative review is to discuss the existing evidence for protein recommendations for supporting muscle and bone health in older adults and to evaluate if a protein intake above the current population reference intake (PRI) for older adults would be scientifically justified. First, this review summarizes the protein recommendations from bodies setting dietary reference values, expert groups, and national health organizations. Next, relevant studies investigating the impact of protein on muscle and bone health in older adults are discussed. In addition, the importance of protein quality for muscle and bone health is addressed. Lastly, a number of research gaps are identified to further explore the added value of a protein intake above the PRI for older adults.

KEYWORDS

protein, amino acid, aging, muscle, bone, physical function

Introduction

Declining muscle mass and strength and bone mineral density (BMD) are common during aging (1). These changes may lead to the development of sarcopenia and osteoporosis. Muscle and bone are interconnected to each other. From a mechanistic point of view, the mechanical forces exerted by muscles during certain activities play a crucial role in stimulating bone formation (2). In addition, osteoporosis and sarcopenia share risk factors and often occur within the same individual (3). Additionally, low muscle mass and strength that occur with sarcopenia increase the risk of falls, and subsequently the risk of fractures, and fractures can in turn accelerate muscle mass loss (4). Protein plays a vital role in supporting both muscle and bone health in older adults; an adequate protein quantity and quality has been proposed to maintain muscle and bone health later in life (5–7).

Recommendations for protein intake for older adults vary across countries (8–12). Some bodies setting dietary reference values and national health organizations make no distinction between adults and older adults (11–14), while others advocate for higher protein intake recommendations specifically tailored to the needs of older adults (8–10). This narrative review first summarizes the current national and international protein recommendations for (older) adults. Next, we describe recent and relevant studies focusing on the impact of protein

on muscle and bone health in older adults specifically. In addition, the importance of protein quality is addressed. Thus, the purpose of this narrative review is to discuss the existing evidence for protein recommendations for supporting muscle and bone health in older adults and to evaluate if a protein intake above the population reference intake (PRI) of 0.83 g/kg/d for older adults would be scientifically justified.

Current protein recommendations

The current protein recommendations from EFSA (13) and the FAO/WHO/UNU (14) are based on a meta-analysis of nitrogen balance studies from 2003, involving 19 studies and 235 healthy adults (15). Hereby the estimated average requirement (EAR) was established at 0.65 g/kg bodyweight/d (g/kg/d) and the PRI at 0.83 g/kg/d. In a stratified analysis comparing younger adults (<40 years, $n = 221$) with older adults (>67 years, $n = 14$), a statistically non-significant difference in the EAR of 27 mg N/kg/d (=0.17 g protein/kg/d) was found (15). Since only 14 older adults were included, the power of this comparison is inadequate. The meta-analysis was repeated in 2014 and led to the same conclusions (16). However, still only 54 older adults (≥60 years) could be included. In both meta-analyses the sample size of older adults was low, so there was limited power to establish if there was a difference between young and older adults. Thus, more nitrogen balance studies involving older adults are needed. Instead of using nitrogen balance studies in which the requirement for protein is based on the lowest amount of dietary protein intake that will balance the nitrogen losses from the body (14), focusing on the effect of protein on physiological variables, such as muscle mass, physical functioning and bone health, in older adults may be of additional value for the estimation of protein recommendations.

The nitrogen balance-based recommendations have been adopted by several national and international organizations (13, 14, 17). However, several expert groups including the PROT-AGE Study Group (5) and European Society for Clinical Nutrition and Metabolism (ESPEN) (6) advise higher protein intakes based on evidence for maintaining muscle mass and function. The PROT-AGE study Group (5) recommends for older adults an average daily intake of at least 1.0 to 1.2 g/kg/d to maintain and regain lean body mass and function. In case of an acute or chronic disease, even higher intakes are proposed (1.2–1.5 g/kg/d). This is in line with the recommendations of the ESPEN expert group (6): at least 1.0–1.2 g/kg/day for healthy older people, and 1.2–1.5 g/kg/day for older people who are malnourished or at risk of malnutrition because they have acute or chronic illness.

Some national organizations also revised their protein recommendations for older adults. For example, the Nordic countries changed the recommended protein intake into 1.2 g/kg/day in 2012 (8), which was maintained in the 2023 version (18), and the nutrition societies of Germany, Austria, and Switzerland revised it to 1.0 g/kg/d for adults >65 years in 2019 (9). In 2021, the Food Safety Authority of Ireland advises older adults at risk of frailty, sarcopenia, or undernutrition to consume a minimum of 1.0–1.2 g/kg/d (10). These increases in national recommendations are based on an overall assessment of metabolic and functional parameters. However, due to different criteria, for example the type of studies used (nitrogen balance studies, cohorts, RCTs), organizations in the Netherlands and United Kingdom conclude that the evidence is still insufficient to increase the recommendation ≥0.8 g/kg/d (11, 12).

Regarding bone health, European guidance provided by International Osteoporosis Foundation (IOF) and European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO) stated in 2013 that 1.0 g/kg/d of protein can be recommended in the general management of patients with osteoporosis (19, 20). However, in the updated guidance of 2019 this number is removed and changed to "sufficient dietary protein" (21). In 2023, the first set of dietary recommendations in the prevention and treatment of osteoporosis have been published by the French Rheumatology Society and the Osteoporosis Research and Information Group (22). Based on evidence from cohort studies, a protein intake of at least 1.0–1.2 g/kg/day (with "high quality" animal proteins) as part of a balanced diet with adequate calories, calcium and vitamin D intakes is advised (19, 22).

Health effects of a protein intake above the PRI in older adults

The most recent systematic review on the health effects of increasing protein intake above the current PRI in older adults was published by the Health Council of the Netherlands in 2022 (11). Data of >1,300 subjects (≥60 years or mean ≥ 65 years) from 18 RCTs were included and only RCT's lasting ≥4 weeks were included. The Health Council concluded that an increased protein intake, combined with physical exercise, has a possible beneficial effect on lean body mass, while an increased protein intake without exercise had *likely no effect* on muscle strength, physical function, and bone health. However, three limitations are of note (23). First, only studies with (relatively) healthy older adults could be included. Studies with older adults living in a nursing home or care home were not eligible and studies in which the study population consisted of hospitalized or immobilized patients or of individuals with a specific disease were excluded. However, the population of older adults is very heterogeneous. Considering the prevalence of malnutrition (9%) (24), frailty (11%) (25), sarcopenia (10–27%) (26), obesity (35%) (27) and multimorbidity (51%) (28), the results of this systematic review only apply to the healthy segment of the older population. Second, no cohort studies were included. In research on bone health, cohort studies significantly contribute to our understanding of factors associated with bone health, since intervention studies are typically too short of duration to identify changes in BMD. Therefore, high-quality cohort studies should have a prominent role in evaluating the role of a protein intake above the PRI on bone health in older adults and should be included in the estimation of reference values for protein. Third, studies with a non-isocaloric control intervention were excluded. This strict criterion excluded many placebo-controlled RCTs, while the protein supplements increase energy intake only by about 80–160 kcal per day. Such a minimal amount of extra calories is not expected to affect fat free mass or physical functioning to an extent warranting exclusion of these important trials.

Muscle health

We have identified five relevant RCTs investigating the impact of enhancing protein intake on muscle health that are excluded from the

systematic review of the Health Council of the Netherlands or published after their systematic search. These RCTs used a non-isocaloric control group but do have added value to the scientific evidence. These include the studies of *ProMuscle* (29, 30), *ProMuscle in Practice* (31, 32), *ProMuscle Implementation* (33), *PROMISS* (34, 35), and *CALM* (36). All investigated the effect of protein on muscle health outcomes, and found beneficial effects compared to control groups (Table 1). In *ProMuscle* (29, 30), there were four arms: supplementation with 31 g of milk protein concentrate or placebo, with or without resistance exercise training for 24 weeks. It was found that lean body mass increased in the protein+exercise group. Strength and physical performance increased in the protein only group and exercise groups. In *ProMuscle in Practice* (31, 32), an increased protein intake of 25 g per main meal in combination with resistance exercise training was investigated for 24 weeks. Lean body mass, muscle strength and the Short Physical Performance Battery score were improved in the protein and exercise group compared to the control group. *ProMuscle Implementation* (33) showed that tailored nutrition advice aiming at 20-25 g protein per main meal for 12 weeks combined with resistance exercise training improved chair-rise performance and leg strength. In *PROMISS* (34, 35), 400-m walking time and leg extension strength improved in the intervention groups (dietary advice to increase protein intake to ≥1.2 g/kg aBW/d with or without advice to time protein intake in close proximity of usual physical activity) compared to controls (no dietary advice). In the *CALM* study (36), the effect of protein supplementation (20 g, twice daily), with or without resistance exercise training for 1 year, was investigated and compared with a placebo (carbohydrate). Quadriceps size and leg strength improved in the exercise+protein group compared to the protein only and placebo group. In the protein only group, there were no improvements in muscle health over time compared to placebo. Despite that the baseline protein intake in these five studies was already >0.8 g/kg/d, beneficial effects on muscle health outcomes were found, especially when combined with exercise training. Even larger effects may be expected in older adults with lower protein intakes (<0.8 g/kg/d).

Bone health

Regarding bone health, the latest systematic review and meta-analysis on dietary protein intake and bone health in older adults was published in 2019 by Groenendijk et al. (7). The systematic review showed a positive trend between higher protein intakes (above the PRI) and higher femoral neck and total hip BMD. The meta-analysis showed that a higher protein intake resulted in a significant decrease in hip fractures of 11%. Since then, new studies investigating the relationship between protein and bone health in older adults have been published (repeated search 02/01/2024) (19). At least one observational study by Weaver et al. (37) and one intervention study by Kemmler et al. (38) would have been included if the systematic review would be repeated (Table 2). Weaver et al. (37) showed that older adults with higher protein intake (mean ± SD: 1.1 ± 0.4 g/kg/d) had 1.8% higher mean hip and 6.0% higher lumbar spine BMD at baseline compared to those with a lower protein intake (0.8 ± 0.3 g/kg/d). While the higher and lower protein intake groups had similar BMD changes over 4 years of follow-up, the higher protein intake group had a 64% (95% CI: 0.14, 0.97) reduced risk of vertebral fractures during 5 years of follow-up. Kemmler et al. investigated the effects of 18-months of high intensity dynamic resistance exercise and whey protein supplementation on BMD in older men with osteoporosis and sarcopenia (38). Total protein intake was aimed at 1.5–1.6 g/kg/d in the intervention group and 1.2 g/kg/d in the control group. While the intervention group followed a supervised high intensity training program twice a week, the control group received no exercise program. In the intervention group, BMD at the lumbar spine and total hip was higher than the control group after 18 months (mean difference 0.012 and 0.013 mg/cm^2, respectively). Both studies support the hypothesis that a protein intake above the PRI improves bone health in older adults (19). Note that only healthy older adults were included in the systematic review by Groenendijk et al. and the two recent studies. Different results may be seen in for example frail, undernourished, or osteoporotic individuals.

Protein quality

As dietary guidelines increasingly advocate for more plant-based diets to address environmental sustainability and health concerns (39–43), a critical view on protein quality is needed, especially for older adults. Bones and muscles require a continuous supply of amino acids for maintenance and repair. The alkaline nature of plant-based diets may offer some bone health advantages by reducing the acid load on the body (by increasing potassium intake), potentially mitigating bone loss (44). However, the lower anabolic potential of plant proteins has been suggested to compromise muscle mass, muscle strength, and bone health (45, 46).

Protein quality, determined by the digestibility and the amino acid pattern of a protein, is generally lower in plant protein sources than in animal protein sources. Such lower quality might lead to reductions in lean body mass, including muscle and bone tissue (45). Some observational studies showed lower BMD values in vegetarians, vegans and older adults with a low animal:plant protein ratio (51:49) (46, 47). In addition, vegetarians and vegans have been shown to have an increased fracture risk, potentially due to intakes below the average requirement of protein, calcium and/or vitamin B12 (47, 48). RCTs with longer term vegan diets in older adults and specific measures of body composition, BMD and bone turnover are urgently needed, and some of these trials have been initiated (ClinicalTrials.gov ID NCT05809466 and NCT06130956).

In response to the lower quality of plant proteins, there's a prevailing recommendation to increase total protein intake (with a factor of 1.3) to meet physiological needs on a plant-based diet (49). However, this approach may not be feasible for older adults, who often experience diminished appetite and energy intake capacity (45). A more viable strategy involves smarter meal planning that leverages the concept of protein complementation—combining different plant protein sources to achieve a complete amino acid profile. This approach can enhance the overall quality of the protein consumed without necessitating increased food intake. For example, combining legumes (high in lysine, low in methionine) with cereal grains (high in methionine, low in lysine) can provide a more balanced amino acid profile, increasing the quality of the total meal protein. Such dietary strategies, however, require careful planning and knowledge, highlighting the need for tools that help dietitians, meal planners and consumers to find optimal combinations of plant-based proteins, which our group is currently working on (50).

TABLE 1 Characteristics and results of five studies investigating the effect of protein on muscle health.

Study [ref]	Population	Intervention	Baseline protein intake (g/kg/d)	Results[1]
ProMuscle ($n = 127$) (29, 30)	≥65 years; frail or pre-frail according to fried criteria	Four arms: supplementation with 31 g of milk protein concentrate or placebo, with or without RET for 24 weeks	Mean (95% CI) Protein: 1.0 (0.9–1.1) Placebo: 1.0 (0.9–1.1) Mean ± SEM Protein+exercise: 1.0 ± 0.0 Placebo+exercise: 1.0 ± 0.0	Protein compared to placebo: physical performance increased, $p = 0.02$[2]; LBM stable, $p > 0.05$[2]; strength improved in both groups, $p < 0.01$[3], but no interaction. Protein+exercise compared to placebo+exercise: LBM increased, $p = 0.006$[2]; strength and physical performance improved in both groups, $p < 0.001$[3], but no interaction.
ProMuscle in practice ($n = 168$) (32)	≥65 years; frail or pre-frail according to Fried criteria or physical inactive and experiencing difficulties in daily activities	Two arms: increased protein intake of 25 g per main meal via conventional and enriched products combined with RET versus control for 24 weeks	Mean (95% CI) Control: 1.08 (1.01–1.15) Intervention: 1.12 (1.05–1.19)	LBM: 0.4 (0.1–0.8) kg, $p < 0.05$[2] Muscle strength: 30.8 (11.5–50.0) N, $p < 0.01$[2] SPPB score: 0.5 (0.1–0.9), $p < 0.05$[2]
ProMuscle implementation ($n = 35$) (33)	≥65 years; room for improving muscle strength or protein intake, or recovery after inactive period	One arm: tailored nutrition advice aiming at 20–25 g protein per main meal for 12 weeks combined with RET	Not measured	Chair-rise performance: −3.3 ± 4.2 s, $p = 0.001$[3] Leg strength: 47.8 ± 46.8 kg, $p < 0.001$[3]
PROMISS ($n = 276$) (35)	≥65 years; habitual protein intake < 1.0 g/kg aBW/d at baseline	Three arms: dietary advice to increase protein intake to ≥1.2 g/kg aBW/d with (PROT+TIMING) or without (PROT) advice to time protein intake in close proximity of usual physical activity, or no dietary advice for 24 weeks	Mean ± SE PROT+TIMING: 0.81 ± 0.01 PROT: 0.82 ± 0.01 Control: 0.82 ± 0.01	Compared to controls: 400-m walking time PROT+TIMING: −4.9 (−14.5 to 4.7) s PROT: −12.4 (−21.8 to −2.9) s Leg extension strength PROT+TIMING: 24.3 (0.2–48.5) N PROT: 32.6 (10.6–54.5) N
CALM ($n = 76$) (36)	>65 years; no medical condition potentially preventing them from safely completing the intervention	Three arms: Supplementation with 20 g of whey protein 2 times/day with or without heavy RET, or placebo (maltodextrin+sucrose) for 12 months	Mean ± SD Protein: 1.1 ± 0.3 Protein+exercise: 1.1 ± 0.4 Placebo: 1.2 ± 0.3	Protein only compared to placebo: no improvements in quadriceps size, lower extremity strength and power, functional capabilities, and body composition. Compared to protein only, protein+exercise improved in: Quadriceps size: +1.68 (0.41–2.95) cm^2, $p = 0.03$[4] Dynamic knee extensor strength: +18.4 (10.1–26.6) Nm, $p < 0.001$[4] Isometric knee extensor strength: +23.9 (14.2–33.6) Nm, $p < 0.001$[4]

LBM, lean body mass; RET, resistance exercise training; SPPB, Short Physical Performance Battery.
[1]Values are mean ± SD or β (95% CI), unless stated otherwise.
[2]p value for time × treatment effect.
[3]p value from paired samples t-tests.
[4]Values are mean between-group difference (95% CI).

It could be that essential amino acids in protein sources exert direct effects on bone health, similar to the way in which leucine can stimulate muscle protein synthesis (51, 52). In 2022, evidence on the potential role of essential amino acids on bone aging was gathered (53). The authors report that *in vivo* and *in vitro* studies showed that several essential amino acids (lysine, threonine, methionine, tryptophan, and isoleucine) can increase osteoblast proliferation, activation, and differentiation, and decrease osteoclast activity, but that conflicts in mechanisms of action exists (53). These findings were partly replicated in human studies. In an observational study ($n = 2,997$, mean age 72 years), higher serum concentrations of valine, leucine, isoleucine and tryptophan were associated with less hip BMD decline after 4 years (OR/SD ranging from 0.83 to 0.92) after multiple adjustments (54). In that cohort, higher serum tryptophan concentrations were also associated with fewer major osteoporotic fractures (HR/SD 0.86) (54), a finding that has been confirmed in another cohort study (hip fracture

TABLE 2 Characteristics and results of two recent studies investigating the effect of protein on bone health in healthy older adults.

Study (ref)	Population	Follow-up period (cohort) or intervention (trial)	Baseline protein intake (g/kg/d)[1]	Results
Weaver et al. (37) ($n = 3,075$)	70–79 years; healthy, white and Black men and women	5 years of follow-up	High: 1.1 ± 0.4 Low: 0.8 ± 0.3	Compared to low protein group, high protein group had: 1.8% higher mean hip BMD at baseline, $p < 0.05$ 6.0% higher lumbar spine BMD at baseline, $p < 0.05$ No differences in BMD after 4 years Reduced risk of vertebral fractures after 5 years, HR 0.64 (95% CI: 0.14–0.97), $p = 0.04$
Kemmler et al. (38) ($n = 43$)	≥72 years; men with osteoporosis and sarcopenia	Whey protein supplementation and high intensity dynamic RET for 18 months	Control: 1.29 ± 0.34 Intervention: 1.10 ± 0.25	Compared to control, intervention group improved in: Lumbar spine BMD: +0.012 (0.001 to −0.020) mg/cm^2, p=0.024[2] Total hip BMD: +0.013 (0.002–0.022) mg/cm^2, p=0.025[2]

HR, hazard ratio; RET, resistance exercise training.
[1]Values are mean ± SD.
[2]Values are mean difference (95%CI). p value from dependent t-tests.

cases $n = 131$; controls $n = 131$) (55). Alternatively, in a study using UK Biobank data ($n = 111,257$; 901 hip fracture cases) that investigated the association between circulating amino acids and incident fractures, an association was found between valine concentrations and hip fractures (HR/SD 0.79) (56). This finding was replicated in the *Umeå Fracture and Osteoporosis hip fracture study* (hip fracture cases $n = 2,225$; controls $n = 2,225$) (56). Although the evidence is starting to suggest a protective role of essential amino acid concentrations, specifically valine and tryptophan, more high-quality evidence is required to arrive at firm conclusions about the role of essential amino acids in bone health.

Discussion

The differences in protein recommendations for older adults between bodies setting dietary reference values, expert groups, and national health organizations stem from the utilization of distinct criteria. When nitrogen balance studies are used, the conclusion is to set the PRI at 0.83 g/kg/d. But if physiological outcomes are taken into account, then it is time to reconsider the values, which is acknowledged by other critical reviews as well (57, 58). Another criterium is if cohort studies are valued to the same extent as RCTs. Especially for bone health, the evidence originates mostly from cohort studies.

Physiological changes that occur with aging, such as sarcopenia, osteoporosis, reduced protein synthesis, and altered metabolism, make it probable that a protein intake above the PRI is needed for older adults compared to adults. To justify this higher protein recommendation for older adults, a number of research gaps needs to be addressed. First, more nitrogen balance studies need to be performed in older adults. Additionally, a distinction between different age groups within the older population should be made, for example between individuals who are between 65 and 80 years and those who are above the age of 80 years. This differentiation allows for a more nuanced understanding of the effects of aging and the potential variations in health needs and outcomes. Secondly, research should focus on the effect of protein on physiological and clinically relevant outcomes. These outcomes are also highly valued by the older population since they directly influence quality of life and overall well-being. Well-designed, large, and long-term RCTs are especially needed to determine if a protein intake above the PRI can support bone health and/or prevent osteoporosis, as evidence from trials is limited. Thirdly, the heterogeneity of the older population needs to be acknowledged. Individuals in this demographic vary widely in terms of health status. For example, different recommendations may be necessary for those who are malnourished or for those who have several comorbidities.

Next, to protein quantity, more studies are needed to investigate protein quality. While plant-based diets offer environmental and health benefits, their adoption among older adults raises concerns regarding protein and nutrient adequacy for muscle and bone health. Unraveling the true effect of plant-based diets on muscle and bone health in older adults is needed, as well as solutions to improve the protein quality of plant-based diets. An exciting topic for future

exploration is the initial evidence that hints at a protective role of essential amino acids in bone health.

In conclusion, considering physiological and clinically relevant outcomes in protein recommendations for older adults is preferable, focusing on both the quantity and quality of protein.

Author contributions

IG: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. LG: Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. IT: Validation, Writing – review & editing. PG: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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In vitro protein digestibility to replace *in vivo* digestibility for purposes of nutrient content claim substantiation in North America's context

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The reliance by North American regulatory authorities on *in vivo* rodent bioassays—Protein Correct-Amino Acid Score (PDCAAS) in the U.S. and Protein Efficiency Ratio (PER) in Canada—to measure the protein quality for protein content claim substantiation represents a major barrier for innovation in the development and marketing of protein foods. Although FAO in 2013 proposed a new method (Digestible Indispensable Amino Acid Score, DIAAS), it is still not used for protein content claim substantiation in any jurisdiction. Together with public health efforts to increase the consumption of plant-based foods, removing hurdles is key to incentivizing the food industry to measure protein digestibility in making food formulation decisions as well as in claiming protein content on product labels. To address this issue, a pathway has been proposed to position alternative methods for *in vitro* protein digestibility in collaborative studies to generate the data necessary for method approval by a certifying body. The latter is critical to the potential recognition of these methods by both Health Canada and the US FDA. The purpose of this article is to briefly summarize the state-of-the-art in the field, to inform the research community of next steps, and to describe the path engaging collaborative laboratories in a proficiency test as the first step in moving forward toward acceptance of *in vitro* digestibility methods. Throughout, a consultative and iterative process will be utilized to ensure the program goals are met. Success will be achieved when the proposed path results in the acceptance of an *in vitro* methods for protein digestibility used for PDCAAS determinations, which will enable increased protein analyses and improved nutrition labeling of protein foods.

KEYWORDS

protein quality, nutrient content claims, *in vitro* protein digestibility, regulatory testing, food labeling

1 Introduction

The definition of protein quality has historically been based on the ability of dietary protein to provide sufficient levels of indispensable (essential) amino acids to meet the metabolic requirements of humans (1, 2). This property is dependent on the protein's amino acid composition and digestibility/availability (3, 4). Protein content claims in the USA and Canada (but not in many other countries) for consumer foods not intended

for special medical uses require the use of *in vivo* animal models to assess the quality of the protein (5, 6). In the US, the Food and Drug Administration (FDA) requires the determination of the protein digestibility-corrected amino acid score (PDCAAS) for protein content claim substantiation (7). The PDCAAS is determined as the product of the amino acid score and the true fecal protein digestibility (TFPD) of the test article in question (2). The amino acid score is determined by dividing the indispensable amino acid composition of the test article by the corresponding reference amino acid requirement values (mg/g protein) (8), with the score established as the lowest ratio value. As such, the AAS is a value that requires only analytical chemistry techniques or published amino acid composition tables for its calculation. However, the second component of the PDCAAS, the true fecal protein digestibility coefficient, requires the use of a rodent bioassay for its determination and represents an estimate of the extent to which the food protein is digested and absorbed. The use of a bioassay to assess protein quality is not unique to the PDCAAS as, in Canada, the protein rating system for content claim substantiation is typically based on the use of the protein efficiency ratio (PER) bioassay (9). The latter compares the growth rate of rats fed test protein compared to casein. The use of PER for general food labeling is unique to Canada and has been reviewed elsewhere (5, 6, 10). In December 2020, Health Canada announced that it would allow the usage of PDCAAS for the calculation of a Protein Rating (PER = PDCAAS × 2.5) for protein content claim substantiation, allowing this method to be harmonized between Canadian and American regulators and food industry stakeholders (11).

It is important to note that international organizations have convened expert panels on numerous occasions to assess measures of protein quality (8). Following a meeting in 2011 in Auckland, New Zealand, an expert panel prepared a document that positioned a refined method for determining the quality of dietary proteins, namely the Digestible Indispensable Amino Acid Score (DIAAS) method (12). Conceptually similar to PDCAAS, DIAAS relies on the use of updated amino acid reference patterns as well as an alternative strategy to assess the utilization of dietary protein. For the latter, the positioned method relies on the assessment of the ileal digestibility of dietary amino acids, instead of total nitrogen/crude protein. Finally, while the optimal subjects for study are humans, for practical purposes, the use of an ileal cannulated swine model has become the standard for the generation of DIAAS data on numerous food products (13, 14). While the DIAAS method has documented advantages over PER and PDCAAS, to date neither Health Canada nor the FDA have indicated an intention to move to this approach for protein content claim substantiation, with PDCAAS remaining the method of choice. This was recently reinforced by Health Canada when, in 2023, they signaled their intent to move even further with the adoption of PDCAAS for protein claims (15).

As reviewed by FAO/WHO (8, 12, 16), there are multiple methods for assessing protein quality, and the literature presents an exhaustive summary of these methods (1, 2, 17). The goal of this current work is to review the challenges that exist for the protein food sector in measuring PDCAAS values as currently required by regulatory authorities, with the primary concern relating to the use of rodent bioassays for measuring true fecal protein digestibility (TFPD). Another purpose of this work is to inform the research community of the options for replacing the TFPD value with those determined by suitable *in vitro* assays with an ongoing program for method validation with the first step being an interlaboratory collaborative study. A line of reasoning supporting the use of *in vitro* versus animal testing for protein quality assessment in North America has been positioned in a recent publication (18).

2 Determination of true fecal protein digestibility

According to Title 21 in the U.S. Code of Federal Regulations, the official method for evaluating protein digestibility for PDCAAS determination is the TFPD method. This method is outlined by the Food and Agricultural Organization/World Health Organization (FAO/WHO) in 1991, originally positioned by McDonough et al. (19), and is mandated by the U.S. Food and Drug Administration. The TFPD (%) of protein is determined as follows:

$$TFPD\ (\%) = \frac{N_I - (F_N - M_N)}{N_I} \times 100$$

where N_I = N (nitrogen) intake (protein in diet), F_N = fecal N and M_N = fecal metabolic loss. M_N is determined from N measured in feces from rats fed protein-free diets. Minimally, this bioassay requires a minimum of four rats per test group (including a protein-free group for metabolic N losses) and 9 days to complete. As such, the TFPD measures the proportion of protein-derived nitrogen that is available (digestible) to meet the protein needs of the respective consumer. As positioned above, the TFPD is used to calculate the final PDCAAS value for a food or food ingredient.

With respect to the measurement of TFPD, the rat fecal balance method is relatively straightforward, and requires processes to ensure adequate measurement of feed intake, total fecal collection, including the use of wire bottomed cages, and feed and fecal nitrogen determinations. However, recognition that rodent nutrient requirements differ from human, that the large intestine microbiota can alter the amino acid composition of the digesta (20) and changes in laboratory animal welfare policies since this method was first positioned, create challenges for the current usage of this method. Although the FDA and Health Canada still requires the use of rodent models for protein quality determination and substantiation of protein content claims, the FDA Modernization Act 2.0 (Bill S.2952) was passed in the USA Congress in 2022, which removes the obligation for pharmaceutical companies to test drugs on animals before human trials. Societal expectations regarding the use of animal testing for regulatory purposes have also evolved. In response to strong external pressures, many food and ingredient companies have adopted policies against the use of animals in research and testing. Additionally, certain third-party validation and front-of-pack labeling systems that provide information to prospective consumers on the nature of the food (e.g., Certified Vegan™; Vegan Action/Vegan Awareness Foundation, 2021) stipulates that animals cannot be used in testing. This can place certain desirable and informative logos out of reach of the food sector, such as "good or excellent protein source" claims. Furthermore, there are global efforts by researchers and government agencies to replace, reduce, and refine (3Rs) the use

of research animals in the safety evaluation of consumer goods, pharmaceuticals, and agricultural and industrial chemicals (21–23). Finally, developing validated *in vitro* methods for assessing protein digestibility will not only enable higher throughput of protein digestibility determinations, but will eliminate the high research costs associated with purchasing lab animals, maintaining animal housing facilities and formulating diets containing the appropriate protein test articles.

In Europe, the United States and Canada, all proposed animal research is reviewed by an appropriate organizational animal welfare committee to justify the use of animals and demonstrate how efforts have been made to comply with the 3Rs (24, 25). The "new approach methodologies" (NAMS) can be applied to this purpose. The NAMS term refers to any non-animal technology, methodology, approach, or combination thereof that can increase testing capacity as a result of significant advances in *in silico*, *in chemico* and *in vitro* method development (26). Completely animal-free methods, which do not use any animal component, are defined as "non-animal methods," whereas replacement methods may still be dependent on animal components such as serum or enzymes (22). When NAMS are to be applied for regulatory purposes, it is important that assay developers and regulators work together to achieve agreement for adequate performance criteria for the specified context of use (26, 27). This holds true for the positioning of alternative, *in vitro* methods for the measurement of protein digestibility for the ultimate calculation of PDCAAS values.

3 *In vitro* protein digestibility assays

Current *in vitro* models used for the evaluation of protein digestibility include the use of both "dynamic" and "static" methods (28). Dynamic gastrointestinal digestion methods are designed to simulate gastro-intestinal digestion phases and nutrient bioaccessibility through the use of sophisticated, computer-controlled, temperature-regulated digestion chambers (28). These models have been used to measure amino acid and nitrogen digestibility (29), but the high cost of system acquisition, limited sample throughput, and high operational costs likely represent major limitations to their routine use for the systematic determination of protein or amino acid digestibility. However, dynamic models offer useful tools for the integrative study of nutrient digestion.

In comparison to dynamic model systems, static models represent "bench top" assays that can be readily implemented across various laboratory settings. Static *in vitro* assays that treat suspensions of food with a mix of digestive enzymes have been used for decades as research tools to study food structure and digestibility, nutrient bioavailability and to provide protein digestibility coefficients (30, 31). Many studies have reported PDCAAS values based on static *in vitro* digestibility for dozens of protein foods (1, 32–38). Static *in vitro* protein digestibility methods have fewer ethical concerns, are less costly, and can be executed more easily and rapidly and with much higher throughput than *in vivo* methods. This would enable a wider variety of raw and processed foods to undergo analyses for protein quality than is possible with the currently approved methods. While *in vitro* methods may not perfectly replicate *in vivo* digestibility, there is good agreement for digestibility values obtained with the two methods (1, 32–39). Recent summative data from the authors' laboratory provide evidence of high R^2 values for PDCAAS values of plant-based protein sources when comparing those calculated via *in vivo* and *in vitro* assays. Sá et al. (40) reviewed various studies suggesting strong correlations between *in vitro* protein digestibility values (performed by pH-drop method) and *in vivo* protein quality measurements (e.g., PDCAAS) for different pulses: green and red lentils ($R^2 = 0.9971$) (36), chickpea ($R^2 = 0.9442$) (37), beans ($R^2 = 0.7497$) (35), and pinto bean ($R^2 = 0.9280$) (34). Furthermore, another study compared *in vitro* and *in vivo* PDCAAS for protein isolates and concentrates from faba beans, lentils, and peas, and the results showed a strong correlation ($R^2 = 0.9898$) (41). Evidence shows that despite the simplicity of *in vitro* models (42), they are often very useful in predicting outcomes of the *in vivo* digestion (43). Thus, *in vitro* protein digestibility could be applied as a surrogate to calculate *in vitro* PDCAAS for determining protein quality. In considering static *in vitro* digestibility models, the methods can vary in complexity, from simple mono-compartmental models to those that simulate multiple gastrointestinal compartments.

3.1 Static, mono-compartmental models for determining protein digestibility

The FAO/WHO report (8) positioned two static, mono-compartmental models for measuring TFPD, namely the pH-drop (PHD) method (44, 45) and the pH-stat (PHS) method (46). These methods have been widely applied by researchers and digestibility measures show good agreement with *in vivo* protein digestibility (30). In general, both methods rely on the principle that as peptide bonds are cleaved during enzymatic digestion, protons are released and cause a drop in pH. Figure 1 represents a scheme of pH-drop and pH-stat analytical measurements for *in vitro* protein digestibility determination. The pH-drop method measures the drop in pH over a specified time, while the pH stat method maintains constant pH by auto-titration of NaOH. This method follows the pH change of the protein digestate over a 10-minute time period, and it has been shown to have a high correlation ($R^2 = 0.90$) with the *in vivo* TFPD as determined in rats (44). A typical methodological approach includes taking 62.5 mg of protein equivalents (N × 6.25, standard nitrogen-to-protein conversion factor) from each test article for digestion. While it is recognized that 6.25 is not the appropriate nitrogen-to-protein conversion factor for all proteins, it is the default currently used until specific, validated and consensus-driven nitrogen-to-protein conversion determinations are established for all proteins (48). Test articles are digested by incubating them, in triplicate, with an enzyme cocktail containing 1.6 mg/ml trypsin [porcine pancreas 13,000–20,000 BAEE (Nα-benzoyl-L-arginine ethyl ester substrate) units/mg protein], 3.1 mg/ml chymotrypsin [bovine pancreas ≥40 N-Benzoyl-L- Tyrosine Ethyl Ester (BTEE) units/mg protein], and 1.3 mg/ml protease (*Streptomyces griseus* ≥3.5 units/mg solid) which are prepared in 10 ml of Milli-Q water and heated to 37°C. A modification of the original method was positioned by Tinus et al. (49) to account for changes in the availability of

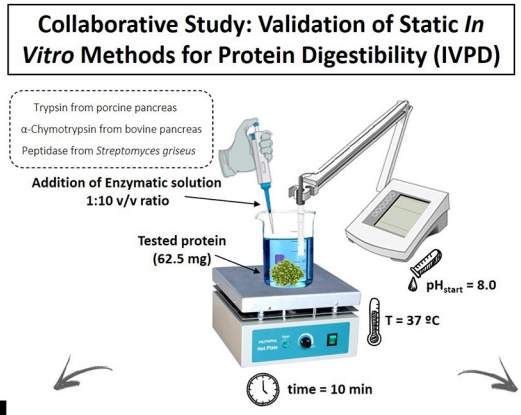

FIGURE 1
Schematic representation of static *in vitro* methods (pH-drop and pH-stat), included in a collaborative study aimed at validating their effectiveness in determining protein digestibility (47).

commercial proteases. The mixture is brought to a pH of 8.0 ± 0.5 with 1 M NaOH or HCl, after pH has stabilized following an hour of solubilization. The PHD is initialized with the addition of 1 ml of the enzymatic cocktail to the protein solution. The initial pH is recorded before the introduction of the cocktail and at 30 s intervals, for a total of 10 min. The PHD *in vitro* protein digestibility (IVPD, %) is calculated using the formula below:

$$IVPD\ (\%)_{pH-drop} = 65.66 + 18.10 \times \Delta pH_{10min}$$

The pH-stat assay, as mentioned, has generated protein digestibility values that agree well with *in vivo* measures with good reproducibility in an interlaboratory study (19). In brief, this method, which follows the protocol set out by Pedersen and Eggum (46), is similar to the pH-drop method. A typical approach involves the incubation of 62.5 mg of protein equivalents (N × 6.25) derived from test articles with an enzymatic cocktail containing 1.6 mg/ml trypsin, 3.1 mg/ml chymotrypsin, and 1.3 mg/ml protease, prepared in 10 ml of Milli-Q water and heated at 37°C. Both the sample protein and the enzyme cocktail are brought to a pH of 8.0 ± 0.5 with 1 M NaOH or HCl, following a 60 min pH stabilization process. Following the addition of enzymes, pH is held at 8.0 using 0.1 N NaOH, and the volume of 0.1 N NaOH used to hold the pH recorded. The pH-stat *in vitro* protein digestibility (IVPD, %) is calculated using the formula:

$$IVPD\ (\%)_{pH-stat} = 76.14 + 47.77 \times \sum 0.1N\ NaOH$$

One clear weakness of the pH-based assays is that foods with remarkably high buffering capacity, including some animal-based protein sources, may yield lower than expected digestibility values. In addition, the pH-based methods do not consider large intestinal fermentation that may contribute to the fecal nitrogen mass measured in the *in vivo* PDCAAS method. However, as indicated previously, the good agreement with published *in vitro* PDCAAS values to *in vivo* determined values suggests that the fecal nitrogen measurements do largely reflect the differences in small intestinal absorption of digestible amino acids from the food proteins. Therefore, the pH-based methods appear to closely calculate the digestible amino acids available for intestinal absorption. One additional limitation relates to the lack of use of pepsin (stomach protease) in these methods, however the use of a bacterial protease provides additional proteolytic activity to enhance overall protein digestibility. Despite these limitations, the documented agreement between the static methods and *in vivo* digestibility estimates supports their consideration for routine *in vitro* protein digestibility assessments for regulatory purposes. The simplicity, ease of implementation and low cost of executing these pH-based methods, however, warrants consideration and will be compared to a third method, the INFOGEST method, which, while modestly more complex, has been developed with the specific goal of standardizing experimental procedures and conditions for an *in vitro* digestion method that can be reproduced globally (50, 51).

3.2 Static, multi-compartment gastrointestinal digestion models

The INFOGEST network (http://www.cost-infogest.eu) was established in 2015 with the aim of "improving dissemination of critical research findings, developing truly multidisciplinary collaborations and harmonizing approaches between groups and discipline areas spanning the main stages of food digestion." The network currently consists of more than 440 research scientists from 45 countries and includes 50 food companies.

The INFOGEST *in vitro* digestion method as originally developed (50) and as recently refined (INFOGEST 2.0) (51) is developed for food digestion in general and has been successfully

used to assess protein digestibility in different foods and food forms (31, 52). This method is representative of gastrointestinal digestibility (GID) models. The INFOGEST method version 2.0 has been fine-tuned based on user feedback and precise and thorough details for the protocol have been published (51). The method is executed in three phases: preparation of the simulated digestive fluids and enzyme reagents, digestion procedure, and sample treatment for subsequent analyses (the latter being specific the specific assay endpoints) (51). The digestion procedure consists of three phases: (a) oral phase (salivary phase), (b) gastric phase, and (c) intestinal phase. Protein digestibility has been evaluated after INFOGEST digestion after each phase by arresting digestion. Different methods can be used to determine total protein digestibility, such as total nitrogen (e.g., Kjeldahl), primary amines (o-phtaldialdehyde, OPA) (53), trinitrobenzene sulfonic acid (TNBSA) (52, 54), and amino acid analysis (e.g., UPLC or LCMS) (55).

Harmonizing digestion techniques, exemplified by standardized INFOGEST protocols, holds a significant promise in advancing food digestion studies and crafting customized food solutions for diverse segments of the population (56, 57). However, while the static protocol improves comparability *in vivo* pig or rodent digestion, it does not fully capture dynamic *in vivo* digestion processes. Thus, direct comparison between *in vitro* and *in vivo* results remains crucial for validation (58, 59). Sousa et al. (55) evaluated the correlation between the *in vivo* and *in vitro* DIAAS, and results showed highly correlated true ileal digestibility values ($r = 0.96$, $R^2 = 0.89$, $P < 0.0001$).

Furthermore, a patented method (U.S. Patent No. 9,738,920 B2) (60) outlines a technique for determining *in vitro* protein digestibility that involves a two-step enzymatic process. Initially, the protein-containing sample undergoes gastric digestion with the enzyme pepsin. Subsequently, the digested sample is treated with trypsin and chymotrypsin to simulate intestinal digestion. After these steps, a spectroscopic compound that binds with the protein's amino and carboxyl groups is added to create a solution suitable for optical analysis, such as the addition of ninhydrin, which produces Ruhemann's purple, and the absorbance is measured at 570 nm using a spectrophotometer. A commercial kit available from Megazyme© contains all necessary enzymes and reagents for this procedure, allowing for the determination of the *in vitro* digestibility score.

4 Toward validation of *in vitro* methods for estimating protein digestibility for PDCAAS measurements

Despite the long-standing use of *in vitro* assays to determine protein digestibility, efforts to validate these methods as a replacement for the *in vivo* rat bioassay for the purposes of calculating PDCAAS have been limited. An interlaboratory study of the pH-stat *in vitro* method (46) for assessing protein digestibility was published in the same journal (61) just before a collaborative study on the *in vivo* method (using the same protein sources) was published. The latter led to the validation of the rat bioassay as an officially recognized method for protein digestibility (19). Notably, the reproducibility and repeatability of the *in vitro* and *in vivo* methods were similar, yet further action was not taken to promote the *in vitro* method presumably since *in vivo* methods were likely prioritized at the time. Additionally, while the pH-drop method has been used recently to determine the *in vitro* protein digestibility of a number of plant-based proteins, including pulses (33–36, 62), the method has been modified since first positioned (49), due principally to changes in the availability of key enzymes. As such, method validation remains a key goal for all of the static *in vitro* methods.

An important concept in developing and approving official methods of analyses is "fit for purpose" (30). The degree of accuracy and precision required for a measure of protein digestibility to enable a food product intended for consumption in mixed diets among the general public to carry a protein content claim must be such that it prevents overestimation of protein content, thereby avoiding the risk of underconsumption of indispensable amino acids. In countries and supranational unions, including the European Union and Australia (5), that do not require protein quality to be measured for protein content claims in foods intended for the general public (i.e., excluding special dietary uses), there have not been any reported safety issues or concerns of misleading consumers regarding choices of protein foods.

The current PDCAAS calculations have other sources of potential error which contribute to the value's inherent uncertainty, namely, the amino acid analyses (63, 64), total protein determination which depends on nitrogen determinations corrected for the protein-to-nitrogen conversion factor (for which there are no standardized factors) (48), and protein digestibility when using published tables of digestibility on a similar, but not the exact food, under study (65, 66). The latter source of error could be significantly reduced if a relatively inexpensive *in vitro*, high throughput, method of determining protein digestibility on the exact food and food forms were available. By convention, the reference protein, casein, used in many methods of assessing protein quality [i.e., Protein Efficiency Ratio (PER), Net Protein Utilization (NPU), and Indicator Amino Acid Oxidation (IAAO)] (30) would be chosen as the standard reference protein in the proposed collaborative study.

As mentioned above, the FAO/WHO has convened expert panels on numerous occasions to assess measures of protein quality and while PDCAAS remains the method of choice due to lack of data on emerging methodologies, recommendations to advance DIAAS were published in two key FAO reports published in 2013 (12) and 2014 (67). The DIAAS method uses the ileal digestibility coefficients of individual amino acids to determine the "true ileal digestibility" of the indispensable amino acids in food, unlike *in vivo* PDCAAS, which uses true fecal digestibility of the entire food protein for calculating protein quality values (68). While the DIAAS method may be a more accurate approach to determine protein quality (5, 13), the use of ileal-cannulated pigs as described is highly impractical to determine digestibility coefficients for large numbers of foods and food ingredients. As a result, several investigators working on standardizing the INFOGEST digestion method have recently published results that offer the promise of developing an *in vitro* DIAAS (IV-DIAAS) method (55, 69). Values for IV-DIAAS are comparable to that observed in the *in vivo* DIAAS

method (69). A major ring-trial and methods validation protocol are currently underway for using INFOGEST 2.0 to determine *in vitro* DIAAS (ISO/NP 24167/IDF 261, Milk and milk products – *in vitro* digestion protocol for the analysis of protein digestibility and *in vitro* DIAAS). Supported by the International Dairy Federation (IDF), the joint IDF/International Standards Organization (ISO) *in vitro* protocol will first be applicable to dairy foods, with ring trial expansion to other foods, including plant-based proteins. The initial validation workflow was recently approved and the protocol has moved into a 36-month development track. While the *in vitro* DIAAS methodology can serve to generate proxy PDCAAS values (53), for the purposes of the remainder of this paper, the focus will be on the development of a collaborative study to determine *in vitro* TFPD for PDCAAS estimation and protein content claim substantiation.

Here, in order to address the major limitation of a lack of approved *in vitro* methods, we report that a collaborative study is ongoing that will evaluate and test the proficiency of candidate static *in vitro* methods to measure protein digestibility based on the currently accepted PDCAAS method. The primary objectives for this collaborative study are (a) to determine the repeatability and reproducibility of the candidate methods, and (b) to demonstrate sufficient agreement to published values of *in vivo* TFPD values to warrant approval as an official method of analysis and acceptance by regulatory bodies for protein quality assessments by PDCAAS. For the ongoing study, the focus will be on the pH-drop and the pH-stat methods, given their long-standing usage, available evidence to support agreement with *in vivo* TFPD data, and relative ease for implementation across multiple laboratory environments, including industry-based research settings.

5 Proposed pathway for validation for *in vitro* protein digestibility

The positioning of an approved *in vitro* method for assessing protein quality would provide an alternative to the use of animal bioassays for the substantiation of protein content claims. At present, protein remains the only nutrient for which the use of a bioassay is required to substantiate a protein content claim on foods in both Canada and the United States. Other nutrients, including energy, folate, niacin, and the fat-soluble vitamins, have established availability coefficients that have been accepted for labeling purposes, thus allowing analyzed food components to be converted to nutrient equivalents (e.g., dietary folate equivalent; Atwater factors for energy). Given that biological responses (PER; TFPD) are required for protein, this creates barriers for the food sector to differentiate both existing and new protein sources in terms of their ability to contribute quality protein for the human diet. The acceptance of an approved *in vitro* method for estimating TFPD would address this challenge. It would provide regulatory agencies assurances that factors influencing the digestibility of dietary proteins, particularly new sources and those derived from new processing methods, have been considered. Additionally, the acceptance of one (or more) approved methods would provide conformity within the food system as to the methods to use when positioning food protein sources for human consumers.

In order to position an *in vitro* method to both Health Canada and the FDA as being a suitable substitute for the TFPD bioassay, the method must first be approved by an accrediting body. Such bodies include the Association of Official Analytical Collaboration (AOAC), the American Oil Chemists' Society (AOCS) or International Organization for Standardization (ISO). The methods approval process employed by authoritative bodies typically involves a series of sequential steps that begin with the submission of a proposed method, either on its own or accompanied by results from a collaborative study. This process allows for an initial review of the method by the certifying body, providing a chance for experts to offer commentary and feedback on the proposal before it undergoes a collaborative study. As such, this approach can mitigate risks associated with methodological concerns prior to the initiation of data generation. Once the method has been approved by the sub-committee, a collaborative study is conducted to generate the data that will then be reviewed by a separate statistical sub-committee enroute to subsequent approval steps. Key to this process is the positioning of a method that has been written and structured according to the style guidance of the approving body. Figure 2 represents the proposed pathway for validation of an *in vitro* protein digestibility method. Regulatory bodies usually mandate the use of approved official methods to meet their scientific requirements for labeling, therefore once the *in vitro* digestibility method is approved as an official method by an accrediting body, the final step would be to petition the appropriate regulatory bodies to accept the official method for PDCAAS calculations.

The following components represent the required elements of an AOCS official method: (1) title of the method; (2) definition of the method including a description of the analyte or component in question; (3) scope of the method, including a description of the test articles to which the method applies; (4) apparatus to be used in the method; (5) reagents to be used, including information on the reagent grade and sourcing as well as pertinent information on the usage of special solutions; (6) procedural information to provide clear instructions for the analysis in question; (7) calculations required for the proposed method, presented in sufficient detail; (8) precision data derived from a collaborative study to support the method; (9) notes that pertain to the method, including safety concerns, data on limits of detection and other comments that are pertinent; and (10) key references, tables and figures.

As the static *in vitro* methods have been published, the methods for both the pH-drop and pH-stat have been based on those key publications (44, 46), with subsequent modifications (49). The draft test methods for both approaches have been submitted to potential study collaborators and consensus on the specific methods has been achieved through a workshop held in the spring of 2023. The Richardson Center for Food Technology and Research is serving as the Central Laboratory and we have successfully recruited a minimum of eight laboratories for the ring test as typically required by authoritative bodies for the generation of data for precision, repeatability, reproducibility, and accuracy. This standard ensures a broad and reliable data set for method validation. Key considerations for establishing a method that could be readily adopted and accepted by regulators include the availability of method reagents and apparatus. For *in vitro* protein digestibility (IVPD) determinations, changes in

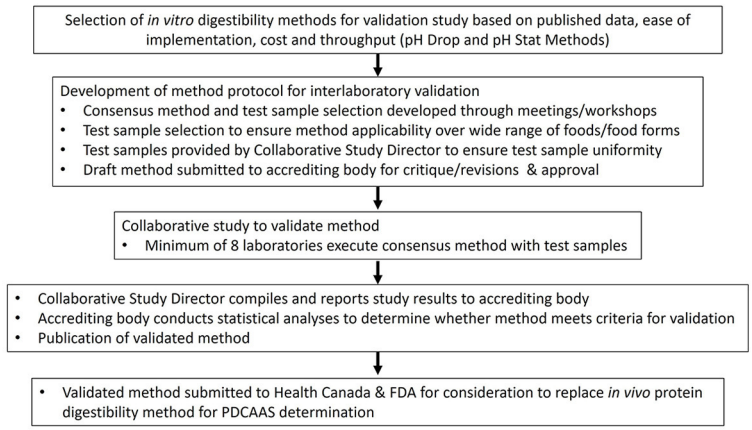

FIGURE 2
Proposed pathway for validation of an *in vitro* protein digestibility method.

TABLE 1 **Potential protein test articles for the validation of *in vitro* methods for the estimation of true fecal protein digestibility.**

Source	Forms	% True fecal protein digestibility	References
Casein	As procured	96–99	(8, 65)
Egg white powder	As procured	97–98	(65)
Skim milk powder	As procured	95	(8)
Beef, ground	Cooked; dried	91–95	(65)
Yellow pea	Thermal treatment; isolate	86–89 (cooked)	(33, 65)
Bean (pinto)	Thermal treatment	63 (baked); 76 (boiled); 85 (extruded)	(34)
Green lentil	Thermal treatment	86–88 (boiled); 86 (extruded)	(36)
Soy	Autoclaved; flour; isolate	84 (flour); 95 (concentrate); 96 (isolate)	(65)
Potato	Raw; boiled; isolate	40 (raw); 83 (boiled)	(70)
Rice	Raw; cooked; isolate	87 (polished); 86 (cooked)	(8)
Wheat	Raw; flour; gluten	87 (whole); 97 (white flour); 98 (gluten)	(65)

availability of enzymes, for example, will require standardization and consensus on suitable alternatives. The consensus method has been submitted and approved by Uniform Methods Committee of the AOCS. The proficiency testing began in the summer of 2023 and once results from all participating laboratories has been received, the data will be analyzed according to standard statistical practices for collaborative studies. Once feedback has been received from the approving organization, the methods would be shared with the regulatory bodies for commentary and critique, with further method refinement as needed in order to gain acceptance for the *in vitro* methods in PDCAAS calculations.

A key consideration for advancing the methods is the establishment of data across a number of test protein sources. The choice of protein food samples was critical for this evaluation to ensure applicability over a wide range of foods and food forms, including plant, animal, and novel protein sources. Casein was included as the standard comparator (as it is the standard in the current official *in vivo* method). A slate of test protein sources determined by consensus among the participating laboratories is positioned in Table 1. The test protein sources were selected to represent a range of plant and animal proteins and levels of processing, and to reflect those samples for which TFPD values are generally available. The latter will be an important criterion for providing evidence to the regulatory authorities on the validity of the generated *in vitro* data for estimating *in vivo* TFPD values. To facilitate the collaborative study, the central laboratory is responsible for the procurement, processing, packaging and distribution of test articles to the collaborating laboratories, as well as the collection of the sample data and statistical analysis in advance of submission to the approving body. During the course of this work and as mentioned above, consultation with the approving body and representatives from the FDA and Health Canada is ongoing and will be critical to ensure that criteria required for regulatory acceptance of the *in vitro* methods are addressed prior to completing the collaborative study execution and analyses.

6 Conclusions

The adoption of validated *in vitro* methods to measure protein digestibility (IVPD) for Protein Digestibility Corrected Amino Acid Score (PDCAAS) assessments will pave the way for innovation in the production and marketing of protein-rich foods in Canada and the United States. This regulatory change, eliminating the reliance on animal-based bioassays to confirm protein quality, is supported by substantial evidence endorsing *in vitro* methods capable of distinguishing between low and high-quality protein sources (18). This report outlines the steps for evaluating potential *in vitro* protein digestibility methods through an ongoing collaborative study, aiming to achieve certification from an authoritative body. Through a continuous and collaborative approach, the ultimate objective is to gain approval for one or more *in vitro* methods for determining protein digestibility in PDCAAS calculations, thereby marking the success of this initiative within North America. Furthermore, it is important to consider the global leadership role that the United States and Canada can play in shaping international standards for protein quality assessment. While the focus has primarily been on the regulatory implications within North America, it is crucial to acknowledge broader perspectives and future directions. Although PDCAAS has been widely used, the limitations associated with certain protein sources underscore the need for considering alternative measures, such as DIAAS, which offer a more precise assessment of protein quality but still currently requires *in vivo* assessment of protein digestibility. The efforts in advancing *in vitro* methods could serve as a model where *in vivo* methods are still required for certain protein foods, contributing to harmonized regulatory frameworks and facilitating international trade of protein-rich foods. In moving forward, it is essential to consider the complexities of inter-individual variability in protein digestibility and the influence of food matrices on digestibility assessments. Future research should explore the applicability of these methods in real food matrices and address population-specific variations in protein digestion.

Author contributions

EK: Conceptualization, Formal analysis, Project administration, Writing – original draft. AS: Validation, Visualization, Writing – review & editing. EG: Validation, Visualization, Writing – review & editing. JH: Supervision, Conceptualization, Project administration, Resources, Writing – review & editing, Funding acquisition.

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Conflict of interest

EK was employed by EKSci. EK is an independent consultant and received remuneration from the Institute for the Advancement of Food and Nutrition Sciences (IAFNS) for providing technical support for this project. She is a member of and serves on several committees for the American Oil Chemists Society. At the time of writing this manuscript, EK also served as a consultant for MOTIF Foodworks Inc., and EverGrain, LLC. EK received technical consulting fees to support protein digestibility standardization from the Institute for the Advancement of Food and Nutrition Sciences (IAFNS) Protein Committee. JH is a member of the IAFNS Board of Trustees and has recently served on the executive of the Canadian Nutrition Society. He currently holds contract and grant support from IAFNS to coordinate the interlaboratory validation studies for the pH-Drop and pH-Stat methods, as well as industry and government sources related to research on protein quality and protein content claims, including both plant- and animal-based foods. He currently holds a Canadian patent for the production of tofu-like products from oilseed press cakes.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Protein quality, nutrition and health

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Dietary proteins are energy macronutrients providing nitrogen, amino acids (AA), and energy. AAs are the main nitrogen-containing compounds in the body and are the precursors for the synthesis of body proteins and of several other AA-derived molecules. Among the 20 AAs included in protein sequence, 9 are classified as "nutritionally essential" or "indispensable" AA (IAA) because they cannot be synthesized in the body and must be provided by the diet. IAAs are limiting components for protein synthesis. An adequate intake of protein is required to support growth, maintenance, body functions, health and survival. Official definition of protein requirement is based on nitrogen balance. Protein quality is related to the capacity of protein to provide an adequate quantity of nitrogen and of each of the 9 IAAs for the different physiological situations in humans. Protein source is considered high quality for humans when the protein is readily digested, simultaneously providing an adequate quantity of nitrogen and of each of the 9 IAAs to maintain an adequate metabolic AA pool. The most accurate assessment of protein quality of foods for humans is through metabolic studies that measure nitrogen balance. The protein quality score is the ratio of the content of each IAA in the food and in a reference profile. This score corresponds to the calculated composition of a protein which, when meeting protein requirements, simultaneously meets the requirements of each of the 9 IAAs. AA scores as predictors of protein quality must be adjusted for protein and AA availability.

KEYWORDS

nutrition, protein for human health, protein quality, protein, amino acids

1 Introduction

Dietary proteins are macronutrients providing nitrogen, amino acids (AAs), and energy. In living organisms, nitrogen is mostly associated to AAs and AAs are mostly in the form of proteins. AAs are the main nitrogen-containing compounds in the body and are the precursors for the synthesis of body proteins and of several other AA-derived molecules, all involved in the structure of tissues and/or in all the functions of the organism.

There is a very large number of proteins in the body (~10,000 types) and each protein is characterized by a specific sequence of AAs encoded in the genetic code. Among the 20 AAs included in protein sequence, 9 are classified as "nutritionally essential" or "indispensable" AAs (IAAs) because they cannot be synthesized in the body and must be provided by the diet (1). The 11 other AAs are "dispensable" because they can be synthesized in the body from precursors available in the organism. In adult humans (female 57 kg, male 70 kg), the protein compartment is 8–12 kg (Figure 1). Despite the large number of body's proteins in the body, about half of these proteins are represented by four proteins—myosin, actin, collagen, and hemoglobin—and among them, about 25% is represented by collagen. Body protein have both

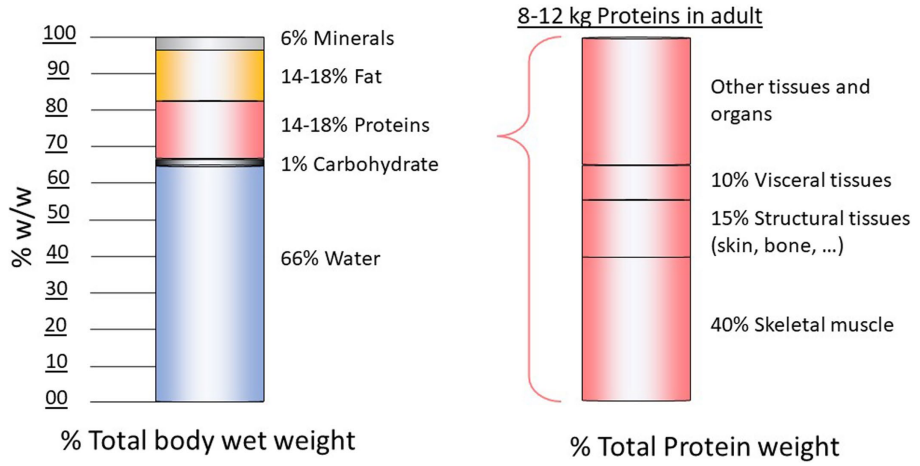

FIGURE 1
Body composition in healthy adult.

structural (muscle, skin, and bone) and physiological function (enzymes, hormones, receptors, antibodies, and cytokines).

Several health outcomes are associated with protein sufficiency such as body weight, body composition, muscle mass and strength, bone health, immune defenses, and most if not all physiological functions. An adequate intake of protein is required to support growth, maintenance, body functions, health, and survival. Protein quality is related to the capacity of protein to provide an adequate quantity of nitrogen and of each of the 9 IAAs for the different physiological situations in humans (1). The nutritive value of proteins from food and diet depends both on the amount of protein provided, but also on the AA composition and concentration, and on the bioavailability of protein-derived nitrogen and AAs. Protein quality matters because there are differences between the different food sources. Moreover, some forms of food storage and processing can affect protein quality (2).

Suitable markers for measuring the need for AAs and proteins and protein quality are derived from the different levels of AA metabolism and utilization in the body and from the functions of protein in the body (Figure 2; Table 1). Since the 1970/80s, the priority for international authorities of the United Nations (FAO/WHO/UNU) has been to define the requirement for nitrogen and IAA as criteria for protein quality to support body protein synthesis (1, 3, 4).

2 Protein and nitrogen requirements

Meeting protein nitrogen needs is required to maintain the body's protein pool that affect body composition and many if not all the functions in the body. Official definition of protein requirement is based on nitrogen balance – the usual protein intake that maintains a nitrogen balance in a person in good health, with normal body composition, normal energy balance and moderate physical activity. Determined by the nitrogen balance method in adult, the mean protein requirement is 0.66 g protein/kg/d (~40–50 g/d) and the recommended protein intake 0.83 g/kg/d (~50–60 g/d) (1). In different physiological situations such as infants, children, adolescents, pregnant women and lactating women, protein needs are derived from a factorial approach including nitrogen balance and additional protein deposition required for growth, pregnancy, or lactation.

Protein concentration or density (i.e., the amount of protein per unit of food) is a factor of food's protein quality (5, 6). Measuring nitrogen content with the Kjeldahl or Dumas methods and using a Nitrogen to Protein Conversion Factor remains the more frequently used approach for protein content in foods (7). The default conversion factor used for a mixture of protein sources is 6.25, corresponding to a nitrogen content of 16%. Specific protein conversion factors range from 5.7 (17.5% nitrogen) to 6.4 (15.6% nitrogen) for the major protein sources in the diet. The protein concentration in different food protein sources shows that animal product protein sources such as meat, milk, eggs, and some animal products are rich in protein with protein content of 30–70% (dry weight, dw). Among vegetables, pulses have the highest protein concentrations, ranging from 20–25% (dw) in most raw beans and peas to 35–38% in soybeans and lupines. Cereal seeds have a protein content of 15–20% (dw). Most nuts and edible seeds contain 8–18% protein (dw). Many oil seeds have 12–20% protein (dw), and the cake that remains after oil extrusion can have as much as 30–40% protein (dw).

3 Indispensable amino acid requirement and protein quality score

Maintaining optimal protein status required to provide in the diet a bioavailable form of an adequate quantity of protein with an adequate IAA profile.

The AA composition of proteins is usually calculated as milligrams AA per gram of protein. If they are reported as milligrams AA per gram of nitrogen, they are converted to the protein equivalents by multiplying by specific Nitrogen to Protein Conversion Factor. To calculate the AA content of a combination of food proteins, as in a food based on several protein sources or in a mixed diet, a weighted mean of the published or analytical results of each component should be used.

The protein required to achieve nitrogen balance must be of high quality. Protein quality is based on the capacity to provide an adequate

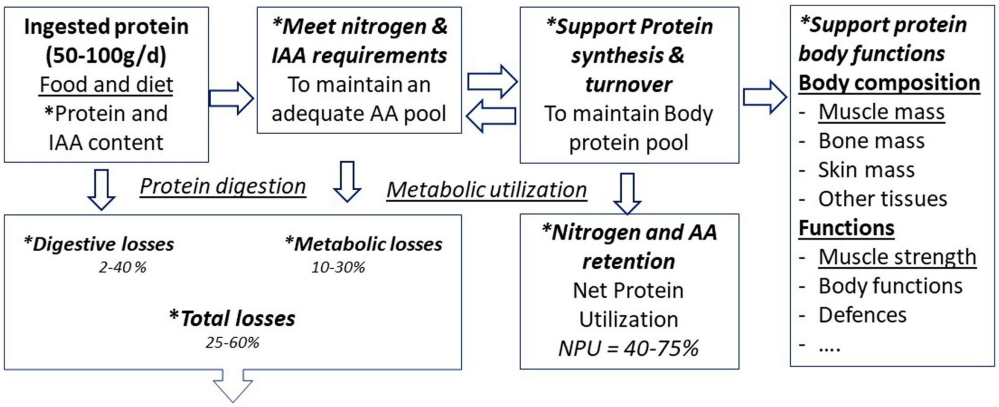

FIGURE 2
Proteinintake and utilization of nitrogen and amino acids (*markers of protein needs and protein quality) and markers and method for assessing protein quality.

TABLE 1 Markers and methods for assessing protein quality.

Protein, nitrogen & IAA requirements		
Protein requirement	Nitrogen balance	Digestive and metabolic nitrogen losses
Amino acid requirement	Amino acid oxidation	Stable isotopes amino acid balance
Protein, nitrogen & AA metabolic fate		
Protein, nitrogen & amino acid bioavailability	Faecal/ileal digestibility Amino acid availability	Faecal/ileal losses, dual isotope, Indicator AA oxidation, *In vitro*/*in silico* methods
Net protein utilization	Nitrogen/AA retention	Nitrogen/AA losses (stable isotopes)
Protein turnover	Whole body Protein synthesis	Stable isotopes amino acid balance and fluxes
	Muscle protein synthesis	Stable isotopes amino acid administration and muscle tissue sampling
Protein body functions		
Body composition	Lean mass, muscle mass, bone mass, …	
Body functions	Muscle strength, defenses, various functions, …	

quantity of nitrogen and of each of the 9 IAAs to achieve nitrogen balance and to support both protein turnover, and synthesis of the various AA derived component in the body. The 9 IAAs not synthesized in the body and limiting factors of AA utilization for protein synthesis must be provided at an adequate quantity and profile. IAA requirement for adult was initially determined by nitrogen balance in 1985 and re-evaluated in 2007 based on stable isotopes methods (1). IAA requirements were also determined for younger subjects by a factorial approach. The protein quality score is based on the ratio of the content of each IAA in the food and in a reference profile. The reference profile is the calculated composition of a protein which, when meeting protein requirements, simultaneously meets the requirements of each of the 9 IAAs. From the 2007 re-evaluation of IAA requirements, many foods such as cereals and legumes previously thought to be adequate in their IAA content, could be partially limited, particularly in lysine and Sulphur AA, respectively.

4 Correction of the score by digestibility

Protein sources are considered high quality for humans when the protein is readily digested, and nitrogen and AA readily absorbed and simultaneously providing an adequate quantity of nitrogen and of each of the 9 IAAs to maintain an adequate metabolic AA pool. A protein may have a good AA composition relative to the reference profile, but if it is not fully digested and its constituent AAs are not absorbed, its capacity to provide nitrogen and IAAs for human function will diminish.

Not all food proteins are digested, absorbed, and utilized to the same extent because of inherent differences in their source (e.g., inside vegetable cells with indigestible membranes), their physicochemical nature (e.g., protein configuration and AA binding), the presence of food constituents that modify digestion (e.g., dietary fiber, tannins, and other polyphenols), the presence of antinutritional factors that interfere with protein breakdown (e.g., trypsin inhibitors and lectins), and processing conditions that alter the nature or release of AAs (e.g., Maillard reaction and formation of polyAAs and methylmercaptan) (2, 8). Protein nitrogen digestibility values and more recently ileal AA digestibility values of specific foods and well-defined diets may be taken from reliable published data or must be determined, preferably in humans (3). When cost and practicality do not permit metabolic studies in humans to be performed, standardized methods in animal models are used (9). Nevertheless, animal data must be used with caution for foods and diets that are known or suspected of being handled differently by the human and animal intestines. When data are not available for a mixed diet, a weighted average can be calculated from the true digestibility of its constituent protein sources.

Consequently, AA scores as predictors of protein quality must be adjusted for protein digestibility and AA availability. The different scores are the "Chemical amino acid score," the "Protein Digestibility-Corrected Amino acid Score" (PD-CAAS), and the "Digestible

Indispensable Amino Acid Score" (DIAAS) (1, 3, 8, 10). Stable isotope-based methods contribute to accumulate values for true protein and IAA digestibility from human food sources, including animal and plant protein sources. The True ileal digestibility assay is the best currently available approach to assess nitrogen and AA absorption. Digestibility measurements at the ileal level may provide a better measure of AA digestibility, however this may pose significant challenges (9). True ileal AA digestibility is assessed by different invasive or minimally invasive procedures in human, or alternatively in animal (pig or rat) models (9, 11–16).

For both IAA profile and bioavailability, plant protein are most often of lower quality than animal protein (6, 17). Digestibility of protein and IAA from plant protein sources are usually lower than for animal protein sources. The difference is more important when plant proteins are consumed in the form of complex flour or whole grains (treatment, matrix, and antinutritional factors) (2). This is particularly sensitive for younger subjects with higher protein and IAA requirements, i.e., a need for high protein quality. Protein quality also matters in the context of climate change (18). Reduction in diet-associated greenhouse gas emissions involves a shift toward plant-based diets that leads to reduce IAA content, particularly lysine and methionine and a risk to not meet IAA requirements.

TABLE 2 Protein digestibility, PD-CAAS and Net protein utilization of different protein sources.

	Protein digestibility %	PD-CAAS % (adult)	Limiting AA	Nitrogen/AA retention NPU %
Animal-source	75–99%	>100	–	–
Bovine Milk	94–99	>100	No	75
Meat (beef)	80–99	>100	No	75
Hen egg	80–97	>100	No	72
Plant sources	60–90%	70	–	–
Soy	75–90	86–100	Met+Cys	~70
Pea	70–90	71–78	Met+Cys	~70
Rice	65–85	50–58	Lys	–
Wheat	65–85	46–51	Lys	~60–65

Adapted from Fuller and Tomé (12), Gaudichon et al. (13), Tome (19), Gausseres et al. (20), Evenepoel et al. (21), Bos et al. (22–24), Gaudichon et al. (25), Tomé and Bos (26), Fromentin et al. (27), Oberli et al. (28), and Oberli et al. (29).

5 Protein quality and protein synthesis

As mentioned above, the most accurate assessment of protein quality of foods for humans is through clinical studies that measure nitrogen balance (1). Food proteins are fed to a group of individuals and nitrogen losses are determined. However, biological assays in laboratory animals have been used to assess food protein quality, based either on a protein's ability to support growth in young rats (protein efficiency ratio, PER) or on nitrogen retention (net protein utilization, NPU) (19). The PER and NPU remain useful indices for screening food protein quality and to validate theoretical models based on the AA composition of the target protein. PD-CAAS and DIAAS values in adults for animal and plant protein sources can be compared to the efficiency of nitrogen retention Net Protein Utilization (NPU) (Table 2).

AAs are the precursors of protein synthesis in the body. The body proteins and free AAs are in a continuous turnover through protein breakdown and synthesis at an overall rate of about 250–300 g/d (Figure 2). AAs in free form, circulating and present in tissues, are a small fraction of all body AAs (less than 100 g). The 9 IAAs are limiting factor of protein synthesis. The major anabolic factors that influence muscle protein synthesis are contractile activity and feeding. AAs, together with insulin, display an anabolic effect and stimulate muscle protein synthesis (10, 30–33). The ability of a protein source to stimulate protein synthesis have thus been used to assess protein quality. Moreover, among AA, the branched-chain AA (BCAA) have many important physiological roles and of the three BCAA, leucine is most notably a key regulator signaling molecules of muscle protein synthesis (MPS), exerting anabolic effects even in the presence of hyper-aminoacidemia (34).

Protein ingestion induces an increase in muscle protein synthesis (MPS, %/h) measured by stable isotopes method in young men (10, 33). For young adults at rest or with low body exercise 10 g or 20 g of high-quality whey protein result in a rise of MPS of 19 and 52%, respectively, from control 0 g while 40 g do not result in higher stimulation beyond consumption of 20 g. However, in young adults following whole-body exercise 40 g of protein did result in significantly higher MPS rate (35) and results in older adults also indicate a greater MPS response to 40 vs. 20 g whey protein (36). From different studies 40 g protein is consistently 10–20% higher compared to 20 g protein, albeit not always statistically significant (37). Lysine deficiency limits the capacity of wheat protein to induce an increase in MPS. Ingestion in older adult of 35 g wheat protein, deficient in lysine, does not induce an increase in MPS and an increase in MPS was induced by 60 g wheat protein, 35–40 g casein, chicken breast fillet, or lysine-enriched wheat and chickpea protein mixture (38). However, in younger adults an increase in MPS was observed in response to the ingestion of 30 wheat protein (39).

Interestingly, 8 weeks resistance training and intake of 46 g/day high-quality whey (WPC), beef (Beef), or hydrolyzed chicken (Chx) protein after workout improves body composition and muscle performance (38, 40). Lean body mass was significantly increased after 8-weeks resistance training with post workout consumption of a 46 g bolus of WPC, Beef or Chx protein, compared with a control (Maltodextrin) (41).

6 Conclusion

Protein requirements relate to the supply of metabolically available nitrogen and IAAs to balance nitrogen and AA losses, to support body protein turnover and synthesis and to maintain the body's protein pool. Several health outcomes are associated with protein and IAA sufficiency, including growth, body weight, muscle mass and strength, bone health, defenses, and most if not all physiological functions. AA scoring is the preferred approach to evaluate the protein quality. It correlates with other approaches of protein quality (nitrogen retention, protein synthesis, physiological functions). The lower IAA content of certain protein sources is at the origin of the risk of protein deficiency

in certain diets. Reference values (data base) on IAA bioavailability of the different protein sources are required.

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Current advances for *in vitro* protein digestibility

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Protein is an essential macronutrient in our diet, source of nitrogen and essential amino acids, but the biological utilization of dietary protein depends on its digestibility and the absorption of amino acids and peptides in the gastrointestinal tract. The methods to define the amount and the quality of protein to meet human nutritional needs, such as the Digestible Indispensable Amino Acid Score (DIAAS), require the use of animal models or human studies. These *in vivo* methods are the reference in protein quality evaluation, but they are expensive and long-lasting procedures with significant ethical restrictions. Therefore, the development of rapid, reproducible and *in vitro* digestion methods validated with *in vivo* data is an old demand. This review describes the challenges of the *in vitro* digestion methods in the evaluation of the protein nutritional quality. In addition to the technical difficulties to simulate the complex and adaptable processes of digestion and absorption, these methods are affected by similar limitations as the *in vivo* procedures, i.e., analytical techniques to accurately determine bioavailable amino acids and the contribution of the endogenous nitrogen. The *in vitro* methods used for the evaluation of protein digestibility, with special attention on those showing comparative data, are revised, emphasizing their pros and cons. The internationally harmonized digestion protocol proposed by the INFOGEST network is being adapted to evaluate protein and amino acid digestibility. The inter-laboratory reproducibility of this protocol was demonstrated for dairy products. The *in vivo/in vitro* comparability results obtained to date with this protocol for several plant and animal sources are promising, but it requires an extensive validation with a wider range of foods and substrates with known *in vivo* digestibility. These *in vitro* methods will probably not be applicable to all foods, and therefore, it is important to identify their limitations, not to elude their use, but to apply them within the limits, by using the appropriate standards and references, and always as a complementary tool to *in vivo* tests to reduce their number.

KEYWORDS

***in vitro* protein digestibility, protein nutritional quality, *in vitro* DIAAS, simulated gastrointestinal digestion, INFOGEST**

1 Introduction

Protein is an essential macronutrient in our diet, source of nitrogen and essential amino acids. In human nutrition, the term protein nutritional quality refers to the ability of a protein to meet human requirements in essential amino acids and fulfill the physiological needs (1). The biological utilization of dietary proteins depends on their digestibility and the absorption

of amino acids and di- and tri- peptides in the gastrointestinal tract. Protein digestibility is linked to its unique amino acid composition, which, in turn, determines the folding state of the protein. For instance, the gastric survival of some globular proteins, such as milk β-lactoglobulin is well known (2), as well as the intestinal resistance of proline-rich protein domains due to the limited intestinal cleavage of the amide bond of proline residues (3). Post-translational modifications, especially glycosylation and phosphorylation, also confer additional gastrointestinal resistance to the protein, as occurs for casein phosphorylated regions that have been found at different sections of the gastrointestinal tract (4, 5). In addition, proteins are often included in supramolecular structures, such as, protein bodies, micelles, fibers, or entrapped in cellular structures surrounded by non-digestible polysaccharides that limit the access of gastrointestinal enzymes (6, 7). Additionally, food products are commonly subjected to different technological processes to improve sensory properties, ensure safety or extend shelf-life, and these processes can also affect protein digestibility. While soft heat treatments denature globular proteins and inactivate anti-nutritional factors which increase digestibility, more severe treatments lead to protein aggregation, cross-linkages, or non-enzymatic browning, decreasing digestibility (8).

Given the complexity of the digestion and absorption processes and the importance to accurately define the amount and the quality of protein required to meet human nutritional needs, protein quality evaluation is being subject to numerous studies and updates. As a result of the FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition held in 2011, a new protein quality index, the Digestible Indispensable Amino Acid Score (DIAAS) was proposed to replace the Protein Digestibility Corrected Amino Acid Score (PDCAAS) (9). DIAAS reflects the balance of amino acid digestibility determined at the terminal ileum, and describes protein quality better than PDCAAS (10). For the calculation of DIAAS, protein digestibility is based on the true ileal digestibility of each amino acid, preferably determined in humans, but if this is not possible, in growing pigs or in growing rats, in that order. In this report, the importance of treating each indispensable amino acid as an individual nutrient was also highlighted, and therefore, the digestibility is calculated as the oro-ileal disappearance of each amino acid. A dataset is being built based on the true or standardized ileal amino acid digestibility of a wide range of foods and ingredients. However, all these indexes, PDCAAS and DIAAS, and other previously used methods like the protein efficiency ratio (PER), include the use of animal models, or human studies. Consequently, dietary protein is the sole food macronutrient that requires animal or human testing for regulatory purposes.

These *in vivo* methods, although are the "gold standard" in protein quality evaluation, have important drawbacks. Animal trials are expensive and long-lasting methods with ethical restrictions. In addition to the policies on experimental animals that lead to follow the principle of the 3Rs (replacement, reduction and refinement), the social demand to reduce the number of animals for experimental purposes is currently growing, as well as, the demand for animal-free food, motivated by environmental and animal welfare reasons. In addition, because protein digestibility is affected by the food matrix and food composition and the technological treatment or the cooking conditions applied, the number of trials to be run exponentially increases, and makes the use of animal or human tests unfeasible.

Therefore, the development of rapid, reproducible and *in vitro* digestion methods that allow the estimation of the protein nutritional quality is an old demand. Despite the huge efforts done, especially in the field of animal nutrition [reviewed by Moughan (11)], these methods have not been sufficiently validated with appropriate *in vivo* data, i.e., ileal and not fecal protein digestibility, to reach sufficient confidence. The aim of this review is to present the actual status of the available *in vitro* methods to calculate protein digestibility with a view on past developments and special focus on the *in vivo/in vitro* comparability. The scope, uses and limitations of these *in vitro* procedures will be discussed, as well as, the work needed for the future application of these methods in routine protein quality evaluation.

2 Challenges of the *in vitro* methods

2.1 Simulate the *in vivo* digestion: a difficult task

Over the last 40 years, there has been interest in simulating human digestion *in vitro*, and specifically protein digestibility, since this knowledge is crucial in different areas, going from the nutritional assessment of novel foods and ingredients to the evaluation of protein allergenicity. However, human digestion and absorption are complex, multistage and adaptable processes in which several factors are involved (12). Thus, *in vitro* simulation of the digestion and absorption is a technically difficult, if not impossible, task. In this sense, although there are conditions that can be reproduced *in vitro*, with more or less success, such as, gastrointestinal enzymes, coenzymes and cofactors, pH, or temperature, there are other variables, such as, mechanical forces, regulation by gastrointestinal hormones, action of the intestinal microbiota or the participation of other organs, that are difficult to reproduce (13, 14). Furthermore, several studies have shown that the digestive capacity is adaptable, and for instance, the enzyme release at different levels of the gastrointestinal tract is regulated by the amount of ingested food (15, 16). This aspect, together with the ability of the products of digestion to modulate the intestinal function, adds extra complexity to the digestive process (17, 18). Although gut microbiota plays a critical role in digestion, its effects on protein quality evaluation could be overlooked in an *in vitro* approximation, since the goal of these methods is to simulate gastrointestinal digestion up to the ileum.

2.2 Simulate absorption and analysis techniques

Another important challenge of the *in vitro* methods to evaluate protein digestibility is the definition of the digestible-, bioavailable- or absorbable-fraction. Because the calculation of *in vivo* protein digestibility is based on the difference between the amount of each amino acid ingested and that non-absorbed, many *in vitro* methods have tried to reproduce or approximate these digestible and non-digestible fractions through dialysis, filtration or protein precipitation (14). However, other methods have estimated protein digestibility in the whole digest, like in the pH-drop or pH-stat methods, as will be described later on. However, as it will be commented, these methods based on pH measurement or monitoring were found to be susceptible to the buffering capacity of components of some food materials (19, 20).

In order to improve the evaluation of protein and amino acid digestibility, the resulting digestible and non-digestible fractions can be analyzed by using the same analytical approaches as the ones used in the *in vivo* assays, i.e., total nitrogen by Kjeldahl or Dumas, and determination of total amino acids by gas chromatography (GC) or HPLC. The different methodologies used for protein quantification can generate discrepancies in the data. The Kjeldahl method is considered the standard method for the estimation of nitrogen in food, because of its universality and good reproducibility in liquid and solid samples. Kjeldahl is a chemical method that determines the nitrogen concentration released during digestion with strong acids (21). Dumas is a high temperature combustion method, and the elemental nitrogen is detected by a thermal conductivity detector. However, because not all of the food nitrogen comes from proteins, these two methods do not give a measure of the true protein. Results obtained with Dumas are usually a little bit higher than those with Kjeldahl due to the detection of nitrogen compounds like nitrates, nitrites and heterocyclic compounds that are not completely quantified by Kjeldahl. In this sense, both methods might need determination of the non-protein nitrogen fraction depending on the food evaluated, and more importantly, an accurate nitrogen to protein conversion factor (NPCF) to convert the nitrogen values into protein. For many foods and ingredients, an overestimation of protein content can result from the use of the standard nitrogen correction factor 6.25 (22), and this will be translated into an underestimated protein digestibility value.

In both, the *in vivo* and *in vitro* assays, an acidic hydrolysis with 6 N HCl at 110°C has to be performed for 18–24 h prior to total amino acid determination by GC or HPLC. The effect of this acidic hydrolysis on amino acid analysis was questioned by Darragh and Moughan (23), who showed that the hydrolysis process can generate an erroneous estimate of the amino acid composition. This is due to the presence of amino acids that require times greater than 24 h to cleave the peptide bond (isoleucine, leucine, and valine) and others, considered labile amino acids, which can be partially destroyed before measurement (serine and threonine). In this context, proteins produce different rates of release and loss of amino acids, depending on their amino acid composition, causing an inaccurate quantification. Furthermore, the effect of acid hydrolysis on the chemical integrity of amino acids has been a topic of great scientific interest for years. It is well known that during thermal processing and storage, some amino acids such as methionine, cysteine, threonine, and tryptophan become unavailable, decreasing their bioavailability between 1 and 10%, as in the case of histidine (24). This aspect is especially notable for lysine amino acids, which are easily damaged during the food processing. The ε-amino group of lysine can react with many compounds such as reducing sugar, vitamins, fats, polyphenols, generating reactions that produce isopeptides and causing a degradation of lysine. Of these reactions, the most important occurs when, during thermal processing, the amino group reacts with the reducing sugar forming early or late Maillard compounds, which generates a decrease in the availability of lysine. When the Maillard reaction is advanced, the lysine is completely destroyed and cannot be recovered. However, during the early stages of the Maillard reaction, a portion of the structurally altered lysine (Amadori products) is partially hydrolysed in the presence of strong acids that reverse to lysine. However, such reversion does not occur during gastrointestinal digestion (25, 26). This fact leads to an overestimation of lysine quantification in processed foods caused by the acid hydrolysis step that takes place during conventional amino acid analysis. For this reason, the ultimate measure of available lysine is considered the absorbed reactive lysine and new methods, such as the isotope method or the oxidation of an indicator amino acid, have been described (27).

Other methods widely used to measure protein hydrolysis degree are those based on the reaction of primary amino groups, such as the trinitrobenzenesulfonic acid (TNBS) or the o-phthaldialdehyde (OPA) procedure. However, the precision of these methods may depend on the method and the protein substrate being hydrolysed. For instance, several studies have shown that OPA and cysteine react weakly due to the sulfhydryl group of cysteine, generating an unstable product (28, 29). This aspect makes the OPA method unsuitable for quantifying the degree of hydrolysis in cysteine-rich substrates. Furthermore, TNBS or OPA do not react with secondary amino acids such as proline or hydroxyproline (30).

2.3 Enzymes and blank of enzymes

One of the critical points in the *in vitro* digestion protocols is the selection of the enzymes and conditions (pH, digestion times, and salt concentration) to mimic physiological digestion. To study protein digestibility, proteases of porcine origin that are commercially available, have been widely used, specifically, porcine pepsin for the gastric phase and porcine pancreatic extracts containing proteolytic, lipolytic and amylolytic activities or individual proteolytic enzymes (31). The physiological protease concentrations at different segments of the gastrointestinal tract have been revised to fix conditions in some *in vitro* protocols (32, 33). In addition, other methods included peptidases of bacterial origin to simulate the carboxy- and amino-peptidase activity of intestinal brush border enzymes (34). It is true that when the food contains a high fat or starch content, an insufficient digestibility of these macronutrients can affect protein digestibility, and amylolytic and lipolytic enzymes are less accessible or are available at high prices. Starch digestion starts in the oral phase and, and although it is inactivated by the low pH in the stomach, some activity may persist within the food bolus. However, oral amylase is not included in most protocols due to its high price. Similarly, lipid digestion starts in the stomach by the action of gastric lipase that reaches activities of ca 120 U/mL in gastric fluid (35). Due to the limited accessibility of human gastric lipase, and taking into account the triglycerol stereospecificity and pH stability, dog or rabbit gastric lipases have been proposed as closest substitutes (36, 37), however gastric lipase is not used in most of the *in vitro* methods to evaluate protein nutritional quality. Bile salts have also been reported to improve the protein hydrolysis by the action of pancreatic proteases (38), however, not all *in vitro* protocols include conjugated bile acids in the intestinal phase. More importantly, as detailed later on (Section 4), the key to ensure batch-to-batch and inter-laboratory reproducibility is the standardization of enzymatic activity. Most of the *in vitro* digestion protocols add a given amount of enzyme or fix an enzyme/substrate ratio (E/S) on weight basis. This adds an important source of variability since the enzymatic activity of commercial enzymes varies enormously from batch to batch and during prolonged or inappropriate storage.

During the simulated gastrointestinal digestion, the use of enzymes, pancreatic extracts or mucins adds a significant amount of

protein that depends on the E/S. In a similar way as occurs in *in vivo* trials, the amount of "added nitrogen" needs to be subtracted in the final calculations of protein and amino acid digestibility. In those methods that separate the digestible and non-digestible protein fractions, this deduction should be done in one or in both fractions. Due to the high degree of autolysis of the digestive enzymes, in particular in absence or in low dietary protein concentrations (39), the use of water or simulated physiological fluids as blank will affect the calculation, underestimating digestibility.

2.4 Other factors that affect protein digestibility

The presence of antinutritional factors (ANF), i.e., lectins, saponins, polyphenols, or trypsin inhibitors, or the fiber content can also influence *in vivo* protein digestibility. ANF could compromise the protein digestibility by inhibiting the accessibility of the digestive enzymes to the protein or inhibiting enzyme activity (40). Trypsin inhibitors, found in field pea, peanut, wheat, lupin, and soybean, have been demonstrated to be capable of reducing the ability to bind the active site of the enzyme, while lectins may interfere with the digestion and absorption of nutrients (41). Furthermore, polyphenols have been shown to generate a complex with the digestive enzymes, inactivating them and therefore reducing the digestibility of proteins (13, 42). Moreover, fiber consumption has shown several effects on the gastrointestinal tract, from reducing enzymatic activity in the lumen and transit time to protecting the enzymes against degradation or stimulating the microbiome activity in the digestive tract. However, the effect of these compounds in the *in vitro* assays is still unknown and will depend on the conditions, especially the E/S used, which makes it difficult to simulate *in vivo* digestion with *in vitro* assays (43).

3 Historical overview

During the last decades, different *in vitro* digestion methods have been developed to evaluate protein digestibility, and thus, nutritional quality of foods and feed. The number of articles dealing with this subject is enormous with more than 5,000 publications from 1990 to date. Therefore, this historical overview will be limited to dietary proteins, and with a special emphasis on those works showing *in vivo*/*in vitro* comparative data. Most of these methods are based on enzymatic hydrolysis, performed in a one- or two-step process, by using a single or a combination of enzymes, often being gastrointestinal proteases from porcine origin. Hydrolysis conditions, pH and temperature, are generally fixed at the maximum for each enzyme with pepsin hydrolysis carried out at acidic pH (around pH 2) and pancreatic enzymes used near neutrality. Some methods to evaluate *in vitro* digestibility of dry matter in feedstuffs proposed to account for microbial degradation at the large intestine by adding a multienzyme step containing a wide range of carbohydrases including cellulase, hemicellulose, arabinose, xylanase and others (44, 45). Differences between approaches are given by the E/S and especially by the method employed to determine protein digestibility. Some methods are based on a measurement of pH (pH drop or pH stat) while others are based on the separation of a digestible or absorbable fraction by various procedures going from ultrafiltration or dialysis to the use of protein precipitating agents. Therefore, in this section, *in vitro* methods are classified by the principle used to evaluate protein digestibility. Table 1 collects different *in vitro* methods used to assess protein and amino acid digestibility in comparison to *in vivo* data where the limitations have been specified.

3.1 Methods based on pH measurement

During protein hydrolysis, release of protons and amino acids from the cleaved peptide bonds results in changes in pH. These methods lie on the correlation between the rate of hydrolysis degree and protein digestibility. Hsu et al. developed a multi-enzyme method (three-enzyme method, trypsin + chymotrypsin + peptidase) for the evaluation of protein digestibility. Specifically, they showed that the pH-drop after 10 min of digestion with the three-enzyme solution of 23 human diets, mainly vegetables and dairy foods, was highly correlated ($r = 0.90$) with *in vivo* PER values in rats. However, substances with high buffering capacities could affect the results. Despite this, the pH-drop methods was able to predict the apparent digestibility of proteins. In addition, the trypsin inhibitory activities and the effect of heat processing on digestion could be detected (19). Two years later, Satterlee et al. slightly modified the Hsu et al. protocol by including an extra 10 min of digestion with a *Streptomyces griseus* protease (four-enzyme method). Numerous authors have evaluated the *in vitro* digestion process of several food sources through the pH-drop method. In 1981, Petersen and Eggum studied the applicability of the three-enzyme (19) and four- enzyme combinations (65) on 61 samples of food and feed. Their results demonstrated a high correlation ($r = 0.89–0.90$) between the pH-drop results and fecal protein digestibility in rats, especially for plant proteins and for mixtures of plant and animal proteins. However, the predicted *in vitro* digestibility value for animal proteins significantly differed from the *in vivo* results (46). In 1983 the same researchers evaluated the *in vitro* protein digestibility of 18 protein sources using the three-enzyme method of Hsu et al. and the four-enzyme method of Satterlee et al. The results showed a greater *in vitro-in vivo* correlation for the three-enzyme method ($r = 0.78$) than for the four-enzyme method ($r = 0.56$). Despite the good correlations obtained, the estimations were significantly affected by the different buffering capacities of some food substances, which was considered a major drawback of the pH-drop method (49). This aspect was further demonstrated by Moughan et al. who compared the *in vitro* digestibility of 20 meat and bone meal samples by the pH-drop method with the values of true ileal digestibility in rats. It was concluded that pH estimation methods may be influenced or affected by the strong buffering capacity of the ash content, mainly mineral content, of food (20). For this reason, it was recommended to determine the pH-drop after a dialysis treatment to eliminate salts with buffering capacities (20). Other authors such as Kim et al. and Wolzak et al. showed *in vitro-in vivo* correlation values of $r = 0.95$ and $r = 0.421$ for soy protein concentrate, and 33 vegetable proteins, respectively (47, 48). However, the difference in the response for different types of food proteins made it necessary to use different regression equations to obtain realistic estimates of digestibility. This task presents a major challenge due to the complexity involved in categorizing foods in each class of food.

Pedersen and Eggum revised the pH-drop method and modified it slightly in order to avoid the effect of substances present in the

TABLE 1 *In vitro* methods used to assess protein and amino acid digestibility in comparison to *in vivo* data.

Food substrate	*In vitro method*	*In vivo* model	Outcome	Limitations	Reference
pH drop					
23 human diets (plant and milk proteins, and food products)	3-enzyme* method	Apparent fecal protein digestibility, PER (Rats)	High correlation (r = 0.90) between pH-drop and *in vivo* apparent digestibility	Affected by buffering capacity of food	(19)
61 samples of food and feed [plant, combination (plant–animal) and animal proteins]	3-enzyme* and 4-enzyme** methods	Standardized fecal protein digestibility (Rats)	High correlation (r = 0.89–0.90), for plant proteins and for combination proteins	Animal proteins were underestimated Correction for buffer capacity of foods is needed 3-enzyme method affected by tannins	(46)
60 vegetable proteins (cereal grains, leguminous seeds, oilseeds, and by-products)	4-enzyme** method	Apparent fecal protein digestibility (Rats)	Overall r = 0.838, but differences between food groups	Distinct equations for different groups of samples	(47)
20 meat and bone meal samples	4-enzyme** method	Standardized ileal protein digestibility (Rats)	r > 0.75	Affected by the buffering capacity of the ash content	(20)
Soy protein concentrate	pH-drop vs. SDS-PAGE 4-enzyme** method *vs* Pepsin 2 h + pancreatin 6 h	Apparent fecal protein digestibility (Rats)	pH-drop correlation r = 0.95	Discrepancies with SDS-PAGE due to protein aggregates	(48)
30 protein samples (animal, plant and combinations of plant–animal proteins)	pH-drop and pH-stat 3-enzyme* and 4-enzyme** methods	Standardized fecal protein digestibility (Rats)	r = 0.78 and 0.56 for pH-drop, depending of the enzyme method r > 0.90 for pH-stat	Pre-digestion with pepsin is suggested for samples containing proteinase inhibitors	(49)
pH stat					
Maize Whole sorghum Pearled sorghum	3 different methods: Pronase Pepsin 3-enzyme* method	Apparent fecal protein digestibility (Rats)	pH-stat procedure correlated better (r = 0.95) than systems containing pronase and pepsin *in vitro*	Multienzyme: highest correlation vs. *in vivo* Pepsin: poor correlation vs. *in vivo*	(50)
17 foods (animal and plant proteins)	Pepsin +4-enzyme** method	Standardized fecal protein digestibility (Rats)	R^2 = 0.61 all foods R^2 = 0.66 without beans and chickpeas	Low correlation values Poor correlation for beans and chickpeas (fecal digestibility)	(16)
10 salmonid diets	3-enzyme* and 4-enzyme** methods	*In vivo* digestibility by chromic oxide method (Fish)	R^2 = 0.82 and 0.64 depend on the pH-stat method used	Overestimation or underestimation depending on diet and method used.	(51)
7 feed ingredients (menhaden, Atlantic menhaden, anchovy, white fish, tuna waste, soybean protein, and langostilla meals)	Shrimp hepatopancreas enzymes or a multienzyme solution**	Apparent fecal protein digestibility (White shrimp)	$R^2 \approx 0.71$ or 0.77 depending on the enzymes used	Low correlation values Additional *in vivo* data are needed	(52)
A veal protein hydrolysate vs. gelatin vs. caseinate	3-enzyme* method	PER and standardized fecal protein digestibility (Rats)	Linear relationship between *in vivo* digestibility and pH-stat method (R^2 = 0.99)	One substrate The use of published regression equations is unreliable	(53)
Soybean and retoasted soybean meals Rapeseed and retoasted rapeseed meals	2 *in vitro* methods: 3-enzyme* pH-stat Pepsin + pancreatin	Standardized ileal protein digestibility (Growing pigs)	Both *in vitro* methods correlated with *in vivo* digestibility (r = 0.95; r = 0.91)	2 plant substrates with 2 treatments	(54)
Precipitation methods					
4 experimental diets (corn, barley, oats, soybean, corn gluten and wheat bran)	1% TCA Pepsin 6 h pH 1 + pancreatin + amylase 1 h pH 6.8	Ileal digestibility (Broilers)	Correlation with digestibility of crude protein r = 0.93 when diets ground to 0.4 mm	Better results with highly digestible diets than diets of low digestibility.	(55)

(Continued)

TABLE 1 (Continued)

Food substrate	*In vitro method*	*In vivo* model	Outcome	Limitations	Reference
7 plant feedstuffs and 16 diets	2% SSA Pepsin 6 h pH 2 + pancreatin 18 h pH 6.8	Apparent fecal digestibility (Growing pigs)	Linear regression with crude protein digestibility but *in vitro* higher than *in vivo* values r = 0.99 for feedstuffs r = 0.95 for diets (r = 0.8 for unextracted diets)	Fat extracted feeds and diets Only N contents	(56)
17 feedstuffs (15 plant-based meals *vs* meat and bone meal *vs* dairy) and 48 feed mixtures	% SSA Pepsin 6 h pH 2 + pancreatin 18 h pH 6.8	Apparent ileal digestibility (Growing pigs)	Linear relationship $R^2 = 0.61$ all feedstuffs $R^2 = 0.92$ excl. Meat and bone meal and barley hull Validation with 48 feeds ($R^2 = 0.57$) *In vitro* AA digestibility (9 products)	*In vitro* protein digestibility > apparent ileal digestibility Relationship generally higher for essential AA, and lower for non-essential AA, than for protein	(57)
28 samples of dry extruded dog foods	2% SSA vs. pH-drop-3 enzyme* method *vs* Near infrared spectroscopy	Apparent fecal protein digestibility (Dogs)	Correlation with *in vivo* crude protein digestibility: Protein precipitation r = 0.81; pH-drop r = 0.78. Near infrared spectroscopy R^2 cv. = 0.53	The ash content affects the accuracy of the pH-drop-method	(58)
Dialysis cell					
Protein diets including beef, casein, rapeseed, soybean and gluten	Dialysis cell-1 kDa Pepsin 0.5 h pH 2 + pancreatin 6 h pH 6.8 *vs* pH stat*	Portal and aortic blood (Rats)	r = 0.92 for plant sources r = 0.70 for animal sources	Variation between protein groups Poor correlation for animal sources	(59)
Heated rapeseed meal, soybean, lupine proteins vs. sodium caseinate vs. gelatin	Dialysis cell-12 kDa Pepsin 4 h pH 2 + trypsin 24 h vs. pH-stat	Fecal digestibility and PER (Rats)	r = 0.88 (true digestibility vs. dialysis cell) r = 0.81 (true digestibility vs. pH-stat)	Comparison with fecal digestibility Only 1 animal protein (gelatin)	(60)
3 feedstuffs: Fish meal, rapeseed meal, cottonseed meal	Dialysis cell-12 kDa Pepsin 4 h pH 2 + trypsin 24 h	Apparent ileal digestibility (Black pig barrows)	Linear regression 0.96 < r < 0.99 Significant linear relationships between ileal apparent digestibilities for crude protein, total AA and 16 individual AA	Comparison with apparent digestibility	(61)
17 grain legumes (faba beans, field pea, lupin)	Dialysis cell-1 kDa Pepsin 0.5 h pH 2 + pancreatin 6 h pH 6.8	Standardized ileal digestibility (Growing pigs)	*In vitro* digestibility higher than *in vivo* $R^2 = 0.73$ for Lys $R^2 = 0.91$ for Cys and Trp	ANF content depress nutrient digestibility *in vivo*	(62)
Dynamic systems					
Standard corn-based diet with coarse ground corn, beet, wheat bran, beet pulp	TIM® Dialysis fluids = absorbed Pepsin+lipase+pancreatin	Standardized ileal digestibility (Growing pigs)	Including all diets: non-significant correlation Excluding corn diet: $R^2 = 0.99$	Starch digestibility was underestimated compared with *in vivo* Digestibility dramatically reduced in the TIM by fibrous ingredients; volume limitation for high-fiber diets.	(63)

(Continued)

TABLE 1 (Continued)

Food substrate	*In vitro method*	*In vivo* model	Outcome	Limitations	Reference
Dairy infant formula *vs* 50% pea proteins *vs* 50% faba bean proteins	DIGDI® SEC < 10Ka N corrected for free AA and secretions *N* = absorbed Pepsin+pancreatin	Digestion (Piglets)	PDCAAS-like score and apparent digestibility comparable with literature	System validated for dairy infant formulas	(64)

AA, amino acids; N, nitrogen; RT, room temperature; SSA, sulphosalicylic acid; TCA, trichloroacetic acid; PDCAAS, protein digestibility corrected amino acid score; PER, protein efficiency ratio; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. *, three-enzyme method (trypsin, chymotrypsin, and peptidase); **, four-enzyme method (trypsin, chymotrypsin, aminopeptidase and protease from Streptomyces griseus). r, correlation coefficient; R^2, determination coefficient.

protein that could influence the drop in pH. In the pH-stat procedure, the pH is kept constant at pH 8 by automatic titration (0.10 M-NaOH titrant) during the incubation with enzymes. At the end of the incubation period, the amount of alkali added is recorded and the value is used as an indirect measure of protein digestibility (43, 46). Using pH-stat, Pedersen & Eggum showed an improvement in the prediction of protein digestibility of 30 samples, compared to the pH-drop. A high correlation coefficient (r > 90) with fecal digestibility in rats was obtained in pH-stat method, improving the one obtained by the pH-drop method (0.56–0.78). However, the digestibility of some foods, such as egg powder, was underestimated, because of the content of trypsin and chymotrypsin inhibitors of egg. A pretreatment with alkali to improve the correlation coefficients was then recommended (49). The pH-stat method has been widely used to evaluate the digestibility of different protein sources. Eggum et al. showed a good agreement *in vitro* vs. *in vivo* (measured by fecal protein digestibility in rats) in 17 foods, with the exception of two legumes, beans and chickpeas. The authors discussed that the discrepancies obtained for these foods, suggesting that it could be due to the high bacterial growth with the consumption of certain legumes in the diet, which caused an increase in the excretion of nitrogen in the feces. Excluding these legumes the obtained *in vitro* and *in vivo* digestibility percentages were similar (86.3–100.0% *in vitro* and 73.1–96.8% *in vivo*), although the correlation coefficient was only acceptable ($R^2 = 0.66$) (16). Better correlation coefficients were obtained by comparing the *in vitro* protein digestibility of maize, whole sorghum and pearled sorghum maize (r = 0.95) and 7 specific foods ($R^2 \approx 0.75$) with their *in vivo* apparent fecal protein digestibility (50, 52). In the same way, good and significant correlations ($R^2 = 0.82$ and 0.64) were obtained when the protein digestibility of 10 salmonid diets were estimated by two the pH-stat *in vitro* assay methods and compared with *in vivo* digestibility in fish (51). Linder et al. measured the protein digestibility of an industrial veal protein hydrolysate, used as a gelatin-replacing ingredient for human consumption. The results showed a high correlation between fecal protein digestibility measured in rats and the pH-stat method ($R^2 = 0.99$), although already published regression equations were used (53). Recently, high correlation coefficients were obtained between *in vitro* protein digestibility of processed soybean meal and rapeseed meal through the pH-stat method and the results obtained from standardized ileal digestibility in growing pigs (r = 0.95) (54).

In summary, the methods based on pH measurement were shown to be suitable for predicting digestibility in many foods, with high correlations in plant substrates. The method was found to be highly reproducible across 6 laboratories that estimated protein digestibility of 17 protein sources by using the 3-enzyme method in a pH-stat (66). However, by using these methods, the results of the entire complex digestion process were evaluated based on a mere measurement of pH or pH-change. In other words, the crucial information on protein digestion that could be extracted from the use of gastrointestinal enzymes was neglected. In addition, the significant differences found for animal proteins, the use of different correlation curves for different samples, and the fact that certain physical and chemical characteristics, such as calcium content or buffering capacity, may prevent an accurate estimation of digestibility, and are important drawbacks for the use of these methods.

3.2 Methods based on protein precipitation

In the methods described in this section, enzyme incubations are followed by measurements of the insolubilized material collected after filtration, although in some cases measurements on the filtrate, or alternatively on one of the separated fractions after centrifugation are conducted. Digestibility is then related to *in vitro* solubility or the definition of an absorbable or bioaccessible fraction and a residue or non-absorbable fraction.

Early methods included one-step incubations giving lower digestible protein values than those obtained *in vivo*, and were rapidly replaced by two-step digestion. In the pepsin-jejunal fluid, a two-step incubation with pepsin digestion for 4 h followed by a further 4 h digestion with pig jejunal fluid was used (67). *In vitro* digestibility of protein was calculated by the determination of dry matter and crude protein on the residue after centrifugation for 10 min at 1,250 × g at 5° C, on the basis of the original protein content of the diet. A two-stage incubation with pepsin for 6 h at pH 2 followed by an incubation with pancreatin at pH 6.8 for 18 h in borate buffer was further developed (68) thus providing an animal-independent method. To calculate the digestibility, 1% TCA final concentration was used, followed by centrifugation for 1 h at 2,000 × *g*. This method was applied for routine analysis in quality control of feeds and feed ingredients for poultry. By reducing the particle size of the test material, passing through a 0.4 mm sieve, the accuracy of predicting *in vivo* digestibility was increased for all the tested diets, that included corn, barley, oats, soybean, corn gluten and wheat bran as protein sources. The correlations between ileal digestibility in broilers and *in vitro* estimates were high (r = 0.93 for crude protein) (55). A modification of this method was presented by Babinszky et al., where pepsin incubation was performed at pH 1 on fat-extracted feed samples, and the residue after pancreatin + amylase incubation and 1% TCA precipitation was centrifuged at 3,500 × g for 15 min, after decanting over a nylon cloth (particle size 40 μm). This method found an improved correlation with

fecal digestible protein in pigs by reaching regression values for feedstuff and diets of 0.99 and 0.95, respectively (56). The additional determination of nitrogen content on the filtrate gave a similar correlation but was abandoned as it was considered to be too laborious.

With the aim to recover solubilized but not fully degraded proteins, precipitation with sulphosalicylic acid was introduced, while undigested materials were submitted to the standardized filtration equipment for measuring dietary fiber (43). Magnetic stirring was included during the enzymatic incubations in order to assure effective starch degradation. By the use of this method, prediction of individual amino acids in eight common feedstuffs showed that the *in vitro* digestibility of the individual amino acids was close to the *in vitro* digestibility of nitrogen. Hervera et al. (58) adapted the last *in vitro* method for estimation of digestible energy of dog foods and compared it with the pH-drop methodology. The results showed a correlation of r = 0.78 between the pH-drop with the three-enzyme method and the apparent fecal digestibility in dogs but higher accuracy, r = 0.81, was shown with the *in vitro* method using precipitation with sulphosalicylic acid (58). Wada and Lönnerdal determined digestibility in infant formulas by using total and non-protein nitrogen (NPN), i.e., soluble fraction in 12% final TCA concentration (69). They investigated the effect of industrial processing with *in vivo* digestibility using a suckling rat pup model in terms of chemical modifications and endurance of intact α-lactalbumin and β-lactoglobulin, but no direct *in vivo-in vitro* comparison was shown.

The role of nitrogen added in the form of enzymes was considered in further developments. When the *in vitro* digestibility of protein was calculated from the difference between nitrogen in the sample and the undigested residue after correction for nitrogen in the blank, it was shown that the resulting amino acid composition of the blank-derived protein was very close to reported values in the literature based on direct measurements of endogenous protein *in vivo*. Apparent ileal digestibility of individual amino acids was predicted in a similar way as for protein. The relationship was generally higher for essential amino acids, and generally lower for non-essential amino acids, than for protein (57). This procedure used precipitation with sulphosalicylic acid (2% final concentration) for 30 min at room temperature followed by rinsing with 1% sulphosalicylic acid of the filtered residues. A close relationship was found for the 17 single feedstuffs but meat and bone meal, and barley hull had to be excluded. The above conditions have been widely used to compare protein digestibility of different products, mainly using sulphosalicylic acid (62, 70) or TCA (71, 72) as precipitating agent.

In summary, the *in vitro* methods based on precipitation of a non-digestible fraction by using different agents such as TCA or sulphosalicylic acid have demonstrated good comparability with ileal digestibility in broilers and in pigs. Precipitation with sulphosalicylic acid after a 3-enzyme digestion protocol has shown higher accuracy than pH-drop when compared with dog fecal digestibility. The main advantage of these methods is the reproducibility of the precipitation step for the definition of a digestible and non-digestible fraction. However, the digestion conditions used by different authors would still require additional optimization and harmonization.

3.3 Methods using ultrafiltration or dialysis

These methods are based on the continuous removal of low-molecular-weight products from digested material by ultrafiltration or dialysis to prevent enzyme inhibition by end products.

A two step-digestion method in which the intestinal digestion products (free amino acids and low molecular weight peptides) were removed through a dialysis membrane was proposed in order to reduce enzyme inhibition by hydrolysis products (73). After a 30 min digestion step with pepsin enzyme: substrate of 1:250 (pepsin activity 3,152 units/mg protein), intestinal digestion took place with pancreatin for 6 h, at an E/S of 1:25, in a dialysis cell of a 1,000 Da molecular weight cut-off, for the continuous elimination of digested products with 10 mM sodium phosphate buffer, pH 7.5, as circulating dialysis buffer. The essential amino acids released during the intestinal phase from beef, casein, rapeseed, soybean and gluten correlated with plasma levels found in portal and aortic blood in rats fed with the same substrates (59). A good correlation was found for plant sources (r = 0.92–0.93), although lower values were reported for beef or casein (r = 0.70). This protocol was also applied to protein mixtures (74) and to 19 selected foods, showing differences between *in vitro-in vivo* amino acid digestibility depending on the protein source. These variations were not related to the amino acid concentration in the protein and it was proposed that the amino acid sequence as the factor leading overall protein and amino acid digestibility (75). The *in vitro* protein digestibility by use of a dialysis cell method and pH stat was compared with the *in vivo* PER and true digestibility of heated rapeseed meal, soybean and lupine proteins (60). *In vivo,* PDCAAS correlated with pH stat and dialysis cell values with r = 0.92 and 0.98, respectively, although PER was poorly correlated with the *in vitro* protein digestibility. Similar strategies but using dialysis tubes in the intestinal phase have been used to predict ileal protein digestibility of pig feedstuffs (61), obtaining linear regression equations between *in vitro* digestibilities and porcine ileal apparent digestibilities. Dialysis cells have been more recently used in the estimation of the protein digestibility of novel food protein sources, such as seaweeds, where the high fiber content affected protein digestibility, likely by reducing the accessibility of the proteolytic enzymes (76, 77).

In some works, the use of chromatography or ultrafiltration with different cut off membranes has been used to characterize the digestible fraction. Besides, the characterization of the non-dialyzed digest has been conducted by ion-exchange or size exclusion chromatography, and ultrafiltration. The undigested residues were separated by ion-exchange chromatography into basic-neutral, lightly acidic and acidic fractions further resolved by sequential ultrafiltration (cut-off 10 and 1 kDa). Interestingly, large proportions of leucine, lysine, arginine, phenylalanine and tyrosine were found as part of peptides smaller than 1 kDa, both in the dialysates and retentates, while glutamine, threonine, serine and asparagine appeared mostly in fractions >1 kDa, while after 6 h with pancreatin, most of the proline appeared in the basic-neutral fraction >1 kDa (78). When this procedure was applied in the comparison of casein, cod, soy and gluten proteins, animal proteins were digested at a greater rate than plant proteins, and more resistant peptides were largely rich in proline and glutamic acid (79).

The impact of cooking on animal and plant protein digestion has been evidenced by the use of this strategy. The increase in protein digestibility of white and brown beans (*Phaseolus vulgaris*) after cooking was found to be related to a higher extent of proteolysis, as monitored by SDS-PAGE and recovery of low molecular weight peptides (< 30 kDa) after ultrafiltration of the digests (80). On the contrary, meat protein digestion in a microreactor fitted with a 10 kDa cut-off membrane in the gastric compartment and 1 kDa cut-off dialysis membrane in the intestinal compartment showed a decrease of protein digestibility with meat cooking (81). This study showed

superior precision with the use of a semi-automatic flow procedure in comparison with the test tube method. Analytical size exclusion chromatography has been used to determine digestibility of casein *vs* modified casein with the glycation product pyrraline. The size pattern was used to show that the digestibility decreased with increasing pyrraline concentration of the peptide mixtures. Moreover, further ultrafiltration of digests using 1 kDa cut-off indicated that 50–60% of pyrraline was included in peptides (82).

The methods based on *in vitro* digestion and dialysis or ultrafiltration have shown good correlation in the prediction of ileal protein digestibility of food and feed. Some of these approaches have been used to characterize the gastrointestinal digests in combination with chromatographic methods. Main weaknesses of these methods would derive from the limited reproducibility of the use of ultrafiltration devices and the unspecific bound of protein material to the membrane material.

3.4 Dynamic systems

Dynamic systems have been proposed as *in vitro* alternatives for human or animal studies as physiologically relevant, including peristaltic mixing of food, computer- controlled pH values and realistic gastrointestinal transit times. Moreover, small molecules are removed from the digesta with hollow fiber membranes. The TNO-developed TIM® system was tested to predict the true ileal digestibility of proteins including dairy, meat, wheat, faba bean or barley, and a linear relationship versus pig or calf data was obtained (83). A standard corn-based diet was compared with the same diet with coarse ground corn, 8% sugar beet pulp, 10% wheat bran, or 8% sugar beet pulp and 10% wheat bran. The dynamic model yielded digestibility coefficients comparable with *in vivo* ileal digestibility in growing pigs for the standard and coarse ground corn but the values were considerably affected by the incorporation of the fibrous ingredients. The linear fitting between the *in vitro* and the *in vivo* results for crude protein digestibility was not significant but resulted in $R^2 = 0.99$ when the coarse ground corn diet was excluded from the regression (63).

Using the tiny-TIM, digestibilities of ovalbumin, cooked and raw chicken egg white, and casein showed similar values to values reported in humans ($R^2 = 0.96$). The true ileal protein and amino acid digestibilities were used by the authors to estimate the DIAAS for immature herring egg proteins (84). More recently, cumulative true ileal digestibility of nitrogen data has been reported during 5 h tiny-TIM, expressed as the percentage of the exogenous nitrogen intake, correcting for nitrogen in gastric residue (85). These values served to calculate DIAAS for different protein ingredients, alongside the corresponding limiting amino acid. The DIAAS values for rice, whey, and pea-based proteins were in agreement with those collected from literature, using pig ileal data. However, for soy and a second source of pea protein with different processing, the values were significantly lower than those previously described in literature. This was ascribed to treatments applied to these specific ingredients during processing, including alkaline or heat treatment, leading to protein aggregation or structural changes. An alternative source of discrepancy was related to differences in the innate protein features due to cultivar of growing conditions. A low (under 50%) bioavailability of the majority of amino acids and low N digestibility was found for the last two products. Isolates with lower DIAAS also showed lower protein solubility and increased protein aggregation, which was identified as a potential cause inhibiting digestion. Indeed, DIAAS positively correlated to protein solubility and N-bioaccessibility. The dynamic system developed at INRAE, DIDGI® was set up to mimic infant digestion upon an extensive analysis of literature on infant physiology and validated with piglet digestion (86). This system provided comparable results *in vitro/in vivo* for a reference dairy infant formula in terms of limiting essential amino acid, PDCAAS-like score and *in vitro* apparent digestibility. The last parameter was determined based on the soluble N lower than 10 kDa, as measured in the peptides by size exclusion chromatography and cumulated to the free amino acid nitrogen (64).

The use of dynamic systems to determine protein digestibility is still limited. Although these systems allow monitoring the progress and digestion kinetics, the calculation of the nitrogen mass balance could be more complex than in static systems. In addition to the difficulties to harmonize conditions in different apparatus, the availability of this sophisticated equipment could be an additional limitation to the extensive use of these methods.

4 INFOGEST static protocol applied to protein digestibility and protein quality analysis (*in vitro* DIAAS)

The INFOGEST static digestion protocol was developed during the COST Action INFOGEST[1] with the main goal to harmonize the highly variable protocols used within the research laboratories interested in food digestion. The first INFOGEST consensus method (32), was followed by an improved and more detailed protocol in 2019 (33). Digestion parameters were based on currently available physiological data. The resultant peptides from the *in vitro* digestion with the INFOGEST protocol have been compared with human and pig peptidomic analysis showing comparable results for milk proteins (Figure 1). Compared to previous published protocols, the following points can be highlighted as the most important advantages, which helped to reduce experimental variability and improve reproducibility (87). Firstly, the protocol includes specific enzyme activity assays in order to harmonize the addition of enzymes based on their activity and not based on weight, as in previous published protocols. Secondly, due to the variable buffering capacity of different foods, the protocol requests to perform a pH test tube where the volumes of HCl to add in the gastric phase (to reach pH 3) and the volume of NaOH (to reach pH 7) in the intestinal phase, are tested for each food sample. And thirdly, the protocol provides indications on how the enzyme activities can be stopped after the gastric and intestinal phase of digestion, depending on the downstream analyses. The protocol was shown to be reproducible and robust in inter-laboratory experiments (87) and the results at the end of the intestinal phase were comparable to *in vivo* results (5, 88) although this has been proved so far only for dairy proteins.

Although the INFOGEST protocol increased harmonization of digestion experiments, critical steps in the protocol and further adaptations were proposed. The INFOGEST sub-group (WG4) tested lipase activity in several collaborative studies and found a high variability due to unprecise descriptions in the original protocol. The

1 http://www.cost-INFOGEST.eu/

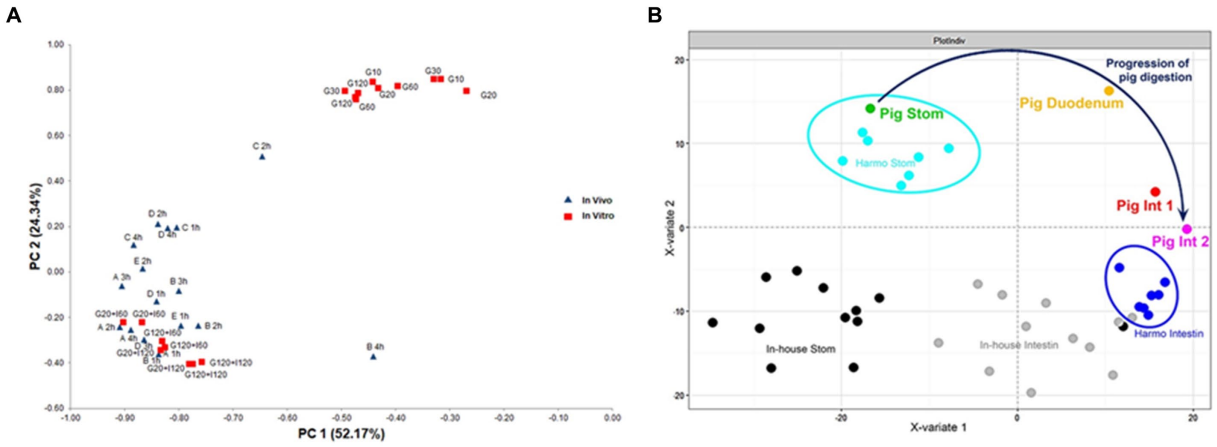

FIGURE 1
Comparison of *in vitro* digestion (INFOGEST protocol) *vs in vivo* (**A**: human jejunal digests; **B**: pig digests). **(A)** Principal component analysis score plot calculated with the frequency of appearance of each amino acid identified as part of a peptide from β-casein and α_{s1}-casein. Different human subjects (blue triangles) are referred to with capital letters from A to E followed by the time of jejunal sampling (1, 2, 3, and 4 h). *In vitro* digests are represented with red squares. G, gastric; I, intestinal, followed by the time expressed in minutes of *in vitro* digestion. Reprinted with permission from Elsevier, by Sanchón et al. (5); **(B)** Partial least square analysis over all peptide patterns identified in the five most abundant milk proteins (β-, α_{s1}-, α_{s2}-, κ-casein, β-lactoglobulin). The average of eight pig samples is shown versus the harmonized or in-house digestion protocol, from previous interlaboratory studies. The arrow indicates the progression of digestion in the pig samples from Stom (stomach)-, Duodenum, Int 1 (proximal jejunum)-, to Int. 2 (median jejunum)- phases **(B)**. Reprinted with permission from Taylor & Francis, by Bohn et al. (17).

detailed protocol elaborated by this group led to a significant reduction in variability and for the study of lipid digestion, therefore these recommendations should be considered (89). A similar work focusing on amylase activity is currently ongoing and in the near future an improved protocol for amylase activity will be proposed and published. Moreover, in order to better simulate digestion in different age groups, both protocols, the static and the semi-dynamic protocol were and are further adapted to infant and elderly conditions.

The static INFOGEST *in vitro* digestion protocol represents a good starting point on which the quantification of the protein digestibility could be based. Several recent publications based on the INFOGEST static protocol are focusing on the quantification of protein digestibility and are listed chronologically in Table 2. In this table, the main adaptations with regard to the original INFOGEST method, the amount of protein input, the separation of non-digestible from digestible material, the use of an enzyme blank, and the calculations of digestibility are compiled. The approaches to overcome the main challenges of the *in vitro* methods are discussed below.

4.1 Enzyme/substrate ratio

The original INFOGEST protocol proposed for each digestion step a 1:1 ratio (w:w) between food and simulated fluid, ending up with a final ratio of 1:8 of food in digesta. No recommendation of nutrient normalization was proposed. However, in order to compare protein digestibility of different foods, a normalization may be needed. In four of the listed publications, protein input was normalized between 4 and 16% in the foods subjected to digestion. Increasing the amount of protein entering into the system reduced digestibility, as was observed in the case of different amounts of TCA soluble casein after size exclusion chromatography (91) and for casein and gluten digestibility, testing 4, 8, and 16% of protein input (94). Another approach to increase the food to enzyme ratio is the adaptation of digestive enzymes as proposed by Ariëns et al. (90) to reduce the background of enzymes. The authors reduced the addition of pancreatin from 100 U/mL of digesta by a factor of 10 to 10 U/mL and observed no impact on released NH_2 during digestion of whey protein isolate, which represents a highly digestible substrate. It would be interesting to test if this observation is also correct for substrates with lower digestibility. Alternatively, a reduction in enzyme background was achieved by Sousa et al., by using the supernatant of the pancreatin suspension after solubilization with ultrasound and subsequent centrifugation (92). This procedure did not reduce the trypsin activity in the pancreatin supernatant.

4.2 Separation of digestible from non-digestible material

At the end of the intestinal phase of the original INFOGEST protocol, all products are in the same container. In order to assess protein digestibility, digestible and non-digestible fractions need to be separated. Different approaches were used by various authors, such as centrifugation (95), or ultrafiltration at different cut-off sizes, such as 5 or 10 kDa (90, 94), corresponding to peptides of 45–90 amino acids in length, assuming an average weight of 110 Da per amino acid. The choice of the rather high molecular weight cut-off compared to *in vivo* (500 Da) was justified by the lack of brush border enzymes in the system (94). Moreover, the use of ultrafiltration could as well lead to loss of material, impacting the mass-balance and in consequence the digestibility of the tested substrates (90). A second approach applied in the different protocols was a precipitation step either with different concentrations of TCA (6–12%) (91, 93) or with MeOH (80%) (92). Depending on the downstream analysis, the precipitation agent could disturb the measurements and it was removed by extraction with diethyl ether (93) or simply be evaporated in the case of MeOH (92).

TABLE 2 *In vitro* methods based on the INFOGEST digestion protocol applied to the evaluation of protein and amino acid digestibility.

Food substrate	INFOGEST protocol, adaptations	Protein input	Separation of undigestible from digestible part	Enzyme blank	Calculation of digestibility	Comparability with *in vivo* data	Reference
9 protein concentrates: blood, corn, mealworm, Mycoprotein®, yellow peas, potato, whey, yeast	pH adjusted continuously by stat titration; 10 U/mL trypsin activity; sodium chloride instead of sodium bicarbonate	5%	Centrifugation + Ultrafiltration 5 kDa	H_2O	Three different calculation strategies using total AA in the filtrate	No direct comparison	(90)
6 food products: cooked beef, raw chicken, wheat flour bread, heated/non-heated pea concentrate, casein	None	17%	Centrifugation + precipitation with TCA 8.3%	H_2O	Small peptides determined by SEC area relative to the total protein	No direct comparison	(91)
7 food products: whey protein isolate, zein, collagen, black beans, pigeon peas, All-Bran®, peanuts	Supernatant of pancreatin suspension after ultrasound and centrifugation	4%	Precipitation with 80% methanol	Protein-free substrate containing fat, carbohydrates, and cellulose	Three analytical workflows: Total N or total AA or primary amines in the absorbable fraction relative to total digest corrected for protein-free substrate blank	Comparison for 7 same substrates with *in vivo* data: Digestibility, average difference: 1.2%, DIAAS, average difference: 0.1%	(92)
12 food products: 6 milk protein products, pea, soy, wheat, zein, cricket, mealworm	none	16%	TCA precipitation (6, 9, 12, and 15% + extraction of supernatant with diethyl ether)	Simulated fluids	N content in digestible vs. whole digesta corrected for N content of the blank and NPN content of the protein material	No direct comparison between foods, correlation of 0.912 for 12% TCA (linear regression)	(93)
Gluten and casein at 4, 8, 16% of the model meal	No oral phase	4, 8, 16%	Centrifugation + Ultrafiltration 10 kDa	Use of ^{15}N labeled substrates	Total N in (<10 kDa) permeate relative to total N in food corrected for blank (<10 kDa) permeate	*In vitro* values below reported *in vivo* values	(94)
5 protein matrices: faba bean, pea flour, soy flour, whey protein isolate, casein	Addition of jejunal-ileal digestion phase, mimicking the brush border digestion: 13 mU/mL leucyl aminopeptidase, pH 7.2, 37°C, 4 h	Dependent on substrate	Centrifugation	H_2O	Total AA in digest relative to total AA in food corrected for total AA in blank	*In vitro* underestimates *in vivo* values	(95)

AA, amino acids; DIAAS, digestible indispensable amino acid score; N, nitrogen; SEC, Size exclusion chromatography; TCA, tricholoacetic acid.

4.3 Consideration of the enzyme background

In both, *in vivo* and *in vitro* situations, the endogenous enzymes and background proteins need to be considered. Different solutions to this challenge have been suggested. Probably the most precise way of differentiating endogenous material from the food of interest represents the use of isotopically labeled food sources as has been used by Ménard et al. (94). In this study, ^{15}N isotopically labeled casein and gluten were digested at different concentrations. Unfortunately, the generation of isotopically labeled substrates is not always possible, in addition to being time consuming and expensive, and therefore other solutions are requested. However, for experiments of proof of principle and validation, isotopic labeling would be the method of choice. As an alternative, the enzyme background was

subtracted by performing a parallel digestion with H_2O (90, 93–95) or using a protein-free food (92). However, as explained in Section 2, in the absence of substrate, a higher enzyme autolysis may occur (39, 94), which would cause an underestimated digestibility value.

4.4 Validation and standardization of *in vitro* protein digestibility protocols

Comparisons between *in vitro* and *in vivo* data were performed by four of the above-mentioned publications (Table 2). A high correlation between *in vitro* and *in vivo* DIAAS, as well as an agreement in limiting amino acid (DIAA) was demonstrated for the investigated substrates, although the *in vitro* digestibility values were below *in vivo* digestibilities in these studies (93, 95). In the same direction, a lower true *in vitro* digestibility value compared to *in vivo* was found for the two investigated substrates, casein and gluten (94). A direct comparison between *in vivo* and *in vitro* digestibility was performed for seven substrates (Figure 2, WPI (A) and black bean (B), representing two of the investigated substrates). The results showed a comparable digestibility with an average bias of 1.2% for all essential amino acids of the assayed substrates (Figure 2C). The DIAAS values were comparable with an average bias of 0.1% and a correlation of r = 0.96 between *in vivo* and *in vitro* results (92). It has to be highlighted that this latter study was carried out *in vivo* and *in vitro* by using identical substrates. In view of the published data, an increased number of substrates of different nature is needed to validate this *in vitro* model for digestibility.

4.5 Method repeatability, reproducibility, and standardization

Until recently, a major drawback of *in vitro* methods was the lack of comparability between different laboratories. In consequence, one

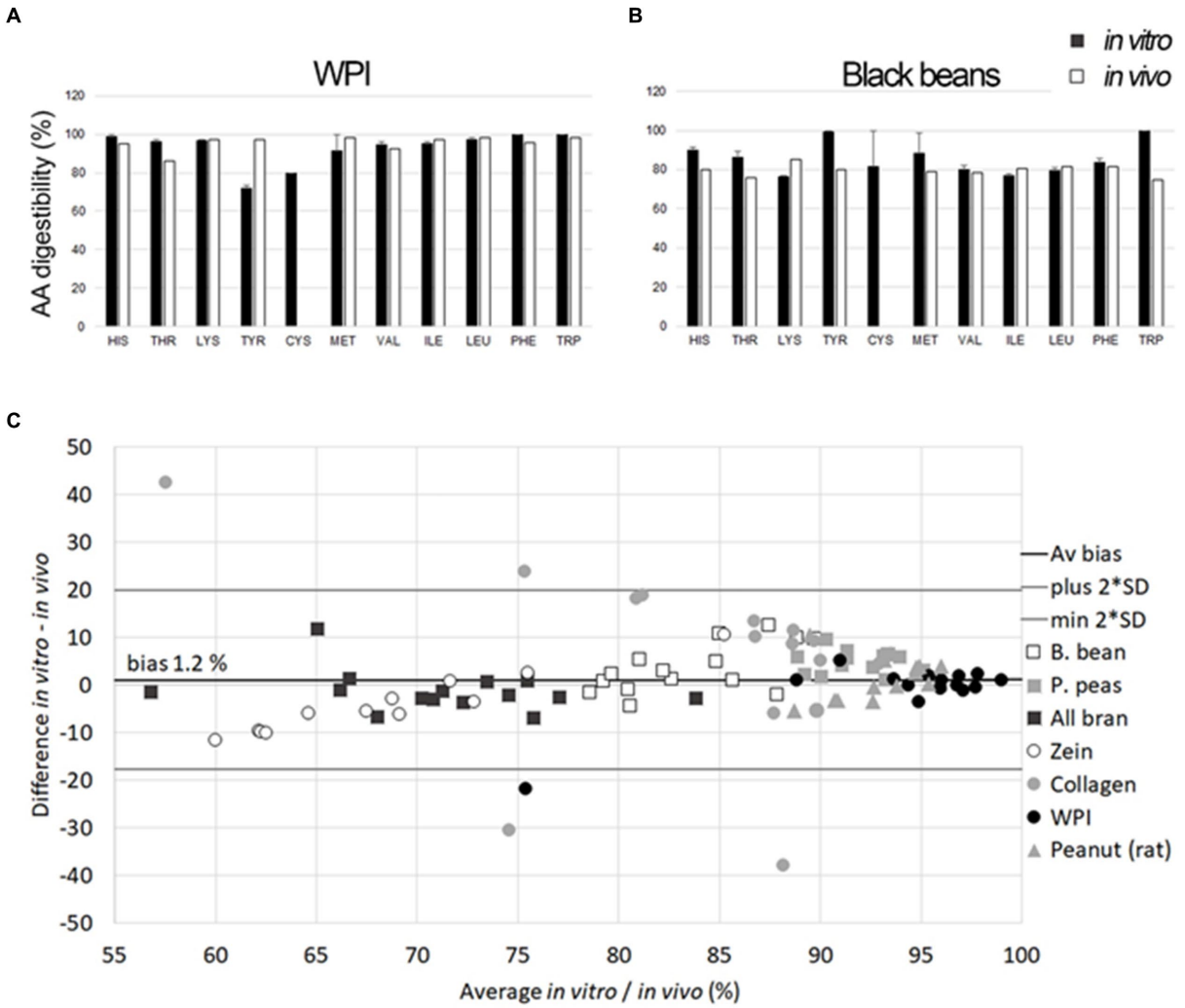

FIGURE 2
In vitro digestibility (y-axis: %) of individual amino acids (black) after IVD compared with *in vivo* data for whey protein isolate (WPI) **(A)** and B. bean **(B)** (mean pig and human values, white). Error bars are SEM of three individual *in vitro* experiments; Statistical comparison between *in vitro* and *in vivo* digestibility of essential amino acids AA **(C)**, according to previous work (96), show the average digestibility of *in vitro* and *in vivo* results (x-axis) versus the differences between *in vitro* and *in vivo* digestibility (y-axis) of all essential amino acids of the comparisons of B. bean, P. peas, All bran, Zein, Collagen, WPI, and Peanut [*in vivo* rat data, (97)]. The mean bias between methods was 1.2% and upper and lower limits indicate ±2 * SD of the average difference. The comparison with *in vivo* DIAAR for SAA could not be calculated due to missing *in vivo* cysteine values.

of the major achievements of the INFOGEST network was to establish harmonized digestion protocols with satisfactory inter-laboratory reproducibility. Within the same INFOGEST network, protein digestibility is currently tested with several dairy products (SMP, whole milk powder, whey protein isolate, yogurt, and gruyere cheese) and with two plant sources (soy protein isolate, chickpea), applying the analytical workflow published by Sousa et al. (92). In parallel to these collaborative studies, a standardization of the method within the International Dairy Federation (IDF) and International Standardization Organization (ISO) was launched. The precision data (repeatability, reproducibility) obtained in the inter-laboratory trials will be included in the future IDF/ISO standard method, with the final goal to obtain a robust and validated protocol allowing the analysis of protein digestibility.

5 Conclusions and future prospects

Several *in vitro* methods to be applied in the assessment of the protein nutritional quality have been developed during the last 40 years. *In vitro* digestion models have been shown to provide a good estimation of protein digestibility and of the nutritional scores, such as the DIAAS, and appear to be a realistic alternative to animal trials in the near future. Some of them have demonstrated good agreement with *in vivo* digestibility data with high correlation coefficients or close protein and amino acid digestibility values. It is important to note that most of these correlations were established protein fecal protein digestibility, while these methods do not take into account the action of microbiota, and thus, when possible, the comparison with standardized or true ileal digestibility data is preferred.

Despite the huge effort done, *in vitro* methods have not reached sufficient confidence to be used for the routine evaluation of protein and amino acid digestibility due to discrepancies in certain substrates. The conditions of static *in vitro* methods are fixed, in the most optimal situation by mimicking as closely as possible the digestive conditions: enzyme/substrate ratios, standardized enzymatic activity, and a digestion time, etc. However, it is highly unlikely that the *in vitro* conditions will be able to simulate all types of foods, matrices, and ingredients without adaptations. For instance, the work performed to date with the INFOGEST method has already detected the need to test protein isolates the same as done *in vivo*, i.e., incorporated in a protein-free food matrix. Similarly, substrates with low protein content or having a high content of trypsin inhibitors will require protocol adaptations. Therefore, it is crucial to carry out *in vitro* protein and amino acid digestibilities of a wide range of substrates with previously measured ileal digestibilities in order to identify limitations and propose adaptations to the *in vitro* protocols. In this sense, new *in vivo* data obtained on biological fluids are needed to refine these *in vitro* digestion conditions. Such work is currently being completed in the frame of a cooperation between INFOGEST and the UNGAP network on drug absorption. Moreover, a large proportion of the studies comparing *in vitro* to *in vivo* values has been made on protein ingredients from animal, plant or alternative sources, although humans do not consume ingredients, but food that adds complexity. More work is needed to apply *in vitro* models to determine protein digestibility on real food where the other constituents of the food matrix can interact with each other, especially when they are processed or ultra-processed, and can limit the accessibility of digestive enzymes to their protein substrates.

Static *in vitro* digestion models are relatively simple techniques with a huge potential for assessing protein digestibility. However, based on the experience within the INFOGEST network, even with protocols extensively described step by step, some slight differences may lead to significant discrepancies. It is important that the validation of these *in vitro* methods is run in different laboratories to generate enough reproducibility and repeatability data. A huge effort is being done in INFOGEST to train people on how to use the model in a proper way and training schools organized in Europe, South America, Australia and Canada. Videos showing the different steps of the model, the digestive enzyme calibration or the quantification of bile salts have been made available on the INFOGEST YouTube channel.[2] All these events and tools will highly improve the reproducibility of the model, leading to more robust interlaboratory data.

Author contributions

GS-S: Writing – original draft, Writing – review & editing, Investigation, Formal analysis. BM: Writing – original draft, Writing – review & editing, Resources. AB: Writing – original draft, Writing – review & editing. DD: Writing – original draft, Writing – review & editing. LE: Writing – original draft, Writing – review & editing. IR: Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Supervision, Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

2 https://www.youtube.com/@fooddigestion

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Progress and challenges in designing dynamic *in vitro* gastric models to study food digestion

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Understanding the mechanisms involved in food breakdown in the human gastrointestinal (GI) tract is essential in food digestion research. Research to study food digestion in the human GI tract requires *in vivo* and *in vitro* approaches. *In vivo* methods involving human or animal subjects are often cost-prohibitive and raise ethical concerns. For these reasons, *in vitro* approaches are becoming more common. Several dynamic *in vitro* models that mimic one or more components of the GI tract have been developed at various research institutions and by commercial companies. While there is evidence of considerable novelty and innovation in the design of these models, there are many differences among them in how the mechanical breakdown of solid foods is accomplished. In some systems, modulating water pressure is used to achieve peristaltic contractions of the gastric antrum, whereas, in other models, the flexible walls of a gastric chamber are compressed by the movement of rollers or clamps outside the walls of the test chamber. Although much progress has been made in standardizing the biochemical environment appropriate to the food digestion process, there is a lack of standard protocols to measure mechanical forces that result in the breakdown of solid foods. Similarly, no standardized methods are available to evaluate the results obtained from *in vitro* trials for validation purposes. Due to the large variability in the design features of *in vitro* models used for food digestion studies, developing consensus-based standards for the mechanical aspects of food breakdown is needed.

KEYWORDS

food digestion, *in vitro* models, gastric digestion, food breakdown, gastric simulator

Introduction

The importance of improving the understanding of food digestion has promoted the need for dynamic *in vitro* models of the gastrointestinal (GI) tract. Within the GI tract, solid foods undergo size reduction to allow the release of nutrients that may ultimately pass through the intestinal walls. Experimental studies on food digestion involving human subjects are often cost-prohibitive and involve ethical and operational barriers. Therefore, *in vitro* models, mimicking the human digestive tract, are necessary to advance the field. Since the early 2000s, considerable progress has occurred in developing *in vitro* models of various parts of the human GI tract for food and pharmaceutical applications (1).

Digestion of solid foods in the GI tract is influenced by the surrounding biochemical media and the mechanical forces created within the tract, such as chewing and mastication in the mouth and peristaltic contractions in the antrum stomach. Biochemical digestion involves exposure of the digesting food to various chemicals and enzymes secreted inside the GI tract. The recipes and protocols for creating biochemical environment in an *in vitro* model to simulate human *in vivo* conditions of the GI tract have been recently standardized (2). However, accurately mimicking the physiologically derived mechanical forces acting on solid foods during digestion remains challenging. While some design features of different *in vitro* models are common, significant differences exist in how the mechanical forces are applied to the digesting food. Almost every *in vitro* model has its own unique mechanism to create mechanical forces. Furthermore, the methods used to validate experimental results vary among researchers. Some researchers measure magnitude of mechanical forces, others rely on indirect procedures involving breakdown of analog materials such as agar gel beads. This paper reviews the design characteristics of selected *in vitro* models with a particular reference to how the mechanical breakdown of digesting foods occurring in the gastric component of the GI tract is accomplished and validated. The need to standardize the methods used to measure and validate mechanical forces in an *in vitro* model will be presented.

Selected dynamic *in vitro* models to study food digestion

This section presents the design characteristics of selected dynamic *in vitro* models of the upper GI tract. These models are selected based on their wide use in food digestion studies or innovative design features to mimic *in vivo* conditions. Schematic diagrams of some models are shown in Figure 1, and some key features are presented in Table 1. For additional details about the *in vitro* models presented in this paper, the reader is referred to review papers (3–6).

TIM (TNO Intestinal Model) dynamic *in vitro* models **(TIM-1, TIM-2, tiny-TIMsg)** were developed at TNO Triskelion, Zeist, the Netherlands, and commercialized at InnoGI Technologies (formerly The TIM Company). **TIM-1** is a multi-component model comprising the stomach, duodenum, jejunum, and ileum (1, 7), **tiny-TIMsg** is a 2-component system comprising a stomach and a single component for the small intestine, and **TIM-2** comprises four independent large intestinal compartments. The stomach component in TIM-1 is represented by a horizontally oriented flexible tube placed in a transparent rigid cylinder (Figure 1). Water at 37°C is circulated inside the annular space between the flexible wall of the tube and the outer rigid cylinder to provide modulated contractions of tube contents. The pressure forces created inside the tubular stomach region have been validated with *in vivo* human data. The operating protocols allow control of temperature and pH. The release rate of secretions of simulated gastric juice is adjustable based on the test food. The operating controls allow the creation of conditions to mimic the stomach functions of neonates, infants, toddlers, adults, and the elderly. The large intestine compartment in TIM-2 model allows inoculation of its inside walls with human fecal samples. Among various dynamic *in vitro* systems, TIM models are notable for most pharmaceutical and food applications. Numerous papers have been published on food digestion using the TIM models (1). A relatively newer *in vitro* model in the TIM series is tiny-TIMsg (smartifcialgut) (8). In this model, the gastric component is represented by three parts: the first two (gastric body and proximal antrum) are vertically oriented, and the other one (distal antrum) is horizontal to mimic the J-shape of the human stomach (Figure 1). Test samples with salivary secretions are introduced in the gastric body, and the simulated gastric juice is injected between the gastric body and the proximal antrum. The connection between the proximal and the distal antrum contains pH electrodes. Similar to the other TIM models, the digesta moves inside flexible tubes contained in a transparent rigid jacket. The water flow in the annular space helps create modulated contractions and is computer controlled to achieve desired motility of the digesta. Sensors are located on the flexible walls to measure the pressure. During digestion of different foods, the pressures in the gastric region are reported to be between 2 and 18 mm Hg (8). The TIM models are now coalesced under a new company, InnoGI Technologies, and are part of the *SurroGut* platform.

DGM (Dynamic Gastric Model) was developed at the Institute of Food Research, Norwich, UK. It was one of the early *in vitro* models incorporating biochemical and mechanical aspects of gastric digestion (9). The fundus part of the stomach is a flexible wall funnel-shaped vessel surrounded by a water jacket. The water flow in the jacket is regulated to provide rhythmic movements of the flexible wall of the vessel (Figure 1). The antrum region comprises a rigid barrel containing a piston. The food bolus transferred from the fundus region by the movement of the piston undergoes breakdown due to shearing action created by the movement of an elastic annulus that moves up and down 3 times per minute. The displacement rate of the annulus is based on data obtained from *in vivo* trials. The outlet valve from the barrel controls the exit of the digested sample at timed intervals. In the DGM, pH electrodes regulate the introduction of gastric secretions. The forces created in the antrum region were validated by studying the breakage of agar gel beads (spherical, 1.27 cm diameter of different fracture strengths) compared to *in vivo* trials conducted with human subjects (10). In addition to validating the results obtained from DGM, these authors also noted that when a USP (United States Pharmacopeia) Dissolution Apparatus II system was used in a similar trial, the agar gel beads did not disintegrate but underwent only surface erosion, emphasizing the shortcomings of the USP system that uses a paddle turning inside a rigid vessel to achieve the breakdown of solid materials. DGM has been widely used in food and pharmaceutical research.

HGS (Human Gastric Simulator), version 1, was designed and fabricated at the University of California, Davis (11). The model consists of a vertically oriented stomach chamber shaped like a cylinder and tapering to the bottom (Figure 1). The flexible walls are made of latex rubber. The bottom of the chamber empties into a plastic tube connected to a peristaltic pump to empty the digesta. A polyester mesh bag with a pore size of 1.5 mm is placed inside the chamber to allow passage of digested content of size less than 2 mm. Simulated gastric fluids are introduced at different locations along the inside wall of the chamber. Peristaltic contractions are created by moving custom-built rollers connected to belts operated with pulleys along the four opposing sides of the chamber. The distance between the opposing rollers decreases as they move

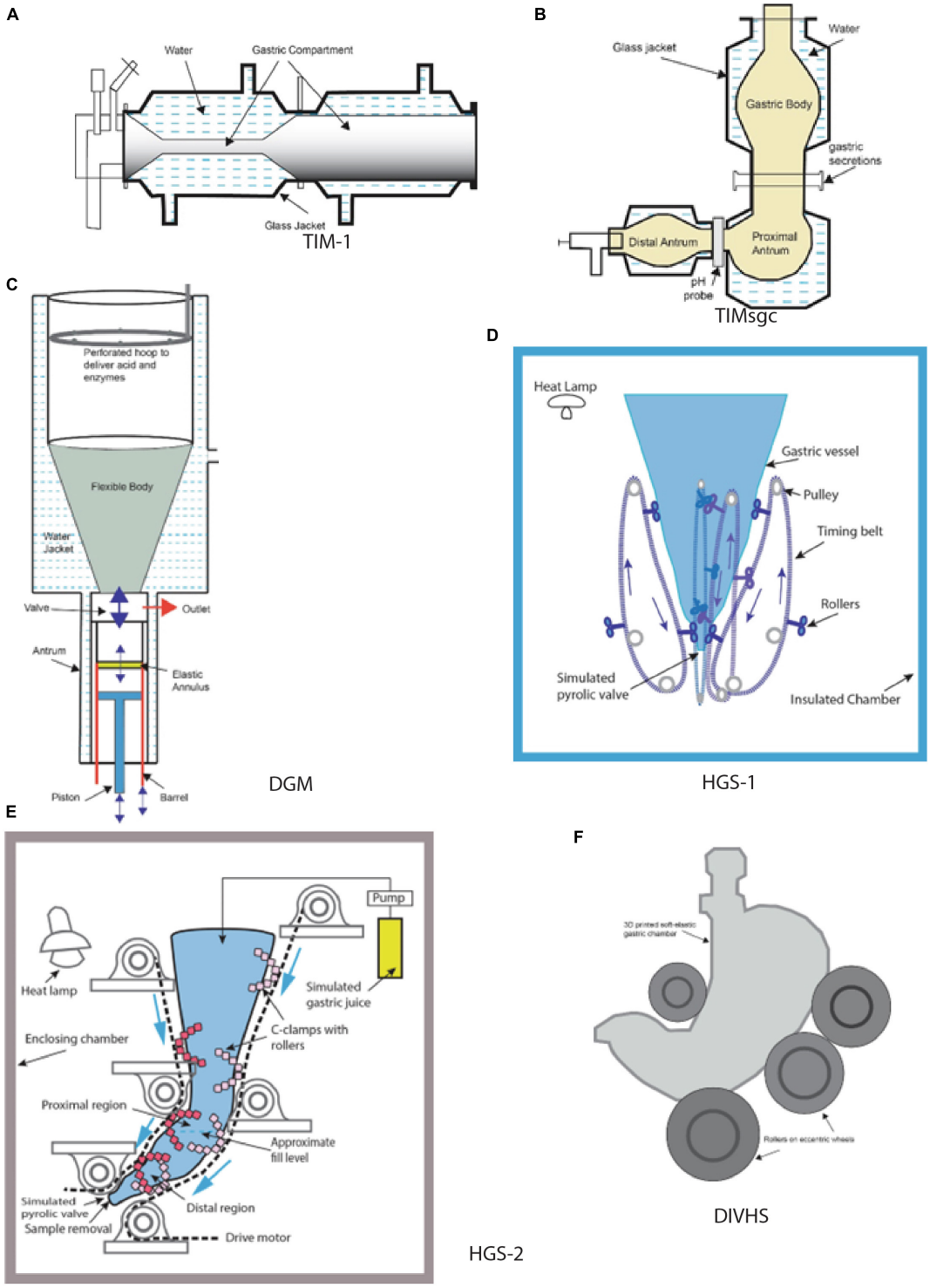

FIGURE 1
Schematic diagrams of selected gastric *in vitro* systems **(A)** TIM-1, **(B)** tiny-TIMsgc, **(C)** DGM, **(D)** HGS-1, **(E)** HGS-2, **(F)** DIVHS. **(A)** Adapted from (32), **(B)** adapted from (8), **(C)** adapted from (9).

down, increasing the contraction in the distal antrum region. The contraction forces created in the model were measured using a thin-walled rubber bulb connected to a pressure manometer. The maximum stress in the distal antrum region was measured to be 6,738 N/m^2 when the gap between the opposing rollers was 12 mm (11). The design of this model has been replicated into multiple units used in research on gastric digestion at the Riddet Institute (Massey University, Palmerston North, New Zealand).

HGS (version 2), the second generation of the original simulator, was designed at the University of California, Davis, to create a J-shaped chamber with circumferential peristaltic contractions. The contractions are obtained using C-clamps

TABLE 1 Physical mechanisms used for solid food breakdown in gastric compartments and selected design characteristics of dynamic *in vitro* models.

In vitro model	Mechanism used for peristaltic contractions in the gastric compartment	Approximate volumetric capacity (mL)	Inside gastric surface
TIM-1	Modulating water pressure around flexible wall compartments	300	Smooth
tiny-TIMsgc	Modulating water pressure around flexible wall compartments	300	Smooth
DGM	Mechanical movement of an elastic annulus in the antrum	800	Smooth
HGS-1	Wheels on a belt moving on flexible walls of the gastric chamber	5700	Smooth
HGS-2	C-clamps with rollers moving on flexible walls of the gastric chamber	900–1000	Smooth
DIVHS	Rollers on eccentric wheels create peristalsis contraction of flexible walls	400	Simulated gastric interior wall
DIDGI	A propeller stirs the gastric content	940	Smooth
ESIN	A shaft stirrer is used with adjustable rotors	Not available	Smooth
GSM	Pneumatically driven syringes compress the gastric wall	600	Smooth
IMGS	Multiple pistons located on the sides of the stomach chamber are used for compressing the gastric chamber	900	Smooth

containing custom-designed Teflon rollers (12). The separation between the opposing C-clamps along the chamber's walls is controlled to obtain the contraction forces inside the distal antrum (Figure 1). Using a rubber bulb attached to a pressure manometer, the maximum contraction force was measured in the gastric chamber as 5.9 ± 0.3 N or normalized by the sectional area of the rubber bulb to be 8347 ± 424 N/m^2 (13). The *in vitro* model was recently validated using data from the digestion of starch-based foods obtained from *in vivo* trials conducted with growing pigs (14). Both versions of HGS have been used for numerous food digestion studies in multiple labs for the past 15 years (15–18).

DIDGI® (Digesteur dynamique gastrointestinal) is a two-component system developed at INRA, France, representing gastric and intestinal regions. Components are made of transparent materials to allow visual observations during a digestion experiment. The stomach is represented by a rigid vessel containing a stirrer. Custom computer software controls the operating parameters, such as temperature, transit times in the gastric and intestinal regions, the addition of digestive secretions, and pH in the two regions. DIDGI® has been used for digestion studies of infant formula, human milk, and various bovine milk products including cheese and skim milk. The *in vitro* system has been validated by digesting infant formula and comparing milk proteolysis in digestion studies with piglets (19). Validation studies have compared the kinetics of casein and beta-lactoglobulins evolution during digestion. With its simple design, the apparatus is robust; however, using a stirrer to accomplish mixing and breakdown fails to mimic the dynamic forces associated with peristaltic contractions in a human stomach.

ESIN (Engineered Stomach and Small Intestinal) was developed at the University of Auvergne, Clermont-Ferrand, France. This system has six chambers, representing an inlet chamber to introduce realistic-sized food particles into the model, a mixing chamber for the test sample to mix with simulated saliva, stomach, duodenum, jejunum, and ileum. The stomach chamber contains a rigid cylinder (methacrylate) containing two pistons moving from opposing sides. The test samples are subjected to mechanical forces generated in this cylinder/piston arrangement. Electronic systems control the temperature, spatial and temporal changes of pH, the input of simulated gastric, pancreatic, and biliary secretions, transit time, and mixing of chyme. The gastric section involves segregated emptying of small-size digested particles (<2 mm) and larger-size particles using peristaltic pumps. Pumps are used to obtain desired emptying rates. Validation trials have largely focused on the digestion of pharmaceutical drugs, such as soluble paracetamol and theophylline (20).

GSM (Gastric Simulator Model) was developed at the University of Georgia, Athens, Georgia, USA (21). The walls of the stomach chamber are made of latex. There are well-defined regions of the stomach, specifically, cardia, fundus, proximal corpus, and distal corpus. The pyloric opening is regulated with an air/vacuum system to allow particles smaller than 1.0–2.0 mm to pass through while retaining larger particles for further breakdown. The peristalsis contractions along the flexible chamber walls are obtained using a series of syringes placed circumferentially along the wall, from the fundus down to the pylorus. A programmable logic control system operates the syringes to obtain the desired regional contractions while creating a forward flow inside the chamber. The intragastric pressure, measured using a pipette rubber bulb with a digital manometer in the antrum region, was around 55 mm Hg. Simulated gastric secretions are introduced at different locations in the corpus using a variable flow peristaltic pump. GSM has been used to measure the breakdown of cooked sausage and the results were compared with those obtained from the conventional shaking bath method (21).

DIVHS (Dynamic *In Vitro* Human Stomach) was developed at Soochow University, Suzhou, China (22). It is based on previous generations of models developed by the researchers and aimed at reproducing human stomach anatomy and biochemical environment. The stomach chamber and the duodenum are fabricated using 3D printing with soft-elastic silicone rubber. The gastric chamber is J-shaped, the size of an adult human stomach (Figure 1). The stomach walls are about 5 mm thick. The peristaltic contractions of the stomach and intestine are created using a series of eccentric wheels and rollers. The amplitude of waves increases toward the distal antrum, by decreasing the distance between the rollers. The mechanical stress in the antrum region was measured to be 8,920 N/m^2. The secretions (simulated gastric juice and intestinal fluid) are delivered using peristaltic pumps. Gastric emptying of smaller particles is achieved by rotating the

platform that supports the gastric model. The entire unit is placed in a controlled-temperature chamber. This *in vitro* model has been used to digest cooked rice and a mixed meal containing beef stew and orange juice (22).

IMGS (*In Vitro* Mechanical Gastric System) was developed at the Universidad Tecnologica Metropolitana, Santiago, Chile. The J-shaped stomach chamber is made of 1.0 mm thick latex wall formed using a polylactic acid mold fabricated with a 3D printer (23). The walls are compressed from opposing sides using four acrylic pistons located on each side. Pistons along the fundus/body region of the chamber operate with 0.3 Nm torque, whereas the other pistons along the antrum employ a torque of 0.88 Nm. A computer control system is used to operate the pistons. Constant temperature is maintained by submerging the flexible gastric chamber in a water bath. Digestion studies have included investigating the role of gastric peristalsis on the intestinal lipolysis of protein-stabilized oil-water emulsions (23).

Other notable *in vitro* models of GI tract developed for food applications use custom designs and operating protocols (24–27).

Discussion

While considerable progress is being made in designing new *in vitro* digestion systems, several opportunities exist for improved representation of *in vivo* conditions. Only a few *in vitro* models mimic the J-shaped anatomy of the gastric chamber. Computational fluid dynamic studies have shown complex flow patterns within a J-shaped space domain when subjected to peristaltic contractions of the flexible walls (28–30). The domain shape uniquely influences the formation and location of eddies and vortical flow. Similarly, inside the J-shaped domain, there is considerable spatial variation of shear forces. Such fluid flow patterns cannot be replicated in tubular or conical-shaped domains. By fabricating the gastric chamber with 3-D printing, an accurate representation of the J-shaped anatomy of a human stomach is now possible (DIVHS, IMGS, GSM). Most of the current *in vitro* models (except DIVHS) use a smooth surface for the inside wall, whereas in the human stomach, the inside wall has numerous odd-shaped wrinkles and small indentations that may influence the surface conditions (such as friction) where solid foods rub against the inside walls during mixing and breakdown. 3-D printing allows creating more realistic surfaces for the inside wall of the gastric chamber (4).

Only a few *in vitro* models have been validated using *in vivo* trials with human or animal subjects. The high costs of *in vivo* trials with animals or human subjects often inhibit such studies. Furthermore, food products have a diverse range of material properties. Therefore, the results obtained from a digestion study of one food may not apply to another. A possible approach is to classify solid foods into broad categories based on their material properties and the rates of solid breakdown (31). Selected *in vivo* trials with foods representing such broad categories may provide useful information to develop reliable operating conditions for different *in vitro* models.

Current *in vitro* models do not incorporate the entire gastrointestinal tract. The TIM models cover most of the GI components except for oral processing. Most models focus on one or two components, and none contain a validated oral component to represent chewing, mastication, mixing multi-food components with saliva and its enzymes, and bolus formation. Simulated oral processing of a food sample fed to the *in vitro* gastric chamber must be clearly described based on the sample's physical properties. While the design and operating features of most *in vitro* models are limited to studies of food digestion in a healthy adult, there is an increasing need for *in vitro* studies of food digestion by the elderly. As the ratio of elderly to adult population increases in many parts of the world, *in vitro* systems specifically designed to mimic the GI tract of the elderly will be required. Similarly, *in vitro* systems appropriate for infants are needed. Some models discussed in this paper (TIM and GSM) suggest modifications for this purpose.

As noted in this paper, many *in vitro* systems have been recently developed in different research laboratories and by commercial companies. In these models, the design characteristics of the GI components and their operating protocols vary significantly. Similarly, there is considerable variation in the validation methods used. Some researchers provide quantitative measures of forces or stresses generated within the system; however, the measurement protocols are not standardized. No published papers were found that present results from digesting the same food or food analog using two or more different *in vitro* models. While each model can yield data on food breakdown, the results from these models will be more reliable if a standardized operational protocol (relevant to the design features) is developed. Inter-laboratory measurements of selected digestion parameters of selected foods using different *in vitro* models would be highly desirable. Currently, there is no standardized method to measure forces developed during digestion in different regions of the GI tract. It would be highly desirable to develop standards for dynamic *in vitro* models and their operating protocols by scientific bodies such as the INFOGEST or the National Institute of Standards and Technology (US Department of Commerce, Washington, D.C).[1] Results using a standardized procedure to measure mechanical forces will increase the credibility of the results obtained from different *in vitro* models. Future improvements in the design and operation of vitro models of the GI tract are expected to enhance our quantitative understanding of the food digestion process and its role in human health.

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1 https://www.nist.gov/

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Protein and amino acid digestibility: definitions and conventional oro-ileal determination in humans

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When assessing protein quality, a correction needs to be made to take into consideration the availability of the amino acids. This correction is based on the digestibility of the amino acids. It is recommended to use ileal (end of small intestine) digestibility as opposed to faecal digestibility. A correction needs to be made for endogenous (gut sourced as opposed to diet sourced) amino acids to give true digestibility as opposed to apparent digestibility. Also, this correction should be made by correcting the amino acid composition for individual amino acid digestibilities as opposed to correcting all amino acids for nitrogen digestibility. Determination of true ileal amino acid digestibility requires the collection of ileal digesta. In the human there are two methods that can be used; naso-ileal intubation and using the ileostomy model. Both are discussed in detail and it is concluded that both are appropriate methods to collect ileal digesta.

KEYWORDS

protein, amino acids, amino acid digestibility, human, ileal digesta

1 Introduction

Typical diets contain a mixture of different protein sources which will vary in nutritional quality (amino acid composition and availability). The aspects that are evaluated when determining protein quality are the amino acid composition and availability of the amino acids. The relationship between the available amino acids and amino acid requirements is then determined. Amino acid digestibility, the disappearance of the amino acids from the gut following consumption of the protein source, is measured to determine amino acid availability. The first part of this work will define terms used in conjunction with amino acid digestibility.

2 Definitions

2.1 Faecal versus ileal digestibility

Amino acid or nitrogen digestibility were traditionally determined based on the difference between the amount of each amino acid or nitrogen consumed and the amount that appeared in the faeces. Faecal nitrogen digestibility is the basis for protein digestibility corrected amino acid score [PDCAAS; (1)] a method that is used commercially in countries such as the United States to evaluate protein quality.

The problem with the use of faecal amino acid or nitrogen digestibility to evaluate protein quality is that the large intestine contains large numbers of microbes which metabolise the amino acids as they pass through. In the pig it has been shown that over 80% of all of the amino acids present in the faeces are of microbial origin rather than dietary origin (2). Moreover, it is generally accepted that there is minimal if any absorption of intact amino acids in the large intestine. The latter has been shown by infusing a single dietary indispensable amino acid (lysine or methionine) into the colon of pigs that had received a diet that was first-limiting in the same amino acid(s) (lysine or methionine + cysteine). If the infused amino acid had been absorbed in nutritionally significant amounts, the nitrogen balance of the pigs would have improved. However, there was no change in the nitrogen balance of these pigs (3). Put together, this means that the absorption of amino acids in a form that can be used for protein metabolism finishes at the end of the small intestine; the terminal ileum. Faecal digestibility values will not represent the amount of amino acids digested and absorbed such that they partake in protein metabolism. Table 1 shows ileal (adult ileostomates) and faecal digestibility coefficients following the consumption of a meat-vegetable-cereal-dairy product-based diet and shows how the difference between ileal and faecal digestibility coefficients can be quite significant. For individual amino acids, differences of up to 0.15 (15% units) were reported. For accuracy, digestibility values must be determined at ileal level (thus giving ileal digestibility) to determine protein quality (1, 5).

2.2 Apparent versus true digestibility

Calculating digestibility values based on the quantity of amino acids consumed in a food and the amount in digesta collected from the terminal ileum gives "apparent" ileal digestibility values. However, while digesta will contain amino acids of food origin, it also contains amino acids of endogenous origin. Endogenous secretions are those that originate from the gut as opposed to the food. Endogenous secretions include digestive enzymes secreted during the digestion process, mucous that lines the gut and enterocytes; the cells that line the gut and are regularly sloughed off and replaced. Serum albumin is also present in the endogenous secretions. Microbes, while not strictly endogenous, are also included in the endogenous category and their potential significance in terms of amino acid homeostasis is reviewed in Metges (6). The majority (around 70–80%) of the endogenous secretions are digested themselves and absorbed before the end of the small intestine (7). However, the remaining endogenous secretions will be present in the digesta.

To determine the amount of the eaten amino acids that are digested and absorbed, a correction needs to be made for the endogenous secretions, thus determining the amount of amino acids of dietary origin that are present in the digesta. When the digestibility is corrected for endogenous secretions (subtracting endogenous secretions from the total amino acid content in ileal digesta), "true" digestibility is determined. Standardized digestibility values are calculated in the same way as true digestibility (see below), thus this is an alternative term used by some research groups for true digestibility.

The equations to calculate apparent and true digestibility are given below.

$$\textit{Apparent digestibility} = \frac{\textit{Dietary amino acids} - \textit{Amino acids in ileal digesta}}{\textit{Dietary amino acids}}$$

$$\textit{True digestibility} = \frac{\textit{Dietary amino acids} - \left(\textit{Amino acids in ileal digesta} - \textit{Endogenous amino acids}\right)}{\textit{Dietary amino acids}}$$

The currently preferred method to quantify endogenous amino acids in ileal digesta, the [e.g., recommended by FAO Expert Working Groups; (8)] involves consuming a protein-free diet before collecting ileal digesta. When a protein-free diet is consumed, all of the amino acids in the digesta must be of endogenous origin. The value for endogenous secretions can be used to calculate true (or standardized) digestibility.

2.3 Correcting for nitrogen or individual amino acid digestibility

When determining protein quality, the correction from the total concentration of amino acids in a food/ingredient to the concentration of available amino acids can be based on nitrogen digestibility or individual amino acid digestibility values. If this correction is based on nitrogen digestibility, the total concentration of each amino acid is multiplied by the same value for digestibility; that for nitrogen. This method of calculation is used when PDCAAS is determined. The principle advantage of carrying out this correction based on nitrogen digestibility is the lower cost for the chemical analyses; it is a lot more economical to determine nitrogen in the samples than to determine the individual amino acids.

In samples both of foods/ingredients and digesta, not all of the nitrogen in a sample will be amino nitrogen. Thus nitrogen digestibility will include more than amino nitrogen. It is important to note that when individual amino acid digestibilities are examined, these can vary markedly in the same food/ingredient. This is especially the case when proteins with a lower average digestibility (60–75%) are considered, such as many cereals and legumes. Table 2 shows the true ileal digestibility coefficients for black beans [data from (9)]. Individual amino acid digestibility coefficients range from 0.302 for cysteine to 0.829 for reactive lysine. Using the nitrogen digestibility value (0.66)

TABLE 1 Mean ileal (determined in ileostomates) and faecal digestibility coefficients in adult human subjects consuming a meat/cereal/dairy – based diet[1].

Amino acid	Ileal	Faecal	Statistical significance	Difference
Serine	0.87	0.92	$p < 0.001$	0.05
Threonine	0.85	0.89	$p < 0.01$	0.04
Glycine	0.72	0.87	$P < 0.001$	0.15
Methionine	0.93	0.83	$P < 0.001$	0.10
Tryptophan	0.77	0.83	$p < 0.05$	0.05

[1]Data from (4).

TABLE 2 Amino acid content, true ileal amino acid digestibility coefficient (TIAAD) and amount of digestible amino acids calculated based on the true ileal digestibility of individual amino acids or the digestibility of *N* for black beans[1] determined in human ileostomates.

Amino acid	Amino acid content mg/g DM	TIAAD	Amount digestible AA (mg/g DM) based on	
			TIAAD	*N* digestibility
Threonine	10.6	0.705	7.5	7.0
Valine	12.4	0.743	9.2	8.2
Isoleucine	10.4	0.784	8.1	6.8
Leucine	18.6	0.797	14.9	12.3
Phenylalanine	13.5	0.809	10.9	8.9
Tyrosine	8.5	0.799	6.8	5.6
Histidine	6.7	0.736	4.9	4.4
Methionine	2.7	0.772	2.1	1.8
Cysteine	2.3	0.302	0.7	1.5
Reactive lysine	13.3	0.829	11.1	8.8
Tryptophan	3.0	0.727	2.2	2.0

[1]Data from (9).

to correct the digestibility of all of the amino acids rather than individual amino acid digestibility values will result in inaccuracy in the digestibility data. Table 2 also shows the amino acid content and amount of digestible amino acids (9) calculated based on either the true ileal amino acid digestibility values of each individual amino acids or N digestibility for black beans. When the amount of true ileal digestible amino acids is calculated based on N digestibility, in this case the values are underestimated for all amino acids except cysteine (which is overestimated by 200%), with the underestimation ranging from 6.4% (threonine) to 20.3% for reactive lysine. In conclusion, to accurately determine protein quality, it is necessary to calculate the amount of available amino acids based on the true ileal digestibility of individual amino acids.

3 Collection of ileal digesta from the human

The biggest complication with determining true ileal amino acid digestibility is that it requires the collection of digesta from the end of the small intestine, the terminal ileum, which is far from straightforward. Two methods that have been developed to collect ileal digesta from the human; naso-ileal intubation and with the participation of ileostomates. These are discussed below.

3.1 Naso-ileal intubation

Naso-ileal intubation is conducted with healthy adult participants. Under local anesthesia, a triple-lumen fine tube is inserted through the nose, down the back of the throat and into the esophagus. The tube then passes through the stomach and moves right to the end of the small intestine, the terminal ileum. One lumen of the tube is used to inflate a small balloon on the end of the tube to facilitate the movement of the tube through the small intestine via peristaltic movements. A non-absorbable marker (e.g., polyethylene glycol) is infused into the intestine through another lumen of the tube and digesta is collected via gentle aspiration through the third lumen, downstream from the site of marker infusion. The tube is radio-opaque and the correct positioning of the tube is checked via X-ray.

Once the tube is in position and after an overnight fast, a test meal with the only source of protein being the food/ingredient being tested (or a protein-free meal to determine endogenous amino acid losses) is consumed by the participant. For the following 8 h, the participant will only consume water, and digesta is gently and continuously aspirated through the tube to provide the ileal digesta sample. Calvez et al. (10) describes in detail the typical protocol for the use of naso-ileal intubation to determine true ileal amino acid digestibility.

The principle strength of the naso-ileal intubation method is that it allows ileal digesta to be collected from healthy "intact" participants. It does, however, have the limitation that it can be considered to be very invasive. Many participants are unable to tolerate the insertion and presence of the tube. Each participant can only partake in the testing of one food (or a protein-free meal). It is not an appropriate method for use in vulnerable groups such as children. It is an expensive technique and must be applied under hospital conditions. As the lumen of the sampling tube is small, if digesta contains many particles, these could clog the tube, which limits the foods that can be tested with this method.

One potential criticism of the method is whether the presence of the tube inside the gastrointestinal tract affects digestive function; such as gastric and/or intestinal transit time. Several studies have determined the effect of an intestinal tube on parameters such as gastric emptying. Some studies have reported a delayed gastric emptying (11–14) while Müller-Lissner et al. (15) reported little or no effect. Whether the presence of the tube affects parameters such as gastric emptying may not be important, however, as Gaudichon et al. (16) reported that amino acid absorption is not influenced by the transit rate of the food.

Overall, the naso-ileal intubation method appears to be a suitable method to collect ileal digesta from the healthy adult.

3.2 Human ileostomates

Human ileostomates are people that, due to medical conditions involving the large intestine, have the end of their small intestine surgically exteriorised via a stoma. Stoma bags are connected to the exterior of the stoma into which all of the digesta that passes through the small intestine are collected. When protein quality is evaluated with the ileostomy model, the participants consume a test meal following an overnight fast. The only source of protein in the test meal is the food/ingredient being tested (or a protein-free meal is consumed to determine endogenous amino acid losses). A fresh stoma bag is attached and all of the digesta that that enters the bag over the next 9 h is collected. While digesta are being collected, the participants can only consume water and sweetened drinks. Moughan et al. (17) describes a typical protocol for determining true ileal amino acid digestibility with the participation of ileostomates.

Working with ileostomates has the advantage that there is no limitation on the types (or particle size) of the foods that can be tested. Numerous protein sources/foods can be tested with each participant,

although, due to the rigorous nature of the testing (no food can be consumed for 9 h after the test meal on study days), participants can reach study fatigue if there are too many study days over a short period of time.

The principal limitation involved in working with the ileostomy model is recruitment due to the low numbers of ileostomised people. Moreover, many ileostomised people have other health conditions or are prescribed medications that could affect digestive functions, so are not suitable for these studies. Nowadays it is common that after the ileostomy surgery and after the large intestine heals sufficiently, the ileostomy is reversed. This means that often there is only a short period of time between healing from the original surgery and having the surgery reversed, further complicating the recruitment of sufficient ileostomised participants for a study.

A potential concern with the use of ileostomised participants in nutritional studies is whether there is increased colonisation of the small intestine with microbes. Several studies have addressed this concern. Englyst and Cummings (18) evaluated polysaccharide digestion in ileostomised participants. The ileostomates consumed metronidazole, which inhibits the metabolism of anaerobic bacteria, with no differences found between before and after the consumption of metronidazole. Sandberg et al. (19) also concluded that there was little if any fermentation occurring in the small intestine of ileostomised participants. Fuller et al. (20) collected ileal digesta from ileostomates after they had consumed a protein-free diet. When antibiotics were administered and ileal digesta collected again, there was no difference in the concentration of amino acids compared with before the administration of antibiotics, also supporting that there is not an increased colonisation of the small intestine by microbes in ileostomised people.

3.3 Comparisons between ileostomised and intact people

Ileostomised participants have been used as models for the "intact" person, particularly to study digestion and absorption to the end of the small intestine both for protein (4, 19) and fiber (21). There is a considerable amount of evidence supporting the ileostomy model as a direct and quantitatively accurate model to evaluate nutrient digestibility in the upper gastrointestinal tract (18, 19, 21–27).

No differences have been found in the gastric or intestinal transit rate between ileostomates and "intact" people; the "head" of the meal has been shown to travel from the mouth to the terminal ileum in the same time in ileostomates as from the mouth to the caecum in "intact" humans (28).

A direct comparison of true ileal amino acid digestibility coefficients determined with naso-ileal intubation and the ileostomy model has been conducted. The true ileal amino acid digestibility of the protein sources zein (relatively low digestibility) and whey protein isolate (WPI, highly digestible) were determined using naso-ileal intubation [results reported in (10)] and with the ileostomy model [results reported in (9)] and the results were statistically compared. No statistically significant differences were determined ($p > 0.05$) between the methods for digestibility of either protein or for any amino acid. The calculated mean true ileal amino acid digestibility coefficients for zein were 0.63 and 0.60 and for WPI were 0.92 and 0.95 (naso-ileal intubation and ileostomy model, respectively). Thus the results for true ileal amino acid digestibility determined using the two methods do not differ. This information taken together supports ileostomised participants as being representative of the "intact" person to study the upper gastrointestinal tract.

The complications involved in collecting ileal digesta from humans mean that while they are useful methods for specific studies, these ileal digesta collection methods (naso-ileal intubation or with ileostomised participants) are not able to be used for routine analyses of multiple foods, for example to generate values required for DIAAS. This has led to the development of animal models, the use of which does have ethical implications. Direct comparisons between the growing ileal cannulated pig and ileostomised human have shown an excellent agreement of ileal amino acid digestibility values for a variety of different types of food (9). Thus the pig has been shown to be an excellent model for the human in terms of true ileal amino acid digestibility for when it is not possible or practical to collect digesta from the human.

4 Summary

Terms related to determining protein and amino acid digestibility are defined with recommendations made on which are considered to be the correct methods to use for protein quality determination, including the difference between faecal and ileal digestibility as well as apparent versus true digestibility. The correction for amino acid availability should be made with individual amino acid digestibility rather than correcting all amino acids for nitrogen digestibility.

There are two methods that can be used to collect digesta from the end of the small intestine; naso-ileal intubation and using the ileostomy model. Both are appropriate methods to collect ileal digesta and they are discussed in detail.

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Digestible indispensable amino acid score (DIAAS): 10 years on

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The objective of the review is to revisit the findings of the 2011 Food and Agriculture Organization of the United Nations (FAO) Expert Consultation on Dietary Protein Quality Evaluation in Human Nutrition, and to report on progress on uptake of the findings. It is evident that since 2011 there has been a concerted research effort to enhance an understanding of the protein quality of foods. The validity of the growing pig ileal protein digestibility assay has been confirmed and numerous studies reported using the growing pig as a model to give true ileal amino acid digestibility values for foods as consumed by humans. This has allowed for the determination of digestible indispensable amino acid scores (DIAAS) for a range of foods. A new non-invasive true ileal amino acid digestibility assay in humans which can be applied in different physiological states, called the dual-isotope assay, has been developed and applied to determine the DIAAS values of foods. It is concluded that DIAAS is currently the most accurate score for routinely assessing the protein quality rating of single source proteins. In the future, the accuracy of DIAAS can be enhanced by improved information on: the ideal dietary amino acid balance including the ideal dispensable to indispensable amino acid ratio; dietary indispensable amino acid requirements; effects of processing on ileal amino acid digestibility and lysine bioavailability. There is a need to develop rapid, inexpensive *in vitro* digestibility assays. Conceptual issues relating DIAAS to food regulatory claims, and to holistic indices of food nutritional and health status are discussed. The first recommendation of the 2011 Consultation regarding treating each indispensable amino acid as an individual nutrient has received little attention. Consideration should be given to providing food label information on the digestible contents of specific indispensable amino acids.

KEYWORDS

digestible indispensable amino acid score (DIAAS), digestible indispensable amino acid ratio (DIAAR), lysine bioavailability, protein digestibility corrected amino acid score (PDCAAS), protein quality, true ileal amino acid digestibility

1 Introduction

In 2011 the Food and Agriculture Organization of the United Nations (FAO) convened an Expert Consultation on the subject of "Dietary Protein Quality Evaluation in Human Nutrition." Fourteen international experts and an FAO Secretariat undertook an in-depth review of aspects pertaining to protein quality evaluation in human nutrition, and the deliberations were published in 2013 (1). The aim of this contribution is to review matters arising from the FAO 2013 recommendations, a decade later. Since 2011, there has been considerable global research effort aimed at improving an understanding of the protein quality of foods.

The 2013 report documented multiple findings, but with two overarching recommendations:

1.1 First overarching recommendation

"In dietary protein quality evaluation, dietary amino acids should be treated as individual nutrients and wherever possible data for digestible or bioavailable amino acids should be given in food tables on an individual amino acid basis."

1.2 Second overarching recommendation

"A new protein quality measure known as digestible indispensable amino acid score (DIAAS) is recommended to replace protein digestibility corrected amino acid score (PDCAAS)." "DIAAS can have values below or in some circumstances above 100%. Values above 100% should not be truncated except where calculating DIAAS for protein or amino acid intakes for mixed diets or sole source foods."

In both cases, it was recommended that the digestibility of each amino acid be given in terms of true ileal amino acid digestibility, and for processed foods where Maillard type damage may have occurred, values for lysine availability (true ileal digestible reactive lysine) should be used. It was recognized at the time of the consultation that there were insufficient published data on the true ileal amino acid digestibility of foods as consumed by humans and rectifying this situation was a key research directive.

In the intervening decade the first overarching recommendation has not received a great deal of attention, possibly because of a primary focus on food scores such as DIAAS. However, it remains an important consideration, especially as further research continues to identify important metabolites associated with specific amino acids, and physiological roles for specific amino acids. The reasoning behind this primary recommendation was firstly that several amino acids have important metabolic fates other than their involvement in protein synthesis and it may be important in this context to have information on the absorbed amount of the amino acid. Secondly, this approach allows for the calculation, where appropriate, of absorbed amounts of conditionally essential amino acids and the dispensable amino acid component. Finally, such data allow for the estimation of the amounts of absorbed amino acids and their adequacy for meeting daily amino acid requirements in the context of meals and dietary patterns. In the latter respect DIAAS values for individual foods are not additive, though true ileal amino acid digestibility values are additive in dietary formulation. Accordingly, it is possible to calculate the DIAAS of a meal or dietary pattern but it is not necessary to do so. DIAAS was designed to meet the need for defining the protein quality of a single food. It gives information as to the ability of that protein to supply available amino acids as if the protein food was the sole source of dietary protein. It is used to compare individual protein sources, particularly for trade purposes and gives a crude estimation of the value of a protein for inclusion in a mixed dietary pattern. Since DIAAS is calculated in isolation from information about the meal or dietary pattern in which it may be consumed, it is necessary to express both amino acid requirements (the reference pattern) and amino acids in the food, relative to protein (the estimated average requirement, EAR, for protein in the case of the reference pattern and the crude protein content of the food in relation to the food amino acids). Although inherently necessary in the case of calculating DIAAS, there are disadvantages in doing this (2).

In the case of ascertaining the adequacy of dietary amino acid intakes in the context of meals or dietary patterns, however, it is not necessary to relate the amino acid contents to protein content. The digestible amino acid contents of the respective dietary proteins in a meal or dietary pattern can simply be multiplied by the amounts of the respective proteins consumed daily (either known or estimated by numbers and sizes of food servings) and each estimated absorbed amino acid intake compared to the required amount. The facility of this approach has recently been demonstrated in the work of Forester et al. (3), who have described a new measure referred to as the Essential Amino Acid-9 Score (EAA-9). Relevant authorities are encouraged to provide newly available information on the digestible amounts of indispensable amino acids on food labels.

The second overarching recommendation has received considerable attention over the past decade with many studies reporting true ileal amino acid digestibility values for a range of foods in a form as consumed by humans, along with the attendant DIAAS values. New methods for determining true ileal amino acid digestibility non-invasively in humans in different physiological states have been developed and animal based ileal digestibility assays have been thoroughly validated. Methodological aspects of DIAAS have been investigated and in some cases aspects of the appropriateness of the DIAAS measure have been challenged. This has occurred largely within a conceptual domain, querying the value of focusing on protein quality to the exclusion of other attributes of a food. These important conceptual issues are discussed. The present overview will mainly focus on this considerable body of work related to DIAAS.

2 Protein quality measures

One objective in evaluating dietary protein quality is to predict the contribution of a food protein, or mixture of food proteins, in meeting nitrogen and amino acid requirements for growth and maintenance for people of different ages and physiological states. The extent to which the amino acids from a food or mixture of foods can be used for protein synthesis, when the total intake of utilizable protein is below the upper limit for protein synthesis and when energy and the amounts of other dietary nutrients and co-factors do not limit protein synthesis, is loosely referred to as "protein quality." Measures of protein quality predict the amount of amino acids from a food that can potentially be utilized for a defined individual and defined physiological state.

Many methods have been developed over the years to enable determination of protein quality (4). Most of these measures are based on biological assays, such as protein efficiency ratio (PER), biological value (BV), net protein utilization (NPU) and net postprandial protein utilization (NPPU), and all these assays have their place.

A more general approach however, has been to estimate protein quality using the chemical score method. Here, a simple model is used to predict the pattern of absorbed dietary amino acids available for protein synthesis, and the estimated amount of utilizable amino acids with reference to an individual's ideal amino acid balance (usually restricted to the indispensable amino acids) required for body protein synthesis. The chemical score approach has great utility and both the previously recommended scoring method, PDCAAS, and the more recently promulgated DIAAS, are forms of chemical score.

The advantages of DIAAS over PDCAAS have been reviewed in detail (1, 5–8). One important attribute of DIAAS is that it is based on true ileal amino acid digestibility and true ileal reactive lysine digestibility rather than fecal crude protein digestibility, and the true ileal amino acid digestibility assay has been shown in *in vivo* animal studies to accurately predict amino acid absorption and tissue amino acid deposition (9). The limitations inherent in using fecal crude protein digestibility values have been shown in numerous studies including more recent work by Rutherfurd et al. (10) and Mathai et al. (11).

Based on the underlying factors (e.g., type of digestibility measure, amino acid as opposed to crude protein digestibility, lysine availability, non-truncation of score), DIAAS is expected to accurately predict the amount of absorbed first-limiting amino acid supplied by most foods in relation to the requirement for that amino acid, and by implication the amount of utilizable amino acids, whenever the protein requirement is met for a defined person.

DIAAS can be described as:

DIAAS (%) = (mg of available first limiting indispensable amino acid in 1 g test protein)/ (mg of the same amino acid in 1 g reference protein) x 100.

PDCAAS is calculated in the same manner as DIAAS, except that a single value for crude protein digestibility is used to correct gross amino acids to digestible amino acids, and lysine availability is not taken into account specifically. DIAAS is based on updated amino acid reference patterns, and PDCAAS values above 100% are truncated to 100%.

Numerous recent studies have generated DIAAS values for foods and the DIAAS measure has been applied to demonstrate the importance of protein quality in meeting protein and amino acid requirements, and in evaluating the environmental footprints of food production expressed on a protein basis.

3 Why is the determination of dietary protein quality important?

3.1 Meeting the daily dietary protein requirement in low-income countries and regions

It is frequently assumed that the dietary protein intakes of adults, estimated from population-based food intakes, exceed the safe level of intake (recommended dietary allowance, RDA) for protein [0.83 g protein/kg/day, (12)], even in low-income countries, and that protein is sufficiently supplied. When such observations are made, however, protein is usually given in units of "total" or "gross" protein, with the potential effects of protein quality being ignored. Rather, the safe level of intake for protein, is given in units of available (high-quality) protein, and for a valid comparison, dietary protein intakes should be corrected for the effect of protein quality (13).

The importance of accounting for protein quality is illustrated here by the re-analysis of a published dataset (Source of data: World Resources 2016 Report: see https://www.wri.org/research/shifting-diets-sustainable-food-future) relating "gross" protein intake (population-based) for an adult to the daily protein requirement.

Daily food protein intakes (based on national food consumption patterns) for India and Sub-Saharan Africa sourced from the Global Agri-WRR model are given in Figure 1A. It is often concluded that in both India and Sub-Saharan African adults receive adequate protein.

These gross dietary protein intakes were then corrected for estimates of dietary protein digestibility (82% for India, and 81% for Sub-Saharan Africa based on data for seven countries) and for DIAAS based on the reference amino acid pattern for the 3-year-old to 10-year-old child as recommended by FAO (1) for application to adults. Lysine was the first-limiting amino and calculated dietary DIAAS values were 93% for India, and 88% for Sub Saharan Africa based on data from seven countries. The corrected protein intakes are shown in Figure 1B.

When the quality of the dietary protein supply is accounted for, the conclusions differ, with protein deficiency now being predicted. This highlights the critical importance of considering protein quality whenever protein intakes are close to required levels. The utility of DIAAS for application in malnourished children in general has been demonstrated by a number of studies including Rutherfurd et al. (14), Manary et al. (15), Manary and Callaghan (16), Shivakumar et al. (17, 18), and De Vries-Ten Have et al. (19), though in one study differences in dietary DIAAS did not relate to growth (20).

3.2 Meeting the dietary protein requirement in mid- to high-income countries and regions

In mid- to high-income countries the average adult has a gross protein intake in excess of the RDA for protein and it would appear that protein requirements would be met regardless of protein quality. There is a proportion of the population, however, having low protein intakes and here protein quality can be an important consideration. Moreover, some people (e.g., weight loss, old age, endurance sports) may have higher dietary protein targets than the RDA, which are often accompanied by lower energy intakes. In these cases, protein quality can be important to ensure that the calories derived from protein as opposed to fats and carbohydrates do not become excessive. Both scenarios have recently been evaluated by Moughan et al. (21), with the results highlighting that protein quality can frequently be an important consideration in the diet of consumers in more affluent nations.

As an example of people with lower habitual protein intakes, Sobiecki et al. (22) concluded that UK vegans had an adequate protein intake of 0.99 g/kg/day. If, however, the plant-based diet had an overall utilization value of 70% (typical value for a plant-based diet), the diet would have been protein deficient (0.69 g/kg/day).

In the population at any one time there will be numerous people receiving protein intakes at or below the RDA (23), and protein quality needs to be considered.

What about individuals purposefully targeting protein intakes higher than the RDA and often with accompanying low-calorie intakes? It can be shown Moughan et al. (21) that at a daily energy intake of 108 kJ/kg/day or lower for an average bodyweight US woman, absolute protein intakes of 1.2 g/kg/day or higher combined with low protein quality scores, can lead to protein intake expressed on an energy basis exceeding the recommended upper limit (30%).

The higher the protein intake target and the lower the energy intake, the more pronounced is the effect of DIAAS. In general, for men and women, lower dietary protein quality (DIAAS <100%) can

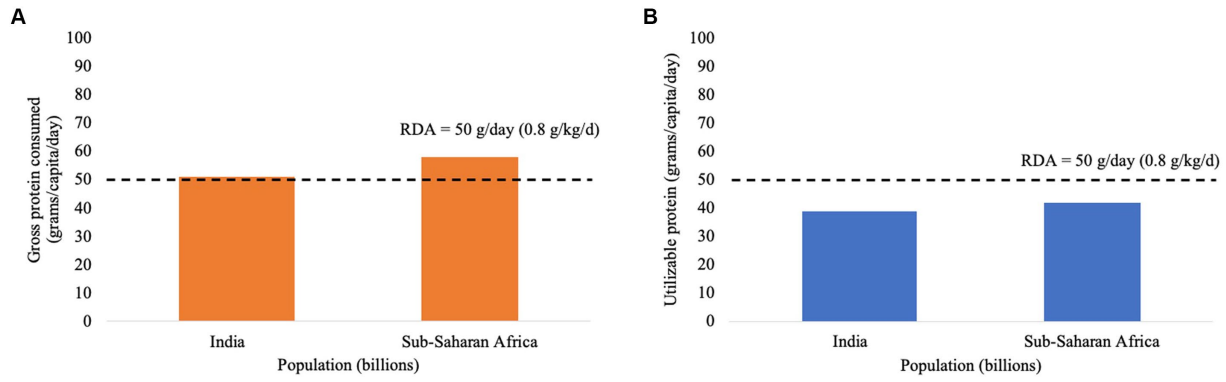

FIGURE 1
(A) Daily protein intake of an adult in India and Sub-Saharan Africa (prior to correction for protein utilizability) compared to Recommended Dietary Allowance (RDA) for protein, as given by World Resources Institute. **(B)** Daily protein intake of an adult corrected for the true ileal digestibility of protein and DIAAS for the diets consumed in India and Sub-Saharan Africa compared to Recommended Dietary Allowance (RDA) for protein, as given by World Resources Institute (DIAAS value for diet based on reference amino acid pattern for the 3-year-old to 10-year-old child).

lead to the need for unacceptably high amounts of dietary gross protein required to meet a target for utilizable protein, at energy intakes below around 120 kJ/kg/day and utilizable protein intakes above around 1.2 g/kg/day. Ciuris et al. (24) have also applied dietary DIAAS values to demonstrate the importance of protein quality for vegetarian athletes to reach dietary protein targets.

4 Correcting environmental footprint data for effects of protein quality

Life-cycle-analysis (LCA) may be used to quantify environmental outputs associated with different types of food protein production. The environmental measures are often expressed per unit gross protein production with no account being taken of differences in the protein quality of food types. Recently several studies have combined measures of protein quality with LCA results (13, 25–31), and demonstrate the importance of considering protein quality in addition to amounts of protein when evaluating environmental footprint data (13).

The results shown in Table 1 are adapted from the study of Moughan (13) whereby published environmental footprint data (annual freshwater consumption and greenhouse gas emissions; Global Agri Model) are expressed per unit gross protein or per unit digestible lysine to factor in the effect of protein quality. The individual DIAAS values of the food proteins were not applied, as this penalizes some foods as it does not account for the complementarity of food protein mixtures. Food proteins are rarely consumed on their own. Lysine is commonly the first limiting amino acid in mixed diets for humans, and in this case true ileal digestible lysine is a useful surrogate measure for DIAAS. When corrected for differences in protein quality (ability to supply digestible lysine), the rankings of the foods change. When no account of protein quality was made, eggs and pork led to much greater freshwater usage, but when protein quality differences are considered the eggs and pork production actually had the lowest levels of water use. Similarly for the greenhouse gas emissions, eggs and pork had much higher emissions compared to corn on a protein basis, but corn production was a higher emitter than both eggs and pork on a lysine basis.

TABLE 1 **Environmental impact of selected plant and animal sources of foods as calculated on a gross protein or digestible lysine basis.**

Freshwater		
Food type	1,000 m^3 per tonne protein	1,000 m^3 per kg digestible lysine
Wheat	18.43	0.80
Corn	14.22	0.65
Egg	25.80	0.24
Pork	51.60	0.25

Greenhouse gas		
Food type	Tonnes Co_{2e} per tonne protein	Tonnes Co_{2e} per kg digestible lysine
Wheat	78.95	3.43
Corn	105.26	4.79
Egg	263.16	3.99
Pork	339.69	4.09

Adapted from Moughan (13). Original data for impact expressed per metric tonne of protein from Ranganathan et al. (32) and are based on the GlobAgri Model.

The protein quality rating of a food in addition to the gross protein content of the food, should be considered whenever evaluating environmental footprints based on life cycle analyses.

5 Development of methods to determine amino acid digestibility and the generation of DIAAS values

5.1 Development of isotope-based methods for determining amino acid digestibility in humans

Traditionally "true" and "real" ileal amino acid digestibility have been determined in adult humans based on the collection of samples

of ileal digesta from the terminal ileum and with correction for ileal endogenous amino acids. Digesta are collected either through the cooperation of ileostomates or following naso-ileal intubation. These approaches are not straightforward however, nor do they readily allow for investigation of the influence of different physiological states on protein and amino acid digestibility. The latter impediment has been addressed by recent work to develop isotope-based methods for determining amino acid digestibility. Two such approaches have been developed, namely the dual-isotope method and the indicator amino acid oxidation (IAAO) based method.

Both approaches have been the subject of recent review (33–39). To the author's knowledge neither method for determining amino acid digestibility has yet been fully independently validated over a wide range of foods, but both approaches show considerable promise to allow the generation of digestibility data obtained in humans and to allow investigation of factors such as age, disease state, pregnancy and lactation on amino acid digestibility. The methods have already been applied in multiple studies to give rise to valuable data on ileal amino acid digestibility and dietary DIAAS values. The recent review by Kashyap et al. (38) gives human true ileal amino acid digestibility data obtained using the dual-isotope method for some 20 foods, including a number of foods commonly consumed in low-income countries.

The different approaches to determining ileal amino acid digestibility and availability lead to coefficients of digestibility that have somewhat different physiological meanings (40), but it has been argued that for practical nutrition purposes the different coefficients (true, real, standardized) can be used interchangeably (35).

5.2 Development of methods for determining amino acid digestibility in humans using animal models

All of the above-described digestibility assays involving humans are costly, time-consuming and have a high ethical cost, and on their own do not provide a routine method for establishing comprehensive databases of amino acid digestibility in diverse foods. To enable the generation of ileal amino acid digestibility data for foods more generally, animal models for protein digestion in humans have been investigated. Over the last decade considerable work has been undertaken to establish the growing pig as a suitable animal model for protein digestion in humans.

The pig, unlike the rat, has the advantage of being a meal-eating omnivore readily consuming typical foods for humans (41). Protein digestion between the mouth and terminal ileum in the growing pig is similar to that in the adult human from both anatomical and physiological perspectives (42), as is protein digestion between the neonatal pig and human infants (43). It is perhaps not surprising then, that close agreement has been found for ileal protein and amino acid digestibility between pigs and humans (44, 45).

Before concluding that the pig is a valid nutritional model, however, the 2011 FAO Expert Consultation (1) called for further pig/human digestibility comparisons to be made over a wider range of foods. This gave rise to the PROTEOS project funded by sectors of the global food industry and coordinated on their behalf by the Global Dairy Platform, which aimed to further evaluate the growing pig as a model for protein digestion in the adult human, and to use the pig assay to generate true ileal amino acid digestibility data for one hundred foods in the form as consumed by humans. The work has established the growing pig as a replicable and valid animal model Hodgkinson et al. (46), thus providing the means experimentally to establish comprehensive databases on the true ileal amino acid digestibility of human foods.

An interesting development with potential application to both the porcine digestibility assay and to humans has been reported (D Wrigglesworth, U.S. Patent for sampling device, patent 10,993,668, May 4, 2021, patent publication number: 20160038086, assignee: Mars Incorporated). A novel orally-administered device containing protease inhibitors was used to collect samples of digesta (around 400 mg) from the intestinal lumen of normal dogs, and ileal and fecal protein digestibility was compared in poorly and moderately digested protein sources. The digesta collections were successful for 59% of the administrations and showed statistically significant differences for ileal amino acid digestibility between the proteins. Incidentally no differences in fecal digestibility were observed. With further improvements the devices offer a more routine means for obtaining ileal amino acid digestibility data *in vivo*.

5.3 Development of *in vitro* methods for determining amino acid digestibility in humans

In vivo animal based digestion assays themselves are inherently time consuming and costly, and have a high ethical cost. It is imperative, therefore, that rapid and relatively inexpensive *in vitro* digestibility assays be developed and validated to allow the prediction of true ileal amino acid digestibility in foods. The *in vitro* digestibility assays may be based on either static or dynamic multi-compartment chemico-physical models (47). Much work in this area is currently underway, with results proving promising (48–50).

The *in vitro* assays developed to date are likely to require more refinement to allow general application (47, 51), and should be comprehensively and independently validated. It is also important that they be validated against appropriate *in vivo* data (52).

5.4 Generation of DIAAS values using the pig model

Over the last 10 years, and coinciding with its validation, and the publication of a standardized methodology (53), the pig digestion model has been applied widely to generate true ileal amino acid digestibility data and food DIAAS values. A Scopus/PubMed literature search, covering the years 2013 to August 2023, reports more than 250 published scientific papers for the keywords of DIAAS/PDCAAS.

The PROTEOS project has led to a digestibility/DIAAS dataset for 100 foods, and these observations have been augmented by numerous other data generated especially at the University of Illinois [see for example (11, 54–56)], and data for typically eastern foods from the Academy of National Food and Strategic Reserves Administration, China (57–59), and data for common Indian foods (14), and foods in Bangladesh (60), along with data from several other studies including the assessment of novel foods (61–63).

Although the PROTEOS study included some foods typically consumed in African countries, more work needs to be undertaken to determine the true ileal amino acid digestibility and DIAAS values of foods from Africa. There is also the ongoing need to evaluate novel foods that are arising due to the valorization of previously poorly exploited food sources and the application of new technologies.

6 Methodological aspects of DIAAS

Several studies have been published addressing methodological aspects related to the DIAAS measure (2, 3, 64–67).

6.1 Reference essential amino acids and their normalization

The amounts and patterns of each indispensable amino acid (IAA) in the reference protein used to calculate DIAAS reflect the amounts considered to meet the daily requirement for each amino acid following the consumption of an amount of the protein equal to the EAR. The reference protein can be viewed as providing an "ideal amino acid balance" such that each IAA is provided in the correct amount and balance in relation to the other IAAs and to the dispensable (non-essential) amino acid component (sum of the dispensable amino acids, DAAs). In practice, however, the reference amino acid pattern is given as estimates of daily IAA requirements expressed on a protein basis. It is normalized by the EAR for protein. It is important to realize, therefore, that the pattern used is not an empirically derived ideal amino acid balance taking into account optimal ratios between individual IAAs and the indispensable and dispensable components, but rather is a composite of daily amino acid requirements and an estimate of the daily protein requirement that have been determined using different approaches.

It is uncertain, therefore, as to whether the reference pattern ratio of IAAs to DAAs is accurate in the context of an ideal amino acid balance. In fact, when compared to ideal IAA/DAA ratios found in other simple stomached mammals the current ratio would appear to be low (2).

If an IAA is first limiting, the DIAAS value reflects this, if however, all of the IAAs are found in a protein in excess of the required amount (no individual IAA is limiting) it is assumed that the excess IAAs are mainly transaminated to DAAs post-absorption and along with the synthesis of dispensable amino acids from ammonium absorbed from the gut, the DAA component is not limiting. Based on this assumption and in recommending DIAAS, it was held that in practice the DAA component is not limiting and that the DIAAS calculation is restricted therefore to the IAAs. It is pertinent to note that although true ileal digestibility is the best approach for predicting the uptake of amino acids during digestion, estimates of fecal crude protein digestibility are needed to model overall nitrogen transactions in the body.

Recently, Adhikari et al. (68) have addressed the potential importance of the DAA fraction, and have modelled the potential effects of the DAA component of a protein, and assumptions around the extent of transamination, on DIAAS and predicted utilizable protein.

While it appears likely that specific DAAs may become limiting in humans under certain conditions (69), it is unclear as to the potential effects of less than "ideal" amounts of the dietary DAA component in total. It is usually assumed that in practice the DAAs are not limiting for protein synthesis. It has been shown, for example, in clinical studies with humans that the ingestion of IAAs alone stimulates muscle protein synthesis equivalently to a mixture of the same amount of IAAs supplied along with additional DAAs (70). DAAs are required for protein synthesis, so when the IAAs were given alone the DAAs were presumably obtained from endogenous sources and from the recycling in the gut of ammonium from blood urea. Ingestion of a mixture of DAAs on their own failed to stimulate muscle protein synthesis. In contrast to these findings nitrogen balance studies have shown an effect of the DAA component on the efficiency of utilization of the IAAs (71).

Regardless, the DAA component does remain an important consideration in the context of DIAAS. A higher IAA/DAA ratio in an ideal amino acid pattern has a large absolute effect on the estimated DIAAS value (2). More accurate estimates of the optimal IAA/DAA ratio and better harmonization between the IAA requirements and the EAR for protein has the potential to increase the accuracy of DIAAS values. An accurate estimation of DIAAS relies on accurate and compatible estimates for both the individual IAAs and the EAR for protein.

6.2 Accuracy of IAA requirement values

The accuracy of the current estimates of IAA requirements used for determining DIAAS has been queried (1, 36, 72, 73). The current estimates are based on a limited number of studies, and often may provide minimal values rather than requirements to optimize organ and body function (74). Further, their generality in application to people in different physiological and nutritional states is also in question. Estimated amino acid requirements are usually given as population averages for a person of defined age (e.g., infant, child, adult) or physiological state (e.g., pregnant, lactating mother). In reality, however, amino acid requirements are influenced by multiple factors (e.g., age of adult, disease and nutritional status, surgery, diet composition) and are dynamic rather than static values (15, 75–77). There is a paucity of amino acid requirement estimates for people in these different physiological and nutritional states.

More and better information on individual amino acid requirements and optimized IAA profiles would lead to enhanced accuracy and versatility in the DIAAS measure. Inaccuracy in the estimated IAA requirements has the potential to affect absolute DIAAS values, but also relative DIAAS values calculated across foods, because the first limiting amino acid differs among foods and any inaccuracy in requirement estimation may vary among the IAAs (2).

6.3 Conversion of nitrogen to protein in foods

In calculating the DIAAS values for a food, each digestible IAA in the food is expressed per unit crude protein which is determined by multiplying the nitrogen content of the food by the generalized conversion factor of 6.25 (78). This approach has been criticized, as using the generalized conversion factor rather than specific factors for each food, can lead to both overestimation and underestimation of DIAAS. This is true, but food nutrient systems need to be consistent, and if a food specific conversion factor is applied in calculating DIAAS

the same factor should be applied to determine the gross food protein intake. The end result is that the estimate of utilizable protein for the food does not differ greatly. Craddock et al. (66) raised this issue in relation to almonds that have a specific conversion factor of 5.2, considerably lower than the generalized factor. If it is assumed that almonds have a digestible lysine content of 5.87 mg/g dry matter (first limiting amino acid) and a nitrogen content of 44 mg/g dry matter, it can be shown that the calculated DIAAS value (6-month to 3-year-old child reference pattern) is 38% using the generalized factor and a considerably higher 45% using the food specific factor. If, however, the factors are also applied consistently to nitrogen intakes from a single serving of almonds (30 g), the differences in estimated utilizable protein intake per serving are negligible (2.26 g when the DIAAS of 38% and the consistent factor of 6.25 were used, and 2.23 g when the DIAAS of 45% combined with the consistent factor of 5.2 were used). Only when the conversion factor is used inconsistently (the low conversion factor of 5.2 is used to generate DIAAS but the higher factor of 6.25 is used to calculate the utilizable protein intake per serving), a higher estimate (2.68 g/serving) is found for utilizable protein intake per serving.

An even more accurate estimate of utilizable food protein intake would be found by correcting food nitrogen content for its non-protein nitrogen content before converting the proteinaceous nitrogen to crude protein using the food specific nitrogen to protein conversion factor, and then calculating DIAAS using the food specific factor to determine the crude protein content of the food. If specific food nitrogen to protein conversion factors are to be used, however, they need to be applied consistently, and this would require the protein contents of foods to also be based on the specific food factors. Conversely to the direction in the DIAAS values, the stated protein content of some plant proteins would decrease and the protein content of some animal-sourced proteins would increase.

The added complexity in the calculations needs to be weighed against any improvement made in the accuracy of the final estimate of utilizable protein intake.

6.4 Processed foods

Processing (e.g., soaking, heating, extracting, extruding) of raw foods often leads to increases in protein and amino acid digestibility as discussed by Craddock et al. (66), especially in plant-based foods where the treatment may deactivate antinutritional factors (ANFs) and lead to beneficial structural alterations in the complex food matrix. It is for this reason that in the PROTEOS study and other recent work to determine true ileal amino acid digestibility in foods, it was ensured that the foods studied were in the form as consumed by human subjects rather than in the raw form. This is an important consideration. An advantage of DIAAS in this respect is that the true ileal amino acid digestibility assay has been shown to be more sensitive than fecal measures of digestibility for detecting changes in amino acid digestibility due to the effects of food processing and ANFs (79, 80).

It is not always the case, however, that the processing of foods enhances protein digestibility and there is an extensive literature documenting deleterious effects of food processing (especially heating and drying) on the amounts of an amino acid (67) and amino acid digestibility and availability, due to complex Maillard-type reactions that can occur under some processing conditions, and during food storage (81, 82). The nutritionally important amino acid, lysine, is particularly susceptible to structural alterations leading to lowered bioavailability (77, 83, 84). As a consequence of this, a lysine bioavailability assay (based on the digestibility of reactive lysine) has been developed (85) and is integral to the calculation of DIAAS for foods susceptible to damage during processing. This step in calculating DIAAS values has been largely overlooked, but is important in describing protein quality (DIAAS) in foods where the protein has been damaged by processing. For some foods, differences between lysine digestibility and availability and thus the calculated DIAAS, can be quantitatively significant (Table 2). DIAAS takes into account the effects of processing, at least to some extent.

Amino acids other than lysine (arginine, methionine and cysteine, threonine and tryptophan) are also subject to structural changes that can affect their bioavailability (88). These amino acids deserve more attention in this context, and bioassays similar to the digestible reactive lysine assay should be developed. However, lysine is the most susceptible amino acid to damage and loss of availability during food processing, and is a sensitive monitor for generalized protein damage. It is also often the first-limiting amino acid in diets.

6.5 Inadequate quantum of ileal amino acid digestibility data

When DIAAS was first introduced FAO (1), a dataset of true ileal amino acid digestibility for some 180 foods was collated (89), see https://www.fao.org/ag/humannutrition/36216-04a2f02ec02eafd4f457dd2c9851b4c45.pdf, but was considered at the time by the Expert Consultation to not be comprehensive enough to allow for the practical implementation of DIAAS. In the interim, other bodies and groups (90, 91) and commentators (3, 30, 64, 92–94) while recognizing the strengths of DIAAS, have also called for the generation of more data on ileal amino acid digestibility.

In the deliberations of the FAO Consultation the global food industry was urged to support research into the ileal amino acid digestibility of a wider range of human foods. This gave rise to the PROTEOS project, involving the cooperation of researchers from four universities and was completed in July 2023. This work has led to the generation of true ileal amino acid digestibility coefficients for a further 100 foods and over a wide range of food groups. It is estimated that other published studies conducted over the last 10 years have generated ileal digestibility data for at least a further 230 foods. Collectively there are now true ileal amino acid digestibility data well in excess of 400 foods including a broad range of plant-based foods including fruits and vegetables.

Moreover, the animal nutrition-based literature provides copious data on the effects of processing on ileal amino acid digestibility. Much of this information can be translated within a human food processing context. There appears to be a surprisingly low overall degree of variability for true ileal amino acid contents and DIAAS within a human food but across multiple factors (e.g., cultivar., batch and sometimes processing or cooking method) (95), suggesting that for regulatory purposes the application of overall fixed conservative digestibility estimates and DIAAS values for foods and food types may be acceptable, as suggested by Marinangeli and House (64). Such food values could be adjusted up or down based on determined *in vitro* digestibility estimates.

There is a need to bring these comprehensive ileal amino acid digestibility data together into a single readily accessible database.

TABLE 2 Mean true ileal digestible total (conventional analysis) and reactive lysine contents (g/kg air-dry) in selected foods.

Food	Digestible lysine[a]		% Difference[d]
	Total[b]	Reactive[c]	
Collagen	36.0	36.0	0
Cooked black beans	13.2	11.3	14.4
Cooked pigeon peas	17.0	16.7	1.8
Heated peas	9.5	8.8	7.4
Split peas	16.1	15.4	4.3
Processed wheat bran	1.9	1.5	20.9
Toasted wheat bread	2.1	1.4	33.7
Wholegrain bread	2.4	2.0	16.7
Popped rice cereal	0.7	0.3	57.1
Grain-based cereal	1.2	0.5	58.3
Whey protein isolate	82.8	82.7	0.1
Heated skim milk powder	19.8	16.6	16.2
Skim milk powder	19.8	16.6	16.2
Whole milk powder	26.2	24.0	8.4
Lactose-hydrolyzed milk powder	27.2	25.1	7.7

Adapted from Hodgkinson et al. (86) and Moughan et al. (87).
[a]Determined in the growing pig or growing rat; from Rutherfurd and Moughan (116).
[b]Based on conventional amino acid analysis.
[c]Based on determination of reactive o-methylisourea lysine in diet and ileal digesta.
[d]% difference = (Total – Reactive)/Total x 100.

6.6 Ethical cost of animal and human digestibility assays

True ileal amino acid digestibility can be determined in humans, and importantly the newly developed dual-isotope digestibility assay gives the means of determining ileal amino acid digestibility in humans of different ages and physiological and disease states. This will be valuable for enhancing an understanding of protein digestion in humans. There is, however, a high ethical cost involved in human research and no human-based assay can be considered routine. Some (64) have also highlighted the ethical cost and increasing opposition around using animal-based digestibility assays such as those involving rats and pigs. It is anticipated that in the future the animal-based ileal digestibility assays will be used to provide generalized tabulated digestibility estimates for foods and food groups and may be applied to generate information on novel foods, but that such assays will increasingly give way to more rapid, non-invasive *in vitro* assays for the routine evaluation of foods.

6.7 Additivity of DIAAS values in meals and diets and associative effects in whole meals

DIAAS was developed to provide information about the amount of the first limiting amino acid supplied relative to the required amount for that amino acid in a protein source, when that protein is ingested at an amount to meet the EAR for protein. A DIAAS of 100% means that each IAA exactly meets the required amounts of IAAs; a DIAAS less than 100% means that one or more of the IAAs are limiting for protein synthesis and the score gives the degree to which the first limiting amino acid is undersupplied relative to the required amount; a DIAAS greater than 100% means that the IAAs are supplied in excess of 100%. DIAAS provides valuable information. If a protein with a DIAAS <100% is ingested as the sole food, the protein will not be fully utilizable and the score provides information about which amino acid needs to be supplied from other foods to enhance utilizability. A protein with DIAAS >100% will be highly utilizable if ingested alone, but can be combined with other proteins to have a complementary effect on the intake of the IAAs. Information provided by DIAAS is practically useful.

In calculating the DIAAS values, the ratio of the amount of each IAA relative to its requirement is calculated (digestible indispensable amino acid ratio, DIAAR), and in addition to DIAAS the DIAAR values themselves provide useful information (65, 67). Table 3 shows the DIAAR calculated for a whey protein isolate. Histidine is supplied at the lowest level (DIAAR = 1.09), but all of the dietary IAAs are estimated to be supplied in excess of requirement. The whey protein isolate may be used to complement amino acid supplies from other proteins that may be limiting in IAAs. The DIAAR values, however, provide additional information. In the case of whey for example, the protein supplies particularly high amounts of tryptophan and leucine, amino acids that have important physiological roles in addition to being building blocks for protein synthesis. The latter information is lost in the single score.

It is important to note that DIAAS values are not necessarily additive, and if information about the DIAAS of a meal or dietary pattern is required this should be calculated based on the amount of each true ileal digestible amino acid supplied by each respective food protein. True ileal amino acid digestibility values are additive across different food proteins. In the case of meals and dietary patterns, however, it is not necessary to calculate DIAAS *per se*. DIAAS was designed to have a specific application to single protein sources. For meals and dietary patterns, the amounts of each IAA provided relative to the daily requirement can be calculated from first principles based on amounts of foods ingested, amino acid contents and the true ileal amino acid digestibility for each food. This is one of the reasons why the FAO (1) Expert Consultation recommended, first and foremost, that information should be provided for all foods on the ileal digestible amount of each IAA provided by a food, and that each amino acid be regarded as a nutrient in its own right. This does not diminish the importance or application of DIAAS values, but rather highlights the need for complete information (including data on the digestible amino acid contents) on food proteins.

The possibility of associative effects between foods has been discussed in relation to true ileal amino acid digestibility (66, 96).

Whereas holistic properties of foods involving the entire food matrix are undoubtedly important and food interactions can influence nutrient uptake and utilization (92, 97–99), the importance of the

overall effect of associative interactions on amino acid digestibility in the context of normal meals may be somewhat overstated, as true (standardized) ileal amino acid digestibility values have been shown in several studies to be broadly additive over a wide range of foods (100–103). This is expected to be particularly so for most foods consumed by humans and given the form in which they are consumed, where ANFs and plant fiber levels are usually relatively low compared to feedstuffs for animals, whereby the additivity of true ileal amino acid digestibility has been demonstrated.

Where a significant associative effect is suspected, and there may be situations where this arises, amino acid digestibility should be determined for the combination of proteins provided as a meal, and the use of *in vitro* digestibility assays may be particularly useful here to determine relative changes in digestibility.

7 Conceptual aspects concerning DIAAS

There has been some discussion, not so much questioning the scientific accuracy of DIAAS, but rather addressing conceptual issues around its application in practice particularly in respect of jurisdictional regulatory frameworks and public health outcomes in wealthier countries.

Marinangeli and House (64) have discussed practical implications of a transition from PDCAAS to DIAAS in industrialized food systems. They note that for several plant-based foods although there are differences between PDCAAS and DIAAS the differences are not always great (2–13% for the limited number of foods in the one study quoted). Given this possibility, the authors appropriately question the practical advantage and cost implications of transitioning to DIAAS.

Although, in general PDCAAS undervalues the protein quality of animal-sourced foods and overvalues plant-sourced foods, it is the case that the differences between PDCAAS and DIAAS are not always high. Nonetheless there are many instances where the difference is of a practically significant magnitude, such as the difference between DIAAS and PDCAAS for unprocessed soya products (DIAAS 86% versus PDCAAS 92%) (81), as well as other plant-based foods (104). This is further illustrated by the data shown in Table 2, and the fecal and ileal digestibility data presented by Adhikari et al. (67).

TABLE 3 Digestible indispensable amino acid ratios (DIAAR) for a whey protein.

Amino acid isolate	DIAAR[a]
Threonine	1.80
Methionine + Cysteine	2.29
Valine	1.21
Isoleucine	2.22
Leucine	2.57
Tyrosine + Phenylalanine	1.71
Histidine	1.09
Tryptophan	3.35
Lysine	2.51

[a]Digestible indispensable amino acid ratio. Based on reference amino acid pattern for the 3-year-old to 10-year-old child.

If the global food supply shifts more towards plant and away from animal protein, as is widely proposed, the need for accurate estimates of protein quality will be even more important, and this may be further exacerbated by enhanced atmospheric carbon dioxide levels potentially leading to lower plant protein contents (105).

The need for accurate estimates of protein quality in the latter context is brought into focus by the recent work of Conzuelo et al. (106) involving modelling the protein quality of daily food patterns as recommended for the "planetary health diet" developed by the EAT-Lancet Commission. For the recommended lower-quality daily dietary patterns, estimated protein quality was low (DIAAS 71 and 76%) and there were large differences between PDCAAS and DIAAS (e.g., DIAAS 76% versus PDCAAS 88%). When higher protein quality foods were added to the pattern, DIAAS was still below 100% (DIAAS 88 and 94%), and practically significant overall differences between PDCAAS and DIAAS (e.g., DIAAS 83% versus PDCAAS 88%) persisted.

Conceptually, a DIAAS-based system of protein quality evaluation mirrors a PDCAAS-based system. The only difference is that DIAAS follows current best practice in describing amino acid requirement patterns and amino acid availability, and thus offers more accurate estimates of protein quality. This is of crucial importance in low-income countries and there is an argument for having one harmonized global system for describing protein quality. In high income countries there may not always be the same imperative around protein quality as is the case in developing nations, but many of these developed economies not only consume the food proteins they produce internally within the economy but also export them widely.

With a new protein quality metric, inevitably the protein quality values and rankings of different foods change to some extent. It is important, therefore, to evaluate if this may lead to unintended consequences in practice. A particular concern relates to food sources of protein that qualify for a protein content claim under PDCAAS but would be ineligible under DIAAS. Is there a risk that the positive attributes of some relatively protein-rich plant-based foods could be downplayed if they have DIAAS scores lower than their PDCAAS, and much lower DIAAS than for animal-sourced foods?

This has been evaluated in the study of Sa et al. (107) for lentils, an important protein source. The authors conclude that with PDCAAS and US standards, lentils qualify for a "good source" claim for protein, but with DIAAS and following the FAO (1) recommendations, such a claim could not be made. The authors discuss how this outcome relates to several plant-sourced foods (e.g., navy beans, yellow peas, tofu) and make the point that with the promulgated DIAAS system, several foods from the categories seeds, nuts and pulses would disappear from the 2019 Canadian Food Guide's ideal plate, which would be inconsistent with current food guidelines. Similar conclusions were drawn in the study of Cargo-Froom et al. (108).

This, however, is not a criticism of DIAAS itself, which is merely a more accurate means of describing protein quality, but relates more to the FAO proposed regulatory system and the cut-off points for making claims. The two components, metric and system, should not be conflated as part of the same issue. Simply because a certain protein fails to make a claim under a particular proposed system, this should not be used as a criterion to judge the suitability of the protein quality metric. Nevertheless, it remains a concern that there may be unintended consequences in adopting the proposed system for making claims. In this respect, it is important to note that the FAO (1) recommendations on the regulatory aspects of DIAAS were couched

as guidelines with the suggested cut-offs only given as examples, and it was stated in the report that: "the DIAAS cut-off points in the context of making claims require careful further consideration (e.g., in relation to national and local dietary patterns)" (1). The Expert Committee recommended the development of a published set of guidelines for Industry. It would appear timely to devote attention to the development of such a set of guidelines that would be acceptable and relevant across multiple jurisdictions, and would take into account specific attributes of foods in the context of providing protein and amino acids.

A bigger picture concern with DIAAS is that because in general DIAAS gives higher scores for animal-based proteins and foods than PDCAAS, leading to designations such as "excellent source," this may encourage the consumption of animal-based foods which may in turn have negative consequences for both the environment and public health (64, 96, 109). The roles of animal-based foods in both latter respects, however, are contentious. Moreover, DIAAS values are restricted to providing information on the delivery of the most limiting amino acid in a food, meal or dietary pattern, and should not be interpreted to mean anything more or less than this. This is important information in its own right, and is restricted to the domain of protein quality.

Many animal and plant foods will be rich sources of other essential nutrients and beneficial compounds (104, 110–112), and may also have specific beneficial holistic properties, while others may contain ANFs and other compounds considered to impair health and function, but a high or low DIAAS in its own right should not be interpreted as providing any information on such properties. Perhaps the somewhat emotive terms such as "poor," "good," "excellent" used in describing protein quality should be replaced by more descriptive and restrictive terms such as "low," "medium," "high" and "complementary." Humans consume foods, not proteins, and the information provided by DIAAS should be restricted to the protein component of a food.

More overarching food and diet quality scores have a place in public health nutrition (109, 113–115), but the components of these scores should not be conflated with protein quality metrics. It remains that consumers and industry require information on protein quality *per se*, and the ability of a particular food to provide utilizable protein, and in this context, it is argued that DIAAS provides that information most accurately. Consumer education and food regulation need to ensure that information on protein quality and other important information on attributes of a food are conveyed to consumers in such a manner as to allow informed decisions. Protein quality metrics should not be used or promoted as proxies for overall food quality attributes, rather the information they convey should relate solely to the estimated delivery of IAAs. It is argued that protein quality is one standalone set of useful information, with a specific purpose.

8 Conclusion

Currently DIAAS is the most accurate means to routinely give a single protein quality value for a stand-alone food. This should not be taken to mean that DIAAS is a perfect measure, and in fact considerable scope exists to improve the accuracy of DIAAS values. Careful consideration should also be given as to how DIAAS is applied in relation to food regulations to ensure that use of the metric does not lead to unintended consequences and misleading representations of certain food types.

The amino acid delivery of meals, dietary patterns and personalized meal plans are best assessed by the direct application of food amino acid contents, and true ileal amino acid digestibility and availability coefficients. For this reason and given the growing importance of having information on the delivery of individual amino acids related to specific physiological roles, consideration should be given to providing information in food labelling on digestible amino acid contents.

True ileal amino acid digestibility coefficients have been shown empirically to be accurate estimates of amino acid absorption in most cases. They have also been shown to be sensitive indicators of changes to proteins incurred during processing and storage. Further, and because such coefficients include relevant corrections for endogenous ileal amino acids, they reflect the effects of most common plant ANFs. None of these claims can be made for the outmoded fecal crude protein digestibility measure. If the intention is to use the world's protein resources more efficiently and to describe available amino acid levels as accurately as possible, then a shift in practice to using DIAAS and ileal amino acid digestibility is a major step forward.

Author contributions

PM: Conceptualization, Writing – original draft, Writing – review & editing. WL: Writing – original draft, Writing – review & editing.

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Determining amino acid requirements in humans

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Amino acids form the building blocks of body protein. Dietary protein sources provide the amino acids needed, but protein sources vary widely in amio acid composition. To ensure humans can meet body demands for amino acids, amino acid intake recommendations are provided by the Dietary Reference Intakes (DRI) and by Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU). Current amino acid intake recommendations, however, are based on data collected predominantly from young adult males. The development of the minimally invasive indicator amino acid oxidation (IAAO) method has permitted the evaluation of amino acid requirements in various vulnerable populations. The purpose of this review is to discuss recent amino acid requirement studies in school-age children, pregnant females and the elderly determined using the IAAO technique. These requirements will help to inform evidence-based recommendations that will help to guide dietary guidelines.

KEYWORDS

amino acids, requirements, humans, IAAO, stable isotope

Introduction

In the human body, protein is the chief functional and structural constituent in every cell (1). During development, dietary protein is necessary for growth plus maintenance and for maintenance alone during all other stages of life. The most important nutritional aspect of dietary protein are the consituent amino acids. Among the 20 amino acids that constitute human body protein 9 are indispensable (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), which means they cannot be made in the body and must be supplied in the diet (1). There are five dispensable amino acids (alanine, aspartic acid, asparagine, glutamic acid, serine) that can be synthesized in the body whereas the remaining six are conditionally indispensable (arginine, cysteine, glutamine, glycine, proline, tyrosine) meaning that they can be made by the body however their synthesis becomes limiting under specific conditions.

The Food and Agriculture Organization (FAO) have acknowledged that indispensable amino acids should be treated as individual nutrients since the amino acid composition of foods vary greatly and *in vivo* amino acids have various regulatory roles (i.e., precursors for coenzymes, hormones, nucleic acids and other molecules) (2). In addition, the nutritional value of dietary protein is determined by the most limiting indispensable amino acid in foods. Therefore, dietary protein sources are categorized as either high- or low-quality. The classification is determined by the amino acid score of the food which is the amount of amino acid supplied by the food relative

to their corresponding amino acid requirements. Animal protein sources (e.g., eggs, meat, fish) provide all indispensable amino acids in quantities and ratios adequate to meet human requirements, whereas many indispensable amino acids in plant foods occur in quantities and ratios that may not meet requirements under all conditions (3). Therefore, in a normal sized meal, plant proteins may fail to fulfil an indispensable amino acid requirement, which is referred to as the limiting amino acid (4). For individual's adhering to a strict plant-based diet, the limiting amino acid will limit the body's capacity to make proteins (3). This is because when an indispensable amino acid is deficient in the diet, all other amino acids, appear in relative excess and will be oxidized since there is no substantial storage of amino acids in the body (5). Over the long term, this may lead to negative consequences on whole-body protein metabolism. Thus, as a first step we need knowledge of amino acid requirements across life-stage groups to understand how to meet their needs with different dietary protein sources. This is especially relevant in today's landscape where plant-based diets are encouraged and increasingly popular.

Current dietary amino acid intake recommendations are outlined in the Dietary Reference Intakes (DRI) issued by the National Academy of Science, Engineering and Medicine [NASEM, formerly—Institute of Medicine (IOM)] (1, 6). Global recommendations were provided by the Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU), and are stated as mean and safe intake levels of amino acids, whereas the DRI uses an estimated average requirement (EAR) and recommended dietary allowance (RDA) (3, 6). However, the mean intake is equivalent to the EAR and the value is set to meet requirements for half (50%) of a healthy population. Similarly, the safe intake level and the RDA are the same and aim to meet the requirements of 97 to 98% of a healthy population. Both the DRI and FAO/WHO/UNU recommendations are provided for adults (19y+) based on values determined in studies conducted in young adults. For all other age groups, including children, pregnant and lactating people a factorial method with maintenance needs from adult data with growth estimates calculated were used to set recommendations. Clearly, data on other populations is lacking and therefore, must be determined directly with a sense of urgency.

Evolution of methods to determine amino acid requirements

Determination of amino acid requirements requires graded levels of the test amino acid above and below the expected requirement to be fed to participants while measuring a definable and relevant biological outcome (7). Currently, the outcome of all existing methods is a surrogate measure of protein synthesis (8). Traditionally, amino acid requirements were determined using nitrogen balance studies (9–15)—measuring nitrogen intake and excretion. Briefly, as the test amino acid intake increases there is a progressive increase from negative to zero-nitrogen balance until the requirement is reached. The limitations of nitrogen balance are well described and include the limited range of test amino acids studied, its cumbersome nature requiring precise measurements of balance among several other isssues (16). Readers are referred to reviews for an extensive background on the limitations of nitrogen balance (16, 17). The lack of amino acid requirement studies in vulnerable groups such as infants, pregnancy and elderly are attributed to these limitations.

As reviewed by Pencharz et al., the advent of carbon oxidation methods using ^{13}C-labelled amino acids—including direct amino acid oxidation (DAAO), indicator amino acid oxidation (IAAO) and 24 h IAAO have allowed for the determination of amino acid requirements in various vulnerable populations (7). Briefly, for DAAO, when the amino acid is fed below the requirement there is no change in oxidation until the requirement is met, after which there is an increase in the oxidation. For the IAAO and 24 h IAAO, the oxidation of an indicator amino acid (another indispensable amino acid) response falls as the test amino acid intake increases, until the requirement is reached after which there is no further change in oxidation (5, 7). The 24 h approach is in essence an adaption of the 8 h, fed state IAAO protocol to include both the fed/fasted states as Indicator Amino Acid Balance (IAAB). According to the FAO/WHO/UNU (2007) report, "*…on theoretical grounds the most reliable approaches are the 24 h indicator/carbon balance approaches.*" As previously reviewed (18) similar amino acid requirement estimates have been derived using both 24 h-IAAB and 8 h fed state IAAO methods with no systematic difference in estimates. Moreover, within the 24 h IAAO studies, when requirements are compared between the 12 h fed and 8 h fed state, there is no difference in requirement estimates (19, 20). Prior adaptation to test amino acid intake to 8 h, 2d, or 6d also did not significantly affect IAAO for lysine (21) or threonine requirements (22). Clearly, the 8 h-IAAO is advantageous in studying AA requirements due its minimal invasiveness including a single day of adaption to the test amino acid (22), oral isotope administration with meals (23), and measurement of $^{13}CO_2$ in breath (23). Given these advantages, the method has been successfully applied to study amino acid requirements in understudied groups like children, elderly and in patients with disease (24–27). As a result, new datasets are emerging on amino acid requirements. The following sections will outline recent IAAO-derived amino acid requirements determined in healthy children, children with disease, pregnancy and elderly groups.

Amino acid requirements in healthy children and children with disease

A comprehensive list of the IAAO-derived amino acid requirement studies in healthy and children with certain conditions are illustrated in Table 1. Healthy children aged 6–10y have similar amino acid requirements compared to adults for total branched-chain amino acids (BCAA, isoleucine+leucine+valine), lysine, total sulfur amino acids (TSAA, methionine+cysteine) and tryptophan, suggesting maintenance needs are the same, considering the fact that the 8 h-IAAO protocol are short-term studies (25). However, in the case of different disease state states amino acid requirements are changed.

Children with liver disease have ~40% increased total BCAA requirements (39). Whereas, post liver transplant, in the same group of children, the requirement is increased by ~17% (39). In patients with maple syrup urine disease, the requirement for total BCAA is much lower because the demand for branched chain amino acids is low, due to BCAA catabolic enzyme defect (41). Thus, this was the first study to estimate a minimum total BCAA needs which are ~69% lower compared to healthy children. The TSAA requirements in children with chronic renal insufficiency are the same as healthy children (42). Yet, the demand for obligatory methionine appears to increase by ~25% in this group relative to healthy controls (42).

TABLE 1 Amino acid requirements in healthy children and under certain conditions determined using the IAAO method.

Population	Amino Acid (mg/kg/d)[a]					
	Total BCAA	Methionine (no cysteine)	Methionine + Cysteine	Lysine	Tryptophan	Phenylalanine
Healthy North American children	147 (28)	12.9 (29)	5.8 (30)	35 (31)	4.7 (32)	–
Healthy North American adults	144 (33)	12.6 (34)	4.5 (35)	36 (36)	4 (37)	9.1 (38)
Cholestatic liver disease children	209 (39)	–	–	–	–	–
Post-liver transplant children	172 (40)	–	–	–	–	–
Maple syrup urine disease	45 (41)	–	–	–	–	–
Chronic renal insufficiency children	–	12.6 (42)	7.3 (42)	–	–	–
Healthy Indian children	–	–	–	33.5 (43)	–	–
Stunted Indian children (with gut parasites)	–	–	–	42.8 (44)	–	–
Stunted Indian children (after treatment of parasites)	–	–	–	35.5 (44)	–	–
Children with phenylketonuria (PKU)	–	–	–	–	–	14 (45)

[a]Values described are mean amino acid requirements.

TABLE 2 Amino acid requirements in early and late-stage pregnancy determined using the IAAO method.

Nutrient[a]	Non-pregnant needs	Early-stage pregnancy (~16 weeks)	Late-stage pregnancy (~36 weeks)	Reference
Protein (g/kg/d)	0.9	1.2	1.52	(47)
Lysine (mg/kg/d)	36	37	50	(48)
Phenylalanine (mg/kg/d)	9	15	21	(49)
Phenylalanine+tyrosine	42	44	50	(49)
Glycine (mg/kg/d)	–	–	40	(50)
Methionine+cysteine (mg/kg/d)	13	11	17	(51)

[a]Values described are mean amino acid/protein requirements.

Healthy Indian children have similar lysine needs as healthy Canadian children (43, 46) while, the lysine requirement is increased ~21% by the presence of gut parasites in under-nourished Indian children (44). Thus, amino acid needs vary depending on the type and severity of disease, and the findings described above lead the way to revising dietary guidelines for disease management.

Amino acid requirements in pregnancy

Amino acid requirements during human pregnancy has been infrequently studied due to the invasive nature of the nitrogen balance method. Due to the minimally invasive nature of the IAAO method, a series of studies across two distinct stages of pregnancy—early (~16 wk) and late (~36 wk) gestation have been conducted (Table 2). The mean protein needs in early-stage pregnancy is 1.2 g/kg/d (47), and increased compared to mean protein needs (0.9 g/kg/d) determined in young males (52). During late stages of pregnancy protein needs increase further to 1.52 g/kg/d (47). However, amino acid requirements do not increase proportionally, compared to protein needs. The findings suggest that while protein needs increase in late stage, not each individual amino acid requirement follows the same pattern. Lysine, and TSAA requirements during early pregnancy stages are similar to non-pregnant needs, however phenylalanine needs (in the presence of tyrosine) increase by 66% compared to non-pregnant needs, as well as the total aromatic amino acid (TAA, phenylalanine in the absence of tyrosine) requirements (49). All determined amino acid requirements (lysine, TSAA, TAA and phenylalanine) increase by late stages of pregnancy (48, 49, 51), albeit at different amounts. Most interestingly, glycine a conditionally indispensable amino acid was shown to be indispensable in human pregnancy by late stages of pregnancy (50, 53). It is of importance to note that in the glycine in pregnancy study, the amount of protein was fed at current pregnancy protein needs (0.88 g/kg/d), which further validates the finding that current protein intake recommendations in pregnancy are underestimates. Further work is required to complete the remaining indispensable amino acid requirements in different phases of pregnancy.

Amino acid requirements in healthy adults >60 years

Amino acid requirements in elderly have also been infrequently studied due to methodoligcal invasiveness. With the global population now aging, there is an increased need to determine amino acid needs in elderly. Table 3 provides a complete list of all amino acid requirements done in healthy males and females >60 years of age. Similar protein needs have been determined for young and older adults using the IAAO-method, although the determined values are higher than current mean recommendations of 0.66 g/kg/d (52, 54, 55). Amino acid

TABLE 3 Amino acid requirements in elderly males and females >60 years of age determined using the IAAO method.

Nutrient[a]	Young males	Elderly males (>60 years old)	Elderly females (>60 years old)	Reference
Protein (g/kg/d)	0.93	0.94	0.96	(54–56)
Phenylalanine (mg/kg/d)	9.1	9.3	8.4	(57)
Leucine (mg/kg/d)	39	77.8	78.2	(58)
Methionine (mg/kg/d)	13	26.2	17.1	(59)
Methionine + Cysteine (mg/kg/d)	4.5	5.4	4.6	(60)

[a]Values described are mean amino acid/protein requirements.

requirements however are not proportionally the same compared to young adults, and are influenced by sex. Phenylalanine requirements were also found to be the same in elderly males and females as healthy young adult male requirement (38, 57). However, leucine requirements in elderly males and females was found to be nearly double that of healthy young males (58, 61) suggesting that while needs for total nitrogen is unchanged with age, there are increased demands for specific amino acids. More recently, the TSAA requirement was affected by sex, with older males having a higher requirement compared to older females and healthy young males (34, 59). Interestingly, the minimum methionine (in the presence of adequate cysteine) requirement was the same between sexes and healthy young adult males (35, 60). These series of amino acid requirement studies highlights the need to assess requirements between sexes. Additionally, the existing studies conducted in older adults include subjects aged 60–69 y old with few aged 70–79 and >80 y old. It has been shown that there is ~5% decrease in whole-body protein turnover when stratified by decade of life with aging (62). As a result, further studies are necessary to experimentally derive the amino acid requirements for these advanced age groups.

Perspective: amino acid requirements and dietary patterns

As summarized above, amino acid requirements determined across a wide range of physiological stages and disease conditions vary based on several factors, and that a factorial method may not be adequate to give amino acid intake recommendations. A few key points must be discussed here with respect to the fact that the experiments to determine amino acid requirements are conducted with adequate energy, following an ideal amino acid composition (egg protein pattern) using a highly bioavailable source (crystalline amino acids). Thus, the determined values represent a true 'minimum' amino acid requirement. Humans consume foods following different dietary patterns – omnivorous, vegetarian, vegan diets etc., and will influence the minimum amino acid requirements. Specifically, following a strict vegan diet would rely on plant-based sources of protein, which would have lower digestibility, lower dietary calorie density and likely a less ideal pattern of all the indispensable amino acids. In theory, amino acid intakes would need to be higher in these instances to meet body amino acid needs. Furthermore, additional nutritional needs and demands would be different based on physiology, for example actively growing children, pregnant females would have increased energy needs, while elderly sedentary individuals would have lower energy needs. In addition, living in poor socio-economic and living conditions might increase the needs for some amino acids, as shown by our lysine requirement study in under-nourished children with active parasitic infection. Thus, translation of our amino acid requirement values to dietary guidelines needs to consider several factors. It is also important to note here that conceptually DRI and FAO/WHO/UNU recommendations as defined by the EAR and RDA for all nutrients are a 'minimum' and not a 'maximum', that ensures populations can consume diets to maintain health and quality of life.

Summary and conclusions

Current amino acid intake recommendations have been determined based on studies conducted in young adult males. For all other life stages a factorial approach was used, primarily due to lack of data. The minimally invasive IAAO method has been successfully applied in vulnerable populations and in different disease states in children. New datasets are also developing for pregnancy and for the elderly population so that we can provide and inform evidence-based recommendations. These datasets are urgently needed since amino acid needs vary based on disease condition, across pregnancy stages and between sexes in the elderly population. At the same time, several key life stages such as adolescents, young female adults and lactation amino acid requirements remain to be investigated. The advent of plant-based diets warrants the need to determine indispensable amino acid requirements with a sense of urgency to appropriately provide nutritional guidelines and recommendations on how to meet individual needs.

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AP: Conceptualization, Writing – original draft, Writing – review & editing. GC-M: Conceptualization, Supervision, Writing – review & editing. RE: Conceptualization, Funding acquisition, Writing – review & editing.

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Dietary protein, amino acids and type 2 diabetes mellitus: a short review

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Diabetes is a widespread metabolic disorder and results from insulin resistance and impaired insulin secretion. Modifiable factors like diet, physical activity, and body weight play crucial roles in diabetes prevention, with targeted interventions reducing diabetes risk by about 60%. High-protein consumption, above the recommended intake of 0.8 g/kg body weight per day, have often explored in relation to diabetes risk. However, the relationship between dietary protein and diabetes is multifaceted. Observational studies have linked high total and animal protein intake to an increased risk of type 2 diabetes, particularly in obese women. Elevated levels of branched-chain amino acids (BCAA), which can result from dietary intake, protein breakdown, as well as an impaired catabolism, are strong predictors of cardiometabolic risk and insulin resistance. With several mechanism linking BCAA to insulin resistance. On the other hand, intervention studies suggest that high-protein diets can support weight loss and improve cardiometabolic risk factors. However, the impact on insulin sensitivity and glucose homeostasis is not straightforward. Proteins and amino acids stimulate both insulin and glucagon secretion, influencing glucose levels, but chronic effects remain uncertain. This short narrative review aims to provide an update on the relationship between increased dietary protein intake, amino acids, insulin resistance and type 2 diabetes, and to describe protein recommendations for type 2 diabetes.

KEYWORDS

diabetes, dietary protein, insulin resistance, branched chain amino acids, high-protein

Introduction

Diabetes is a prevalent metabolic disorder worldwide, characterized by elevated blood glucose levels. Its prevalence has been steadily increasing, with an estimated 537 million adults aged 20–79 living with diabetes globally in 2021, and a further rise expected (1). Type 2 diabetes comprises the majority of cases, accounting for around 90% of all diabetes cases. Disruptions in insulin action and secretion contribute to the characteristic hyperglycaemia (2). Cells become resistant to insulin's actions, most notably insulin-resistant glucose uptake in skeletal muscle which results in elevated blood glucose levels. Initially, the pancreas may produce extra insulin to compensate for insulin resistance. However, over time, the pancreatic beta cells may fail, leading to decreased insulin production and exacerbating high blood glucose levels. Both the hyperglycaemia as well as the chronic exposure to elevated insulin levels can in their turn further diminish insulin-mediated glucose uptake, potentiating insulin resistance and eventual development of type 2 diabetes mellitus.

Diet, physical activity and body weight are key modifiable factors for the development of diabetes. Targeted interventions aiming at these three factors have shown to reduced diabetes risk by ~60% in those at risk for the disease (3–5). Various diets aimed at weight reduction and improving insulin sensitivity are advocated for patients with diabetes, among them high-protein diets. With high protein diets or high protein consumption being defined as a protein intakes above the general recommended level of 0.8 g/kg BW /per day for healthy adults (6). Already a century ago dietary protein intake was investigating in relation glycemic control (7), due to its glucogenic properties as well as its stimulation of insulin and glucagon secretion (8). In addition, high-protein diets are recommended for weight-loss and maintenance (9), which might benefit diabetes risk reduction. Also, a –too—low protein intake, for example as part of protein-energy malnutrition in older adults, is associated with morbidity and mortality (10). However, the impact of high-protein diets or high protein consumption on insulin sensitivity and – risk of – diabetes is not straightforward, as we reviewed 10 years ago (11). Observational studies have identified a high protein intake as a risk factor for type 2 diabetes mellitus, and elevated circulating branched-chain amino acids (BCAA) levels are among the strongest predictors of future cardiometabolic risk. Also, some amino acids can have a direct impact on hormones and pathways that control glucose homeostasis.

This short narrative review aims to provide an update on the relationship between increased dietary protein intake, amino acids, insulin resistance and type 2 diabetes, and to describe protein recommendations for type 2 diabetes.

Population studies: high-protein diets are associated with type 2 diabetes

Besides calorie intake, fiber and carbohydrate consumption, also protein intake has been investigated in relation to diabetes risk in large scale observational studies. Within the setting of EPIC-InterAct, a large scale pan-European type 2 diabetes case-cohort, we were able to study the association between protein intake and risk of type 2 diabetes (12). After adjustment for important diabetes risk factors and dietary factors, the incidence of type 2 diabetes was higher in those with high intake of total protein and animal protein, not plant protein (12). Associations were stronger in women, more specifically obese women. A meta-analysis of several large population studies, including the large Nurses' Health Studies and the Melbourne collaborative cohort study confirmed these observations: higher intakes of total and animal protein were both associated with increased risks of T2D, whereas higher plant protein intake tended to be associated with lower risk of T2D (13). High (animal) protein intake is in 'free-living' conditions in general related to a higher (saturated) fat intake, a lower fiber and vitamin intake, increased BMI and lower physical activity levels (14). These are known diabetes risk factors and could contribute to the positive association between protein intake and diabetes risk. Indeed, including these – and other – factors into subsequent models attenuated the association, but the positive association between total or animal protein and diabetes risk did not disappear (12, 13).

In particular the BCAA attracted attention, as several studies identified high plasma levels of BCAA predicting diabetes risk (15, 16), and decreased levels were associated with improvement in insulin resistance (17). BCAA, which include leucine, isoleucine and valine, are all essential amino acids with a relative high presence in various protein sources, in particular those from animal origin. BCAA have, next to protein synthesis, multiple important roles in human metabolism and several metabolic diseases. Although acute infusion of BCAA can introduce insulin resistance (18), plasma BCAA levels are not simply a consequence of a high (animal) protein intake. Elevated circulating BCAA levels can have multiple origins, including increased appearance in plasma due to food intake, protein breakdown and gut microbial synthesis, and/or alteration in disappearance due to protein synthesis, excretion and BCAA catabolism. With the latter, a dysfunctional—repressed—BCAA catabolism has been proposed as playing a large role (19). Thus the relationship between BCAA, insulin resistance and type 2 diabetes is much more complex, and characterised as a "two-way street" (20), with diabetes, obesity and insulin resistance contributing to elevated BCAA levels and vice versa. Several strategies are considered to lower BCAA levels and/or boost BCAA catabolism, which includes diet and exercise interventions, next to pharmaceutical approaches (19). Interestingly, combining a high-protein diet with a high fiber intake diminished the correlations of AA with IR (21), which may be related to the effect of fiber on digestion and absorption of dietary protein.

Thus, observational data clearly identified a high (animal) protein intake under 'free living' non-restricted conditions to be associated with an increased risk of developing diabetes, with elevated circulating BCAA levels as biomarkers of disease risk. However, these associations do not immediately identify high protein consumption as a cause of diabetes, due to multiple other – dietary – factors being associated with a high protein consumption, and elevated circulating BCAA levels.

Intervention studies: protein intake to support weight-loss and improved metabolic control

Protein intake is recommended to support weight-loss and weight maintenance, in particular to preserve lean mass when on calorie restriction. It is estimated that high protein intake (e.g., >1.0 g/kgBW/day) compared to normal protein intake (0.8 g/kgBW/day) can prevent a loss of 0.5–1.0 kg lean mass with moderate weight-loss (22, 23). Adequate protein intake stimulates muscle protein synthesis and hence supports lean mass. In addition, dietary protein is more satiating than fat or carbohydrate, and dietary protein stimulates thermogenesis, both also facilitating weight loss and maintenance. Interestingly results from the POUNDS LOST study suggest that optimal diet composition for weight loss depends on metabolic state of an individual. Those with normoglycemia lost the most body weight on a low-fat/high-protein diet, while subjects with insulin resistance lost the most on a high-fat/high-protein diet, most likely due to difference in the satiating effects of carbohydrates (24).

As body weight, and body weight-loss, is a key factor in insulin resistance and glucose homeostasis, high-protein diets may improve cardiometabolic risk factors. A recent systematic review and meta-analysis of 54 randomised controlled trials in populations without diabetes confirmed the impact of high protein diets (i.e., 20–45 Energy%) versus low-protein diets (i.e., 10-23E%) on weight-loss and fat mass loss (25). But also systolic blood pressure, total cholesterol, triacylglycerol and fasting insulin levels, a marker of insulin resistance, were lower on

HP diets. No significant differences were seen for glucose, HbA1c and insulin resistance as estimated by HOMA-IR, although fewer studies assessed these effects (25). In patients with diabetes, a high-protein, low carbohydrate weight-maintaining diet improved fasting plasma and 24-h glucose and HbA1c level (26). Although protein is known for its effect on insulin (and glucagon) secretion by the pancreas, the low glucose availability is probably the key in the impact of this diet. In a moderate weight-loss trial in obese women, a high-protein (1.2 g/kg/day) regular carbohydrate diet compared to a low protein (0.8 g/kg/day) diet reduced the WL-induced decline in lean tissue mass, but it also prevented the WL-induced improvements in muscle insulin signalling and insulin-stimulated glucose uptake (23). Thus without a significant decrease in CHO intake the effect of weight-loss was blunted by a high protein intake.

Next, to total protein intake also protein source could play a role. In a randomised cross-over trial we compared two weight-maintenance high-protein diets (22En%) with different protein sources (27). Substituting 30 grams of protein daily from meat products with soy products in postmenopausal abdominally obese women led to improvements in various cardiometabolic risk factor, including insulin sensitivity as measured with an frequently sampled intravenous glucose tolerance test (FSIGT). Whether the protein itself explains these findings or the isoflavones associated with soy protein could not be concluded. From a systematic review on the effects of plant protein versus animal protein in healthy humans and those with a metabolic impairment, it was concluded that there is some evidence that the intake of plant protein, in particular soy protein associated with isoflavones, may prevent the onset of cardiometabolic risk factors like hypercholesterolemia and hypertension, but an effect on glucose homeostasis could not be concluded (28). From studies with individuals with diabetes, it was concluded that replacing sources of animal with plant protein leads to modest improvements in glycaemic control (29) Again, an important note is that changing plant-protein intake is in general part of changes in plant-based foods consumption with subsequent changes in other nutrients and dietary factors, like fiber, fat, micronutrients and energy. Attributing effects to (plant) proteins perse should therefore always be done careful.

Altogether, in controlled intervention studies, high-protein (energy restricted) diets improve body weight and composition as well as multiple cardiometabolic risk factors including insulin resistance and glycaemic control. High plant protein intake, as part of a plant-based diet, may have stronger effects compared to animal protein, as part of an animal-based diet, but mainly on lipids, not on glucose homeostasis. With other dietary factors than plant protein, like fat, fiber and micronutrient intake explaining the beneficial effect on in particular LDL cholesterol (30). In addition, a reduced glycaemic load associated with a higher protein intake may be an important factor explaining the effect on glucose metabolism.

Protein, AA, and insulin and glucagon secretion

Protein and AA are known to stimulate insulin and glucagon secretion from the pancreas (8, 31). Insulin stimulates peripheral glucose uptake, in particular in muscle tissue, and hence can lower glucose levels. Indeed, when co-ingested with glucose protein and AA can stimulate insulin secretion, and can attenuate the glucose response, although these effects are relatively small in young healthy adults (8). The metabolic effects differ per AA, with isoleucine and phenylalanine resulting in the largest attenuation of glucose levels while leucine had the largest impact on insulin secretion when co-ingested with glucose (8). In type 2 diabetes, where insulin secretion after carbohydrate ingestion is severely impaired, amino acid and protein co-ingestion were shown to substantially increase plasma insulin responses (32), and can assist in acute metabolic control. Whether this acute stimulatory effect of AA on insulin is conserved over time, as well as whether this is desirable knowing the effect of chronic hyperinsulinemia on worsening of insulin resistance, needs to be established. Next to stimulation of insulin secretion, AA are also known to induce a rise in glucagon and to attenuate the glucose lowering effect of glucagon response (8), with again a different effect for different AA. Glucagon has multiple metabolic effects. Glucagon opposes insulin and stimulates gluconeogenesis and hepatic glucose output, resulting in maintenance or elevation of plasma glucose levels and availability for peripheral tissues. Elevated fasting glucagon levels (hyperglucagonemia) are present in obese individuals with (pre-)diabetes and are predictive of future diabetes development (33). The effect of protein and AA on glucagon secretion and circulating glucagon could be one of the mechanism underlying the observation of an increased diabetes risk associated with a high (animal) protein consumption (33). But glucagon also stimulates insulin secretion which in the prandial state could assist in glycaemic control (34). In addition glucagon activated hepatic lipolysis which lower hepatic lipids, a condition known to be associated with insulin resistance (35). Both insulin and glucagon responses are modulated by incretin responses, i.e., gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Nutrients as well as mixed meals trigger incretins, but the endogenous incretins do not seem to play a major role in the hyper glucagon secretion seen after a mixed meal in type 2 diabetes (36). It is proposed that targeted combinations of AAs that maximise insulin secretion and mitigate (fasting) hyperglucagonaemia, could be considered for clinical applications in patients with diabetes (31).

BCAA, mTOR, and mitochondrial function

Elevated blood BCAA levels have bene identified as predictor of diabetes risk, and direct infusion of BCAA induces rapidly insulin resistance (18). Elevated BCAA levels are not just a simple reflection of a high protein intake. Next to food intake, also protein breakdown in tissue, a process which is inhibited by insulin, and gut microbial synthesis contribute to BCAA appearance and levels in the blood. Disappearance of BCAA on the other side is a consequence of protein synthesis, excretion and BCAA catabolism. All these processes together define the levels of BCAA in plasma, and in particular a dysfunctional or impaired breakdown of BCAA is thought to be an important factor in the elevated circulating BCAA levels in patients with (pre)diabetes (19). BCAA, and potentially toxic metabolites such as BCAA-derived acylcarnitines, can however directly or indirectly interfere with insulin action and contribute to the development of insulin resistance and diabetes (19, 20, 37).

A direct effect could be the persistent activation of the mTOR pathway. The mTOR pathway is a nutrient-sensing pathway, which integrates nutrient sensing and insulin signalling to coordinate cell growth and metabolism and it could have a crucial role in understanding the association between BCAA and insulin action (11). BCAA and other nutrients activate mTOR, which is well-known for its role in dietary protein stimulated (muscle) protein synthesis.

Among other signals, this activation can also lead, in a negative feedback loop, to phosphorylation of the insulin receptor substrate 1 (IRS1), leading to a decreased insulin action (37).

Indirectly, BCAA, but in particular its potential toxic metabolites, may result in an impaired mitochondrial function, with reduced oxidation of lipid substrates resulting in accumulation of lipid in (muscle) cells (19, 20, 37). Lipid accumulation in muscle tissue is associated with insulin resistance, as lipid intermediates can interfere with insulin signalling, known as 'lipotoxicity', resulting in a reduced insulin stimulated glucose uptake and impaired glucose homeostasis (38). But lipotoxicity affects also normal function of other tissues, including the pancreatic (B-cell) and cardiac tissue.

Protein recommendations for adults with – an increased risk of – type 2 diabetes

Several national bodies have in recent years released guidelines or statements for those with diabetes are at risk for developing diabetes ('prediabetes'), including the American Diabetes association, ADA (39), Diabetes Canada (40), Diabetes UK (41) as well as the Dutch diabetes Federation, NDF (42). General consensus is that diabetes can be delayed or prevented by a healthy diet and increased physical activity, accompanied by weight loss. Dietary protein intake is not a key target in nutritional guidelines for patients with diabetes across the different countries. These dietary guidelines, supported by strong evidence, recommend restricting energy intake and weight-loss, increasing fiber intake, including low glycaemic index foods, and reducing saturated fat intake.

According to Nutrition Therapy guidelines of Canada Diabetes Association (40), there is no evidence that the usual protein intake for most individuals (1 to 1.5 g per kg body weight per day), representing 15 to 20% of total energy intake, needs to be modified for people with diabetes. This level of intake, that is generally observed in western countries, can already considered to be high-protein compared to the 0.8 g/kg BW recommendation of the FAO, although experts recommend an intake of ~1.2 gram/kg BW for older adults (43). Importantly, this intake in grams per kg per day should be maintained or increased with energy-reduced diets to maintain lean mass during weight-loss. As reviewed above, protein quality could be an another consideration, as replacement of animal protein with sources of plant protein could improve A1C, FPG and fasting insulin (29).

Finally, some words of caution are made for diabetes patients with chronic kidney disease (CKD) and when using a low-protein diet. In CKD patients a level of intake at 0.8 g/kg ideal BW is advised (40, 42), as protein restriction reduces end stage renal disease. However, harm due to protein malnutrition, in particular in older adults, should not be ignored (43), and quantity and quality of protein intake must be optimized at the individual levels to meet requirements for essential amino acids.

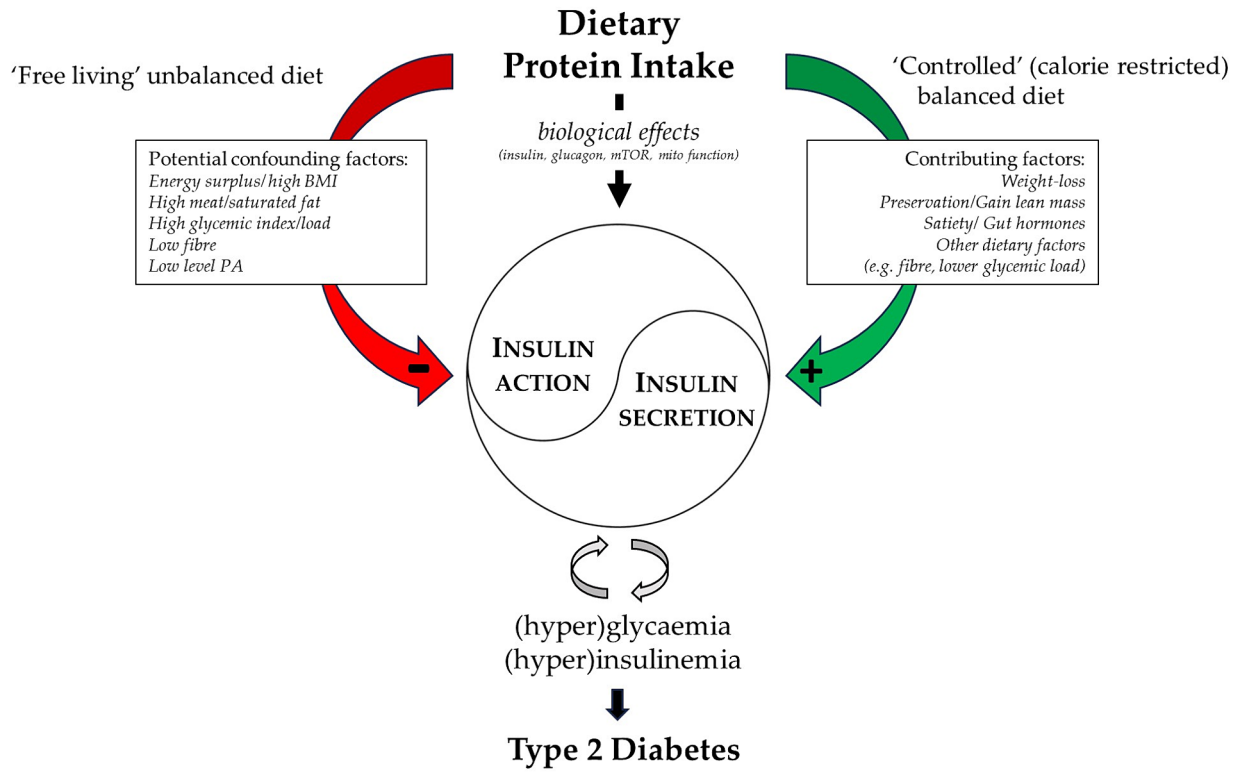

FIGURE 1
Visual summary of the different links between dietary protein intake and type 2 diabetes. A high protein intake associates with several potential confounders in "free living" (observational) conditions and is linked to an impaired insulin and glucose regulation and increased diabetes risk. While in controlled energy restricted (intervention) conditions several factors contribute to the beneficial link between high protein diets and an improved glucose metabolism. With central the biological effects that could either facilitate glycaemic control (i.e., insulin secretion), or have potentially detrimental effects (i.e., hyperglucagonemia, mTOR activation and/or impaired mitochondrial function).

Concluding remarks

Protein and AA play a crucial role in human physiology and metabolism. Low protein diets and diets with a low or inadequate protein and/or essential AA uptake are associated with severe health risks. The effect of high protein diets and high protein consumption on cardiometabolic disease, in particular type 2 diabetes, is much more complex and multifaceted (Figure 1).

Proteins and AA can have multiple biological effects. Acutely, the insulin secretory properties of AA can improve glycaemic control in patients with diabetes, while its effect on glucagon secretion has the potency to impair glucose regulation and is associated with insulin resistance. Protein intake, in particular when combined with exercise, stimulates protein synthesis and can improve body composition during weight-loss with beneficial health effects, including an improved glucose metabolism. But BCAA dysmetabolism and as a consequence elevated BCAA levels and its metabolites can on the long-term have detrimental effect on insulin action and mitochondrial function facilitating the development of insulin resistance and an impaired glucose regulation.

Under 'free living' condition a high protein consumption is in general associated with an unbalanced 'western' diet, with an excess of calories and increased BMI, a high meat and saturated fat intake, a high glycaemic load and low fiber intake. This results in observational studies identifying a high protein consumption as a risk factor for type 2 diabetes. In controlled settings with a (calorie-restricted) balanced diet a high protein intake, together with a low glycaemic load, high fiber intake and weight loss with preservation of lean mass can improve insulin resistance and metabolic control (Figure 1).

The conclusions from our review 10 years ago (11) remain valid: high protein, non-energy restricted diets seem not warranted to reduce insulin resistance or prevent diabetes. Long-term high-protein intake maybe even having deleterious effects, when diets are unbalanced. High-protein energy restricted diets to support weight loss and lean muscle accretion can be considered to improve metabolic control in those with—or at risk for—type 2 diabetes. To improve glycaemic control other dietary changes like restricting energy intake, reducing total and saturated fat intake and increasing fiber intake should however be of higher priority.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nutritional importance of animal-sourced foods in a healthy diet

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Animal-sourced foods, such as meats, poultry, eggs, milk, and fish are nutrient-dense foods that are rich sources of protein, essential amino acids, and micronutrients that can be challenging to obtain solely through plant-based foods. Animal-sourced protein foods provide crucial nutrients that support the growth and development in children, maintenance of muscle mass and function in adults, gain in muscle mass and strength in exercising individuals, and mitigation of sarcopenia in the elderly. The *2020–2025 Dietary Guidelines for Americans* have identified the important role of animal-sourced foods in the diet at every stage of life. Animal-sourced foods are consumed worldwide and contribute to global food security.

KEYWORDS

meat, essential amino acids, protein, diet, animal-sourced foods, nutrition

Nutrient density of animal-sourced foods

Animal-sourced foods are considered nutrient-dense in that they can be a single source of high-quality protein, vitamins, and minerals; this can be challenging to obtain through consumption of only plant-based foods. Proteins are the basis of all metabolically active tissues in the body and are comprised of dispensable (nonessential) and indispensable (essential) amino acids. Dispensable amino acids can be synthesized by the body from other amino acids or nitrogen-containing molecules and do not need to be obtained from the diet whereas indispensable amino acids must be acquired through the diet. Most animal-sourced protein foods contain all the required essential amino acids in proportions that are suitable for meeting human requirements and are considered complete proteins. Many plant-derived proteins, on the other hand, contain low amounts of one or more essential amino acids such as leucine, lysine, or methionine and are considered incomplete proteins. A deficiency of a single essential amino acid will limit the use of all other amino acids for protein synthesis.

In addition to the amino acid profile of a protein-containing food, its digestibility is an important determinant of its protein quality because it determines the amount of dietary amino acid that is biologically available. Protein digestibility is determined by its amino acid composition, and protein structure can be modified by the method of processing, storage, and cooking, which also influences digestibility. Protein quality is defined by the Digestible Indispensable Amino Acid Score (DIAAS), which calculates a score for each indispensable amino acid, based on the concentration of each indispensable amino acid (per g protein) corrected for its digestibility measured at the end of the small intestine. The DIAAS for a protein is the lowest score of the individual indispensable amino acid that is most limiting as defined by its requirement in the reference population (1–3). Because most animal-sourced foods are more digestible than plant-based foods, the individual amino acids from their

proteins are more readily absorbed. The combination of greater digestibility and an amino acid composition that better meets human requirements when consumed in appropriate amounts, is optimal for sustaining the body's anabolic processes (1–3).

Animal-sourced foods are an excellent source of other nutrients that are often deficient in plant-based foods, such as zinc, iron, vitamin B12, selenium, and phosphorus (4, 5). For example, nutrient recommendations for zinc, potassium, copper, thiamin, and choline are more likely to be met in pork consumers than non-pork consumers (6). Iron, zinc, vitamin A, and vitamin D are also present in more bioavailable forms in foods from animal compared to plant sources (7). In addition, animal-sourced foods may increase absorption of iron and zinc from plant-based foods, leading to a higher probability of meeting nutrient requirements (8). However, plant-based foods do provide essential nutrients, such as dietary fiber and vitamin C that are not present in animal-sourced foods. Thus, once weaned, a healthy, balanced diet will be comprised of a variety of plant-based and animal-sourced foods that complement each other to meet nutrient requirements.

While even a food containing relatively low amounts of micronutrients or an unbalanced amino acid mixture, if eaten in sufficient amounts, will meet an individual's requirements, the implications for total energy intake cannot be ignored. For example, for the average adult, 100 g of cooked lean beef (approximately 3 ounces) provides half of the daily needs for protein, selenium, niacin, and vitamin B12 and is an excellent source of iron and zinc, while only contributing to 10% of daily energy and fat intake (9). On the other hand, one would need to consume 250 g of peanut butter (15 tablespoons) to get the same amount of zinc, and this would provide 75% of the daily energy requirement. For adults consuming a 2,000 kcal diet, 735 g (3 cups) of low-fat milk that are recommended in the *2020–2025 Dietary Guidelines for Americans* (DGA) provides all the calcium and phosphorus, half of the protein, and a significant proportion of the riboflavin, potassium, magnesium, as well as other micronutrients required each day (4, 10). Thus, 3 servings of dairy foods help meet recommendations for nutrients which are frequently in shortfall in the diet (11). For young, physically active males, approximately 25 g of high-quality protein is needed at each meal to maximize the rate of protein synthesis in skeletal muscle (12). However, the energy content of dietary protein sources required to provide this amount of protein vary greatly. For example, 96 g (6 tablespoons) of peanut butter or 555 g (3 cups) of quinoa provide about 25 g of protein, but at a high energy value of approximately 600 kcal (4). However, only 100 g of lean beef provides the same amount of protein while contributing just 150 kcal to energy intake. Thus, lean beef has the caloric advantage of providing fewer calories but more essential nutrients, including high-quality protein.

Currently in the United States, approximately 70% of daily calories come from plant-derived foods that are derived primarily from refined grains and foods with added sugar (13). While only 30% of daily calories are obtained from animal-sourced foods, they provide nearly 100% of daily requirements for vitamin B12, calcium, and vitamin D and about 60% of requirements for zinc, iron, vitamin B6, and niacin. As Americans shift to more plant-based diets, most of the protein comes from grains, predominately refined wheat that is low in both protein quality and nutrient density, and much less from legumes that have a higher protein quality and content among the plant foods (14, 15). Thus, the reduction in nutrient density associated with a shift to a more plant-based diet will require continuous and careful consideration of diet preparation to ensure that the quantity and quality of protein consumed, and micronutrient intake meet the requirements for sustaining optimal health.

Animal-sourced foods are important for populations in low- and middle-income countries who are vulnerable to undernutrition and the consumption of these foods has been increasing (16). Using an aggregated global food composition database to calculate recommended nutrient intakes for iron, zinc, folate, vitamin A, calcium, and vitamin B12 in population groups with varying requirements, researchers found the top sources of these priority micronutrients include animal organs, beef and other meats, eggs, milk, fish, and dark green leafy vegetables (17). Populations with increased nutritional needs, such as infants and children, benefit greatly from animal-sourced foods due to the nutrient density of these foods. Indeed, addition of even small amounts of nutrient dense animal-sourced foods to the diet could alleviate deficiencies of several of "the most common shortfall nutrients in the world" (18).

Children

Animal-sourced foods provide a rich source of nutrients that are critical at all stage of the life cycle. Nutrient-dense, protein-rich foods are critical to the growth and development of infants and children. Animal-sourced protein foods are excellent sources of many of the essential vitamins and minerals for which deficiencies are prevalent world-wide (9, 19, 20). Indeed, the World Health Organization has described animal-sourced foods as the best source of high quality nutrients to reduce stunting in toddlers and young children (21). The provision of meat as a complementary food in low-income settings is associated with less stunting in toddlers (22), and in the United States results in increased linear growth without excessive weight gain or adiposity (23). Moreover, cow milk consumption compared to plant-derived beverages is associated with greater childhood height (24). In children, consuming milk is associated with increased overall body protein balance (25), increased lean mass, and decreased body fat (26). This is particularly important when, worldwide, 20% (390 million) of children and adolescents, ages 5–19 years, are overweight and 8% (160 million) are obese. In the United States, 16% of children, ages 2–19 years, are overweight, 18% are obese, and 6% are severely obese (27, 28). Children with obesity are very likely to become obese adults and are at increased risk for cardiovascular disease, diabetes, osteoarthritis, cancer, and other chronic diseases (27). Animal-sourced foods have a role in preventing obesity in children. In overweight children, eating a high protein breakfast reduces hunger and increases fullness, fat oxidation, and energy expenditure (29), all of which are key when considering the long-term treatments for and the prevention of obesity in children. Thus, animal-sourced protein foods can play an important role in reducing the risk of obesity in current and future generations.

Malnutrition is a pervasive global health problem, especially in children. Twenty-one percent (144 million) of children less than 5 years of age are stunted, 7% (47 million) are wasted, whereas 6% (38 million) are overweight (30). Poor nutrition is associated with cognitive and behavioral impairments during adolescence and adulthood (31) and contributes to 45% of all child deaths (30). In undernourished populations, consumption of animal-sourced

protein foods can promote greater length-for-age (32–34) and cognitive development and function in children (35). Thus, children in low- and middle-income countries could benefit from increased consumption of animal-sourced foods to ameliorate nutrient deficiencies and mitigate the occurrence of undernutrition (20).

Animal-sourced foods for weight management and muscle mass anabolism in adults

In the United States, 42% of adults aged 20 years and older are obese, and 9% are severely obese (36). It is projected that by 2030, nearly 1 in 2 adults in the United States will be obese, and the prevalence will be higher than 50% in most states (37). Worldwide, obesity has doubled since 1990 and in 2022, more than 43% (2.5 billion) of adults over the age of 18 were overweight, and 16% (890 million) were obese (27). Annually, approximately 5 million deaths globally are linked to overweight and obesity (27).

The World Health Organization (WHO) recommends healthier food choices and physical exercise as the simplest techniques to prevent overweight and obesity (27). Protein rich foods can play an important role in weight management. A meta-analysis of 24 weight loss trials of approximately 12 weeks duration showed that when a portion of the carbohydrates in the diet was replaced with protein, thereby increasing the protein content from 15 to 30% of energy in the diet, participants in the studies lost more weight while preserving lean mass (38). High protein diets containing animal-sourced foods also can promote satiety, reduce food motivation and reward, and improve diet quality (39, 40).

Dietary protein is instrumental for promoting the effects of resistance exercise training on muscle protein synthesis and accretion (12, 41, 42). Consumption of egg protein after resistance exercise can promote muscle protein synthesis in a dose-dependent manner in healthy adults (12). Maximal stimulation of protein synthesis could be achieved with 25–40 g of protein, depending on protein quality (12, 43, 44). Consumption of increasing amounts of either beef alone or with exercise increases muscle protein synthesis and maximum rates of protein synthesis are achieved with 170 g of beef providing about 35 g of protein (45). Comparison of the response to 110 g (4 ounces) of beef vs. soy demonstrated that beef compared to plant-based proteins increases protein synthesis more at rest, as well as with exercise (46). Similar muscle protein synthesis rates can be achieved with 30 g of a balanced plant-derived protein blend and milk protein (47) suggesting that if plant-based proteins are combined and consumed in high doses, equivalent protein anabolism can be achieved.

The timing with which dietary protein intake is distributed over the course of the day also impacts the utilization of dietary protein for protein synthesis. Data from the National Health and Nutrition Examination Survey (NHANES) in 2017–2018 showed that most people in the United States consume protein in a skewed pattern with small portions of protein at breakfast, slightly more at lunch, and the bulk of protein at dinner (48). However, consumption of a moderate amount of high-quality protein, about 30 g, 3 times a day stimulates muscle protein synthesis to a greater extent than the common practice of skewed protein consumption (49). Thus, establishing a dietary pattern of moderate amounts of high-quality protein at each meal is a viable strategy to promote muscle mass anabolism in adults.

Older adults

It is estimated that the percentage of adults aged 65 years and older will grow from 9% in 2015 to 17% by the year 2050 and account for 1.6 billion of the projected 9.4 billion total world population (50). Older adults may require more dietary protein than younger adults to preserve muscle mass, support health and disease recovery, offset inflammatory and catabolic conditions, protect against frailty and falls, maintain functionality, and help ensure independent living (51, 52). For example, a dose–response study investigating the effect of protein intake on muscle protein synthesis revealed that 30 g of dietary protein are needed to maximally stimulate muscle protein synthesis in older men whereas only 20 g of dietary protein are required in younger men (53). The difference in response has been attributed to the development of anabolic resistance with aging.

Age-related loss in muscle mass may be due in part to anabolic resistance to the stimulation of protein synthesis by feeding (54). However, animal-sourced foods may play an important role in promoting anabolism in muscle. Ingestion of an omnivorous meal containing beef results in higher rates of protein synthesis in skeletal muscle compared with an isonitrogenous vegan plant-only meal in older adults (55). Moreover, a higher intake of animal-sourced foods over a 20-year period is associated with the protection of muscle mass and functional performance in older adults (52). When compared to plant proteins, animal-sourced protein foods alone and with exercise are associated with the preservation of muscle mass and functional performance in older adults (56). Greater protein intake and an even mealtime distribution of protein is associated with increased muscle mass and strength in older Canadian adults (57). However, ingestion of balanced protein meals evenly distributed across the course of the day may be challenging for older adults whose energy intake is reduced. However, consumption of milk protein at breakfast or in the evening can promote higher rates of muscle protein synthesis in older adults (58, 59). Thus, supplementation with animal-sourced proteins, especially when combined with exercise, may be a viable strategy to preserve muscle mass with aging.

Potential health risks

Red and processed meat consumption has been associated with an elevated risk of noncommunicable diseases, including cardiovascular disease, cancer, and obesity (60, 61). However, the observational data to support these assertations are weak and not confirmed by studies with more robust designs (18). Moreover, confounding dietary and lifestyle factors may also play a role in the purported link between meat consumption and disease risk. Higher red and processed meat consumption has been associated with lower vegetable and dietary fiber intakes, increased body weight, and less physical activity. Although saturated fat has been purported to be a mediator of many adverse effects of red meat on health outcomes, recent studies have called into question recommendations to limit saturated fat intake consumed within a whole food matrix such as unprocessed meat and whole-fat dairy (62, 63). A meta-analysis of 945 studies found that red meat did not influence blood lipids, lipoproteins, or blood pressure and, thus, did not adversely impact cardiovascular disease risk (64). Four systematic reviews of cohort and randomized trials of more than 6 million participants found that red and processed

meat consumption has little to no effect on cardiometabolic outcomes, cancer, and all-cause mortality (65–68). Based on these systematic reviews, a panel of experts recommended that adults do not need to change their meat-eating habits (69).

Due to the above concern of the potential health risks of red and processed meat consumption, dietary guidelines have recommended the consumption of lean meat such as poultry and fish that are also a source of high-quality proteins, vitamins, and essential omega-3 fatty acids. There is some concern with fish consumption as it relates to the presence of heavy metals which accumulate in their bodies from the marine environment (70). While on average, levels are not of significant concern, for high fish consumers and pregnant women, they could pose a health risk (71).

The 2020–2025 Dietary Guidelines for Americans

The *2020–2025 Dietary Guidelines for Americans (DGA),* published by the United States Department of Human and Health Services (HHS) and the United States Department of Agriculture (USDA), recommends that Americans "Make Every Bite Count" (10). The DGA highlights four ways to achieve this goal: following a healthy dietary pattern at every stage of life; customizing and enjoying nutrient-dense foods and beverage choices to reflect personal preferences, cultural traditions, and budgetary considerations; focusing on meeting food group needs with nutrient-dense foods and beverages, while staying within calorie limits; and limiting food and beverages high in added sugars, saturated fat, and sodium, and limiting alcoholic beverages (10). The DGA stresses the importance of nutrient density throughout the lifespan, and animal products are nutrient-dense foods that provide not only high-quality protein but also vitamins and minerals. The Guidelines recommend 735 g (3 cups) of dairy and 150 g (5 ½ ounces) of lean meats, poultry, eggs, or seafood per day in a healthy 2,000 kcal diet.

The 2020–2025 edition of the DGA is the first to investigate the nutritional habits of infants and toddlers. The Guidelines recommend feeding infants exclusively human milk during the first 6 months and supplementing with vitamin D soon after birth (10). As complementary foods are added, protein foods, including meats, poultry, eggs, seafood, nuts, seeds, and soy products, that are considerable sources of iron, zinc, protein, choline, and long-chain polyunsaturated fatty acids, should be included. In the second year of life, the DGA recommends the provision of dairy products, including milk, yogurt, cheese, and fortified soy beverages and soy yogurt, to provide a good source of calcium, along with vitamin D-fortified cow's milk or soy beverages (10). Plant-based milk alternatives have significantly less protein than cow milk and are not natural sources of calcium. Thus, the DGA recommends cow's milk or fortified soy beverages to meet the dairy recommendations (10).

Although the DGA uses "ounce equivalents" when recommending protein needs, animal protein and plant protein sources may not be metabolically equivalent. A recent study in young, healthy adults demonstrated that consumption of "ounce equivalents" of animal-sourced protein foods (beef sirloin, pork loin, and eggs) results in a greater gain in whole-body net protein balance than the ounce equivalents of plant-based protein food sources (tofu, kidney beans, peanut butter, and mixed nuts) and the response is correlated with the essential amino acid content of the food source (72). The improvement in whole-body net protein balance is due to an increase in protein synthesis with all the animal protein sources, whereas egg and pork consumption also suppresses protein breakdown compared with the plant protein sources.

Americans are also starting to increase their consumption of animal-sourced foods (73), similar to the global increase in consumption of animal-sourced foods (16). From 2020 to 2022, the percentage of adult respondents to a food and health survey who reported eating more red meat in the past 12 months increased from 13 to 19% (73). Additionally, about one-quarter (24%) of respondents reported that they actively tried to consume animal proteins during the studied timeframe. The percentage of consumers who perceived that animal proteins were unhealthy was only 15% in 2020. The data suggest that the majority of Americans believe that there is a role for animal-sourced foods in a healthy diet.

Conclusion

Animal-sourced foods are nutrient-dense foods that provide high-quality protein and are rich sources of vitamins and minerals. Meat and other animal-sourced foods provide crucial nutrients that support the growth and development of children, maintain muscle mass and function in adults, and help mitigate several chronic diseases, such as those associated with aging and obesity. Thus, animal-sourced foods encompass a noteworthy role in a healthy diet.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The pig is an excellent model to determine amino acid digestibility of human foods and to generate data needed to meet human amino acid requirements

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The protein value of any food item is determined by the quantity and ileal digestibility of indispensable amino acids in that food. To determine the ileal digestibility of amino acids, an animal model needs to be used, and the pig is the preferred model because values for ileal digestibility obtained in pigs are representative of values obtained in humans. In addition, pigs are omnivorous animals like humans, they are meal eaters, they consume most diets that humans consume, they are easy to work with, and they can be used for repeated determinations of digestibility in many foods. It is, therefore, possible to use pigs to establish a database with digestibility values for human foods and by correcting digestibility values obtained in pigs for the basal endogenous losses of amino acids, it is possible to calculate true ileal digestibility values that are additive in mixed meals. As a consequence, the protein quality of a meal consisting of several food items can be calculated based on digestibility values obtained in pigs. Future work needs to focus on expanding existing databases for amino acid digestibility in foods to include more food items, which will make it possible to estimate the amino acid value of more mixed meals. It is also necessary that the amino acid values in mixed meals be related to requirements for digestible indispensable amino acids in the individuals consuming the meals. The current contribution describes the basic steps in determining amino acid digestibility in human foods using the pig as a model and also outlines future steps needed to further improve amino acid nutrition in humans.

KEYWORDS

additivity, amino acids, digestibility, pig, protein

1 Introduction

Animals have been used as models for humans in nutrition research for centuries and a number of important discoveries in nutrition were based on animal studies. The earliest recorded nutrient digestibility experiments were conducted approximately 270 years ago by de Reaumur who fed small, perforated metal tubes filled with grass to sheep [cited from Sauer et al. (1)]. Among animals, pigs are attractive models for humans because diet and intake patterns as well as diurnal patterns are similar to humans. The anatomy of the digestive system in pigs, secretions of enzymes and hormones, and absorption mechanisms in pigs are also very similar to the human digestive system (2, 3). Pigs are also omnivorous

animals like humans, they are meal eaters, and they will eat pretty much anything humans eat, which makes it possible to study digestion, absorption, and post-absorptive metabolism of nutrients in pigs and apply results to humans (2). Indeed, in experiments where the same proteins were consumed by pigs and humans, it was demonstrated that for all indispensable amino acids, the true ileal digestibility is very similar (3, 4). Given that pigs are easy to work with, easily tolerate procedures to collect fluids from the distal ileum, and can be fed human diets without modifications, it is natural that the pig has emerged as the preferred model to study amino acid digestibility in humans (5, 6). The digestibility of amino acids is less in newly weaned pigs than in older pigs (7), whereas no differences between growing pigs and mature pigs have been observed (8), and a growing female pig between 30 and 100 kg has, therefore, been proposed as an appropriate model for humans (6). There are no indications that amino acid digestibility is different between male and female pigs, but because most male pigs in commercial units are castrated, female pigs are usually utilized in experiments to determine digestibility of amino acids in human foods. There are no indications that differences in ileal digestibility of amino acid among commercial breeds of pigs exist and the choice of breed is likely not going to influence digestibility. During the last decade, there has, therefore, been a number of experiments conducted in which pigs were used to determine digestibility of amino acids in human foods and results have been used to calculate the digestible indispensable amino acids score (DIAAS) in a large number of human foods. As a consequence, a large set of data with values for the ileal digestibility of amino acids in human foods determined in pigs is now available (9), and more data will undoubtedly be generated in the future. There is, therefore, a need to highlight some of the consequences of determining amino acid digestibility in human foods using pigs as models. The practical aspects of preparing, managing, and feeding pigs used in digestibility experiments have been highlighted in two recent publications (6, 10). Likewise, detailed procedures for calculation of true ileal digestibility of amino acids and values for DIAAS have also been provided (5, 11) and detailed descriptions of the factors used to calculate DIAAS have been provided (12). There is, however, a lack of information about the application of digestibility data for amino acids obtained in pigs into practical recommendations for human consumption. It is, therefore, the objective of the present contribution to provide examples of how the pig model can be used to not only generate digestibility values for amino acids, but also how these data can impact formulation of meals for humans to meet requirements for amino acids. It is not the objective to give an exhaustive review about factors affecting amino acid requirements in humans, nor is it the objective to discuss post-absorptive metabolism of amino acids. Instead, the focus will be on discussing why amino acid digestibility is important and how data for amino acid digestibility obtained in pigs may be used in human food formulation.

2 The importance of amino acids in nutrition

Although it is generally assumed that humans have requirements for protein, this is not entirely true, because humans, like other monogastic species, have requirements for indispensable amino acids and not for protein *per se* (13). Of the 20 amino acids that are needed for protein synthesis, the body can synthesize only 10 in quantities that are sufficient to meet the requirement, whereas the remaining amino acids need to be supplied in the diet; these amino acids are, therefore, called dietary indispensable. There is no storage in the body of excess amino acids, and the 10 indispensable amino acids, therefore, need to be provided in the diet each day. In fact, recent evidence indicates that providing approximately one third of the daily requirements for indispensable amino acids at each meal supports muscle protein synthesis to a greater extent than providing the majority of the amino acids in one daily meal (14, 15). It is therefore most important that sufficient quantities of the indispensable amino acids are provided in each meal every day. However, not all amino acids in food proteins are digested, but only the amino acids that are digested and absorbed contribute to the protein status of the individual. It is therefore the digestible quantity of each indispensable amino acid in each meal that determines if the requirement for protein synthesis can be met. Whereas there are estimates for requirements of total amino acids by different age groups (13) there is a lack of estimates for requirements for ileal digestible amino acids. However, most experiments conducted to determine amino acid requirements used diets that were high in animal proteins, and the true ileal digestibility of amino acids in animal protein is generally very high (16–19). As an example, in 23 beef and pork ingredients, the true ileal digestibility of all indispensable amino acids was between 92 and 99% (18, 20, 21), and the same was the case for the digestibility of amino acids in whole milk (19). Assuming that human requirements for total amino acids are based primarily on animal proteins, the requirement for true ileal digestible amino acids may be estimated to be around 95% of the requirement for total amino acids (Table 1). The challenge, therefore, is to mix dietary food items at each meal to meet requirements for digestible quantities of each amino acid. As a consequence, a database with values for the digestibility of each amino acid in each food item is required (9).

TABLE 1 Calculated requirement for true ileal digestible amino acids, mg/kg body weight per day.[a]

Age, years	0.5	1–2	3–10	11–14	15–18	>18
Histidine	21	14	11	11	10	10
Isoleucine	34	26	22	21	20	19
Leucine	69	51	42	42	40	37
Lysine	61	43	33	33	31	29
SAA[b]	29	21	17	16	15	14
AAA[b]	56	38	29	29	27	24
Threonine	32	22	17	17	16	14
Tryptophan	9.0	6.1	4.6	4.6	4.3	3.8
Valine	47	34	28	28	27	25

[a]Data were calculated from World Health Organization (13) assuming a true ileal digestibility of 95% of amino acids used to determine requirements for total amino acids.
[b]SAA, sulfur amino acids, i.e., methionine and cysteine; AAA, aromatic amino acids, i.e., phenylalanine and tyrosine.

3 Procedures for determining digestibility of amino acids in food items fed to pigs

Proteins cannot be absorbed but need to be digested by gastric, pancreatic, and intestinal proteases to liberate the individual amino acids, which can then be absorbed. However, absorption of amino acids takes place only in the small intestine and proteins that have not been digested prior to the distal part of the small intestine, which is called the ileum, make no contribution to amino acid absorption because amino acids are not absorbed from the hindgut (8). It is therefore necessary to gain access to the digesta leaving the small intestine at the end of the ileum and a number of techniques have been suggested for this purpose (22). However, installment of a T-cannula at the distal ileum, which was first suggested 50 years ago (23), has been recognized as the most practical way to gain access to ileal digesta. A cannula in stainless steel or titanium is usually used although cannulas in polyethylene have also been proposed (23, 24). However, the inflexibility of the stainless steel or titanium cannulas has proven to result in better stability of the cannulas and less problems with dislodgements (25). The cannula consists of a flange that is inserted in the small intestine, and a barrel that penetrates the body wall. The upper part of the barrel is threaded, and the cannula is secured on the outside with a washer that is screwed onto the barrel. A screw cap is used to close the barrel and is removed when digesta is collected from the barrel (25). Cannulas with different dimensions can be used for different sizes of pigs, but for pigs from 30 to 100 kg, a cannula with an inner diameter of 2.24 cm and a barrel length of 6 cm is usually used (Table 2). The cannula is installed using a simple surgery that can be performed in less than 30 min by a trained surgeon (24, 25). Following surgery, pigs are housed individually to prevent other pigs from disrupting the cannula. Pigs are placed in a pen that should provide at least 1.25 square meter of space, and it is recommended that floors are fully slatted to prevent accumulation of fecal materials in the pen. If pens are not fully slatted, frequent cleaning is necessary to prevent coprophagy because if pigs ingest even small amounts of feces, which have a high concentration of the indigestible marker, calculations of amino acid digestibility will be inaccurate. No bedding is provided (6) because that may interfere with endogenous amino acid secretions and calculations of amino acid digestibility.

Pigs are typically given 7 days to recover after the surgery and feeding of experimental diets can then be initiated. An adaptation period of 5 days to experimental diets is recommended with ileal digesta being collected for 9 h per day on days 6 and 7 (6, 10). This schedule fits a normal work week, and collections can be scheduled to take place in the middle of the week. However, because amino acid digestibility is rapidly adjusted to the diet being provided, 3 days of adaptation is sufficient to obtain steady state in terms of marker and amino acid flow (26). In cases where the amount of an ingredient is limited, a 3-day adaptation period can, therefore, be considered.

TABLE 2 Dimensions of intestinal cannula installed in pigs from 30 to 100 kg and used to determine ileal digestibility of human foods.

Item	Cannula for 30–100 kg pig
Barrel length, cm	6.00
Barrel outer diameter, cm	2.54
Barrel inner diameter, cm	2.24
Flange length, cm	7.00
Flange width[a], cm	2.54

[a]The width of the flange is 2.54 cm in the middle, but the flange is tapered toward the extremes where the width is only 1.50 cm.

Collection of ileal digesta from the cannula will not result in total collection and it is, therefore, necessary to include an indigestible marker in the diets being fed and ileal digestibility is subsequently calculated using the marker to estimate the flow of amino acids to the distal ileum (11). The assumption for using this procedure is that the marker is completely mixed with the test diet and that the marker flows through the intestinal tract at the same speed as undigested material, and these assumptions have been confirmed in several experiments. In most circumstances, titanium dioxide is used as the marker to determine the ileal digestibility of amino acids in human foods and an inclusion rate of 0.50% (dry matter basis) is often used (10). Where diets are provided in a meal form or as a porridge, it is usually not a problem to ensure a complete mixture of the marker and the diet (16, 27). Likewise, if the digestibility of amino acids in baked products such as bread or bagels is determined, the marker can be mixed into the dough and consumed along with the diet and subsequently analyzed in the ileal digesta. The marker can also easily be mixed into liquid diets such as milk or juice. However, for food items such as meat products, nuts, vegetables, and others, a complete incorporation of the marker with the meal may not be possible. It is recommended to provide all meals to pigs in the same form as they are usually consumed by humans (6), but to ensure a complete mixture of the marker with the meal, a gentle grinding may sometimes be necessary (28) in which case the ingredients are not fed to the pigs exactly as they would be consumed by humans. However, because mixing of the marker with the diet is critical for correct calculation of digestibility values, this compromise may sometimes have to be made and because amino acid digestibility is not impacted by the particle size of the ingredient ingested (29, 30), it is unlikely that this modification will have any impact on results. Another approach that can be used to ensure that the marker is well mixed into the meal is to incorporate the marker into a protein free mixture that is usually added to the diets to provide vitamins and minerals. Sometimes, it is also necessary to add protein free ingredients such as starch, lactose, oil, or sugar to this mixture to provide sufficient calories to the animals along with the protein food that is used (6). In this case, the protein food, which can be a meat product, can be cut into small squares prior to feeding and then gently mixed with the protein free mixture that also contains the marker. Because pigs are fed restrictedly and usually consume their meals quickly after feeding, this approach results in satisfactory incorporation of the marker in the meal and digestibility values using this approach, therefore, are associated with low errors (20, 21, 31).

After collection of the ileal digesta, it is critical that microbes in the digesta are quickly inactivated to prevent fermentation of amino acids after collection. It has been suggested that microbial activity can be prevented by adding an acid to the collection bags (6), but results of recent research demonstrate that this is not necessary because the acid is not mixed with the digesta flowing into the bags.

Instead, if collection bags are frequently changed (i.e., every 30 min) and if the collected digesta are stored at −20°C immediately after collection, there is no advantage of adding acids to collection bags (32). Having a freezer located in the barn where pigs are kept is, therefore, critical.

At the conclusion of the collection period, the frozen digesta need to be thawed, mixed, and subsampled, and a subsample of around 200 mL is lyophilized. It is important to lyophilize these samples rather than oven dry them, because oven drying results in loss of amino acids and subsequently inaccurate calculation of amino acid digestibility (33). The lyophilized sample is ground using a coffee grinder, mixed, and a subsample is collected for analysis of dry matter, crude protein, amino acids, and titanium. Following analysis, values for apparent ileal digestibility, true ileal digestibility and digestible indispensable amino acid scores are calculated (5, 11).

4 Additivity of values for amino acid digestibility

Both animals and humans usually consume diets that consist of more than one source of amino acids and to meet requirements for digestible amino acids, it is critical that the values for amino acid digestibility that are determined are additive in mixed meals. However, values for the apparent ileal digestibility of amino acids are not additive in mixed diets (34), which prevents the use of such values in calculating the intake of digestible amino acids from a given meal. The lack of additivity of values for apparent ileal digestibility is caused by the influence of the endogenous amino acids on the ileal output of amino acids. The presence of endogenous nitrogen, or metabolic fecal nitrogen, in the feces of rats fed protein free diets was demonstrated in some of the earliest experiments to determine amino acid digestibility (35, 36). It was later demonstrated that the amount of endogenous nitrogen in the feces as a percentage of total fecal nitrogen output of rats fed a protein-containing ingredient depended on the inclusion rate of that ingredient in the meal (37), and subsequent work confirmed that values for the apparent ileal digestibility of amino acids are also influenced by the inclusion level of the ingredient in the diet (38, 39). As a consequence, it is necessary to correct values for the apparent ileal digestibility of amino acids for the pre-cecal endogenous loss of amino acids and subsequently calculate values that are independent of the inclusion rate of each ingredient in the diet. Values for endogenous losses of amino acids that are needed for this correction are obtained after feeding a protein free diet, and factors influencing ileal endogenous amino acid loses have been reviewed (40). Correcting values for apparent ileal digestibility for endogenous losses results in calculation of values for standardized ileal digestibility values, which is the term mostly used in animal feeding (11) whereas in human nutrition, values calculated after correction for ileal endogenous losses are termed true ileal digestibility values (5). However, strictly speaking, correction for values obtained after feed a protein free diet does not result in calculation of values for the true ileal digestibility of amino acids (11). Additivity of values for the standardized ileal digestibility of amino acids in mixed diets fed to pigs has been demonstrated multiple times (34, 41). Likewise, additivity of values for true ileal digestibility of food proteins in a mixed meal has also been demonstrated (19, 31, 42) and it is, therefore, possible to calculate the digestibility of indispensable amino acids in mixed meals from digestibility values for each amino acid in individual ingredients (43, 44). As a consequence, establishment of a database with values for the true (or standardized) ileal digestibility of amino acids for individual food items will allow dietitians and food professionals to calculate the quantities of digestible amino acids that are present in mixed meals and by comparing these quantities to requirements for amino acids, it can be determined if the meal is adequate in all indispensable amino acids. Establishing a food database with digestibility values for as many food proteins as possible, therefore, is critical (9, 45), and the only practical way to generate such a database is to use pigs to determine values for digestibility. As an example, ileal digestibility values for some food items determined in the authors laboratory using the procedures outlined above are presented in Tables 3, 4. However, there is a need to extend this database to contain a much larger number of food items.

5 Application of amino acid digestibility values to human foods

The concept of determining protein quality in human foods is not new and was first attempted by establishing the protein efficiency ratio (PER) in foods (48). This procedure was based on determining the growth of rats fed different proteins and the PER value was calculated by expressing the growth over 28 days relative to the protein intake of the rats during those 28 days. A later modification to the procedure involved comparing all proteins to the PER of rats fed a casein-based diet and resulted in calculation of the casein-corrected PER. A different procedure called the biological value of proteins was based on the proportion of retained nitrogen relative to absorbed nitrogen and offered some advantages over the PER procedure (49). However, because of the very high requirement for the sulfur-containing amino acids by rats compared with humans, procedures using growth or nitrogen retention in rats have been criticized for not being reflective of the protein quality of foods for humans (50). As a consequence, protein evaluation based on the digestibility of nitrogen rather than retention or growth was introduced and this procedure also for the first time introduced values for the digestibility of individual amino acids (50). The procedure was called the "Protein Digestibility Corrected Amino Acid Score" (PDCAAS) and is used for regulatory purposes in the United States. The PDCAAS procedure also introduced the concept of scoring values of proteins by comparing quantities of digestible indispensable amino acids to the profile of amino acids required by children from 2 to 5 years (50), and therefore, recognized that humans have requirements for individual indispensable amino acids rather than for protein. The limitations of the PDCAAS procedure have been highlighted (16, 51) and resulted in recommendation of calculating DIAAS of proteins (5). The DIAAS procedure corrects some of the flaws in the PDCAAS procedure including measuring the ileal digestibility of each individual amino acid rather than the total tract digestibility of nitrogen. There are also several other advantages to the DIAAS procedure over the PDCAAS procedure, and one of the consequences of determining DIAAS values is that the pig is a more natural model for humans than the rat for reasons outlined above. In addition, because DIAAS is based on the ileal digestibility of each individual amino acid after correction for endogenous losses, the

methodology for determining amino acid digestibility is identical to that used to determine digestibility of feed ingredients used in the feeding of animals. As a consequence, because values for true ileal digestibility are additive in mixed diets, DIAAS of meals consisting of several food items can be calculated, which is a great advantage because more than one protein item is included in most meals. However, DIAAS values, like PDCAAS values and PER values, only indicate the quality of a specific protein or meal, but do not indicate anything about the quantity needed to meet amino acid requirements. There is, therefore, a need for a further refinement of the DIAAS concept to directly link values to quantities of digestible amino acids required by different groups of humans. As a consequence, future work needs to focus on not only measuring digestibility of individual amino acids and calculating DIAAS values, but also on developing methodologies that can calculate the quantities of specific meals needed to meet amino acid requirements for humans.

6 Conclusion

Protein evaluation of human foods needs to start with determining the true ileal digestibility of each individual indispensable amino acid. The pig has proven to be an accurate model for humans in terms of amino acid digestibility and because pigs are easy to work with and easily tolerate the procedure of installing and maintaining an intestinal cannula in the distal ileum, it is easy to conclude that the pig is the preferred model for humans when it comes to amino acid digestibility determinations. Pigs easily consume most human foods in the form they are consumed by humans and can be used for multiple measurements of digestibility of amino acids. Detailed procedures for determining ileal digestibility of amino acids in pigs are available and the ileal digestibility of a number of food items determined in pigs have been published and can be used to determine DIAAS values in mixed meals. Future work will focus on development of methodologies that can connect DIAAS in individual ingredients and meals to the requirements for digestible indispensable amino acids in different groups of humans.

Author contributions

HHS: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

TABLE 3 True ileal digestibility (%) of indispensable amino acids in selected plant food items determined in the authors laboratory.[a]

Food item	His	Ile	Leu	Lys	Met	Cys	Phe	Tyr	Thr	Trp	Val
Grains											
Maize	83	76	84	75	90	77	83	80	71	70	75
Barley, de-hulled	81	76	79	74	78	80	82	77	72	84	77
Oats, de-hulled	88	87	87	85	90	77	88	81	81	82	85
White rice, polished	91	92	94	92	95	94	95	92	91	95	94
Rye	77	72	74	67	81	76	79	66	94	75	71
Sorghum	74	74	76	69	77	68	76	71	68	73	74
Wheat	84	79	81	73	85	83	84	77	65	84	75
Processed grain products											
Corn flakes	88	93	97	78	98	93	95	95	93	91	94
Quick oats	88	87	88	83	89	90	88	88	85	85	86
Burger bun	92	91	93	64	93	92	94	90	88	95	90
Grain protein concentrates, isolates											
Oat protein concentrate	81	83	85	86	83	86	86	86	82	95	82
Brown rice protein concentrate	80	79	78	74	71	68	80	74	78	90	79
Pea protein concentrate	94	92	93	95	88	73	93	91	89	91	89
Rapeseed protein isolate	91	71	73	82	79	85	72	66	72	76	72
Rapeseed protein isolate, heated	98	95	97	95	98	94	97	96	94	98	95
Soy protein isolate	97	94	93	97	95	91	95	96	91	98	93
Nuts											
Pistachio nuts	89	87	88	87	87	88	87	88	88	92	88
Pistachio nuts, roasted	79	78	79	77	80	80	77	78	77	85	78
Plant based meat analogs											
Impossible burger patty	96	94	94	96	95	77	95	95	90	99	94
Beyond burger patty	90	90	90	94	84	64	92	92	86	98	89

[a]Data from the following references: Fanelli et al. (19), Cervantes-Pahm et al. (27), Bailey and Stein (28), Fanelli et al. (31), Abelilla et al. (46), Bailey et al. (47).

TABLE 4 True ileal digestibility (%) of indispensable amino acids in selected animal food items determined in the authors laboratory.[a]

Food item	His	Ile	Leu	Lys	Met	Cys	Phe	Tyr	Thr	Trp	Val
Pork products											
Pork belly, raw	99	98	98	99	98	93	98	98	98	99	97
Smoked bacon	97	97	97	97	97	89	96	97	96	97	95
Smoked cooked bacon	95	96	97	98	97	88	96	96	96	96	95
Ham, non-cured	94	94	95	96	96	77	94	95	93	92	93
Ham, cured	96	97	97	98	98	89	97	97	97	95	96
Pork loin, cooked to 63°C	96	96	97	97	97	85	96	97	96	95	95
Pork burger patty, 80% lean	98	96	97	98	97	79	96	95	95	100	96
Beef products											
Salami	95	96	96	96	96	91	95	96	96	96	96
Bologna	97	97	97	97	96	91	96	96	95	98	96
Beef jerky	96	97	97	96	97	90	97	97	96	97	96
Ground beef, raw	99	98	99	98	98	96	98	99	99	98	97
Ground beef, cooked	96	97	97	98	98	88	97	97	97	97	97
Rib eye roast, cooked to 63°C	96	96	96	96	97	88	95	96	94	95	95
Beef burger patty, 80% lean	93	94	94	96	96	63	93	92	89	95	92
Milk products											
Whey protein isolate	100	98	99	98	98	98	98	99	94	100	97
Whey protein concentrate	97	97	98	96	97	95	96	96	91	98	95
Milk protein concentrate	99	96	98	96	97	85	97	98	96	97	94
Dried skimmed milk	99	95	98	96	99	99	99	99	96	97	97
Skimmed milk powder	94	89	94	95	96	73	94	95	82	91	90

[a]Data from the following references: Mathai et al. (16), Fanelli et al. (19), Bailey et al. (20, 21), Fanelli et al. (31).

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Critical variables regulating age-related anabolic responses to protein nutrition in skeletal muscle

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Protein nutrition is critical for the maintenance of skeletal muscle mass across the lifecourse and for the growth of muscle in response to resistance exercise – both acting via the stimulation of protein synthesis. The transient anabolic response to protein feeding may vary in magnitude and duration, depending on, e.g., timing, dose, amino acid composition and delivery mode, which are in turn influenced by physical activity and age. This review aims to: (i) summarise the fundamental metabolic responses of muscle to protein feeding, (ii) discuss key variables regulating muscle anabolic responses to protein feeding, and (iii) explore how these variables can be optimised for muscle anabolism in response to physical activity and ageing.

KEYWORDS

protein, nutrition, muscle, ageing, exercise

Muscle mass regulation in health and ageing

Skeletal muscle plays an integral role in maintaining health throughout the life-course; an illustration being the close links between low muscle mass/strength and all-cause morbidity and mortality. Reduced muscle mass and strength are also predictive of declines in activities of daily living and increased dependence (1). Such functional limitations and dependence have been highlighted as key factors in reducing quality of life in older individuals (2), emphasising the importance of preserving muscle mass to maintain quality of life.

Beyond physical function, muscle is critical to whole-body metabolism through its role as an amino acid (AA) reservoir, the utilisation of fat and glucose, and the storage of glucose as glycogen (3, 4). Skeletal muscle contributes to energy expenditure through various means, including basal metabolism, physical activity and thermogenesis (5, 6). Reduced energy expenditure is associated with increased risk of obesity, metabolic syndrome, type II diabetes and cardiovascular disease (7), meaning it is critical to maintain metabolically active skeletal muscle tissue to sustain energy expenditure. The quantity of skeletal muscle mass relative to body weight has also been shown to be inversely associated with insulin resistance and pre-diabetes even in populations with healthy quantities of muscle mass (8). However, these detrimental effects are most concerning in more vulnerable individuals such as those with age-related declines in skeletal muscle mass and function (sarcopenia) (9), where increased insulin resistance and a lower contribution of muscle mass to total energy expenditure have

been associated with increased risk of metabolic disease (10) and type II diabetes (11).

Given the crucial role of muscle in both physical function and metabolic health, maintaining muscle mass and strength throughout the life course is vitally important. Muscle mass exists in a state of constant turnover that is determined by the processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (12). Changes in net balance are primarily driven by changes in MPS, which are approximately four to five times greater than changes in MPB in response to protein nutrition and resistance exercise (RE) (13). This, combined with the greater technical challenge of quantifying MPB compared to MPS, means that research assessing the anabolic effect of nutritional and RE interventions is focused largely on alterations to MPS (14). In young, healthy individuals, MPS and MPB exist in a dynamic equilibrium. Under these conditions, AAs leave the muscle in the postabsorptive state and are utilised, e.g., for hepatic gluconeogenesis or synthesis of proteins in other tissues (4). This is balanced by the synthesis of new muscle protein in the postprandial state when there is excess availability of building block AAs from protein intake (12).

It follows that disruption of this equilibrium in muscle wasting conditions (such as ageing), skews it towards reduced net protein balance, driven largely by reductions in MPS (15). This age-related blunted anabolic response to key stimuli, namely protein feeding (16) and physical activity (17), has been termed "anabolic resistance" (18, 19), which incipiently chips away at muscle mass, contributing to the onset and progression of sarcopenia. While the mechanisms of anabolic resistance remain at large, age-related inflammation (20) and increased splanchnic uptake of AAs (21–23) are purported contributors implicated in reducing the MPS response to protein and activity. Due to age-related anabolic resistance, there is consensus in the literature that the current recommended daily protein intake of 0.75 g protein/kg/day in the UK (24) or 0.8 g protein/kg/day internationally (25) is insufficient for older adults (26–30), which is unsurprising given the recommended protein intake is meant as a guideline for *all* adults regardless of age. As such, recent think tanks and consortia have recommended a protein intake of approximately 1.0–1.5 g/kg/day for older individuals (26, 29), which, in the face of age-associated reductions in appetite (31), may still be achievable without excessive feeding via increasing the proportion of protein in the diet. Similarly, during acute or chronic illness or injury, MPS rates are suppressed and MPB rates may be elevated, resulting in more rapid skeletal muscle atrophy (32). This is concerning in older populations, as the onset and development of sarcopenia may be accelerated due to illness through various disease-mediated mechanisms (33), while prolonged hospital stays and inactivity following injury result in poorer functional outcomes in the long-term (34, 35).

Maximising anabolic potential is necessary to delay the onset and progression of sarcopenia and maintain function and quality-of-life throughout all stages of the life course. In addition to physical activity, the foundation of achieving this is good nutritional practice, namely in relation to dietary protein intake. Numerous variables impact the anabolic effectiveness of protein intake, including, e.g., the timing, type and quality of the protein source delivered (36), alongside external factors such as the combination of feeding with physical activity (12) and the effects of ageing (37). Given this complexity, there is no universal recommendation that can optimise protein intake for all individuals in all conditions. Instead, these variables should be considered carefully and protein feeding adapted to meet the needs of different individuals (30). As such, the aims of this review are to (i) summarise the fundamental metabolic responses of muscle to protein feeding, (ii) discuss key variables regulating muscle anabolic responses to protein nutrition, and (iii) explore how these variables can be optimised for muscle anabolism in response to physical activity and ageing; all in the context of the human literature.

Temporal anabolic response to, and timing of, protein nutrition

The anabolic effects of protein feeding are temporally regulated. There is approximately a 30–45 min delay between the consumption of a protein source and subsequent increases in MPS; the magnitude of which is approximately 200–300% compared to postabsorptive (i.e., fasted) rates (38, 39). This delay can be attributed to the time taken for digestion of the protein source and subsequent absorption of AAs into the blood before being transported to the target muscle tissue where it acts as both the stimulus and substrate for increases in MPS. When AAs are provided intravenously (i.e., negating the need for digestion), there is still some latency in the MPS response as the AAs are transported to, and accumulate within, the muscle (40). After this period, MPS rates remain elevated for approximately 90 min, beyond which time there is a rapid decline back to postabsorptive rates, a phenomena termed "muscle full" (38). The muscle full effect is consistent with the understanding that muscle hypertrophy in adulthood cannot be achieved in the absence of accompanying physical activity (12) no matter the protein quantity consumed. Instead, replenishment of muscle protein lost during breakdown in the postabsorptive state is the homeostatic endeavor. To date, the mechanisms regulating the muscle full effect remain elusive. The reduction of MPS rates back to baseline occurs despite continued elevated plasma and intramuscular essential amino acids (EAAs), meaning that these are not responsible for MPS resetting. This also cannot be attributed to de-phosphorylation of key mTOR substrate signalling proteins including p70S6K1, 4EBP1 and EIF4G as these have all been shown to remain elevated after MPS returns to baseline (38). One speculated mechanism is endoplasmic reticulum (ER) stress caused by misfolding of proteins (41), which leads to the unfolded protein response (UPR) to limit further translation of misfolded proteins (42). UPR activity has been implicated in skeletal muscle loss (42) and ageing (43), but has also been found to be sensitive to physical activity (42), which could be relevant given the capacity of activity to delay the muscle full effect.

The duration of the refractory period (i.e., the duration before another MPS stimulation may be achieved) is speculated to be ~3–4 h (44); this being based on findings from Witard et al. (45) showing maximal increases in MPS following 20 g protein feeding approximately ~4 h after consumption of a high protein (0.54 g/kg) breakfast. This is also supported by a study in young people during recovery from physical activity, which showed that 20 g of protein feeding every 3 h produced greater increases in MPS over a 12 h recovery period compared to 10 g every 1.5 h and 40 g every 6 h (46). While these results do represent an interesting starting point for investigating the time-course of regaining sensitivity to protein feeding, physical activity has been well documented to increase the magnitude and duration of the MPS response to EAAs – essentially

delaying the muscle full effect (47, 48). Consequently, it is possible that the optimal strategy of protein feeding approximately every 3–4 h exhibited in the studies by Witard et al. (45) and Areta et al. (46) may not apply to the rested state due to these alterations in the muscle full phenomenon. Further, it would be useful to consider the potential implications of factors such as ageing on this refractory period in the rested state, as ageing is pertinent to the onset of sarcopenia, meaning that establishing optimal nutritional strategies for the ageing population is highly important. In sum, the anabolic response to protein nutrition is regulated by the muscle full effect, meaning that, to maximise our anabolic potential, it is important to appropriately time protein feeding around the refractory window.

Protein dose

The optimal protein quantity needed to elicit a maximal anabolic response has been well researched, with a consensus now established (37, 49). In healthy, recreationally active young adults, a per meal dose of roughly 20 g of "high-quality" protein (or 0.24 g/kg), or 10 g EAAs (roughly equivalent to 20 g intact protein) is sufficient to elicit a maximal and transient MPS response (49). Demonstrating this dose–response relationship between protein intake and MPS, 20 g of whey protein elicited greater MPS responses compared to 10 g whey, with no further anabolic effect observed with 40 g whey except increased AA oxidation and ureagenesis (45). Importantly, these protein quantities determined using isolated protein sources such as whey/EAAs, translate into realistic meal-like settings whereby a moderate portion of lean beef, providing ~30 g of protein (~10 g EAAs), elicits maximal MPS, with no further anabolic benefit seen with much larger portions providing ~90 g protein (~30 g EAAs) (50). It should be noted that physical activity may influence the protein dose needed to elicit a maximal anabolic response and is discussed in the "Physical activity" section.

Due to well-established anabolic resistance to protein intake seen with ageing, the protein dose needed to evoke maximal MPS responses is different in older age. A comprehensive retrospective analysis of multiple studies estimated that the dose of protein required to maximally stimulate MPS in older adults is ~68% greater compared to younger counterparts, resulting in a recommendation of 0.40 g protein/kg body mass for older individuals (49). In practice, ~40 g protein or ~20 g EAAs would be required to achieve an MPS response in older adults that resembles that of younger adults (49). While the mechanisms regulating anabolic resistance to nutrition remain to be precisely defined, older adults have been shown to exhibit hyperphosphorylation of mTORC1, potentially manifesting as a reduced ability of aged muscle to phosphorylate mTOR and activate MPS in response to protein, and is thus one plausible regulator (51). In the context of lower than maximal doses, it has been shown that whey protein, delivering 14.86 g total AAs, elicits greater muscle protein accrual compared to EAAs, delivering ~6.72 g total (E)AAs, demonstrating the importance of protein *amount* in older adults, and that when given in these doses, the mechanisms of protein accrual may go beyond EAAs (52). In addition to protein dose, recent evidence has highlighted the importance of protein per meal for ageing muscle, by finding that the number of meals with either ≥20 g or ≥ 30 g of protein were significantly associated with greater *m. vastus lateralis* cross sectional area and appendicular lean mass (53). Protein dose across the day must therefore be carefully considered to maximise diurnal muscle anabolism. Thus, in healthy younger adults ~20 g of high-quality protein (e.g., whey) or 10 g EAAs is sufficient to elicit maximal MPS responses, with this amount increasing to ~40 g of high-quality protein or 20 g EAAs to evoke similar anabolic responses in older adults.

Protein "quality"

According to the World Health Organization, protein quality can be defined by the amount and proportion of individual EAAs that can be absorbed from the diet and used by the body (54). Until recently, protein quality was estimated using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), which estimates protein quality based on fecal nitrogen, up to a value of 1.0 (55). Due to limitations with the method, PDCAAS has since been replaced by the Digestible Indispensable Amino Acid Score (DIAAS), which scores protein quality based on ileal digestibility and a theoretical reference protein, which results in foods having a similar score to PDCAAS but without truncating the value at 1.0 (55, 56). As such, despite whether evaluated using PDCAAS or DIAAS, protein foodstuff that provides all 9 EAAs such as meat, chicken, fish and dairy (e.g., milk) are all deemed "high-quality" protein sources (55). In depth discussion and comparisons on PDCAAS and DIAAS is beyond the scope of this review, and so we direct the readers to the following rich resources for further reading (36, 55–59).

With respect to muscle, early studies confirming EAAs as the principal nutritional stimulators of MPS (60) were later refined to reveal phenylalanine, valine and leucine as the most anabolically potent of these EAAs (61, 62). Since then, multiple human studies have repeatedly shown leucine as the most potent EAA for stimulating MPS (63, 64), while also demonstrating leucine as the EAA that elicits the most robust anabolic signaling responses mediated via Sestrin2 sensing and the mTORc1–p70S6K1 pathway (65, 66). Combined, these findings have led to the consensus that leucine is a multifunctional EAA, which can act as the main trigger for the initiation of MPS, in addition to being a substrate for the synthesis of *de novo* proteins (64, 65). The importance of leucine in anabolic responses to feeding has been demonstrated both in isolation and in combination with EAAs. In isolation, as little as 3.42 g of leucine has been shown to *maximally* stimulate MPS by ~110% (64). To place this in the context of more traditional protein feeding regimes, a large protein meal of 48 g whey resulted a ~ 150% increase in MPS (38). Comparing these anabolic responses directly over a 2.5 h measurement period (i.e., time taken for peak MPS and return to baseline) results in a similar overall protein accretion, via increases in MPS (64), demonstrating that leucine alone can evoke maximal MPS, at least until other EAAs become rate limiting (63).

As a branched chain amino acid leucine is metabolised within skeletal muscle, implicating its metabolites in muscle anabolism, with one metabolite, β-hydroxy-β-methylbutyrate (HMB), demonstrating anabolic facets. In humans, 3.42 g of free-acid HMB, providing 2.42 g of pure HMB, becomes rapidly bioavailable in plasma and muscle, and has been shown to stimulate MPS (+70%) and inhibit MPB (−57%); the latter in an insulin-*independent* manner (64). While isolated leucine and its metabolite, HMB, are anabolically effective, if provided in isolation repeatedly overtime other EAAs must become rate limiting for MPS

(63). As such, leucine enriched amino acid (LEAAs) strategies have been trialed to exploit the anabolic potency of leucine without compromising MPS in the longer term. When compared with a standard feed of 20 g whey, a much smaller LEAAs feed (3 g, 40% leucine) resulted in a similar temporal MPS response, despite greater insulinemia and aminoacidemia in response to whey. This suggests that whey offers no trophic advantage over LEAAs – or in other words – LEAAs are equally anabolic to larger whey doses (67). This was further confirmed using even smaller doses, with only 1.5 g of LEAAs containing 0.6 g leucine, robustly, and possibly maximally, stimulating MPS, with negligible anabolic advantage of greater doses of LEAA (6 g, 40% leucine) or whey (40 g) (68). Similarly demonstrating the anabolic significance of leucine, a lower-protein but leucine-matched feed (10 g) induced similar increases in MPS compared with a higher-protein feed (25 g) (69), indicating that leucine – and not total protein content – is the primary determinant of anabolic responses in muscle. This has important ramifications for certain cohorts who have, for example, reduced appetite (i.e., ageing), whereby leucine-enriched supplements/feeds may represent an advantageous approach to evoke maximal muscle anabolic responses (69). In a further study trialing the efficacy of leucine "top-ups" in humans, 15 g of EAAs compared with 15 g EAAs plus a 3 g leucine top up 90 min after feeding elicited a similar temporal MPS profile, whereby MPS increased until the onset of the "muscle-full" state ~180–240 min after feeding (70). Thus, while leucine can be used effectively to supplement meals containing suboptimal protein levels when leucine is given shortly after adequate EAAs feeds it has no further anabolic effect. The time frame in which protein/EAAs/leucine re-feeding is capable of re-stimulating MPS remains to be defined. Together, these data suggest that the composition of protein/EAAs, notably the presence of leucine, rather than amount of protein/EAAs is most crucial for stimulating muscle anabolism.

Protein delivery profile

Bioavailability of EAAs in the circulation and subsequently at the muscle tissue is of paramount importance for stimulating MPS and are variables that can be impacted by the protein delivery profile. Skewed protein feeding, where most of the daily protein intake is fed in a single meal, has been proposed as an alternative to the traditional even diet where protein intake is distributed similarly across multiple meals throughout the day. This maybe particularly relevant in older populations who have greater first pass splanchnic sequestration of AAs, resulting in a reduced hyperaminoacidemia in response to protein feeding compared to young individuals (21–23). It should be noted, however, that others have shown similar AA delivery to muscle across age despite a greater first-pass splanchnic sequestration in older age (23). Skewed protein feeding has also been applied to older hospitalised patients over a six-week period and it was suggested that this diet produced greater plasma AA availability (i.e., aminoacidemia) compared to even feeding (71). While this is noteworthy, plasma AA concentrations were only recorded for 3 h following the midday meal, where the even feed diet provided 30% of total daily protein intake, compared to 78% for the skewed diet. Therefore, there is no consideration in these results for any potential reduced hyperaminoacidemia observed following the three other meals where the even protein feed supplied more protein than the skewed protein feed. Other studies have also reported benefits of a skewed protein feed pattern compared to even feeds, with enhanced retention of fat free mass, greater whole body protein turnover and improved nitrogen balance (72). However, this is in contrast to the findings of Mamerow et al. (73), who reported greater 24 h MPS responses with an even protein feed than a skewed protein feed. Importantly, the study by Arnal et al. (72) was done in older individuals (average age 68 ± 1 y), whereas the study by Mamerow et al. (73) was carried out in younger individuals (average age 36.9 ± 3.1 years). The differences in findings may be reflective of the increased first pass splanchnic sequestration of AAs in older people, though this could best be confirmed by a study design with four experimental groups assessing both even and skewed protein feeds in both young and older participants.

Using more direct (i.e., stable isotopic tracers) methods to capture the transient responses to different protein delivery methods, Mitchell et al. (39) reported that young adults consuming EAAs as a single bolus (15 g) displayed rapid aminoacidemia and insulinemia, whereas smaller repeated "pulse" doses (4 × 3.75 g every 45 min) achieved gradual low-amplitude aminoacidemia and blunted insulin responses. Despite the different systemic profiles, the muscle anabolic response was the same across both delivery methods, demonstrated by the identical MPS temporality (i.e., latency period and return to baseline) and similar MPS rates (39). This data suggests that EAA delivery profile is not an important determinant of muscle anabolism and also implies that rapid aminoacidemia is not a key factor for maximising MPS (39). A follow-on study in older adults consuming EAAs as a single bolus (15 g) or as smaller repeated "pulse" doses (4 × 3.75 g every 45 min) reported that bolus feeding resulted in rapid essential aminoacidemia and insulinemia, which was accompanied by robust mTOR signaling (74). By comparison, pulse feeding resulted in a gradual low-amplitude aminoacidemia and diminished insulin responses, with undetectable mTORC1 signaling changes (74). Despite these attenuations, similar MPS responses were observed, where in fact MPS was sustained beyond 3 h following the pulse feed, by which point MPS had returned to baseline in response to bolus feeding (74). As such, in line with the prior study in young adults (39), there was no anabolic benefit of rapid aminoacidemia in older adults, which is despite greater overall EAA exposure and enhanced anabolic signaling. Instead, the benefit of low-grade-sustained EAA exposure elicited by pulse feeding seems to be the apparent delay in the onset of "muscle-full" permitting equal MPS responses, compared to bolus feeding. As such, the data so far suggests that the protein feed delivery method is not a crucial consideration so long as the protein quantity is sufficient to maximise MPS.

Protein blends

Protein blends are a mixture of two or more protein sources fed simultaneously. These may be different animal proteins (e.g., whey, casein), plant proteins, (e.g., soy, wheat), collagen proteins (e.g., gelatin) or combinations of these (36). Animal protein sources have complete EAA profiles (i.e., contain all 9 EAAs) compared to most plant protein sources, meaning that they produce more robust EAA hyperaminoacidemia and MPS responses, while plant proteins are either (i) incomplete protein sources meaning they contain some but not all 9 EAAs, or (ii) contain all EAAs but in insufficient quantities (e.g., pea protein contains all 9 EAAs but is insufficiently low in

methionine and cysteine), but represent (on average) a more sustainable source of protein (36). Collagen proteins, on the other hand, are a rich source of non-EAAs but a poor source of EAAs, thus limiting anabolic potential. For a comprehensive review on protein sources, readers are directed to the following resource (36). Surprisingly few studies have assessed the anabolic role of protein blends in the rested state. This is an important consideration as many individuals, particularly those who are most at risk of sarcopenia such as older adults, are less (or unable to be) physically active (75). Some recent studies have taken this approach when assessing the efficacy of plant-based protein blends compared to milk protein in the rested state (76, 77). Interestingly, these studies have reported that there was no difference in MPS responses between plant-based protein blends and milk protein, despite the milk protein producing a significantly greater increase in plasma EAAs in both studies. Considering the potency of even small doses of leucine, it is perhaps unsurprising that plant-based protein blends containing greater than 1.8 g leucine were able to stimulate a maximal increase in MPS in young individuals. Importantly, the reduced availability of EAAs in plant-based proteins means that large quantities of protein will be required to achieve the same hyperaminoacidemia as can be achieved with animal proteins, potentially creating challenges for older individuals with reduced appetite who may not want to consume more protein, as well as somewhat counteracting the sustainability advantages of these sources. In the context of ageing, protein source is also important to maximising MPS responses with previous findings favouring whey protein over other protein sources such as casein and soy for older adults (78–80). The mechanisms behind the enhanced MPS response are likely two-fold: a combination of the rapid digestion of whey protein and its higher overall leucine content compared to other protein sources. This rapid digestion also elicits more pronounced hyperaminoacidemia, particularly in older individuals who experience greater splanchnic sequestration of AAs.

The interaction between protein blends and acute RE have also been assessed, e.g., where milk (a casein and whey protein blend) elicited a greater MPS response in the 3 h post-exercise period than soy protein (81). Moreover, when extended to a chronic training period over 12 weeks, there was a greater increase in type II muscle fibre area and fat-and bone-free mass in the milk group than the soy group (82). Other studies have demonstrated similar findings in the post-exercise period comparing a 25% soy, 25% whey and 50% casein protein blend with both a protein (83) and a leucine content matched whey protein isolate (84). These studies both demonstrated no differences in MPS rates between the feeds, suggesting that both performed equally. It is worth noting that the study by Borack et al. (83) only found a significant increase from baseline in the whey protein group and not the protein blend group. As the authors suggest, this is likely a result of a higher baseline in the protein blend group caused by high variance rather than reflective of a difference in the capacity of the drinks to stimulate MPS, given the similarity between the performance of both drinks across the postprandial period. Reidy et al. (84) reported a prolonging of the MPS response in the protein blend group reflected by elevated fractional synthesis rate at 2–4 h, which was not observed in the whey protein group. While it is true that, at the 4 h time point, fractional synthesis rates were only higher than baseline in the protein blend group and not the whey protein group, there were no differences in MPS rates between the groups at any given time or when analysed across the entire 4 h postprandial period. Any suggestion of a prolonging effect of a protein blend based on these findings should be cautiously interpreted, but it is noteworthy that this may somewhat corroborate the findings of Hartman et al. (82). Overall, the current evidence suggests some promising applications for protein blends, including those containing plant-proteins, to produce robust MPS responses as individual protein sources and animal protein blends.

Physical activity

The capacity to go beyond muscle maintenance with nutrition and achieve muscle growth (hypertrophy) is dependent on the addition of contractile activity, particularly RE (85). RE essentially shifts the muscle full set-point to the right when in proximity with protein nutrition (12), increasing both the duration (86) and magnitude (87) of the MPS response. Notably, RE in the postabsorptive, fasted state, increases muscle protein turnover owing to a ~ 100% increase in MPS rates and a ~ 50% increase in MPB rates (88). Thus, net protein balance becomes less negative in the postabsorptive state following RE, but without the provision of protein, RE does not produce a positive state of protein balance. This highlights that neither protein nutrition nor exercise alone are sufficient to achieve hypertrophy; it is the synergistic combination of these anabolic stimuli that is paramount to increasing muscle mass.

Research points to the timing of protein intake in relation to exercise not being the most important factor in hypertrophy or strength gains, with most hypertrophic differences likely explained by the quantity of protein intake (89). This is perhaps because the enhanced anabolic window achieved through RE persists for up to 48–72 h, meaning that sufficient EAA provision throughout this time period will still produce a robust increase in MPS (90). This is not to suggest that there is no effect of the timing of protein intake, and reflecting this, previous work has shown greater increases in strength and hypertrophy following 12 weeks of RE training with post-exercise protein intake compared to 2 h post-exercise protein intake in older individuals (91). Perhaps more important than the timing of the first protein feed relative to a RE bout is to take full advantage of the enhanced anabolic window within this 48–72 h post-exercise period with repeated protein feeds. As already highlighted, there is a refractory period in response to protein feeding following RE in young, trained individuals which is approximately 3 h in duration (86). These findings should be considered in the context of nutritional practices and recommendations, as a large proportion of the population will typically consume three protein-containing meals a day with upwards of 6 h between protein feeds, which is clearly suboptimal. Further, protein consumption is often skewed between meals, with many individuals consuming less protein at breakfast than other meal times, which is shown to result in reduced MPS rates (73), and may be associated with reduced muscle mass and strength. That said, further research is needed (92), particularly in light of contradictory data in older adults demonstrating no significant changes in post-absorptive MPS over 24 h when protein was consumed evenly or skewed throughout the day (93). In the 48–72 h post-exercise window, prolonged postabsorptive periods are essentially wasting some of the anabolic potential from RE training, and feeding following an overnight fast should provide adequate protein to achieve a state of anabolism following prolonged overnight MPB. Therefore, it is critical to regularly consume an adequate amount of protein roughly every 3 hours where possible to maximise hypertrophy following RE.

Regarding dosing, ~20–30 g of high-quality protein is required following RE to maximally stimulate MPS, with excess protein intake beyond this catabolised via AA oxidation (45, 94, 95). There is clearly capacity for reducing this quantity with leucine enriched protein feeds, with 6 g of LEAA supplements able to stimulate similar post-RE MPS rates up to 4 h compared to 40 g whey protein feeding in older women (68). Although these findings were not replicated in the study by Churchward-Venne et al. (63), who found that only 25 g whey protein post-exercise was able to sustain elevated MPS rates over 3–5 h, whereas 6.25 g whey protein supplemented with either leucine or EAAs did not. This disparity between studies is perhaps surprising, as the study by Wilkinson et al. (68) was carried out in older women while the study by Churchward-Venne et al. (63) assessed young male participants. Given the established anabolic resistance that accompanies ageing, it would perhaps be expected that the younger participants would have had a more sustained MPS response even with the lower dose of whey protein fortified with either EAAs or leucine. Potentially, there was no sustained anabolic response to the EAA enriched whey protein feed in this group due to the low leucine content of this feed (0.75 g) despite the high overall EAA content. However, this does not apply to the leucine enriched whey protein feed, which had a comparable leucine content to the feeds in the study by Wilkinson et al. (68). Instead, the difference in post-exercise assessment durations (5 h in Churchward-Venne et al. (63) and 4 h in Wilkinson et al. (68)) may account for some of this disparity, as it is possible that MPS remains elevated under post-exercise conditions with lower quantity leucine fortified feeds, for approximately 4 hours before rapidly declining to baseline. Additionally, it could also be that the differences in lower leg RE protocols between the studies may explain some of the disparity in findings. The study by Wilkinson et al. utilised 6 × 8 repetitions of unilateral leg extensions, compared to 4 × 10–12 repetitions of both unilateral leg extensions and leg press. The addition of the leg press exercise would have resulted in the stimulation of additional muscle groups not utilised in leg press, resulting in a greater post-exercise AA demand compared to leg extension alone, which perhaps could not be sufficiently met by the low dose leucine or EAA feeds. Therefore, lower dose leucine fortified protein may be a robust post-exercise protein source for prolonged anabolism, particularly in older individuals who have reduced appetites and would subsequently prefer smaller protein feeds (31).

While there is general consensus regarding the maximal magnitude and duration of the MPS response to protein feeding following exercise, it was reported that there are greater increases in whole body net protein balance with 70 g mixed meal protein intake compared to 40 g following RE training (96). However, this same study reported no differences at the muscle level, with no effect of meal size, or even exercise, on MPS rates. The lack of effect of exercise can likely be attributed to measuring MPS over a seven-hour post-exercise period, as the peak in MPS rates that would be expected from RE training and protein feeding would largely be masked by prolonged periods of lower MPS rates based on the muscle-full phenomenon. More importantly, post-exercise MPS rates being the same between the 40 g and 70 g protein feed brings into question the relevance of the findings of greater net protein balance with the higher protein feed. Kim et al. (96) suggest that the anti-catabolic benefits of higher protein intakes are important but given that all measures made were of whole-body protein turnover, it is unknown how much of this breakdown can be attributed to muscle compared to other tissues, with the gut being a primary candidate for this. Other studies have reported similar findings following endurance exercise, with a 45 g protein feed producing a significantly greater whole body protein balance than 30 g, but similarly to RE, with no differences in myofibrillar fractional synthesis rates between the protein feeds beyond 30 g (97). Overall, the relevance of a greater whole body protein balance in the absence of any further increases in MPS needs to be considered. It may not be advantageous to compromise other feeds with suboptimal protein quantities that do not produce robust increases in MPS, in order to provide more of a post-exercise pulse feed which may produce greater whole-body protein balance but does not provide further benefits at the muscle level.

Only a single study has suggested that there is no upper limit in magnitude or duration of the anabolic response following RE, reporting a dose-dependent relationship between quantity of protein feeding and increases in myofibrillar MPS rates (98). In this study, it was reported that 100 g protein feeding stimulated greater increases in post-exercise MPS rates than 25 g protein over a 12 h post-exercise period. These findings contrast with previous research showing that post-exercise MPS rates are maximally stimulated with approximately 20 g of protein, with no further increase in MPS with 40 g protein (45, 95). One potential explanation for the differences in these findings could be the type of exercise employed. The study by Trommelen et al. (98) assessed MPS following whole body RE, whereas the studies by Moore et al. (95) and Witard et al. (45) used leg based RE. There is some precedence for this, with previous research indicating that 40 g of protein feeding may be more effective at increasing MPS than 20 g of protein feeding following whole body RE training (99). This is likely caused by the increased demand for AAs following whole body compared to isolated leg-based training, with the 20 g protein dose potentially not supplying enough AAs to meet the demands of the greater number of muscles utilised in whole body RE. It should also be noted that the adults recruited were untrained, which may also contribute to the observed anabolic response, which is known to be attenuated in trained individuals (100). However, these explanations are speculative, and there is currently no study directly comparing MPS responses to different protein doses following different types of RE. Moreover, this study likely simply reflects "on / off / on" of MPS in line with muscle full – due to the lingering large quantities of EAA in the circulation owing to gradual oxidative elimination.

Indeed, the suggestion from Trommelen et al. (98) there is no upper limit in magnitude or duration of the anabolic response to protein feeding should be interpreted with caution. The authors observed a ~ 20% increase in MPS rates in the 0–4 h post-exercise period when comparing 100 g protein feeding to 25 g, which matches the 20% increase also observed in the study by Macnaughton et al. (99) in the 0–5 h post-exercise period when comparing 40 g protein feeding to 20 g, with both studies using whole body RE. This would indicate that there is indeed an upper limit in magnitude of the MPS response, as MPS peaked at approximately the same relative increase compared to a lower dose (40 g vs. 20 g and 100 g vs. 25 g protein, respectively (98, 99)) and absolute value (approximately 0.06%/h), following 100 g protein feeding compared to 40 g protein feeding (98), both following whole body RE. Regarding duration, there was no difference in MPS rates between the 100 g protein group and the 25 g protein group between 8 and 12 h post-exercise, despite the fact that only the 100 g protein group was significantly elevated compared to the 0 g protein group. Given that there was no difference between the two protein feeds over this later time period, the physiological reality of this supposed extended duration of anabolism should be questioned. This is particularly important as the authors suggest that this provides mechanistic insights into the potential benefits of larger, less frequent

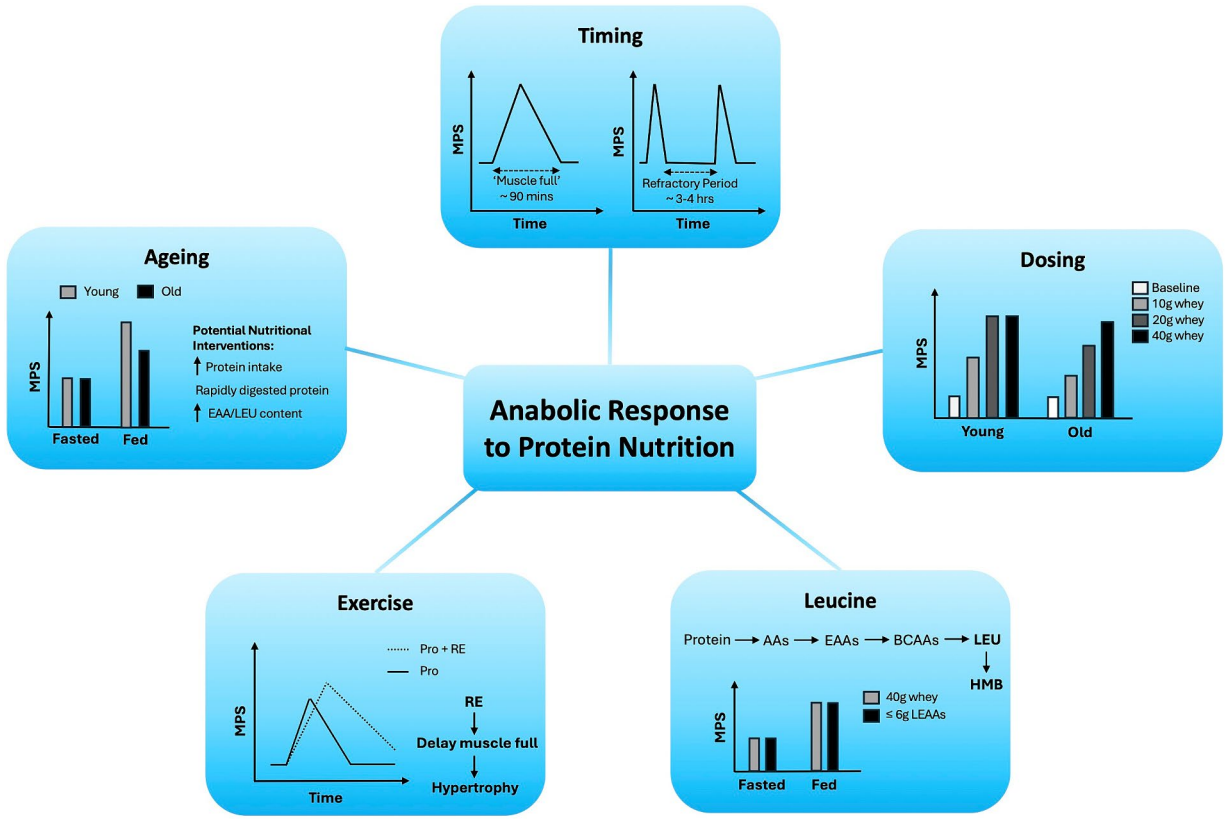

FIGURE 1
Variables regulating age-related anabolic responses to protein nutrition in skeletal muscle.

protein feeding patterns, which is directly in contrast to the findings of Areta et al. (46). There may be some validity in assessing even more doses with higher protein quantities as an extension to the findings of Areta et al. (46) based on the results of Trommelen et al. (98). However, given that four 20 g protein feeds were more efficacious than two 40 g protein feeds (46), it would be surprising to see a shift in favour of an even higher dose, but less frequent protein feed. In sum, RE is a key stimulator of muscle anabolism, but the intricacies of maximising the MPS response are still debated. Repetitive protein feeding containing EAAs/leucine within the 48–72 h post-exercise period should be the primary aim of anyone pursuing muscle hypertrophy, with any additional benefits of large quantities of protein intake (> 40 g) in response to whole body RE requiring further validation.

Conclusion and future directions

Protein nutrition is essential for the maintenance of skeletal muscle mass across the lifecourse and for the growth of muscle in response to RE via the stimulation of MPS. Many critical variables contribute to the duration and magnitude of this MPS response, including the protein dose, timing, EAA/leucine content, and delivery method, which are further impacted by age and exercise, all of which we have summarised in Figure 1.

Based on the reviewed evidence, we provide the following highlights, which contain practical recommendations for, and relevant to, protein nutrition practice:

- Maximal MPS can be achieved with ~20 g high quality protein (e.g., whey) or 10 g EAA in young healthy weightbearing adults.
- Older adults display anabolic resistance to protein nutrition (and exercise), requiring larger amounts of protein, ~40 g high quality protein or 20 g EAA, to elicit a maximal MPS response.
- As an anabolic signal and substrate, small doses of leucine (3 g) can evoke a maximal MPS response. This has significant application in cohorts who cannot, or do not, consume sufficient protein throughout the day to stimulate maximal MPS (e.g., older adults).
- Animal-delivered protein sources contain all EAAs in high quantities, eliciting robust MPS responses. By relative comparison, plant-derived protein sources contain lower EAA levels and in some cases do not contain all EAAs, eliciting less robust MPS responses. Nonetheless, with appropriate protein blending, plant-derived protein feeds can elicit maximal MPS.
- The delivery method of protein feeds (i.e., bolus versus pulse) is not a major determinant of the MPS response, so long as sufficient protein is consumed.
- Following a protein feed, the duration before which another MPS stimulation may be achieved (i.e., the refractory period) is estimated to be ~3–4 h but remains to be precisely defined.
- Consuming a protein feed in close proximity to exercise will ensure a maximal MPS response, although it is not critical as long as sufficient protein is consumed within the 72 h post-exercise period.

TABLE 1 Future research directions regarding variables regulating age-related muscle anabolic responses to protein nutrition.

Directly investigate the role of the unfolded protein response in regulating the muscle full response
Precisely define the refractory period timeline in healthy young and ageing scenarios
Characterise the anabolic impact of alternate protein sources at rest
Understand whether skewed diurnal protein intake impacts muscle mass/strength
Test the efficacy of chronic bolus versus pulse diets for muscle anabolism in older adults
Re-define the upper limit of magnitude and duration of the MPS response to different protein doses
Characterise the effects of chronic consumption of protein blends, particularly those containing plant proteins, on longer term MPS and muscle mass
Update recommended protein intakes for older adults to reflect that 0.75-0.8 g/kg/day is insufficient for muscle mass maintenance

- The current protein recommendation of 0.75–0.8 g protein/kg/day is insufficient for older adults and should be increased to 1.0–1.5 g/kg/day.

In addition to these practical tips, we have highlighted future research directions which we consider worthy of research attention in Table 1.

Author contributions

CD: Conceptualization, Investigation, Project administration, Writing – original draft, Writing – review & editing. JC: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. PA: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Analysis of global nutrient gaps and their potential to be closed through redistribution and increased supply

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Global food systems are crucial for sustaining life on Earth. Although estimates suggest that the current production system can provide enough food and nutrients for everyone, equitable distribution remains challenging. Understanding global nutrient distribution is vital for addressing disparities and creating effective solutions for the present and future. This study analyzes global nutrient supply changes to address inadequacies in certain populations using the existing DELTA Model®, which uses aggregates of global food production to estimate nutrient adequacy. By examining the 2020 global food commodity and nutrient distribution, we project future food production in 2050 needs to ensure global adequate nutrition. Our findings reveal that while some nutrients appear to be adequately supplied on a global scale, many countries face national insufficiencies (% supply below the population reference intake) in essential vitamins and minerals, such as vitamins A, B12, B2, potassium, and iron. Closing these gaps will require significant increases in nutrient supply. For example, despite global protein supply surpassing basic needs for the 2050 population, significant shortages persist in many countries due to distribution variations. A 1% increase in global protein supply, specifically targeting countries with insufficiencies, could address the observed 2020 gaps. However, without consumption pattern changes, a 26% increase in global protein production is required by 2050 due to population growth. In this study, a methodology was developed, applying multi-decade linear convergence to sufficiency values at the country level. This approach facilitates a more realistic assessment of future needs within global food system models, such as the DELTA Model®, transitioning from idealized production scenarios to realistic projections. In summary, our study emphasizes understanding global nutrient distribution and adjusting minimum global nutrient supply targets to tackle country-level inequality. Incorporating these insights into global food balance models can improve projections and guide policy decisions for sustainable, healthy diets worldwide.

KEYWORDS

systems modeling, micronutrients, sustainability, nutrient adequacy, mathematical modeling, inequality

Introduction

The global food system is the most critical human activity, essential for sustaining the lives of everyone on the planet by providing the necessary nutrition (1). It also serves as a major economic activity and is responsible for a significant portion of the anthropogenic impact on the environment (2).

The ability of a country to secure food and nutrients for its population depends on factors such as agricultural production, trade dynamics, and import economic capacity (2, 3). This is impacted by international trade patterns, regional and national economic conditions, domestic food production and the resilience of food systems to external shocks from climatic events or political issues (3).

Previous study (4–6) aligning food production with the nutrient requirements of the global population has shown that with equitable global distribution, there was sufficient food produced in 2018 to meet nutrient requirements for everyone for 27 of 29 nutrients considered within the DELTA Model®. Projecting into the future, 2018 production included sufficient protein—and indispensable amino acids—to meet the requirements of the expected 2050 population if these were equally distributed (5).

These global-scale approaches assume equal access to nutrients for everyone on the planet and lead to the development of scenarios describing the minimum food production necessary to meet nutrient needs. This is itself a valuable insight as it gives us the minimum conditions under which it might be possible to adequately nourish everyone on the planet. However, the distribution of food and nutrients is not equitable, and with a level of global food production that could provide adequate nutrition for all, many are undersupplied. Developing scenarios for future food systems that accommodate some degree of inequality requires an understanding of how food commodities and nutrients are currently distributed.

The global food system is known for its complexity and wide-reaching impacts (2), illustrated by a variety of health outcomes. People may have protein-energy malnutrition, obesity due to excess energy consumption and lifestyle choices, and/or micronutrient deficiencies (often termed as 'hidden hunger'). Micronutrient deficiency and obesity can exist at the same time as 'hidden hunger' or exist separately. To add further complexity, these issues can exist in the same country and the same household as those who do not have these health issues (7). Globally, in 2021, 768 million people were affected by hunger, 3.1 billion people were unable to afford a healthy diet, and simultaneously, 40% of all adults were overweight or obese (8). Dietary choices, availability, and affordability of food within a country play a significant role in these health outcomes and adequate nutrition through food and lifestyle choices is an effective strategy for avoiding long-term health consequences (9, 10).

By 2050, global populations will increase, and demographics will shift, leading to changes in global nutrient requirements. National food supplies are expected to encounter pressures due to the impact of climate change on domestic food production, impacting the ability to consistently meet market demands and uphold nutritional requirements (4, 11).

Bell et al. (12) investigated several aspects of inequality in global food, nutrition, and health between 1970 and 2010. These included energy intake from animal-sourced foods, energy intake from fruits and vegetables, intake of vitamin A, zinc, and iron, and health indicators like child stunting and the prevalence of overweight and obesity in men and women. Their study determined and compared global distributions of these nutrients and health metrics at both ends of this period, while also considering factors such as food production, land use, and GDP *per capita*.

In our study, we seek to understand current (2020) inequality in nutrient supply and use this to consider the impact on future food system scenarios with a view to better-informing conversations about how the food system might change to deliver sustainable development goal #2 of Zero Hunger. Understanding the present state of global nutrient distribution is a crucial step in identifying the areas of disparity and creating credible solutions. Food supply is linked to nutrient distribution and is a key component of both sustainable food systems and sustainable healthy diets.

Initially, the study modeled the current distribution of nutrient supply at the country level against population requirements. This process generated both a global sufficiency distribution for each nutrient and country-specific sufficiency patterns across all the nutrients. Subsequently, the research aimed to utilize this information to address the following questions:

1 In a scenario where the total global nutrient supply is sufficient, what adjustments are necessary to close nutrient inadequacies by redistribution from those who have more than enough? What approaches, or foods, could help countries with shortages to secure adequate nutrient supply?
2 Looking into the future, what is the impact of unequal distribution of food and nutrients on the food production required to deliver sufficient nutrition to everyone on the planet? How much more of each nutrient is required to accommodate the reality that many people consume more than the minimum requirements for health, thus potentially depriving others of an adequate intake?

This study provides a method to set revised minimum supply targets for future scenarios in a manner that accommodates inequality at a country level and to apply these in global food nutrient balance models such as the DELTA Model® (5) to deliver more realistic projections for the future. For example, an oft-quoted statement is that we need to increase global protein production by 70% by 2050 to meet the "needs" of the changing population (13), yet the study by Smith et al. (5) shows we could make do with current production. Which is correct? This study provides another approach to answering the future supply question for protein and other nutrients.

Methods

Quantifying nutrient gaps

Data from the DELTA Model® (version 2.2) were used for this analysis. The methodology used to calculate the nutrient supply at a country level is detailed in a previous study by Smith et al. (5) but is described briefly here. In this study, global supply values from 2020 were used.

The DELTA Model® used the food balance sheets from the United Nations Food and Agriculture Organization (14), which contains the total supply of food items intended for human consumption after trade, non-food uses, and supply chain losses. Food item quantities

are further adjusted for consumer waste using a second FAO source (15).

The food items are matched to food composition data from the United States Department of Agriculture to calculate the total quantity of nutrients on a country basis. For protein and the indispensable amino acids (IAAs), the values are adjusted for digestibility using true ileal digestibility coefficients from literature sources (16, 17). The results can be compared to national nutrient requirements, which are calculated using demographic data for the age and sex proportions of the population and the European Food Safety Authority (EFSA) nutrient reference values (16, 17). The target intake values are defined as the population reference intakes (PRI, where available) or adequate intakes when PRI is not available. IAA requirements are determined from the protein requirements (g/kg body weight) and the reference amino acid patterns (g/kg protein). The method is the same as used in the DELTA® model, and the corresponding section of the supplementary material from (5) is included in the Supplementary material.

The ratio between the current supply of a country of a given nutrient and its target intake determines the sufficiency ratio. This sufficiency ratio is used as an indicator to display whether current nutrient supplies are adequately meeting national target intake values. Figure 1 is introduced here to illustrate the method. The set of national sufficiency ratios provides a sufficiency distribution across the global population as illustrated by the solid "Initial" line. This does not consider the impact of within-country variation in food intake and nutrient supply, which adds additional variation. Country-level nutrient sufficiency is a necessary—but not sufficient—condition for nutrient adequacy within a population.

In a utopian scenario, with equitable global distribution and consumption, the sufficiency ratio of all countries for a given nutrient is equal. Global sufficiency is achieved by ensuring the average global supply exceeds the average global requirement. This is the default use of the DELTA Model®, which leads to the design of bare minimum scenarios for global food and nutrient supply scenarios, in which it is "possible" to meet global nutrient requirements.

The variation that exists between countries means that even when average global sufficiency is well above 1.0 there may be a significant proportion of the global population that live in countries that have an insufficient supply. For the years covered by the food balance sheet data—in this case 2020—the global nutrient gap can be calculated as the additional amount of a nutrient required to have brought all countries with insufficient supply up to a sufficiency value of 1.0 without changing the supply to the other countries. This is expressed as a percentage increase in the global supply of the nutrient.

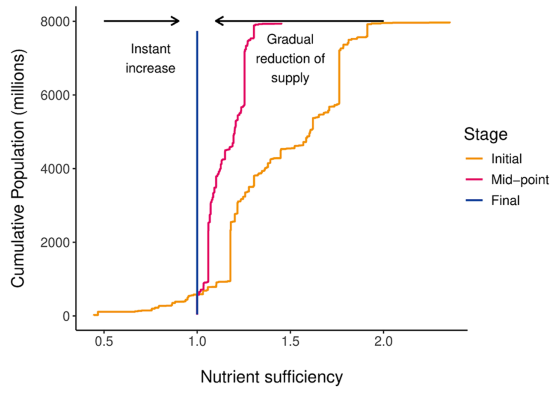

FIGURE 1
Conceptual diagram of nutrient distribution and re-distribution across the global population. Colors and linetype represent the stage of supply reduction and text labels, and arrows indicate the direction of increase or decrease.

Global scenarios for nutrient adequacy

When creating food production scenarios for future years, the challenge is setting realistic and practical nutrient supply targets that have the potential to ensure that all global citizens have access to an adequate supply. The first aspect of this is straightforward in setting the minimum sufficiency target for nations with an inadequate supply to 1.0.

The second aspect is setting expected future sufficiency levels for countries currently enjoying a more than adequate supply. Consumption of most nutrients above the required level does not cause harm to the individual, and people derive considerable pleasure from eating food. However, at high intake levels, some nutrients may be toxic (18). Apart from reducing energy intake the nutritional benefit of an individual reducing intake of a nutrient that is currently oversupplied is abstract and remote—more nutrients available for someone else—which limits the drive for rapid change, unless driven by external forces (availability, affordability). Even when individual change occurs rapidly, an extension of change over groups, countries, and globally takes much longer and changes are complex to implement due to the inherent interconnected nature of food systems (2). A reduction in nutrient intake by those currently enjoying a surplus should realistically be seen as a decades-long process and, to be "practical," future scenarios should recognize this. Changing systems at a gradual rate, as one of the options presented in this study, would present less long-term stress on the food system while allowing for the necessary changes.

One approach is to set the nutrient "needs" of countries currently enjoying more than sufficient supply to linearly reduce from current levels to converge with the basic requirement in a future year (e.g., 2050). Combining these two aspects enables future minimum sufficiency targets for countries to be set by Eq. 1.

$$S_{i,j}(k) = 1 + \max\left(\frac{y-k}{y-2020}\left(S_{i,j}(2020)-1\right),0\right) \quad (1)$$

where S is the sufficiency ratio, i is the nutrient, j is the country, k is the year of interest, and y is the convergence year (e.g., if $S(2020) = 1.6$, $y = 2050$ and $k = 2040$ then $S(k) = 1.2$).

A slightly modified approach that allows setting a sufficiency convergence point $S_i(y)$ that is greater than 1.0 is given by Eq. 2.

$$S_{i,j}(k) = S_i(y) + \frac{y-k}{y-2020}\left(\max\left(S_{i,j}{}^{*}(2020),1\right) - S_i(y)\right) \quad (2)$$

Summing across the globe, we can calculate the required global nutrient sufficiency to achieve the specified transition path. This involves summing up the required alterations in supply for each country.

Results

Quantifying nutrient gaps

The distribution of nutrients for the global population is displayed in Figures 2A–E. Results show a range of nutrient disparities across the global population.

The macronutrient results (Figure 2A) show considerably narrower ranges of nutrient sufficiency compared to other nutrient groups such as the amino acids, seen in Figure 2E. This is not that surprising as macronutrients are linked to food bulk and satiety, limiting consumption at the upper end, and shortages (particularly of energy and protein) have acute and severe consequences at the lower end. Fat was the most limiting macronutrient in this analysis with 2.84 billion people in countries with an inadequate supply although approximately 1.7 billion of these are within 10% of the target level. Protein was the least limiting macronutrient with 570.3 million people short, and fiber showed the largest range among the macronutrient results with some groups having a supply 3.5 times greater than the target intake value.

Figure 2B shows that calcium is the most limiting nutrient overall with approximately 6.7 billion people in countries that appear to have insufficient dietary calcium supply based on calcium

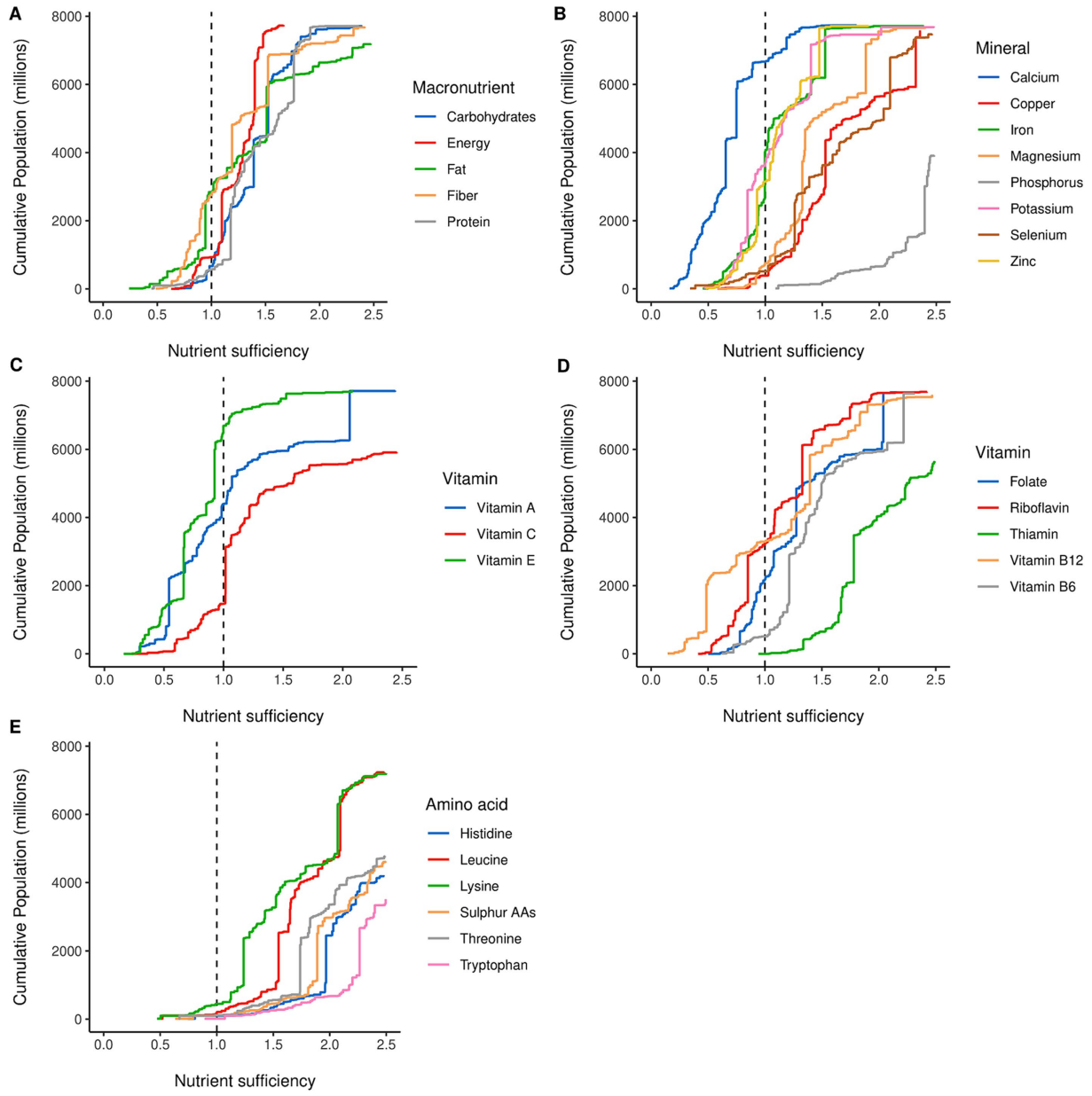

FIGURE 2
Cumulative distribution of nutrients across the global population in 2020. The *x*-axis denotes the nutrient sufficiency at a country level; the *y*-axis is the global cumulative population. **(A)** Distribution for macronutrients, **(B)** distribution for minerals, **(C,D)** distribution for vitamins, separated between non-B and B vitamins, and **(E)** distribution for indispensable amino acids. Colors are individual nutrients indicated by the subplot legend.

derived from primary food products and without considering fortification or supplements. This contrasts with phosphorous where the global population is well supplied. The remainder of the minerals studied form two distinct groups: iron, zinc, and potassium show very similar distribution curves with approximately half of the global population at or below an adequate supply; copper, magnesium, and selenium form another cluster with much lower national deficits.

The vitamins were split into two groups to increase visibility as can be seen in Figures 2C,D. Tail of vitamin B12 is the result of Mongolia and Hong Kong having sufficiency ratios of 8.4 and 9.2, respectively, which stems from the large amount of meat (including offal) reported as available as food in these countries. Vitamin B12 had the lowest minimum, and highest maximum, nutrient sufficiency ratios of all the nutrients considered in this study. Vitamin E had similar results to calcium, with approximately 6.7 billion people in countries currently undersupplied with reference to the target intake. Thiamin had one of the lowest nutrient gaps with approximately 210.5 thousand people affected by nutrient insufficiency worldwide (0.003% of the global population).

The IAAs show similarly shaped distributions. This group is the least limiting compared to the other nutrient groups, with most countries having an adequate supply for almost all the IAAs. Lysine was the most limiting IAA with 443 million people (5.7%) in countries currently undersupplied. When comparing lysine and overall protein sufficiency ratios, all countries with sufficient protein also had sufficient lysine, except for Afghanistan where the lysine sufficiency ratio was 0.8.

Table 1 shows the summary results for 2020 and includes a calculation of the minimum increase required to close the nutrient gap for all countries with insufficient supply without reducing the supply for those who were above a sufficiency ratio of 1.0. For the IAAs, only small increases in supply are needed, as most of the population is well supplied and the high global sufficiency levels indicate that by redistribution it could be possible to decrease supply and yet maintain the entire global population above 1.0. The minimum increases in supply were less than 10% for the other nutrients, except for calcium (51%), vitamin E (30.9%), vitamin A (17.8%), and vitamin B12 (16.3%).

Comparing the percentage of the global population undersupplied and the change in supply required to address this provides some interesting results. Iron, for example, showed that while 51.9% of the population did not have adequate supply, a 6% increase in supply would be sufficient to close the gap as many countries were very close to an adequate supply. In comparison, vitamin B12 showed a lower value for the proportion of the population undersupplied and a higher overall global sufficiency than iron; however, as there was a much wider distribution between countries, a much larger increase (+16.3%) was required to close the undersupply gap. These results demonstrate the importance of examining the inter-country distribution of nutrients as well as global adequacy when considering the performance of the current or a proposed future food system. Upon ranking based on global sufficiency and minimum change criteria, the order of priority shifted. Specifically, vitamin B12 ascended from eighth to fourth place, while zinc descended from fifth to ninth.

The same data can be used to look across the supply of all nutrients for a single country. For example, Figure 3 shows the 2020 nutrient sufficiency estimates for Kenya. These show supply gaps for five minerals and four vitamins in addition to protein and total calories. Targeting nutrient adequacy here requires foods that are good sources of calcium, vitamin E, zinc, vitamin B12, etc. The equivalent charts for the other countries covered by the FAO Food Balance Sheets are available within the latest version of the DELTA Model®.

Global scenarios for nutrient adequacy

Figure 4 shows the possible changes for a selection of nutrients when insufficiencies are resolved in countries that have them and oversupplied countries have their supply reduced to 1 over a 30-year convergence period (from 2020 to 2050). Results for all other nutrient groups can be found in the Supplementary material. A comparison of the curves shows the impacts of the linear reduction model application. For calcium and vitamin E, much of the population was undersupplied, and filling the shaded area dominates future changes. This contrasts with the protein, phosphorous, lysine, and thiamin where the changes are dominated by a reduction in supply above the sufficiency line.

The effects of redistributing nutrients from all countries, as depicted in Figure 3 (with Kenya as an example), on total nutrient requirements can be observed in Figures 5A–D. This is shown as a percentage change in global supply over 2020–2050 required to deliver the minimum nutrient requirements of the 30-year transition model. The starting point for all the curves is the 2020 supply that would have been required to meet minimum requirements for all countries without decreasing supply where this was more than adequate.

The curves show interesting differences. For some nutrients, supply must increase between 2020 and 2030 as the nutritional demands of changing global demographics exceed the amount released by reductions elsewhere. For calcium (Figure 5B) and vitamin E (Figure 5C), a decrease in minimum supply is never achieved. A significant shift in the current supply of calcium and vitamin E is required to achieve nutrient sufficiency. Energy and fiber show a peak between 2030 and 2040 before decreasing to a final supply near the 2020 starting point (after closing gaps). Minerals and vitamins in Figures 5B,C show a diverse set of changes where some nutrients show significant increases (calcium and vitamin E) and others such as phosphorus and thiamin show substantial decreases. The IAAs (Figure 5D) show a significant reduction as the redistribution effects allow less nutrients to be produced, despite increases in population compared to 2020. Another way of reading these curves is that when values decrease over time in this manner the nutrient is unlikely to be limiting for global nutrition.

Discussion

Intercountry comparison of nutrient distribution

Results showed the current distribution of nutrients (both macro and micro) across the global population. Our study shows that the world's 2020 food supply could—with the exceptions of calcium and vitamin E—nourish the world's population, but that unequal distribution of food means that for almost all nutrients there is a portion of the population that is not adequately supplied. Studies by Wood et al. (19) and Wang et al. (20) have come to similar conclusions.

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TABLE 1 Summary results by nutrient showing global sufficiency value in 2020, the proportion of global population living in countries without a sufficient supply, the minimum increase in global supply required to bring all countries to basic sufficiency, and top 10 rankings based on the sufficiency score and minimum change.

Nutrient	Global sufficiency	Top 10 ranking by global sufficiency	Population in countries undersupplied as %	Minimum change required as %	Top 10 ranking by minimum change
Macronutrients					
Energy	124%	10	12.1%	1.5%	
Carbohydrate	139%		8.6%	0.5%	
Fat	137%		36.8%	4.1%	10
Fiber	122%	9	35.5%	5.4%	8
Protein*	143%		7.4%	1%	
Amino acids*					
Histidine	244%		1.3%	0.1%	
Leucine	183%		2.1%	0.3%	
Lysine	171%		5.7%	0.7%	
SAA (Cys + Meth)	233%		1.5%	0.1%	
Threonine	222%		1.5%	0.2%	
Tryptophan	272%		0.2%	~0%	
Minerals					
Calcium	68%	1	86.1%	51%	1
Copper	168%		5%	0.4%	
Iron	110%	6	51.9%	6%	7
Magnesium	145%		9.7%	0.5%	
Phosphorous	268%		0%	0%	
Potassium	109%	4	46.6%	8.2%	5
Selenium	166%		6.7%	1.1%	
Zinc	110%	5	40.6%	5.4%	9
Vitamins					
A	108%	3	56.8%	17.8%	3
B1—Thiamine	212%		0%	0%	
B2—Riboflavin	114%	7	42.1%	8%	6
B6—Pyridoxine	152%		6.6%	0.9%	
B9—Folate	135%		28.3%	3%	
B12—Cobalamins	115%	8	42.5%	16.3%	4
C	154%		19%	3%	
E	80%	2	86.5%	30.9%	2

*Protein and amino acid sufficiency values are given after adjusting for ileal digestibility.

In the study by Wood et al. (19), the global food system, including food trade, waste, and conversion of food to non-food uses, was examined to highlight current trends and identify the nutritional potential of the food system. The authors found that there was sufficient food globally to meet global nutrient demands if equally distributed for the year 2018, with folate being the most limiting nutrient. However, Wood et al. (19) also suggest there is significantly more capacity in the food system, including the ability to meet the protein needs of an additional 11 billion people by redistribution of excess consumption, which does not align with our findings.

A more recent study by Wang et al. (20) found that calcium, vitamin B12, vitamin B6, iron, vitamin A, and zinc were undersupplied globally, which aligns with the trend in our results. However, they arrived at significantly lower figures for global food and nutrient supply, despite using similar data sources and methods. It appears that Wang et al. (20) subtracted pre-harvest or on-farm losses from the Food Balance Sheet production data, where it is our understanding that the FBS production figures represent the commodities that leave the farm or fishery (and thus enter the government production statistics used as the basis of the FBS) and have already accounted for such losses. Wang et al. (20) presented results depicting the sufficiency of nutrients across global populations, aggregated by regions, where we have taken a per-country approach.

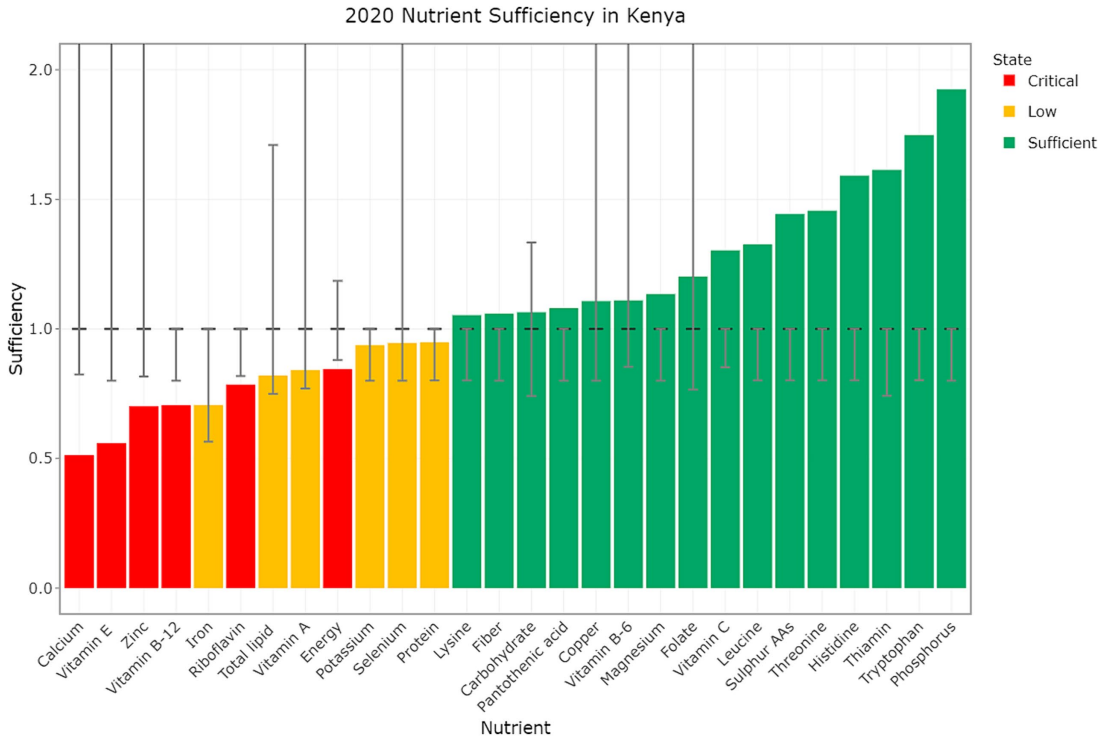

FIGURE 3
Nutrient sufficiency in Kenya for the Year 2020. The bars represent the level of sufficiency, while the dashed black line indicates the threshold for nutrient sufficiency based on PRI. The lower limit is either the EAR if this is available or 20% below the target if not. Error bars denote upper and lower limits where applicable, and colors signify different states of sufficiency with values below the lower limit shown as critical (red), values between the lower limit and the target as low (gold), and values at or above the target as sufficient (green). Upper limits are only shown where these fit within the *y*-axis bounds.

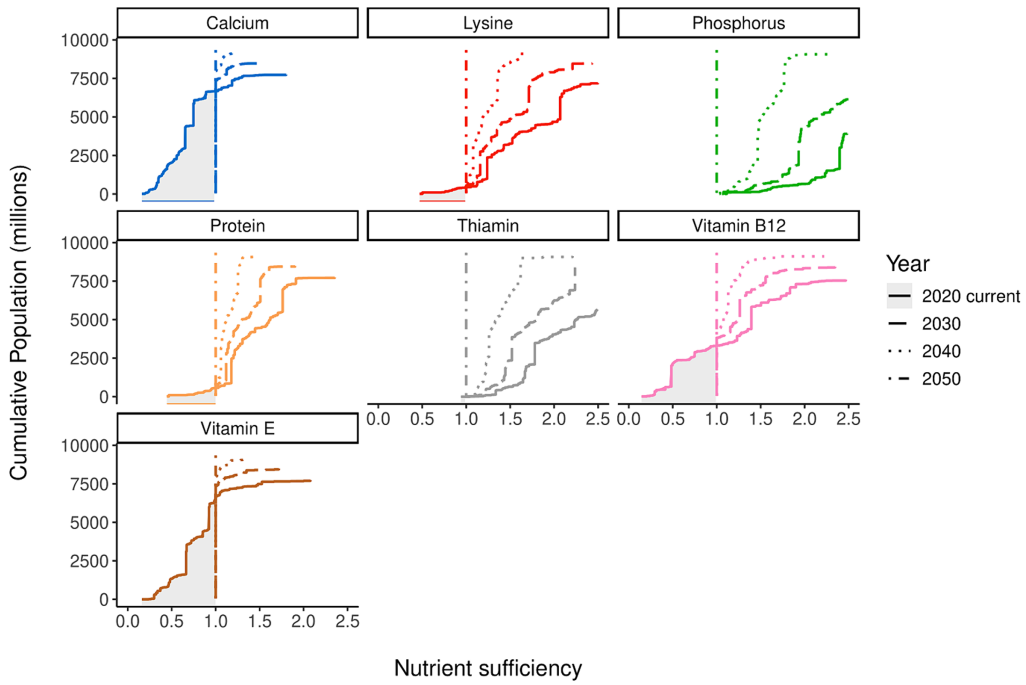

FIGURE 4
Potential change in country-level nutrient sufficiency based on a future convergence date of 2050. Colors indicate nutrients, with the linetypes indicating the year, which includes the population demographics and prospects in that year. The *y*-axis is the cumulative population; the *x*-axis is the nutrient sufficiency ratio.

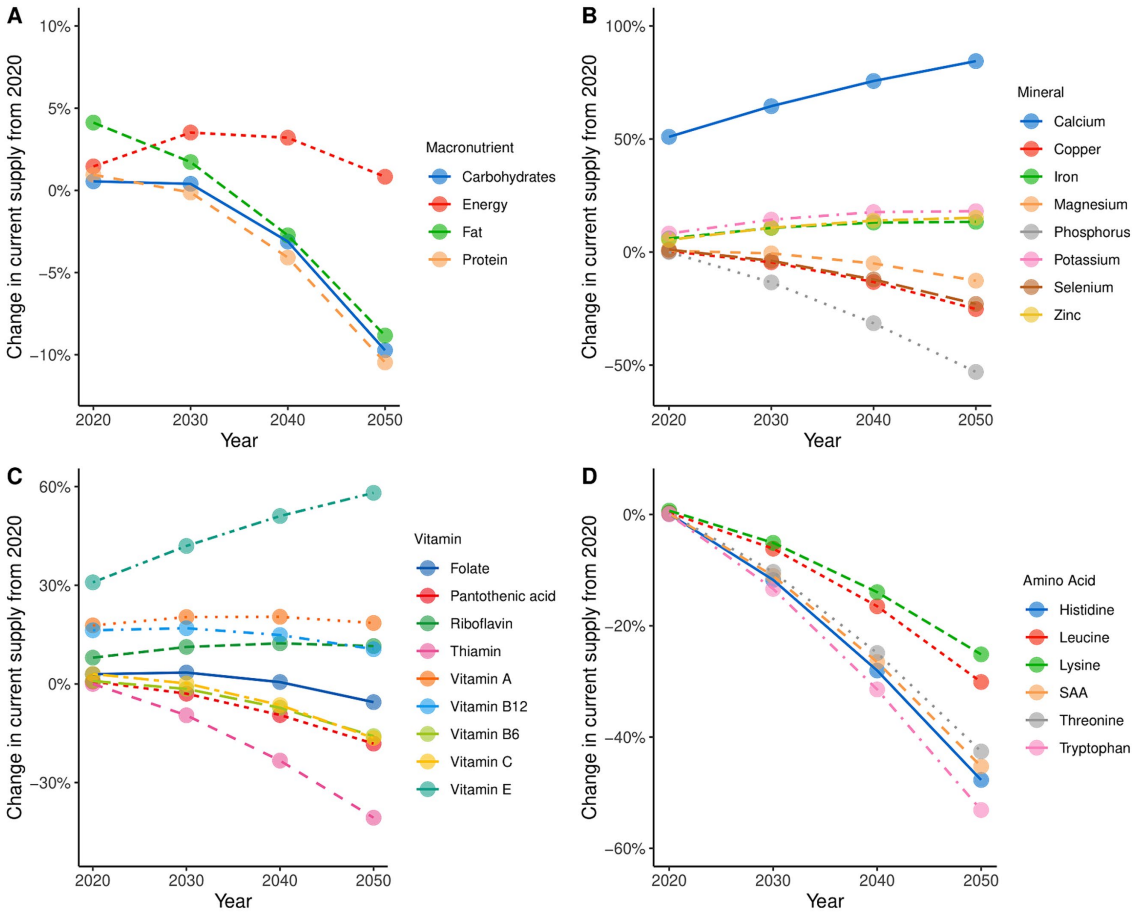

FIGURE 5
Required changes to global nutrient supply compared with 2020 in order to meet the minimum requirements for all countries. **(A)** shows the changes in macronutrients, **(B)** shows the changes in minerals, **(C)** shows the changes in vitamins, and **(D)** shows the changes in amino acids. The *y*-axis shows the percentage change with reference to the current 2020 supply and is scaled to the nutrient group. The *x*-axis is the year. New supply values are based on the change in the target intakes for the future population. The new target intakes are based on the linear reductions from the oversupplied parts of the population and the increase for those who are undersupplied based on a convergence date of 2050. The *y*-axis scales vary significantly between the nutrient groups.

A significant outcome of our study is highlighting the connection, and contrasts, between global nutrient sufficiency and the impact of food distribution. Take iron, for instance; the global supply is 110% of requirements, yet 51.9% of individuals are in countries that are undersupplied to varying degrees. Wang et al. (20) indicated that iron was almost sufficiently supplied but identified moderate deficits in Southeastern Central Asia, Oceania, Sub-Saharan Africa, and Latin America—consistent with the shortfall for iron observed in our results. The study at hand did not incorporate a regional analysis, contrasting with the approach of Wang et al. (20). However, future research endeavors could aim to identify regions experiencing nutrient insufficiency alongside high trade activity and elevated GDP *per capita*, similar to the study presented by Bell et al. (12). This endeavor could begin to create a perspective on the linkage between nutrient supply distribution and broader global system dynamics. Insights from the nutrient trade dynamics presented by Smith et al. (5) may offer valuable guidance for such prospective investigations.

A further layer of complexity exists when considering the variability within countries due to dietary choices, food availability, and affordability. To examine the intra-country nutrient distribution, Passarelli et al. (21) took a bottom-up approach and used dietary data sets from 31 countries to model nutrient adequacy against estimated average requirements (EAR) for a range of nutrients. This demonstrated significant within-country variation and significant differences between women and men, with women generally having less adequate intakes. Within-country intake distributions tend to be skewed with a tail toward the upper end, these distributions require at least three parameters (mean, coefficient of variation, and skewness) to be properly characterized, and the shape of the distribution has a significant impact on predictions of the portion of the population with inadequate intakes. Even where national-level supply appears to be adequate, a large portion of the population may have inadequate intakes (22). This within-country variation is additional to the between-country variation we have characterized.

Within this study, we have used PRIs as these represent the amount of nutrient required per person to meet the needs of 97.5% of the population within each of the gender and age bands. If a country has sufficient supply to meet the demographically weighted PRI, then in the absence of distribution inequality within the country this provides enough for the needs of almost every citizen. The alternative

approach of using EARs would imply that for any country that just meets the target level for a nutrient, 50% of the population would be adequately supplied and 50% undersupplied, even without considering the impact of internal distribution effects.

Duro et al. (23) constructed a simplified food index to assess the resource use of food supply of different countries based on the portion of food energy from plant and animal sources, with the animal fraction multiplied by five to reflect the greater feed and thus cropland demand and presented this by country income category between 1990 and 2013. For high-income countries, this remained almost constant, low-income countries had a slight increase, and intermediate categories showed larger increases—in particular, China. They used the Theil index (a measure of inequality) which showed a decrease in inequality over the period of study—from 0.075 to 0.05. D'Odorico et al. (24) calculated Gini coefficients for food at a country level using a calorie-based analysis and showed that the level of inequality in food production (0.57 in 2010) was significantly greater than for food availability (0.23 in 2010). Our findings suggest that food availability remains a significant concern, and there is potential for increasing inequality if we persist on the current trajectory. D'Odorico et al. (24) also noted "Although the existence of country-average food availability above the malnourishment level is an important prerequisite for food security, within-country inequalities may still prevent part of the populace from having adequate access to food." Future research in this field could explore nutrient distribution within countries to determine whether well-supplied nations are more likely to have adequate nutrient provision across their regions, even when the overall food supply meets nutrient sufficiency criteria. In a similar study Bell et al. (12) investigated inter-country distributions of agriculture production and health status metrics, and a range of nutrients between 1970 and 2010. To measure inequality, Gini coefficients were given for all variables in both 1970 and 2010 and largely show a reduction in inequality at a country level over this period. Our study starts with the level of nutrient inequality present in 2020 and generates global nutrient supply targets that would bring all countries to an equal and adequate supply in the chosen convergence year of 2050.

A challenge in changing nutrient intakes is that we consume foods, not nutrients, and changes need to be considered from the perspective of an individual's food intake or the production of foods at regional, national, and global scales. The apparent oversupply of many nutrients is often the consequence of consuming foods that are critical to achieving sufficiency of less abundant nutrients, and large reductions are unlikely to be realized, unless the constrained nutrient is delivered from an alternate source. Translating this back into food production or dietary scenarios requires the use of tools such as the DELTA Model® or dietary nutrient models that link nutrient supply to foods produced or eaten.

Limitations to these results include uncertainty on the final form in which the foods are consumed, which may impact the nutrient content, both from potential loss of nutrients through processing and food preparation, and not allowing for fortification of micronutrients where this is common practice. Beal et al. (22) showed that fortification has reduced micronutrient deficiencies in many developed countries; however, many low-income countries—where the need is greatest—do not have fortification legislation in place. Selection and development of crop varieties with high levels of micronutrients (biofortification) is also an option that is not currently considered in the modeling. Another limitation of this study is the variability in food composition data, particularly for minimally processed foods (25–27). Addressing this variability could involve using location-specific food databases, instead of assuming global consistency, though this approach would introduce complexity to the modeling process.

Another limitation is modeling in-home waste and the inedible portion of foods uses data that is comparatively coarse and dated and may not reflect practices in all countries—especially where nutrients are scarce. For example, fish bones are considered part of the inedible portion, but could be a significant source of calcium in some countries. Canned fish containing fish bones has a very high calcium density score, whereas canned fish with bones removed is low (28).

Only the bioavailability of protein and the IAAs have been included in the analysis as these are largely driven by the protein source itself, rather than other dietary factors. The absorption of calcium, iron, and zinc is impacted by anti-nutritional factors such as phytate and oxalate that are more prevalent in plant-rich diets. This would potentially further reduce the effective supply of these nutrients in some countries. The short-term impact of protein intake and IAA content at the meal level is also outside the scope of this analysis, which assumes all available foods are equally distributed across all meal occasions.

Impact on protein supply

While much research has emphasized increasing protein supply, our findings indicate that the micronutrients often accompanying protein should receive greater attention. Regarding protein, our results reveal that 570 million people (7.4% of the global population) reside in countries where the protein supply falls short of meeting the adult requirement of 0.8 g of protein per kilogram of body mass per day. Most of these are poorer countries in Sub-Saharan Africa or Latin America. Any steps to increase protein supply must first fit the needs of these people and the supply chains that serve them. This drives toward solutions early in the supply chain, such as improving domestic agricultural productivity.

It is also important to consider the other nutrients that are lacking in these countries to focus on protein sources that are also rich in these nutrients. Using the example of Kenya (Figure 3), in addition to a protein gap there are significant gaps in eleven other nutrients that must also be considered. Selecting the right combination of protein-rich foods may help to address these gaps also, either through a shift in domestic production or trade, noting that some of these gaps exist because the readily traded staple food grains are not good sources of these nutrients.

Global scenarios for nutrient adequacy

Figure 4 shows the impact of linearly reducing supply targets in countries where there is currently a surplus, on future global requirements for each nutrient. The results shown are based on 2050 convergence to a just adequate global supply of all nutrients.

Future protein supply

Under the base scenario, global digestible protein requirements decrease through 2050 ending 10% below the total 2020 supply, aligned with the conclusions of Smith et al. (5). For

many countries, this represents a significant reduction in protein; for example, the USA would decrease from a sufficiency value of 1.9, China would decrease from 1.75—and given free choice by consumers is unlikely to be realized over the 30 year convergence period, if ever. The sufficiency target is also based on current recommendations of 0.8 g of protein per kg of body weight per day. The 2013 ESPEN expert group (29) suggested a range of 1.0–1.2 g for healthy older adults and 1.2–1.5 g for those malnourished or at risk of malnourishment due to illness.

Using Eq. 2 for target setting and changing the parameters, we can explore a range of different protein supply scenarios for 2050. As noted above converging on a just adequate intake using the current targets, we require 10% less protein in 2050 than was available as food in 2020. If we allow more time to make this transition by pushing the convergence date out to the end of the century, extending the adaptation period by 50 years, this requires a 12% increase in protein by 2050. If the only change is to close the supply gaps where they currently exist, and all other countries maintain their current level of consumption then we require a 26% increase in protein supply by 2050. Adopting an increased 2050 target of 1.2 g/kg/day for everyone, we need an increase of 34%. If everyone on the planet had the protein supply available to China in 2020 ($S = 1.75$) then we require an increase of 57%, and if we converged on the 2020 sufficiency of the USA ($S = 1.9$) then the increase becomes 70%, which is similar to the high-end scenario proposed by Henchion et al. (13).

In all these scenarios, the required increase in bioavailable lysine is smaller than for total protein. For example, increasing the protein target to 1.2 g/kg/day only requires a 12% increase in bioavailable lysine, compared with a 34% increase in digestible protein. This indicates that the increased protein supply could come from lower-quality sources and still meet the required amino acid supply if there was a redistribution of higher-quality protein; that is, many people currently oversupplied could substitute a portion of their animal-sourced protein intakes with plant protein without limiting their protein utilization (as they are likely to be total protein, not IAA limited), making additional animal-sourced protein available to improve the diets of others, or reducing the global need for its production.

Other nutrients

Looking outside of protein and IAAs, calcium, vitamin A, and riboflavin all showed significant gaps in 2020 supply, requiring increases of 51, 17.8, and 8%, respectively, to close the existing gaps. Using Eq. 2 and projecting forward to 2050, with the added impact of global population growth, we can suggest a range of possible scenarios. Converging on adequate supply for everyone we arrive at an 88% increase in calcium, a 20% increase in vitamin A, and a 13% increase in riboflavin compared to 2020. Closing gaps where they exist now and maintaining current levels of supply where these are above adequate gives calcium +89%, vitamin A +45%, and riboflavin +34%. The comparatively small difference in calcium between these scenarios reflects the small portion of the global population that have a more than adequate food-based calcium supply. If everyone enjoyed the 2020 sufficiency levels of the USA, we get calcium ($S = 1.18$) +121%, vitamin A ($S = 1.07$) +27%, and riboflavin ($S = 1.75$) +95%.

Addressing current and future micronutrient gaps potentially requires much larger food system changes than meeting the basic needs for energy and protein. The scale of change required for many of the micronutrients requires emphasizing foods that are nutrient-dense—have a high level of important nutrients per unit of food energy—to fully nourish people and not just transition from protein-energy malnutrition to hidden hunger and/or obesity.

As previously discussed, converting these targets into realistic food system scenarios requires connecting nutrient requirements back to changes in food production and consumption. This drives toward prioritizing the production of nutrient-dense foods in the most environmentally efficient manner. Beal et al. (22) concluded that countries with adequate energy supply, but inadequate micronutrient intakes should focus on increasing the nutrient density of the foods consumed via a range of different approaches. They found that in most regions of the world, the micronutrient density index has improved over the last 50 years, except for sub-Saharan Africa.

Understanding current levels of inequality provides additional information for scenario models, especially for the near term when the extent of change will necessarily be limited. Changing food consumption patterns globally is a challenging process and is embedded in complex interactions that include prices, preferences, culture, location, and socio-economic status (30), none of which will be resolved rapidly, and potentially, the 30 year convergence period we have used is too optimistic. By setting the length of the adaptation period and a final convergence point for each nutrient, we can set targets for tools like the DELTA Model® that better reflect reality.

Conclusion

Modeling country-level sufficiency provides valuable insights into the availability of nutrients globally and provides additional perspectives on nutrient undersupply. Many nutrients that appear adequately supplied in global scenarios are undersupplied in many countries, including vitamins A, B12, and B2, and the minerals potassium and iron. Significant increases are required to close some of these gaps.

While the protein supplied in foods globally is already more than sufficient to meet the base needs of the 2050 population if equally distributed, the scale of the inter-country variation means there are significant shortages. A relatively modest production increase of 1%—targeting the needs of countries in deficit—would have closed the 2020 gap. In the absence of any changes in consumption patterns global food protein will need to grow 26% by 2050. A large portion of this growth must be focused on the needs of low-income countries in the form of affordable protein foods that also contain other nutrients that are in short supply, rather than the development of expensive high-tech protein food ingredients.

Any redistribution of nutrients, enabled by reductions in countries currently enjoying an abundant supply, will be a gradual process. Applying a multi-decade linear convergence to country-level sufficiency values provides a useful framework for enabling global food system models such as the DELTA Model® to move from utopian minimum production scenarios toward more realistic assessments of future needs.

While understanding nutrient needs is critical, it is also critical that we translate these into foods produced and diets consumed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AF: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – review & editing. RL: Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. WM: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1396549/full#supplementary-material

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Diet affordability: a key dimension in the assessment of sustainable food systems and healthy diets

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A promulgated global shift toward a plant-based diet is largely in response to a perceived negative environmental impact of animal food production, but the nutritional adequacy and economic implications of plant-sourced sustainable healthy dietary patterns need to be considered. This paper reviews recent modeling studies using Linear Programming to determine the respective roles of animal- and plant-sourced foods in developing a least-cost diet in the United States and New Zealand. In both economies, least-cost diets were found to include animal-based foods, such as milk, eggs, fish, and seafood, to meet the energy and nutrient requirements of healthy adults at the lowest retail cost. To model a solely plant-based least-cost diet, the prevailing costs of all animal-sourced foods had to be increased by 1.1 to 11.5 times their original retail prices. This led to the inclusion of fortified plant-based foods, such as fortified soymilk, and a plant-based diet that was considerably (34–45%) more costly. The first-limiting essential nutrients were mostly the vitamins and minerals, with special focus on pantothenic acid, zinc, and vitamin B-12, when transitioning from an animal- and plant-containing least-cost diet to a plant-only based least-cost diet. Modeled least-cost diets based on contemporary food costs include animal-sourced foods, at least for developed high-income US and NZ food economies, and potentially for developing low- and middle-income countries, such as Indonesia. Modeling of least-cost diets that consist exclusively of plant-based foods is feasible, but at a higher daily diet cost, and these diets are often close to limiting for several key nutrients. Diet affordability, as a key dimension of sustainable healthy diets, and the respective economic roles of animal- and plant-sourced foods need to be considered.

KEYWORDS

diet cost, diet optimization model, linear programming (LP), nutrient adequacy, adult, protein, animal-source foods (ASF), plant-based food (PBF)

1 Introduction

Sustainable food systems and healthy diets should be considered around four interconnecting dimensions: environment, society and culture, nutrition, and affordability (1). While the environmental dimension is well studied (2, 3), the nutritional quality and particularly economic affordability of sustainable diets are often overlooked (4, 5). Recently, the perceived environmental impact of food production and consumption is underlying a move toward a planetary sustainable healthy diet that is mostly plant-based. This is largely argued for, based on the high-level comparison that animal-sourced foods give rise to more greenhouse gas emissions per kg of food than plant-sourced foods (6). However, the sustainable plant-forward EAT-Lancet diet has been found to be nutritionally inadequate for

calcium, iron, zinc, and vitamin B-12, and unaffordable at a median global cost of US $ 2.84 per person per day (2011 food prices) for 24% of the world's population (7, 8). Diet affordability needs to be taken into account when considering globally sustainable dietary patterns, as the monetary cost of foods is a crucial determinant of food choice, diet quality, and food and nutrient security (5, 8–11).

The modeling of cost-minimized diets that meet recommended energy and essential nutrient requirements and are most affordable, is routinely conducted (9–18). The global median cost of a nutritionally adequate least-cost diet was found to be US $ 1.35 per day for the year 2011 (14), and US $ 2.32 per day for the year 2017 (17). These diet costs were based on food retail prices converted from local currency into US dollars in terms of purchasing power parity (PPP). The modeled diet was considered nutritionally adequate when it met the energy (2,329 kcal) and nutritional requirements for half of the population of healthy non-pregnant, non-lactating, 30-year-old adult women, as defined as estimated average requirements (EARs) or harmonized average requirements (H-ARs) (14, 17). In comparison, when nutritional requirements were based on recommended dietary allowances (RDAs), to estimate intake levels adequate for 97–98% of a healthy population, or adequate intakes (AIs), the median daily diet cost for men and women aged 19–50 years, was found to be US $ 2.62 and 2.45 (US $ PPP for the year 2017), respectively (18). On the other hand, the average daily diet cost for an average adult aged 19–50 years was US $ 2.71 (18).

A common approach to evaluate diet affordability is to use Linear Programming (LP) as a mathematical dietary optimization tool to minimize dietary cost under a given set of linear constraints (19–21). Here, LP provides unique solutions for the mixtures of foods available in the market that meet all the nutritional requirements of the adult, but do so at the lowest price possible. The commonly applied LP does not necessarily give rise to practical dietary patterns, but rather highlights the role of key food groups in assisting to meet nutrient needs at the lowest cost. LP allows the interrogation of multiple food mixtures and identifies the one dietary combination that meets all the stated nutrient requirements of the adult at the lowest cost. The purpose of this paper is to review recent country-specific LP modeling studies to determine the inclusion levels of animal- and plant-sourced foods in the formulation of nutrient adequate dietary patterns at the lowest dietary cost. This paper brings together our previously reported LP modeling work in the United States (US) (22) and New Zealand (NZ) (23), and ongoing LP modeling research in developing countries. Moreover, diet cost is an important focal point of attention when transitioning from a diet that contains animal- and plant-sourced foods to a plant-only based vegan diet. The extent to which the relative prices of animal-sourced foods needed to be increased to be excluded from nutrient adequate least-cost dietary patterns and the economic feasibility of plant-only nutrient adequate least-cost dietary patterns were evaluated (22, 23).

2 Modeling of least-cost dietary patterns

Dietary optimization using the LP approach involves the minimization or maximization of a linear function of a set of decision variables, while subjected to several linear constraints (19–21). LP can take into account simultaneously food costs, the supply of locally consumed foods, food serving sizes, food nutritional compositional data, and energy and nutritional intake requirements, in the formulation of least-cost (most affordable), nutrient adequate, and culturally acceptable dietary patterns. Here, the LP model aimed to minimize the cost of the optimal dietary solution by changing the decision variables, which were the quantities and corresponding costs of selected foods, according to the following equation:

$$f(x) = \sum_{i=1}^{N_f} c_i x_i$$

where $f(x)$ is the diet cost, N_f is the number of foods included in the LP analysis, c_i is the cost per unit quantity of food i, and x_i is the unit quantity of food i. The linear constraints applied in the LP model were daily estimated energy requirement, daily minimum and upper intake limits of nutrient requirements, and maximum limits on daily food serving sizes, and can be expressed using the following Equations 1–3, respectively.

$$\sum_{i=1}^{N_f} e_i x_i = E \tag{1}$$

$$m_j\left(j = 1,2,\ldots,N_n\right) \le \sum_{i=1}^{N_f} n_{ij} x_i \le u_j\left(j = 1,2,\ldots,N_n\right) \tag{2}$$

$$0 \le x_i \le 3r_i\left(i = 1,2,\ldots,N_f\right) \tag{3}$$

where N_f is the number of foods included in the LP analysis, e_i is the energy value per unit quantity of food i, x_i is the unit quantity of food i, E is the daily estimated energy requirement to meet, m_j is the daily minimum required intake level of nutrient j, N_n is the number of nutrients included in the LP analysis, n_{ij} is the amount of nutrient j per unit quantity of food i, u_j is the daily upper intake limit of nutrient j, and r_i is the daily recommended serving size for food i.

At a country-specific level, the LP approach was used to model nutrient adequate least-cost diets for adults in the US (22), NZ (23), and developing countries, such as Indonesia. An empirical approach was used for the linear constraints on food serving sizes, based on the assumption that individuals commonly consume three main meals per day, to limit the daily maximum allowable amount of each food or food subgroup to be no more than three servings per day. In the US (22), some additional pragmatic constraints were applied, to limit energy-rich foods (bread and bread rolls, tortillas, and rice) to no more than 2 servings per day, and to limit fat-rich foods (margarine and vegetable spreads, peanut butter, mayonnaise and salad dressings) to no more than 1 serving per day. Moreover, in NZ (23), margarine was limited to no more than two servings per day, rather than 3 servings per day, for the least-cost modeled diet to be within the acceptable macronutrient distribution of 20–35% of energy from fat. As the modeling study in Indonesia is preliminary, each food or food subgroup was initially constrained to be selected to no more to one serving per day. The constraints for daily energy and nutrient requirements of average adults aged 19–50 years, that were applied in

the LP modeling studies in the US (22), NZ (23), and Indonesia are given in Table 1. Several least-cost dietary scenarios were explored in a step-wise manner, to evaluate dietary LP model outcomes for nutritional adequacy and cost.

2.1 Least-cost diets in the United States

The modeled nutrient adequate baseline least-cost diet in the US, using the most up-to-date, reliable, and comprehensive data on foods and food prices, was shown to have a daily diet cost of US $ 1.98 (2009–2010 US food prices), and comprised dairy milk, eggs, and fish as animal-sourced foods among the 15 foods in the diet (22). Milk (26%), fortified breakfast cereals (14.2%), potatoes (12.6%), and legumes (12.4%) largely contributed to the total diet cost. The fat-rich foods, such as margarine (1.4%) and mayonnaise (1.5%), and the carbohydrate-rich foods, such as corn tortillas (1.4%) and bread rolls (2.3%), accounted the least to total diet cost.

Increases in the baseline national retail prices of all animal-sourced foods by 5, 10, 15 or 20% were found to marginally change dietary composition, and to gradually and slightly increase diet cost up to US $ 2.14 per day (22). To model a dietary scenario whereby all animal-derived foods were no longer included in the least-cost diets by incrementally (5%) increasing food prices, the prices of selected

TABLE 1 The nutritional constraints applied in the linear programming modeling analyses of nutrient adequate least-cost dietary patterns in the United States (22), New Zealand (23), and Indonesia, as daily energy and minimum level of nutrients required by average adults aged 19–50 years.

	United States	New Zealand	Indonesia
Energy	2,600 kcal	2,665 kcal	2,400 kcal
Carbohydrate	130 g		
Dietary fiber	31.5 g	27.5 g	33.75 g
Linoleic acid	14.5 g	10.5 g	
α-linolenic acid	1.35 g	1.05 g	
Protein	50.8 g	55 g	62.5 g
Calcium	1,000 mg	1,000 mg	1,000 mg
Chromium		30 µg	
Copper	0.9 mg	1.45 mg	0.9 mg
Iron	13 mg	13 mg	13.5 mg
Magnesium	355 mg	362.5 mg	
Manganese	2.05 mg	5.25 mg	
Molybdenum		45 µg	
Phosphorus	700 mg	1,000 mg	700 mg
Potassium	4,700 mg	3,300 mg	4,700 mg
Selenium	55 µg	65 µg	
Sodium	1,500 mg	670 mg	1,500 mg
Zinc	9.5 mg	11 mg	9.5 mg
Biotin		27.5 µg	
Choline	487.5 mg		
Folate	400 µg (DFE)	400 µg (DFE)	
Niacin	15 mg	15 mg	15 mg
Pantothenic acid	5 mg	5 mg	
Riboflavin	1.2 mg	1.2 mg	1.2 mg
Thiamin	1.15 mg	1.15 mg	1.15 mg
Vitamin A	800 µg (RAE)	800 µg (RE)	625 µg (RE)
Vitamin B-6	1.3 mg	1.3 mg	
Vitamin B-12	2.4 µg	2.4 µg	
Vitamin C	82.5 mg	45 mg	82.5 mg
Vitamin D	15 µg	5 µg	
Vitamin E	15 mg	8.5 mg	
Vitamin K	105 µg	65 µg	

DFE, Dietary Folate Equivalent; RAE, Retinol Activity Equivalent; RE, Retinol Equivalent. Average adult daily nutrient requirements values were sourced from the Institute of Medicine (24), National Health and Medical Research Council (25), and Ministry of Health (26), for the United States, New Zealand, and Indonesia, respectively.

TABLE 2 The extent by which prevailing prices of animal-sourced foods selected in the linear programming modeling analyses of least-cost dietary patterns needed to be increased for their exclusion, in the United States (US) and New Zealand (NZ).

Food group	United States (US) 2009–2010 food prices	New Zealand (NZ) 2020 food prices
Milk	8.0x	2.20x
Eggs	11.5x	1.80x
Fish	6.5x	2.30x
Seafood		10.30x
Chicken	5.0x	1.95x
Turkey	3.0x	
Beef	5.5x	
Pork	2.5x	
Lamb		1.25x
Cold cuts and cured meats	2.0x	
Sausages		1.05x
Cheese	3.0x	3.95x
Yogurt	2.5x	
Ice cream	2.0x	
Mayonnaise (containing eggs)	5.0x	
Bread rolls (containing milk and eggs)	4.5x	
Mashed potatoes (containing milk and/or butter)	2.0x	
Egg noodles	2.0x	

Data adapted from Chungchunlam et al. (22) and Chungchunlam et al. (23).

animal-sourced foods had to be increased by 2.0 to 11.5 times their baseline costs (Table 2). The resulting plant-only least-cost diet contained 14 foods and had a daily diet cost of US $ 3.61 (22). The greatest contributors to total diet cost were fortified soymilk (37%), legumes (13.3%), fortified breakfast cereals (12.7%), and cabbage (9.7%). Unsurprisingly, energy-dense foods, such as corn tortillas (0.8%), margarine (1.1%), and vegetable oils (2.5%), contributed the least to total diet cost.

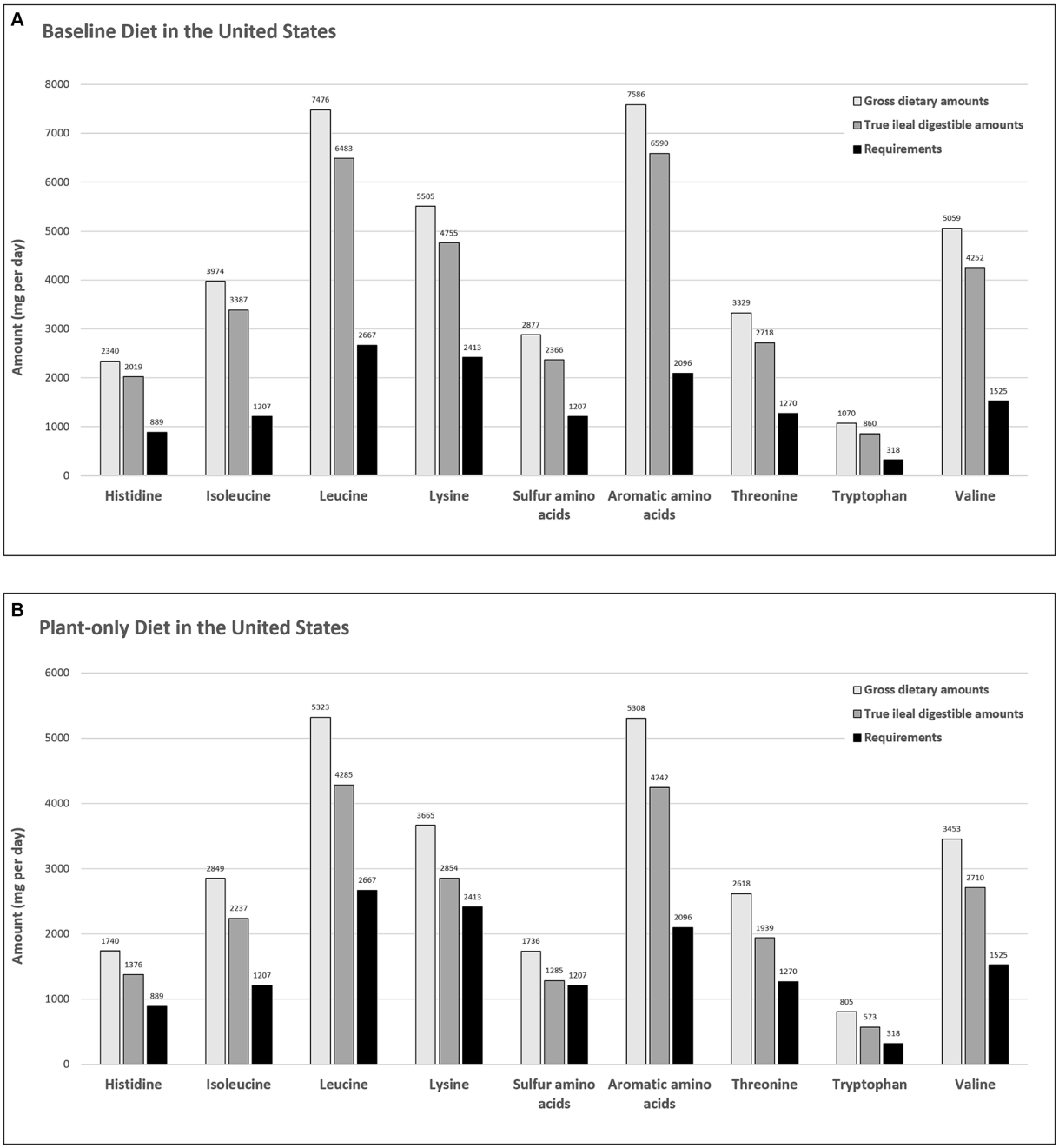
The nutrients that were supplied by both baseline (animal- and plant-containing foods) and plant-only modeled least-cost dietary scenarios at exactly their minimum requirements, were the essential fatty acid α-linolenic acid, potassium, choline, vitamin D, and vitamin E. Compared to being close to limiting in the baseline least-cost diet, vitamin C and vitamin K were adequately provided by the plant-only least-cost diet. A nutrient that was supplied at its minimum required level by the plant-only least-cost diet was pantothenic acid.

2.1.1 Protein quality of least-cost diets in the United States

Protein quality is considered to be a potentially important factor for assessing the inclusion levels of animal and plant food protein sources in least-cost dietary patterns. The protein quality of a food is dependent on its amino acid composition and the bioavailability of the dietary protein and dietary indispensable amino acids (27). Amino acid bioavailability in humans is best expressed as true (standardized) ileal amino acid digestibility, determined at the end of the small intestine rather than over the total digestive tract and corrected for endogenous amino acid losses (27, 28). The protein quality of least-cost diets in the US was not reported in our previous study (22). In our previous work, we described the amino acid composition of the foods on a gross, and not on a digestible basis, and it is thus relevant to explore potential effects of differences in amino acid digestibility among food types. To this end, amino acid contents of foods found in the LP modeled least-cost dietary patterns were corrected here for true ileal amino acid digestibility (29), and digestible indispensable amino acid scores (DIAAS) were calculated (27) and used to estimate the amount of utilizable protein in the least-cost diets.

The recommended dietary allowance (RDA) for protein for an average US adult, with a reference body weight of 70 kg for US adult men and 57 kg for US adult women, and a recommended protein intake of 0.80 g/kg body weight/day, is estimated to average 51 g of utilizable protein, as given in terms of bioavailable amounts of dietary protein that the adult human body can use (30). Based on the gross dietary protein and amino acid contents, the requirements for protein were sufficiently met by the baseline least-cost diet (89.4 g of gross protein, 176% of RDA) and the plant-only least-cost diet (77.2 g of gross protein, 152% of RDA). Dietary protein was mostly provided by legumes (27.6%) and milk (26.8%) in the baseline least-cost diet, and by legumes (27.8%) and soymilk (19.2%) in the plant-only least-cost diet, respectively.

Similarly, and when based on the LP analysis using gross dietary protein and amino acid contents, the indispensable amino acids were well supplied by the baseline least-cost diet (228–362% of RDA) and plant-only least-cost diet (144–253% of RDA). When corrections were made independently for true ileal amino acid digestibility, the indispensable amino acids still exceeded their nutritional requirements, but by lower proportions than when expressed on a gross dietary basis (Figure 1). True ileal digestible amino acid



Sulfur amino acids = Methionine + Cystine; Aromatic amino acids = Phenylalanine + Tyrosine

FIGURE 1
Daily indispensable amino acid requirements for an average adult (mg per day) vs. gross and true ileal digestible dietary amounts (mg per day) for the baseline **(A)** and plant-only **(B)** least-cost diets in the United States.

requirements were adequately met by the consumption of the baseline least-cost diet (196–314% of RDA) and plant-only least-cost diet (106–202% of RDA). Importantly, true ileal digestible sulfur amino acids (methionine + cystine) were at only 106% of their required level, when supplied by the plant-only least-cost diet.

DIAAS is the currently recommended method for dietary protein quality assessment and for calculation, requires a reference amino acid scoring pattern for the indispensable amino acids (Table 3). The Food and Agriculture Organization (FAO) has published amino acid scoring patterns for the calculation of DIAAS (27), and comparisons were made here using the recommended reference patterns for adults aged over 18 years old (FAO adult). It is important to note, however, that DIAAS is often based for regulatory purposes, on the amino acid reference pattern of a child aged 6 months to 3 years (FAO young child) (27), and this reference pattern was also applied in the present analysis. It was estimated that the DIAAS for the baseline least-cost diet, in relation to the FAO amino acid scoring patterns for the adult and young child, was 120 and 95%, respectively (Table 3). The baseline

TABLE 3 Calculation of digestible indispensable amino acid score (DIAAS) values for the baseline and plant-only least-cost diets in the United States, and the recommended reference amino acid scoring patterns against which DIAAS was calculated.

	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val	
True ileal digestible amino acid content of least-cost diets (mg/g protein)										
Baseline Diet	22.9	38.4	73.5	53.9	26.8	74.7	30.8	9.7	48.2	
Plant-only Diet	18.0	29.3	56.1	37.4	16.8	55.6	25.4	7.5	35.5	
Reference amino acid scoring patterns (mg/g protein)										
FAO adult[1]	15	30	59	45	22	38	23	6.0	39	
FAO young child[2]	20	32	66	57	27	52	31	8.5	43	
Digestible indispensable amino acid reference ratio[3]										**DIAAS (%)[4]**
Baseline diet										
FAO adult	1.53	1.28	1.25	1.20	1.22	1.96	1.34	1.62	1.24	120
FAO young child	1.14	1.20	1.11	0.95	0.99	1.44	0.99	1.15	1.12	95
Plant-only diet										
FAO adult	1.20	0.98	0.95	0.83	0.76	1.46	1.10	1.25	0.91	76
FAO young child	0.90	0.92	0.85	0.66	0.62	1.07	0.82	0.88	0.83	62

His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; SAA, Sulfur amino acids; Methionine + Cystine; AAA, Aromatic amino acids; Phenylalanine + Tyrosine; Thr, Threonine; Trp, Tryptophan; Val, Valine; DIAAS, digestible indispensable amino acid score.

[1]FAO adult reference pattern is based on the amino acid scoring patterns for adults aged over 18 years old, as recommended by the Food and Agriculture Organization (FAO) (27).

[2]FAO child reference pattern is based on the amino acid scoring patterns for young children aged 6 months to 3 years, as recommended by the Food and Agriculture Organization (FAO) (27). For regulatory purposes, this scoring pattern for young children is recommended for the calculation of DIAAS.

[3]Digestible indispensable amino acid reference ratio is obtained from the true ileal digestible indispensable amino acid content in 1 g of dietary protein (mg/g protein) divided by the same indispensable amino acid in 1 g of reference protein (mg/g protein), for a given reference amino acid scoring pattern.

[4]DIAAS, expressed as a percentage (%), is the lowest calculated digestible indispensable amino acid reference ratio multiplied by 100, for a given reference pattern.

least-cost diet provided adequate amounts of utilizable protein and indispensable amino acids, though true ileal digestible lysine was supplied at its lowest level. However, for the modeled plant-only least-cost diet, the DIAAS was 76% for the FAO adult reference pattern, and 62% for the FAO young child reference pattern (Table 3). The plant-only least-cost diet was estimated to contain respective amounts of 58.4 and 47.6 g of utilizable protein, and was potentially limiting for the sulfur amino acids in their digestible form. The current analysis highlights that protein quality is an important consideration when assessing sustainable diets, particularly for plant-sourced diets, and amino acid digestibility needs to be taken into account.

2.2 Least-cost diets in New Zealand

While the LP modeling study focused only on the US (22), the US government provides economic subsidies to the animal-sourced food sector (31). This may distort the US food market and affect the relative prices of animal-sourced foods compared with the retail prices of plant-based commodity crops (32, 33), which in turn would influence the outcomes of our LP analyses. Using food prices in the New Zealand (NZ) market, another LP modeling study was conducted, where the eating habits and food economic status are similar to the US, but where food subsidies imposed on animal-sourced foods are not found.

In agreement with the US LP modeling study, foods sourced from animals, such as dairy milk, eggs, and seafood, were found in the least-cost diet in NZ. The nutrient adequate baseline least-cost diet had a daily diet cost of NZ $ 3.23 (2020 NZ food prices; US $ 2.14), and the main contributors to total diet cost among the 13 foods were legumes (29%), milk (21%), and seeds (13.0%) (23). A plant-only nutrient adequate least-cost diet, with a daily diet cost of NZ $ 4.34 (US $ 2.87) (23), was modeled after 1.05 to 10.30-times increases in the prevailing retail prices of selected animal-sourced foods (Table 2). The majority of the total diet cost contribution by the plant-based foods was from fortified soymilk (47%), seeds (12.6%), pasta (10.5%), and legumes (7.3%). The essential nutrients that were commonly first-limiting in both dietary scenarios were calcium, selenium, biotin, pantothenic acid, vitamin A, and vitamin C, with the plausible addition of potassium. While molybdenum was found to be supplied well in excess of requirements, zinc, vitamin B-12, and vitamin D were found to be first-limiting when the least-cost diet was formulated with plant-sourced foods only.

2.3 Least-cost diets in developing countries

The above findings are specific to high-income countries, such as the US and NZ, and may not apply to developing low- and middle-income countries (8–17, 34, 35). In developing countries, the prices of animal-sourced foods may be relatively higher than the prices of plant-sourced foods. A LP modeling study in Indonesia has shown that dairy milk, chicken liver, and clams are needed in a least-cost diet, for the adequate provision of calcium, sodium, potassium, and vitamin A, for a daily diet cost of Rp 16,189 (US $ 1.09). These results

are preliminary, and should be viewed with some caution, but do mirror the results found for developed economies. Similar LP studies are currently being undertaken by our research group for the Philippines, Kenya, and Tanzania. Such countries are highly vulnerable to changes in food prices (8–11, 14, 17, 34, 35).

3 Discussion and conclusion

The economic dimension of sustainable diets that have a low environmental impact and provide socio-culturally acceptable and nutrient-dense foods, is often not considered. The focus of this paper was to review how the economic (monetary) cost of animal- and plant-sourced foods influences their inclusion in affordable least-cost mixed diets. Using the LP approach to identify foods included in modeled economically optimal least-cost diets that meet the nutrient requirements of a healthy average adult, animal-sourced foods were selected under current market conditions in the US and NZ. Foods originating from animals, such as dairy milk, eggs, fish, and seafood, were often key components of the least-cost diets. Legumes, milk, potatoes, and seeds were the greatest contributors to diet cost, whereas fats, oils, sugars, and starchy staples were low-cost rich sources of energy. As these findings are relevant to developed high-income countries, as exemplified by the US and NZ, there is an urgent need for LP modeling studies to test the premise that animal-sourced foods will be included in such least-cost diets in developing countries. Preliminary modeling studies in Indonesia indicate that animal-sourced foods, such as dairy milk, chicken liver, and seafood, are required for the least-cost diet, and the same LP modeling approach is currently being applied in the Philippines, Kenya, and Tanzania.

Concomitantly, in these studies, a number of dietary scenarios were analyzed that involved relaxing food price constraints around the foods included. The magnitude of food price elasticities by which the prices of animal-sourced foods needed to rise to be excluded from least-cost dietary patterns was estimated to formulate an explorative scenario of a plant-only least-cost dietary pattern. In the US, the prevailing retail prices of all animal-based foods had to be increased by 2.0 to 11.5 times their baseline costs to generate a plant-only least-cost diet, that had a diet cost that was 45% higher than that for the least-cost diet that contained animal- and plant-derived foods. Similar results were found for NZ, where the market prices of animal-based foods are not subjected to government subsidies to the same extent as in the US (31–33). When the baseline prices of animal-sourced foods were increased by 1.05 to 10.30 times, a least-cost diet with only plant-based foods was modeled, with a daily diet cost that was 34% more than that of the least-cost diet that contained animal- and plant-sourced foods. These results, representative of the US and NZ markets, give a clear indication of the leeway of food price variations of these animal foods for their complete exclusion from least-cost dietary patterns. Such food retail price interventions in developing low- and middle-income countries merits more investigation. As diet costs were limited to average annual national retail food prices, more in-depth country-level food prices are needed to consider regional diversity, seasonal and monthly variations, and affordability differences at a household level. In addition, while the cost of diets in this case relates to the market cost of food to the consumer, externality food costs include cost associated with food production, food processing and transportation, and food waste. Trade-offs may be appropriate to potentially cover these wider food costs.

The foods selected in the LP modeled dietary patterns are not meant to be necessarily included in realistic diets for consumption, but merely were identified to fulfill the arbitrary requirements for energy and country-specific dietary nutrient recommendations for almost all individuals in an average adult population aged 19–50 years (22, 23). Further modeling research for the elderly, pregnant or lactating women, adolescents, and growing children, who are most susceptible to inadequate nutrient intakes and increases in food prices (9–11, 16–18, 36, 37) warrants investigation. The first-limiting nutrients were found to be mostly the vitamins and minerals, notably calcium, potassium, selenium, vitamin A, vitamin C, vitamin D, and vitamin E. Particular additional nutrients that were first-limiting in the plant-only dietary scenarios were zinc, pantothenic acid, and vitamin B-12 (22, 23, 38, 39). It is of considerable note that most plant-sourced foods in the modeled least-cost diets were enriched with essential vitamins and minerals. For instance, fortified soymilk was the predominant source of plant-based vitamin B-12 in NZ (23). Fortification of plant-sourced foods has secured their place in the modeling of plant-based nutrient adequate least-cost dietary patterns.

Nutritional adequacy depends on the dietary supply of nutrients and bioavailability, that can be described as the proportion of an ingested nutrient that is available for utilization in metabolic functions (39). Natural food products originating from animals often contain protein and key vitamins and minerals, in higher amounts and greater bioavailability, than those of plant origin (27, 29, 39–44). Regarding protein quality, in general, least-cost diets that included animal proteins scored higher on DIAAS than least-cost diets that contained only plant-based protein sources. When all animal proteins were replaced with plant proteins in the US, utilizable protein intake was greatly reduced. Consideration of such protein quality metrics suggests that animal proteins play a critical role for ensuring sufficient provision of utilizable protein and indispensable amino acids (45, 46). The question also remains as to whether incorporating bioavailability of vitamins and minerals, that varies greatly among animal and plant food sources, will significantly impact the composition and cost of nutritionally adequate least-cost dietary patterns. The outcomes and conclusions may substantially change when diet cost is expressed per g of nutrient, and more importantly per g of bioavailable nutrient (46–48), in the LP modeling studies.

Taking an economic sustainability perspective toward dietary patterns in the US and NZ, and preliminarily in Indonesia, animal-sourced foods needed to be included in least-cost diets, to sufficiently meet basic nutrient requirements of the adult population, at the lowest retail dietary cost. Our results show that animal-derived foods are economically valuable sources of first-limiting essential key vitamins and minerals, and there is a considerable margin whereby the prevailing prices of animal-sourced foods need to increase to ensure their exclusion. Furthermore, when all animal-based foods were substituted with plant-based foods, the modeling of exclusively plant-sourced nutrient adequate least-cost dietary patterns was dependent on nutrient fortification and was relatively expensive. The respective roles of animal and plant food sources for the affordable and adequate provision of essential nutrients, and the often-missing economic dimension in the context of sustainable nutrition security, has been addressed.

Author contributions

SC: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. PM: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Potential impact of climate change on dietary grain protein content and its bioavailability-a mini review

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The changing global climate brings a gradual yet constant and adverse shift in crop production. Grain crop plants, particularly cereals and legumes, respond varyingly to adverse climate, including reduction in grain yield and changes to their nutrient densities. An understanding of specific changes to crop systems under differing climatic conditions can help in planning diets to meet human nutrient sufficiency. Grain protein content is also affected by adverse environmental factors. Deficits in protein yield, linked to changes in grain or seed protein and antinutrient concentrations, have been reported in major food crops when exposed to elevated carbon dioxide, high temperature, drought, and humidity. These changes, in addition to affecting the quantity of indispensable or essential amino acids (IAA), also impact their bioavailability. Therefore, it is important to assess consequences of climate change on grain protein quality. An important tool to measure grain protein quality, is measuring its digestibility at the level of the ileum and its IAA concentration, linked to a metric called the Digestible IAA Score (DIAAS). A minimally invasive technique called the dual isotope tracer technique, which measures IAA digestibility after simultaneous administration of two different intrinsically labelled protein sources, one a test protein ($^{2}H/^{15}N$) and one a reference protein (^{13}C) of predetermined digestibility, has been used in evaluation of grain protein IAA digestibility, and promises more in the evaluation of changes based on climate. This review discusses climate induced changes to grain protein quality through the prism of IAA digestibility, using the dual isotope tracer technique.

KEYWORDS

climate change, grain protein, antinutrients, protein digestibility, dual isotope tracer technique

1 Introduction

Agriculture and sustainable food production depends, in the short term on the weather and in the long term on climate (1). Recent trends in climate change have largely been attributed to emission of greenhouse gases like carbon dioxide (CO_2), methane, nitrous oxide which has led to various important consequences such as increased atmospheric temperature, drought, and changes in rainfall pattern to name a few (2). Unseasonal environmental changes

might impact seasonal crops as they require optimal conditions to achieve their vegetative and reproductive potential (1, 2). When crop plants fail to adapt to the changing environment or activate mechanisms to conserve organic matter, this results in a lower grain yield (3). Further, the mobility of reserves from leaves and roots to the grain is altered and this causes modifications in nutrient densities (4). For instance, macronutrients such as protein and micronutrients such as zinc and iron deposition in major food crops such as wheat, corn, rice, and soy decreased by approximately 3 to 9% when grown under high CO_2 (5). Variations in nutrient densities co-occur with changes in secondary metabolite concentrations in the grain; for example, the concentration of antinutrients, such as polyphenols, can affect nutrient bioavailability from the grain (6). These shifts are also found in the vegetative parts of the crops which serve as the fodder for livestock and can potentially impact productivity in terms of meat, eggs, and milk (7, 8). These effects on food systems can be multifaceted, and therefore the evaluation of climate induced changes in food systems can help plan global food production to achieve nutrient security and sufficiency.

More specifically, the impact of climate change on protein nutrition needs evaluation as protein is critical for growth and maintenance of body structural and functional proteins encoded by the human genome (9). The grain protein quantity of cereals and legumes, grown under a predicted atmospheric CO_2 that would occur in 2050, would decline by 4% (10). It also is important to determine the effect of climatic changes to the grain components and protein composition, and antinutrient quantity which can affect protein digestibility and hence its quality (11). The quality of a protein is dependent on the ability of its indispensable or essential amino acid (IAA) content to satisfy age-specific requirement (amino acid score) and its digestibility (digestion and absorption) (12, 13). The measurement of 'protein digestibility' is now recommended for individual IAA as this can vary for each IAA, either due to the difference in their interactions with the food matrix, which could include antinutrients, like anti-proteases, or due to a varied effect of food processing on different IAA (14). The digestibility of grain proteins becomes critical to measure in different food matrixes, to define protein quality and the ability of specific plant foods to meet daily protein and IAA requirements. The present review aims to understand the effect of changes in three major environmental factors such as atmospheric CO_2, temperature and water availability, which are critical for plant growth and productivity, on grain protein yield and content, protein composition, and antinutrient concentrations. Further, the review also examines a minimally invasive method of protein digestibility measurement in humans which can be used to assess protein quality against the background of climate induced grain composition changes.

2 Effect of climate change on grain protein yield

Crop plants grown under elevated atmospheric CO_2 have increased grain yield resulting from higher carbon assimilation (15, 16). Free-air-CO_2 enrichment field experiments show that the quantum of yield increase was greater in C3 cereals, such as rice and wheat (10–12%) at CO_2 exposure of 500 to 700 ppm compared to C4 plants such as maize (16–18). This is mainly due to increased photosynthetic efficiency of C3 plants under elevated CO_2, while C4 plants such as maize which are already efficient photosynthetic assimilators do not respond equally to elevated atmospheric CO_2 (15). However, increased yield is associated with ionomic imbalances and changes to protein concentration in most crop plants (5). A meta-analysis of 228 studies with elevated CO_2 showed a reduction in the grain protein content (GPC) of major food crops such as rice, wheat, barley and soyabean (16). The decreased GPC in rice and wheat (7 to 15%) could be due to its dilution by higher quantities of carbohydrates synthesized or due to decreased leaf protein concentration which is the main source of cereal grain protein (16–18). The reduction of GPC was lower in legumes compared to non-leguminous C3 plants, except for chickpea (8–10%). Smaller but significant decreases of GPC were found in soybean (4.8%), lentil (2%), and field pea (3%) (19–22). This could be attributed to nodule-based nitrogen fixation and protein translocation to the seeds in legumes. Although GPC decreases under elevated CO_2, the overall protein yield/hectare may not be reduced as it is compensated by increases in grain yield/hectare (23).

Decreased grain protein yield is associated with total crop yield losses. An analysis of the cereal yield, the largest contributors for global protein, over two decades (1990–2010), has shown a production plateau in major cereal producing areas across the world. This yield gain plateau could be due to maximum yield potential of these areas or due gradual climatic changes over the two decades (24). In Europe, between 1991 and 2015, cereal production decreased by 7.3% mainly due to extreme weather conditions such as heatwaves and drought (25). An increase in temperature by 1°C during cultivation of different varieties of wheat can reduce its yield by 3–10% (26). This is not limited to cereals, as the crop yield of legumes decreased by 4–31% with increases in temperature of 1–4°C (27, 28). Crop plants experience heat stress on exposure to increased temperature; and nutrient composition of grains are particularly affected if this exposure is during the reproductive and the seed filling stage as it reduces the seed filling time and impairs starch and protein synthesis (29–31). The protein content of rice and wheat on a dry weight basis increased under heat stress, but the amount of protein/grain was not altered when compared to control (32–34). However, in spring dry pea, protein/seed decreased when compared to control plants with every 1°C rise in temperature, but the magnitude of this decrease was lower compared to other dry matter components of the seed (0.032 mg of protein vs. 0.8 mg of other dry matter components/seed), such that the rise in temperature eventually resulted in an increased total grain protein on dry weight basis (35). Mixed results were observed for legumes, for instance in mung bean, lentil and chickpea, GPC reduced on an average by 7, 14, and 19% respectively, while it increased by 6.7% in soybean (36–39) (Table 1).

Water deficit (drought) which often accompanies increases in temperatures also has varying effects on grain protein yield. A meta-analysis of 48 studies on effect of drought on wheat showed a decrease in grain yield and grain protein yield/hectare by 57.32 and 46.04%, respectively, but GPC increased by 9.38% (40, 41). This effect is similar to that observed under high temperature. Decreased protein yield/hectare was also reported in chickpea and mung bean under drought where protein yield reduced by 41 and 88%, respectively, (42). While GPC decreased in chickpea by 5% and faba bean by 12%, it increased in mung bean, and a few common bean varieties by 10% and 6–10%, respectively, (42–44). In sorghum, while heat stress on an average decreased GPC in 24 different cultivars by

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TABLE 1 Effect of different abiotic stresses on crop grain protein content.

Stress[a]	Crop	Treatment	Treatment stage	Grain protein content	Reference
Elevated CO_2					
	Wheat	553 ppm	Reproductive phase	↓ 7.4%	17
	Wheat	500 ppm	Full growth cycle	↓ 14.9%	18
	Rice	500 ppm	Full growth cycle	↓ 7.0%	18
	Barley	550 ppm	Full growth cycle	↓ 11.5%	58
	Maize	550 ppm	Full growth cycle	↑ 2%	58
	Chickpea	580 ppm	Full growth cycle	↓ 8.4–10.2%	19
	Soybean	700 ppm	Seed filling	↓ 4.8%	20
	Lentil	550 ppm	Full growth cycle	↓ 2%	21
	Field pea	550 ppm	Full growth cycle	↓ 3%	22
Elevated temperature					
	Rice	↑ 5°C average air temperature	Grain filling	↑ 21%	32
	Wheat	From 24°C/16°C to 35°C/25°C	25 days post anthesis	↑ 6.6%	33
	Spring dry pea	↑ 5°C average air temperature	Grain filling	↑ 6.2%	35
	Mung bean	↑ 4°C average air temperature	Full growth cycle	↓ 4.1 to 9.3%	36
	Chickpea	From 25/15°C to 32/20°C	Grain filling	↓ 19%	37
	Lentil	From 11.4–30.6°C to 22.4 to 43°C	Reproductive phase	↓ 14%	38
	Soybean	↑ 6°C average air temperature	Grain filling	↑ 6.7%	39
	Sorghum	From 22.5°C to 30.6°C	Full growth cycle	↓ 9%	44
Drought					
	Wheat	Half optimal irrigation	Full growth cycle	↑ 15–18%	84
	Mung bean	No irrigation	Reproductive phase	↑ 10%	41
	Chickpea	Gradual evaporative water deficit	Full growth cycle	↓ 5%	41
	Common bean	No irrigation	Reproductive phase	↑ 6–10%	42
	Faba bean	No irrigation	Full growth cycle	↓ 12%	43
	Sorghum	No irrigation	Full growth cycle	↑ 8%	44
Combined stress					
Heat and CO_2	Rice	↑ 5°C and 700 ppm	Reproductive phase	↓ 4–6%	45
Heat, CO_2, and ozone	Wheat	↑ 5°C, 700 ppm and 80–100 ppb	Reproductive phase	↑ 4.6%	46
Heat and drought	Lentil	↑ 19°C and without irrigation	Reproductive phase	↓ 57.2%	38

*CO_2, carbon dioxide; ppm, parts per million; ppb, parts per billion.
[a]Values are a mean if multiple varieties are evaluated by a study.

9%, drought increased it by 8% (45). Although it is important to understand the effect of individual climatic changes, cultivated lands experience multiple stresses together; therefore, their simultaneous interaction on the GPC needs to be considered. For instance, rice cultivars grown under elevated CO_2 and heat had 4–6% lower GPC when compared to elevated CO_2 alone (46). In wheat, combined stress of ozone (80–100 ppb), elevated CO_2 (700 ppm), and higher temperature (5°C) increased GPC by 4.6% (47) (Table 1). Combined

heat and drought stress reduced GPC by 57% in lentil; however, this reduction in GPC was 14% when grown under heat stress alone (38). Overall, grain protein yield is affected by reduction of crop yield or reduction of GPC from exposure to various weather/climatic conditions. Selection of cultivars which are tolerant to different abiotic stresses with minimum yield and grain protein penalty can help in future climatic conditions. However, these varieties might also have increased quantities of antinutrients such as polyphenols and phytic acids which accumulate in grain in response to abiotic stresses (48, 49).

3 Effect of climate change on grain protein composition, amino acid and antinutrient concentration

The shifting climate not only changes the proximate nutritional make-up of the grain, but also leads to changes in the protein fractions deposited in the grain. The main storage proteins of cereals and legumes are albumin, globulin, prolamin and glutelins with crop specific variations in their type, proportions and subfractions (50). Climate change induced changes can co-occur in all the storage protein fractions, however the extent to which each protein fraction is affected defines the grain protein digestibility. This is because each protein fraction is digested with different efficiencies in different crops. In rice bran protein when assessed *in vitro*, glutelin had highest digestibility followed by albumin and globulin, and prolamin (51). Whereas in barley, hordeins (prolamins) had the highest digestibility followed by albumin and globulin, and glutelin (52).

A change in gliadin to glutenin ratio (reduction by 4.8%) was observed when wheat was grown under elevated CO_2 conditions (53). The reduced gliadin to glutenin ratios could decrease the protein digestibility of wheat as the glutenin fraction is 6% less digestible than the gliadin fraction (54). On the contrary, the protein digestibility of maize grown under elevated CO_2 was 9% higher in pigs, although the changes in the protein fractions were not reported in this study (55). Elevated CO_2 decreased albumin (34%), prolamin (21%), glutelin (17%) and globulin (16%) concentrations in rice (56). A concurrent decrease in of all IAA concentrations in the range of 4.8 to 9.0% in wheat and 1.6 to 5.0% in rice was observed when grown under elevated CO_2 (18). Heat stress that is experienced after anthesis in wheat also resulted in changes of gluten protein composition by decreasing the ratio of gliadin to glutenin (5.5%) (57). In rice, exposure to high temperature caused a reduction in prolamin by 12% and increase in glutelin by 31% (58). Changes in protein fractions were also observed in other crops including maize, barley, mung bean, lentils, and chickpea, at elevated CO_2 and under heat stress (Table 2) (36, 37, 59, 60). It is important to note that the changes in protein fractions of the grains are dependent on various factors including the length and time of stress induction, variety of the crop, the type of stress induced and combination of environmental stresses that the crop experiences. In total, the alterations in AA concentration and protein fraction of grains have potential to influence their protein digestibility and quality.

Environmental changes affect the quantity of antinutrients such as phytic acid (PA) and phenolics in grains. For example, PA concentrations increased by an average of 13.7% in 22 rice genotypes when exposed to heat stress, and the extent of this increase varied among different cultivars (61). Increases in PA concentration were found in lentil and sorghum while there was no change in wheat (62, 63). Elevated CO_2 decreased PA content in wheat, while increases were observed in rice (5, 64) (Table 2). High temperature increased total phenolic content (free and bound) in wheat genotypes (4–33% for every 5°C increment) while elevated CO_2 increased total phenolics in faba beans by 50% (65, 66). Both PA and phenolics can decrease protein digestibility by 3–31%, although the extent of this decrease varies on the concentration of PA and the type of polyphenols (11). A higher quantity of non-structural carbohydrate is observed in a few forms of abiotic stress. Under elevated CO_2, wheat grains had significantly higher concentrations of fructose (5%) and fructan (4%). The concentrations of other carbohydrates such as sucrose, raffinose and maltose also increased, though this the change was not significant (17) (Table 2). The presence of these sugars, in excess, indirectly influences protein quality as they can be involved in Maillard reactions with grain protein during processing for consumption and form products which are not utilized functionally (11). Non-structural polysaccharides which impact protein digestibility such as arabinoxylan has been shown to increase in spring wheat by 11 and 10% under heat and drought stress, respectively, (67). Together these changes can result in a decrease of protein quality. While limited *in vitro* studies examine the digestibility under different abiotic stresses, there are no studies which examine the effect of climate on grain protein digestibility in humans. This is important as in addition to the above-mentioned factors, food matrices in which protein is habitually consumed can further affect bioavailability.

4 Protein digestibility measurement and the digestible indispensable amino acid score

The changing landscape of dietary protein quantity in the background of differing grain components make it important to measure protein digestibility in widely cultivated and emerging abiotic stress resistant crop cultivars, and alternative protein sources. It is also important for understanding the effectiveness of food processing techniques on improving protein digestibility. A protein quality metric which takes into account the amino acid score as well as its digestibility (digestion and absorption till the terminal ileum), called the digestible indispensable amino acid score (DIAAS), is currently recommended for protein quality assessment (12, 13), and briefly described below.

Protein digestibility was earlier measured by oro-fecal intestinal balance, as the difference in the protein content between intake and fecal excretion, expressed as a proportion of the intake (14). The colonic microbial protein transactions that trap body nitrogen in the colon (urea for example) can confound these measurements (14). Since digestion and absorption of dietary protein is mainly considered to occur in the small intestine, measurement of ileal amino acid digestibility (oro-ileal balance) is recommended, corrected for the contribution from endogenous protein secretions (14). Ileal digestibility measurements are invasive as the ileum is not easily accessible and requires measurement of the endogenous intestinal protein secretions as well. It is measured by naso-ileal intubation technique or fistulation of the terminal ileum to collect ileal effluents required for quantification of the amount of ingested protein that

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TABLE 2 Effect of different abiotic stresses on grain protein composition, amino acid and antinutrient concentration.

Stress[a]	Crop	Treatment	Treatment stage	Protein composition, IAA and antinutrient concentration	Reference
Elevated CO_2					
	Wheat	550 ppm	Full growth cycle	↓ 4.8%: gliadin to glutenin ratio	52
		500 ppm	Full growth cycle	↓ 7%: threonine, ↓ 7.5%: valine, ↓ 7.1%: methionine, ↓ 9%: isoleucine, ↓ 8.1%: leucine, ↓8.2%: phenylalanine ↓ 4.8%: lysine	18
		553 ppm	Reproductive phase	↑ 5%: fructose, ↑ 4%: fructan	17
	Rice	↑ 200 ppm	Full growth cycle	↓ 34%: albumin, ↓ 21%: prolamine, ↓ 17: glutelin, ↓ 16%: globulin	55
		500 ppm	Full growth cycle	↓ 1.6%: threonine, ↓ 4.5%: valine, ↓ 5.0%: methionine ↓ 1.9%: isoleucine ↓ 1.7%: leucine, ↓ 1.5%: phenylalanine, ↓ 2.6%: lysine	18
	Barley	550 ppm	Full growth cycle	↓ 34%: albumin, ↓ 2.3%: globulin, ↑ 12%: glutenin, ↑ 10%: horedins	58
	Maize	550 ppm	Full growth cycle	↓ 32%: albumin, ↓ 62%: globulin, ↑ 37%: glutenin, ↑ 14%: zien	58
	Faba bean	700 ppm	Full growth cycle	↑ 53%: total phenolic	65
Elevated temperature					
	Wheat	24/17°C to 37/28°C	At anthesis	↓ 5.5%: gliadin to glutenin ratio, ↓ 40%: albumin and globulin	46
	Rice	↑ 1.6°–3.1°C	Grain filling	↓ 12%: Prolamin, ↑ 31%: Glutelin	57
	Chickpea	From 25/15°C to 32/20°C	Grain filling	↓ 37.6%: globulins, ↓ 14.6%: glutenins, ↓ 29%: prolamins, ↓ 27.8%: albumins	37
				↑ 43% glucose and ↑ 49.5%: fructose	37
	Lentil	From 28/23°C to 33/28°C	Post anthesis	↓ 21%: albumin, ↓ 14%: globulin, ↓ 22%: glutelins, ↓ 28.2%: prolamins	59
				↓ 19.2: methionine + cystiene, ↓ 21.3%: phenylalanine and tyrosine ↓ 14.7: threonine, ↓ 8.4%: tryptophan	59
	Rice	↑ 6°C	At anthesis	↑ 13.7%: phytic acid	60
	Lentil	↑ 10°C	Reproductive phase	↑ 11%: phytic acid	61
	Sorghum	From 32°C/21°C to 38°C/21°C	Full growth cycle	↑ 29.2%: phytic acid	62
	Wheat	↑ 5°C and ↑ 10°C	Full growth cycle	↑ 15.6% and ↑ 30.6%: total phenolic	64
	Wheat	From 22/12°C to 32/22°C	Reproductive phase	↑ 11%: arabinoxylan	66
		Reduction in soil moisture by 60%	Stem elongation stage	↑ 10%: arabinoxylan	

*CO_2, carbon dioxide; ppm, parts per million.[a]Values are a mean if multiple varieties are evaluated by a study.

disappeared after digestion and absorption until the terminal ileum (14). As stated above, substantial amounts of endogenous protein, which are secreted into gastrointestinal tract, mix with the dietary protein leading to an underestimation of oro-ileal digestibility (14, 68). Therefore, an additional measure of endogenous protein losses on a separate day becomes necessary (14). When corrected for endogenous protein losses, the ileal IAA digestibility is termed as "true ileal IAA digestibility." Using stable isotopically labelled test dietary protein which distinguishes it from the endogenous protein helps avoid this additional measurement (14). Due to the invasiveness of the ileal-balance method, it cannot be used widely to determine ileal protein digestibility.

A relatively recent, minimally invasive technique called the dual isotope tracer technique is promising for measurement of true IAA digestibility in different populations and age groups (69). In this technique, two intrinsically stable isotopically labelled protein, one a test protein ($^{2}H/^{15}N$) and another a differently labelled reference protein (^{13}C) of known digestibility, are simultaneously administered in a plateau feeding protocol. The ratio of postprandial plasma enrichment of $^{2}H/^{15}N$ IAA from the test protein and ^{13}C-IAA from the reference protein at plateau corrected for amounts ingested and the digestibility of the reference protein provides a measure of test protein digestibility (69). The equation used for the calculation of true IAA digestibility by dual isotope tracer technique is given below:

$$\mathrm{Dig_{test}} = \left[\mathrm{Plasma}\,^{2}\mathrm{H-IAA\,(APE)} / \mathrm{Meal}\,^{2}\mathrm{H-IAA\,(APE)}\right] / \left[\mathrm{Plasma}\,^{13}\mathrm{C-IAA\,(APE)} / \mathrm{Meal}\,^{13}\mathrm{C-IAA\,(APE)}\right]^{*} 100^{*}\,\mathrm{Dig_{ref}}\,/100.$$

Where, $\mathrm{Dig_{test}}$ is digestibility of test protein, $\mathrm{Dig_{ref}}$ digestibility of reference protein, and APE is atom percent excess.

The dual isotope tracer technique makes two important assumptions, first, that the absorption kinetics of labelled IAA from both the test and reference protein are similar. Second, differently labelled IAA's undergo similar splanchnic extraction and metabolism (14). Both of these assumptions are reasonable as an isotopic effect for these processes is not conclusively known. Several aspects of the measurement, to be considered while using this technique, such as intrinsic labelling of the test protein, selection of reference protein and feeding protocol have been discussed before (70).

To obtain intrinsically labelled plant protein, different methods are available, through foliar or soil applications of ^{15}N labelled ammonium/potassium salts or by administration of heavy water ($^{2}H_2O$) to the soil, or through hydroponics during the reproductive and seed development phase of the plant (69, 71–73). ^{15}N-salts label the amino groups of all IAA, while deuterium atoms from $^{2}H_2O$ are fixed at different position into IAA during their synthesis (73). The extent of labelling of the IAA depends on the quantity of the precursors administered and the length of application. The incorporation of ^{15}N into IAA is more efficient than ^{2}H because ^{15}N is incorporated into fewer molecules such as protein, and nucleic acids whereas ^{2}H is incorporated into all molecules. It is important to consider losses of label which can occur during metabolic reactions, for instance, ^{15}N from α-amino groups of labelled IAA are replaced by ^{14}N during the reverse reaction of transamination and the α-^{2}H of AA is replaced with H from body water (74, 75). Transamination correction factors can be derived in separate studies to account for these losses (69, 76).

The reference protein used could either be a ^{13}C-labelled bound protein of known digestibility or free ^{13}C-AA mixture (69, 77). Commercially available U-^{13}C spirulina has been previously used as the reference protein in dual isotope tracer technique; however, the interindividual variability of spirulina IAA digestibility was found to be high (1–12%) (69). Animal source protein (egg, milk, whey, or casein) with high digestibility and lower inter-individual variability can also be used as reference protein. The other option is to use ^{13}C-AA mixture which is considered to have 100% digestibility. A protein comparator as a reference is preferred as peptides have an absorption advantage over free AA (78).

The dual isotope tracer technique has not yet been validated with ileal-balance methods in an appropriately designed protocol. However, the true ileal IAA digestibility of animal source foods measured by dual isotope tracer technique was similar to that measured by other ileal balance methods (79–82). Previous studies have shown that the mean true ileal IAA digestibility of *desi* chickpea and *kabuli* chickpea was estimated to be 56 and 74.6%, respectively, and extrusion increased this by 89% (69, 77, 83). Climate change induced increases in grain anti-nutrients, such as polyphenols and phytate, (65, 66) can potentially decrease grain protein digestibility through covalent or non-covalent interactions with either the grain protein or the gastrointestinal tract proteases which hydrolyze them. This effect on digestibility can be shown through traditional processing techniques such dehulling, which has been shown to increase the mean true IAA digestibility of whole mung bean by 7.7% (77). Dehulling removes antinutrients such as tannins and polyphenols present in the seed coat which can reduce protein digestibility. Further, the true mean IAA digestibility of egg protein decreased by 17% when it was co-ingested with black tea (84), with a polyphenol content of 4.6 mg/mL.

The main advantage of the dual isotope tracer technique over the traditional oro-ileal balance methods of digestibility measurement is that it is minimally invasive. The plateau feeding protocol employed in the dual isotope tracer technique minimizes the number of blood samples collected and hence it can be used across age groups. The use of intrinsically labelled protein prevents confounding by endogenous protein secretions and allows measurement of true digestibility of all IAA of a grain protein on a single study day. The technique is also sensitive to changes in true IAA digestibility of different crop varieties, varying food matrices, and food processing techniques. Therefore, it has the potential to be used to assess the effect of climate change on true IAA digestibility of different dietary grain protein sources across geographical locations in different populations.

5 Conclusion and future research directions

Climate change has the potential to reduce protein quality particularly in crop grains, by reduction in protein yield, protein content, IAA content, and decreased digestibility due to protein compositional changes and varied antinutritional factors. Systematic field-based analysis of effect of combined abiotic stresses on protein content and protein quality is required to plan a sustainable approach for improving protein nutrition. Mitigation strategies including

selection of cultivars which are tolerant to environmental stresses, introgression of multiple abiotic stress tolerant traits in new cultivar by crop breeding, and alternative sources of protein needs to be evaluated. In addition to traditional crop breeding techniques, newer approaches such as quantitative trait loci mapping and marker assisted section, and CRISPR-Cas9 genome editing can be used to introduce multi-stress tolerances in important food crops. Selection of alternative crops to suit the climate of a particular production area to diversity cropping systems can help in sustaining or increasing nutrient production/hectare. Combination of different food processing techniques or development of processing techniques to reduce the effect of anti-nutrients can further improve protein quality. The dual isotope tracer technique, while expensive, is minimally invasive and can be used to evaluate the effect of changing climate on the protein digestibility of crops in humans and to determine the effectiveness of mitigation strategies in improving protein digestibility. It is also important to determine the fate of higher quantities of undigested crop protein which can enter the colon in background of climate change with controlled intervention studies, as several beneficial and adverse effects on health outcomes have been associated with protein fermentation products in the human colon.

Author contributions

SK: Writing – original draft, Writing – review & editing. BR: Writing – review & editing. SD: Writing – original draft, Writing – review & editing. AK: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Evolution and significance of amino acid scores for protein quality

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Amino acid scores have become very popular protein quality scores since their definition and recommendation by FAO expert groups. The chemical score is the central pillar of this method, and has been refined with digestibility correction factors, such as protein digestibility for the PD-CAAS and amino acid digestibility for the DIAAS. Several elements need to be taken into account to properly determine these scores, not only from a methodological point of view but also in order to reconcile regulation, pragmatism, accuracy and also biological significance. This review offers a reminder of the main points raised in the FAO reports on protein and AA requirements in 1995 and 2007, and on protein quality in 1991 and 2013. It also highlights the factors that most impact score metrics, and in particular the choice of reference pattern and protein determination in the food. Lastly, the scores are compared, and versus another quality score based on the physiological response, the protein efficiency ratio.

KEYWORDS

chemical score, amino acids, FAO, PD-CAAS, DIAAS

Introduction

Amino acid scores have been designed to reflect the ability of dietary protein to satisfy amino acid requirements. They are primarily based on the indispensable amino acid (IAA) content of dietary protein related to human amino acid requirements. They can secondarily include correction factors to account for the digestibility of protein (Protein Digestibility Amino Acid Score, PD-CAAS) or individual amino acids (Digestible Indispensable Amino Acids, DIAAS). A single composite figure resulting from these scores then summarizes this capacity.

Reference patterns

Amino acid requirements have evolved since the FAO reports in 1985 (1) and 2007 (2) after methods based on the oxidation of ^{13}C amino acids were recognized as being more accurate than the N balance method, leading to values up to three times higher for some AAs such as lysine. Briefly, the N balance method consists in determining digestive, urinary and miscellaneous N losses in response to various intake of the amino acid which requirement is to be determined (3). The AA requirement is assumed to correspond to the intake for which N intake is equal to N losses (null balance). In the nineties, two tracer methods, namely Direct AA Oxidation (DAAO) (4) and Indirect AA Oxidation (IAAO) (5) emerged. They were based on the intravenous infusion of a ^{13}C labeled AA, which oxidation was measured in expired air in response to various intakes of the AA of interest. When the AA intake is adequate, ^{13}C

oxidation reaches a minimum through a breakpoint that is considered to correspond to the AA requirement. In children, the factorial method is used to determine the maintenance and the growth components of the requirement. These methods have been described in detail in the FAO report in 2007 in which AA requirements for adults were reevaluated on the basis of ^{13}C oxidation methods. AA requirement (expressed per body weight unit) decreases rapidly from the age of 0–6 months to 3 years of age after which AA requirements are very similar to those of adults. To generate a so-called reference pattern, AA requirement values are divided by the protein requirement, which in adults has been established as 0.66 g/kg/d, based on N balance studies (2). The resulting reference pattern is then used to calculate the chemical score. Because AA requirement values differed markedly between the 1985 and 2007 FAO expert reports, the reference patterns published in the reports regarding protein quality evaluation in 1991 (6) and 2013 (7) also differed, as shown in Table 1. In 1991, it was recommended that the reference pattern for infants or preschool children aged 2–5 years should be used. In 2013, three reference patterns were proposed, for infants 0–6 months, children 0.5–3 y and individuals older than 3 y, because of the small difference between AA requirements at 3 y and 18 y. When comparing the FAO 1991 pattern for preschool children and that for individuals >3 y from FAO 2013, both being used for adults, the pattern from 2013 was more favorable, particularly for lysine and aromatic AA (Table 1).

Calculation of the chemical score

For each indispensable AA (IAA), the ratio between the AA content in the dietary protein and that in the reference pattern is calculated. A ratio above 1 signifies that the AA is present in sufficient quantities to satisfy the AA requirement. Among the ratios obtained for each of the nine IAAs, the lowest is retained as the chemical score which quantifies the degree of effects of the most limiting AA. Higher than 1, there is no limiting AA. Below 1, there is at least one limiting AA whose degree of insufficiency is reflected by this score. A score of 0.8 therefore means that the most limiting AA is 20% below the amount of this AA required in the target group of individuals. It may be noted that an increase of protein intake by 20% above the requirement could compensate this deficiency. Moreover, the scoring metric is a simplistic approach as it only reflects the ability of one dietary protein to satisfy *per se* the requirement, but in practice several protein sources compose the diet.

The choice of reference pattern is therefore a crucial factor in score calculation. The publication by Sa et al. (6) clearly showed the impact of the reference pattern used on the chemical score distribution for 1,200 lentil samples. For instance, the distribution of the ratios for sulfur AAs ranged from 0.6 to 0.83 for preschool children (i.e., profile 1991), 0.55 to 0.78 for 0.5–3 years (children) and 0.64 to 0.9 for 3 y and older (~adults). For tryptophan, these ranges were 0.63 to 0.75 for preschool children (FAO 1991), 0.84 to 0.97 for children and 1.08 to 1.25 for "adults".

Impact of the N to protein conversion factor

The conversion factor applied to extrapolate protein from nitrogen (N) has a marked impact on the chemical score. Indeed, the AA composition is determined in an ingredient or food and needs to be related to the mass of protein. To achieve this, one classic and universal strategy is to measure N and apply by default a conversion factor of 6.25. However, this factor overestimates the protein content of almost all protein sources. Specific factors exist for different protein sources and are more relevant (8), but from a regulatory point of view, a factor of 6.25 should be used. By overestimating the real protein content, this default conversion factor penalizes the chemical score. One compromise is to provide both values using both the default and

TABLE 1 Reference patterns in mg/g protein from FAO reports on protein quality evaluation.

	FAO report 1991				FAO report 2013			Difference between "preschool children 1991" and "older than 3y 2013"
	Infant (0–1 y)	Preschool children (2–5 y)	Older children (10–12 y)	Adults	Infants (0–6 m)	Infants (6 m – 3 y)	Children (>3 y), adolescents, adults	
Histidine	26	**19**	19	16	21	20	**16**	3
Isoleucine	46	**28**	28	13	55	32	**30**	−2
Leucine	93	**66**	44	19	96	66	**61**	5
Lysine	66	**58**	44	16	69	57	**48**	10
Sulfur AA	42	**25**	22	17	33	27	**23**	2
Aromatic AA	72	**63**	22	19	94	52	**41**	22
Threonine	43	**34**	28	9	44	31	**25**	9
Tryptophan	17	**11**	9	5	17	8,5	**6,6**	5
Valine	55	**35**	25	13	55	43	**40**	−5

Bold values indicate the reference pattern recommanded for "adults".

TABLE 2 Chemical score, PD-CASS and DIAAS of protein sources assessed during clinical or pig studies.

	CS	Non-truncated PD-CAAS	DIAAS
Casein (10)	1.48 (SSA)	1.42	1.45 (SSA)
Whey (14)	1.08 (His)	0.99	1.03 (His)
Pea isolate (15)	1.06 (SSA)	0.98	1.00 (SSA)
Sunflower isolate (16)	0.99 (Lys)	0.85	0.86 (Lys)
Flaxseed isolate (11)	0.74 (Lys)	0.68	0.58 (Lys)
Faba beans (17)	0.78 (His, Trp)	0.66	0.66 (His, Trp)
Oat concentrate (18)	0.80 (Lys)	0.69	0.67 (Lys)
Soy flour (19)	0.97 (SSA)	0.93	0.89 (SSA)
Wheat (19)	0.56 (Lys)	0.51	0.45 (Lys)

For score calculation, protein content was determined using N x 6.25. Reference pattern used: individual >3 y (FAO 2013).

specific factors. Another possible strategy is to sum up the amounts of AAs determined analytically, after correcting the mass by the hydration factor of free AAs vs. in-chain AAs, and to use this value as the true protein content of the ingredient or food. The first strategy of using both the by default and a specific conversion factor better ensures homogeneity among studies than the second strategy because inter-laboratory variability exists when measuring AAs. In particular, acid hydrolysis destroys a given proportion of AAs that might be heterogeneous in AAs, ingredients, laboratory conditions, etc. The accuracy of the correction applied to take account of this loss cannot be certified because no internal standards exist to control the hydrolysis yield.

The chemical score, i.e., the AA composition related to the reference pattern, is the principal determinant of a scoring quality index so that particular attention should be paid to this analytical component.

Digestibility correction factors

To take account of the bioavailability of nitrogen or AAs, the chemical score can then be modulated by a digestibility factor. When corrected for whole protein (i.e., nitrogen) digestibility, the appropriate index is the PD-CAAS, which was recommended by the FAO in 1991. In their report, the experts stated that "for practical reasons, the rat balance method is the most suitable practical method for predicting digestibility by humans." This is often interpreted as "digestibility must be measured at the fecal level in rats," but in fact, if more accurate values have been obtained in pigs or humans at the ileal level, they can be used. Another interpretation of the PD-CAAS that could be discussed concerns the appropriate reference pattern. During the expert consultation in 1989, the reference pattern was established on the basis of the AA requirement in 1985 (preschool children, as referred to above). However, because AA requirements were markedly revised in 2007, and subsequently the reference pattern, it might be more logical to use the 2013 reference patterns to evaluate the PD-CAAS. In the same way as the N to protein conversion factor, the main reason put forward for using the 1991 reference pattern is regulatory.

In the 2011 expert consultation, the digestibility of each individual AA was proposed as the digestibility correction factor in place of protein digestibility. The main methodological difference between the DIAAS and PD-CAAS is that ileal values of AA digestibility are necessary, which is much more complex than measuring fecal protein digestibility. This challenge resulted in greater interest in the digestibility methodology and several alternative approaches, where *in vitro* (9) or minimally invasive *in vivo* (10), have been developed during the past decade. Another aspect that has been extensively debated is truncation of the PD-CAAS to 1 as this index was designed to reveal limiting AA but not to inform on excess AAs. It is however possible to indicate the non-truncated PD-CAAS, especially for comparisons with the DIAAS. The latter is not truncated, so that the ability of protein sources to offset each other can be acknowledged. Table 2 presents an internal comparison of these different scores for some protein sources.

Table 2 reveals the relatively low impact of digestibility correction factors on the scores compared to the AA composition that is the main determinant of the quality scores. Moreover, one can notice the good consistency between DIAAS and PD-CAAS values, except for the study on flaxseed where a particularly low digestibility of the limiting AA (namely lysine) was observed; it was suspected to be ascribable to Maillard reactions in the food matrix, in that case a biscuit (11). As a result, a small difference between PD-CAAS and DIAAS values could be presumed for low processed ingredients or foods but greater discrepancies are probable for ultra-processed foods because specific AAs such as lysine or SSA are more sensitive to technological treatments. As for the issue of the reference pattern, if the FAO 1991 pattern for preschool children had been used to calculate the PD-CAAS, the latter would have been drastically lower; for instance 0.51 for Faba bean (Trp) or 0.7 for sunflower (Lys). This illustrates that the use of different reference patterns to compare PD-CAAS and DIAAS is biased, and the 2013 reference patterns for any quality score metrics should clearly be recommended in order to ensure consistency between the different quality indicators.

Thresholds for claims regarding protein quality

Another novelty concerning the DIAAS metric was the proposal of thresholds in order to claim a good (DIAAS>0.75) or excellent (DIAAS>1) protein quality. Herreman et al. (12) reported DIAAS data on 17 protein sources, each involving several observations. Surprisingly, only casein and pork satisfied the criteria for an excellent

source in both the older than 3 y and 0.5 y-3y patterns, but not in infants. A third of the sources, all from plants except gelatin (for which the DIAAS is null), did not reach the threshold for good quality, even under the >3y pattern. To appreciate the biological significance of this 0.75 threshold, it is necessary to compare DIAAS values with physiological markers of protein quality. In a recent review, Nosworthy et al. (13) collected values for DIAAS (using the 0.5–3 y reference pattern) and the Protein Efficiency Ratio (PER), which indicates the ability of protein to sustain growth in growing rats. The correlation between the two indexes was good ($R = 0.84$, $p < 0.001$) and all the products (except tofu) with a DIAAS value <0.75 had a low PER (<1.6), whereas a DIAAS higher than 1 was associated with a high PER. A more exhaustive collection of data may be necessary, especially for products with DIAAS values ranging from 0.75 to 1, but it appears from this rough analysis that a DIAAS score lower than 0.75 is associated with impaired growth.

Conclusion

Quality scores are mainly dependent on the AA composition of the protein to which specific attention must be paid. The reference pattern applied, and determining the protein content of an ingredient or food, will also have a significant impact on quality scores. Digestibility correction factors have been complexified from PD-CAAS to DIAAS, resulting in a considerable growth of interest in digestibility methods. The technical challenges have been faced and interestingly, numerous data have been produced since the FAO report in 2013. DIAAS and PD-CAAS values are often very close because although some differences exist between N and individual AA digestibility, these correction factors exert limited influence on the quality scores, because the digestibility values of N and AA in various protein sources mostly range from 75 to 95%.

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Amino acid requirements of the infant: the amino acid composition of human breast milk

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The recommended amino acid requirements of the infant are based on the amino acid composition of mature human breast milk. The amino acid composition of breast milk is usually determined following either acid or alkaline (for tryptophan) hydrolysis. For accuracy, however, the known effect of hydrolysis time on amino acid composition should be accounted for. Also, ideally the amino acid composition of breast milk should be given in units of digested (assumed to be absorbed) amino acids. A review of the literature is presented which gives mean total amino acid concentrations in mature human milk ($n = 26$ studies), mean hydrolysis correction factors ($n = 3$ studies) and mean true ileal amino acid digestibility coefficients ($n = 3$ studies, suckling piglet). There were differences between the estimates of amino acid concentration corrected for hydrolysis time and digestibility, and current FAO (2013) recommendations that were not corrected for these factors. The values based on the published literature up until 2023 (mg/g true protein) corrected for hydrolysis time and digestibility gave higher values (more than 16% higher) for leucine, lysine and threonine, and considerably higher values (greater than 30%) for histidine and tryptophan. Current recommendations may need revision.

KEYWORDS

breast milk, human milk, human milk protein, indispensable amino acid, infant nutrition, lactation, protein hydrolysis, true ileal amino acid digestibility

1 Introduction

Human breast milk is a complex biological fluid and in nature is the sole source of nutrients for a baby for the first few months of life. The protein composition and consequent amino acid composition of breast milk is the result of millions of years of evolution, and as such, it is generally assumed that the amino acid composition of breast milk from healthy well-nourished women, provides a suitable basis for estimates of the amino acid requirements of the baby postnatally (1, 2). It is of utmost importance to know the amino acid requirements of the infant with accuracy as they provide the building blocks of proteins synthesised during growth and development, and many of the amino acids have important specific physiological roles (3–5).

Breast milk is the preferred source of nutrition for the newborn baby, but for numerous reasons in practice many infants receive infant formula as their sole source of nutrition. It is

important, therefore, to have accurate estimates of the amino acid composition of breast milk.

Human milk contains hundreds of different proteins of which the concentrations are variable, and although the amino acid sequences of some of the more common milk proteins are known, not all of the proteins have been sequenced. Moreover, a significant proportion of breast milk amino acids are in the free form. It is for these reasons that the amino acid composition of milk is usually determined by chemical analysis.

Since the development of ion-exchange chromatography and other methods such as HPLC and UHPLC with precolumn derivatization to separate amino acids in complex mixtures, many studies have been reported determining the amino acid composition of human milk. Common to these studies is the need to firstly hydrolyse the breast milk proteins to their constituent free amino acids to allow quantitation. This commonly involves acid hydrolysis (usually 6 M HCl) of the defatted material in an oxygen free environment for 20 to 24 h at 110 degrees Celsius. It is well established, however, that with strong acid hydrolysis, methionine (particularly if oxygen is present), cysteine and tryptophan can be destroyed. Accordingly, methionine and cysteine are usually determined as methionine sulphone and cysteic acid, respectively following performic acid oxidation undertaken before the hydrolysis step, and tryptophan after an alkaline hydrolysis. Also, during hydrolysis tyrosine can become halogenated but this can be prevented by adding phenol to the hydrolysis mixture. What is less widely appreciated, however, is that regardless of the type of hydrolysis, a hydrolysis time longer than 24 h is required for the full release of some amino acids (for example leucine, isoleucine and valine), while others (for example serine, threonine, cysteic acid, tryptophan) can be progressively oxidized (6). The degree of underestimation can be practically important urging some authorities to adopt correction factors (e.g., TNO, the Netherlands: threonine 1.05; serine 1.10; valine 1.07; isoleucine 1.08). For some applications such a degree of underestimation may be acceptable, but it is important that infant formulas mimic the amino acid composition of human milk as accurately as possible.

An approach to determining amino acids that is more accurate than using a set hydrolysis time, is to subject the protein to multiple hydrolyses (different durations of hydrolysis) and then apply a curvilinear mathematical model to allow the prediction of the amounts of amino acids present in the protein, accounting for simultaneous rates of both amino acid release and destruction (7, 8). The Robel and Crane model has been modified (9) to allow for complex mixtures, such as breast milk, that have a free amino acid as well as a bound proteinaceous amino acid component.

Another consideration when equating milk amino acid contents with amino acid requirements for the infant is that not all proteins in human milk have a primary nutrition function, but rather some proteins (e.g., immunoglobulins; lactoferrin; transferrin; lysozyme) may have primary immunological and developmental roles. It appears that these types of proteins are only partially digested between the mouth and end of the small intestine and complete fragments of such proteins can be detected in faeces from breast-fed babies (10–12). Consequently, not all human milk amino acids are absorbed and thus a more refined estimate of amino acid requirements is given by the digestible (assumed absorbed) amino acids in human milk (13). It is the profile of absorbed rather than gross amino acids that needs to be mimicked by the digestible amino acids in infant formulas.

It is well established that the absorption of intact amino acids in humans is essentially complete by the end of the small intestine and that amino acid digestibility should be determined between the mouth and the terminal ileum using a true ileal amino acid digestibility assay (14, 15). Such a measure cannot be readily obtained using human infants, necessitating the need for animal models of digestion. The three-week-old suckled piglet, ingesting milk at an amount per unit stomach volume to mimic the human infant, has been shown to be a suitable candidate model for the three-month-old human baby, from an anatomical and physiological perspective (16–19). A study directly comparing the protein and organic matter digestion of milk in the piglet and human baby, provides empirical evidence for the suitability of the suckled piglet model (20). Other models such as the rat pup have been successfully used to study the digestion of milk proteins (21) but such models rely upon intubation of the milk, and thus exclude suckling, which may affect digestion. The suckled piglet model has been used in several studies to determine the digestibility of amino acids in breast milk.

The objective of this contribution is to review the published literature on the amino acid composition of human milk with an emphasis on the effect of amino acid losses and gains during hydrolysis, and on the absorbability of the breast milk amino acids. A profile of absorbed amino acids in human milk is put forward as the current best estimate of the amino acid needs of the newborn term infant. This amino acid profile is compared with the current FAO recommendations (2). The latter recommended amino acid profile does not consider the effects of hydrolysis time during amino acid analysis, nor the effects of differences in amino acid digestibility.

2 Methods

A systematic review of the literature was conducted to identify publications that reported total amino acid concentrations in human milk, including publications up until the end of 2023. A search was performed using Scopus and Google Scholar. Keywords used were "PubMed", "amino acid," "protein composition," "human milk composition," "human milk," "breast milk," "human milk nutrition," "characterisation of human milk," "standardisation of human milk," and "factors affecting human milk composition." Reference lists of the selected publications were further searched manually to identify any other relevant articles. A total of 74 articles were identified for potential inclusion.

Within each publication, milk collection methods and methods for amino acid analysis were reviewed. Inclusion criteria included the collection of mature milk from healthy women, defined as collection periods extending between beyond 1 to 10 months post-partum. For publications that presented total amino acid concentrations for multiple lactation periods, those that were within the specified collection period were averaged.

Exclusion criteria included collection from a single donor or from non-healthy women or results from the collection of non-mature milk (defined as less than 1 month post-partum). Studies that conducted amino acid analysis using non-standard methods were also excluded. Publications in which the milk collection methodology or amino acid analysis methods were not well-described were excluded.

When the results from one study were reported in several publications (determined according to the description of methods), these data were only included in the database once. Table 1 lists the studies that were not included in the database and the reason for their exclusion.

A total of 26 studies were included in the database (13, 22–46).

TABLE 1 Studies that report the amino acid (AA) concentration in mature human milk that were published before 2023 and not included in the present dataset.

Study	Exclusion criteria
Atkinson et al. (52)	Data from collection periods earlier than 1 month post-partum
Beach et al. (53)	Non-standard amino acid analysis (microbiological)
Block and Bolling (54)	Non-standard amino acid analysis (microbiological)
Chathyushya et al. (55)	Only breast milk of 1 week post-partum collected
Close and Van De Walle (56)	Inadequate information on breast milk sample
Darling et al. (57) and Darling (58)	Data from collection periods earlier than 1 month post-partum
Davis et al. (59)	Data reported in another publication that was included (duplicate data)
DeSantiago et al. (60)	Health criteria of lactating mothers not met
Faus et al. (61)	Total AAs not determined
Feng et al. (62)	Data reported in another publication that was included (duplicate data)
Ferreira (63)	Data from collection periods earlier than 1 month post-partum
Filippova and Aronova (64)	Method for AA determination not reported
Giuffrida et al. (65)	Total AAs not determined
Guo et al. (66)	Single milk donor
Hanning et al. (67)	Data from collection periods earlier than 1 month post-partum
Heine et al. (51)	Data reported in another publication that was included (duplicate data)
Jarvenpaa et al. (68)	Data reported in another publication that was included (duplicate data)
Lemons et al. (69)	Total AAs not determined
Macy (70) and Macy and Kelly (71)	Non-standard amino acid analysis (microbiological)
Miller et al. (72)	Non-standard amino acid analysis (microbiological)
Mitton and Garlick (73)	Inadequate information on breast milk sample
Moya-Alvarez et al. (74)	Health criteria of lactating mothers not met (many with malnutrition)
Motil et al. (75)	Total AAs not determined
Nagasawa et al. (76)	AA profile of breast milk casein only
Nayman et al. (77)	Non-standard amino acid analysis (microbiological)
Nwachoko et al. (78)	Results reported in mg AA/100 g protein but no total N or protein data provided
Pang et al., 2019 (79)	Inadequate information on methodology; text in Mandarin
Picone et al. (80)	Inadequate information on methodology
Purkiewicz et al. (81)	Data from collection periods earlier than 1 month post-partum
Räihä et al. (82)	Data reported in another publication that was included (duplicate data)
Rassin et al. (83)	Lactation stage unclear
Renner (84)	Data reported in another publication that was included (duplicate data)
Rigo et al. (85)	Data reported in another publication that was included (duplicate data)
Saben et al. (86)	Total AAs not determined
Saito et al. (87)	Non-standard amino acid analysis (microbiological)
Sarwar et al. (88)	Data from collection periods earlier than 1 month post-partum
Scott et al. (89)	Inadequate information on methodology
Shaikhiev (90)	Text in Russian (Cyrillic)
Soupart et al. (91)	Lactation stage unclear
Tarján et al. (92)	Total AAs not determined
Tikanoja et al. (93)	Lactation stage unclear
Van Sadelhoff et al. (94)	Total AAs not determined
Volz et al. (95)	Data reported in another publication that was included (duplicate data)
Wei et al. (96)	Total AAs not determined; non-standard amino acid analysis; not representative of mature milk
Williamson (97)	Non-standard amino acid analysis (colorimetric)
Woodward (98)	AA profile of breast milk casein only

The amino acid composition of human milk was reported using different units in the publications so these were converted when required, to mg amino acid/L milk, mg amino acid/g dry matter (DM) and mg/g true protein (TP). Data that were presented only in moles were first converted to mg of amino acid per L milk by multiplying by the molecular weight of each amino acid (x 10). For each publication, the reported dry matter (DM) content of milk given in that publication was used to convert each amino acid value between mg/L and mg/g DM. When the DM content of the milk was not reported in the publication, the average DM content of milk calculated from publications that reported this value was used (121.2 mg DM/L milk). To convert between mg/g and mg/L, the conversion factor of 1.032 mg milk/L was used (47).

Values were also converted to mg/g true protein (TP) with the TP content of the milk samples calculated as reported by FAO (2) where TP = nitrogen concentration × 6.38 × 0.75. The factor of 0.75 is used as the non-protein content of human milk (comprising mainly urea and free amino acids) is around 25% of the total nitrogen content (2). Where necessary, as different publications used different conversion factors between nitrogen concentration (which is chemically analysed) and crude protein, reported protein concentrations were first converted back to nitrogen concentrations according to the reported conversion factor in each publication.

A review of the literature (until 2023) was also undertaken to identify studies addressing the effect of hydrolysis time on amino acid yield in human breast milk, and studies determining the true ileal digestibility of amino acids in human breast milk.

3 Results and discussion

3.1 Amino acid composition

The overall mean total amino acid compositions of human milk reported in the 26 studies included in the database are given in Table 2. Zhang et al. (48) also conducted a systematic review of the total amino acid concentration in human milk, and their values are included in Table 2.

The rigorous and detailed review of Zhang et al. (48) covering the literature published up to 2009 provides an important benchmark against which to compare the presently derived data. Data included in the Zhang et al. (48) study related to breast milk samples from complete 24 h collections or at least collections of the entire amount of milk from one or both breasts at a feeding, or pooled or banked milk. The milk was from healthy mothers receiving "free-living" diets and who had delivered healthy mainly term babies. Studies employing microbiological

TABLE 2 Amino acid composition of human milk (based on 20 to 24 h amino acid hydrolysis period) collected from women between 3 and 42 weeks post-partum from data published before 2023 (n = 26 studies) and values reported in the systematic review of Zhang et al. (48).[1]

	Amino acid composition			
Amino acid	mg/L	mg/g DM[2]	mg/g TP[3]	Zhang et al. (48) mg/L[1]
Indispensable amino acid				
Histidine	280.8 ± 12.65	2.3 ± 0.11	31.2 ± 1.47	278.0
Isoleucine	572.7 ± 15.68	4.7 ± 0.15	63.6 ± 1.81	597.5
Leucine	1084.4 ± 29.47	9.0 ± 0.26	120.0 ± 3.10	1117.0
Lysine	755.0 ± 23.85	6.2 ± 0.20	83.8 ± 2.73	755.5
Methionine	162.2 ± 6.60	1.3 ± 0.06	18.0 ± 0.71	172.0
Phenylalanine	420.8 ± 16.60	3.5 ± 0.14	46.8 ± 2.00	425.0
Threonine	499.0 ± 14.22	4.1 ± 0.13	55.2 ± 1.48	510.5
Tryptophan[4]	196.1 ± 11.84	1.5 ± 0.18	21.6 ± 1.30	222.0
Valine	596.7 ± 19.31	4.9 ± 0.19	66.3 ± 2.18	625.0
Dispensable amino acid				
Alanine	422.1 ± 16.36	3.5 ± 0.15	46.9 ± 1.72	436.5
Arginine	411.2 ± 19.80	3.4 ± 0.17	45.6 ± 2.14	409.5
Aspartic acid	971.5 ± 26.13	8.0 ± 0.23	107.8 ± 2.88	990.5
Cysteine[5]	233.2 ± 863	1.8 ± 0.15	26.3 ± 0.98	237.0
Glutamic acid	1898.2 ± 37.87	15.7 ± 0.38	211.1 ± 4.96	1952.5
Glycine	253.8 ± 12.59	2.1 ± 0.11	28.1 ± 1.38	266.0
Proline	937.1 ± 26.35	7.8 ± 0.26	103.9 ± 3.02	976.0
Serine	492.2 ± 14.29	4.1 ± 0.13	54.4 ± 1.23	499.5
Tyrosine	456.6 ± 24.97	3.8 ± 0.21	51.0 ± 2.96	515.0

Values are given as mean ± SEM between studies.
[1]Data averaged from milk collected between 3 and 20 weeks postpartum, Zhang et al. (48).
[2]DM, dry matter; when no dry matter content of breast milk was given in the original publication an average of the collected data (n = 6) was used.
[3]TP, true protein = N × 6.38 × 0.75.
[4]Tryptophan values from published studies were only included when an alkaline hydrolysis was performed (n = 12).
[5]Cysteine values from published studies were only included when an initial treatment of the sample with performic acid was applied (n = 15).

methods of amino acid determination were excluded, and only studies using ion exchange chromatography, HPLC and UHPLC with precolumn derivatization or similar validated methods were used in the analysis. Results largely relate to 20 to 24 h hydrolysis of protein. Attention was paid to ensuring that the studies used appropriate consistent methods for the determination of methionine, cysteine and tryptophan. Overall mean amino acid concentrations in milk were determined for each study and least squares means generated with stage of lactation fitted as an effect in the ANOVA model. Mature milk in the Zhang et al. (48) work was defined as milk from 21 days of lactation up to >136 days of lactation. Lactation stage significantly ($p < 0.05$) influenced total amino acid composition and data were presented separately for mature milk relating to 21 to 58 days of lactation; 59 to 135 days of lactation and 136 to 540 days of lactation. Most studies used the units of weight of amino acid per 100 ml milk. Where data were given as weight per 100 grams milk, the volume-weight correction, which is quantitatively minor, was not undertaken. The study reviewed human milk composition data from 83 published scientific papers, from 18 countries, with publication dates ranging from 1941 to 2009. For total amino acid content, 26 papers providing 79 mean values from 3,774 subjects were selected by the authors for analysis. The total N concentration of breast milk and the amino acid content of breast milk declined ($p < 0.05$) moderately for milk from around 2 months of lactation to milk from 5 to 18 months of lactation. This is consistent with the conclusions reached by Lönnerdal et al. (49) and Ren et al. (47) that human milk amino acid content is relatively stable from around 3 to 4 weeks after birth and onwards. For our purposes and to align with the lactation period used in the present work, the mean concentrations calculated over 21 to 135 days of lactation were taken as an estimate of the amino acid composition of mature human breast milk (see Table 2).

There is close agreement between the values for the amino acid composition of human breast milk between Zhang et al. (48) and the present estimates, though differences were found for some of the amino acids. This gives confidence in the presently derived estimates. The presently reported estimates are preferred, as these incorporate the most up-to-date published information (studies published up to 2023, as opposed to 2009).

3.2 Correction for the effect of time of hydrolysis

Three studies were identified that conducted amino acid analysis with multiple hydrolysis intervals (13, 44, 45). The difference between

TABLE 3 Determined correction factors[1] for breakdown or incomplete release of amino acids during hydrolysis, and concentration of amino acids in breast milk corrected by these values.

Amino acid	Correction factor (%)	Value before correction mg/L (20 to 24 h hydrolysis)[2]	Value after correction		
			mg/L	mg/g DM[3]	mg/g TP[4]
Indispensable amino acid					
Histidine	4.7	280.8	294.0	2.4	32.7
Isoleucine	−0.3	572.7	571.0	4.7	63.4
Leucine	−1.3	1084.4	1070.3	8.8	118.7
Lysine	−0.7	755.0	749.7	6.2	83.2
Methionine	−0.7	162.2	161.0	1.3	17.8
Phenylalanine	−2.0	420.8	412.4	3.4	45.9
Threonine	6.2	499.0	529.9	4.4	58.8
Tryptophan	2.0	196.1	200.0	1.5	22.0
Valine	1.5	596.7	605.6	5.0	67.2
Dispensable amino acid					
Alanine	−1.9	422.1	414.0	3.4	46.0
Arginine	−1.4	411.2	405.4	3.4	45.0
Aspartic acid	0.2	971.5	973.4	8.0	108.0
Cysteine	−3.7	233.2	224.5	1.8	25.3
Glutamic acid	−0.4	1898.2	1890.6	15.6	210.2
Glycine	−2.7	253.8	247.0	2.0	27.3
Proline	0.5	937.1	941.8	7.8	104.5
Serine	5.0	492.2	516.8	4.3	57.1
Tyrosine	3.2	456.6	471.2	3.9	52.6

[1]Correction factors are mean differences between concentration of amino acids determined with 24 h hydrolysis and multiple hydrolysis intervals (modelled value), based on observations from Darragh and Moughan (13), Charton et al. (44) and Hodgkinson et al. (45). Values using the equation below for each amino acid in each of the three studies expressed as a percentage were averaged: Correction value = (Modelled concentration of amino acid) − (Concentration of amino acid using 20–24 h hydrolysis)/Concentration of amino acid using 20–24 h hydrolysis.
[2]Data from Table 2.
[3]DM, dry matter; when no dry matter content of breast milk was given in the original publication an average of the collected data ($n = 6$) was used.
[4]TP, true protein = N × 6.38 × 0.75.

TABLE 4 Published mean true ileal amino acid digestibility of amino acids in breast milk determined using the piglet as a model for the human infant.

Amino acid	Study		
	Charton et al. (44)	Darragh and Moughan (13)	Hodgkinson et al. (45)
Indispensable amino acid			
Histidine	0.979	0.950	0.952
Isoleucine	0.963	0.980	0.955
Leucine	0.982	0.990	0.942
Lysine	0.984	0.980	0.920
Methionine	ND[1]	1.000	0.956
Phenylalanine	0.963	0.930	0.879
Threonine	0.892	0.860	0.842
Tryptophan	0.955	ND[1]	1.000
Valine	0.931	0.900	0.883
Dispensable amino acid			
Alanine	0.931	0.950	0.812
Arginine	0.965	1.010	0.842
Aspartic acid	0.949	0.950	0.905
Cysteine	ND[1]	ND[1]	0.678
Glutamic acid	0.976	0.980	0.945
Proline	0.941	0.920	0.876
Serine	0.938	0.950	0.774
Tyrosine	0.959	1.000	-

[1]ND, not determined.

TABLE 5 True ileal amino acid digestibility coefficients (TIAAD)[1] and amounts of true ileal digestible amino acids in human milk presented in different units.

Amino acid	TIAAD	Amount of digestible amino acids[2]		
		mg/L	mg/g DM[3]	mg/g TP[4]
Indispensable amino acid				
Histidine	0.960	282.3	2.3	31.4
Isoleucine	0.966	551.5	4.6	61.3
Leucine	0.971	1039.3	8.6	115.2
Lysine	0.961	720.5	6.0	80.0
Methionine	0.978	157.5	1.3	17.5
Phenylalanine	0.924	381.1	3.1	42.4
Threonine	0.865	458.4	3.8	50.8
Tryptophan	0.978	195.6	1.5	21.6
Valine	0.905	548.1	4.5	60.9
Dispensable amino acid				
Alanine	0.898	371.8	3.1	41.3
Arginine	0.939	380.7	3.1	42.2
Aspartic acid	0.935	910.2	7.5	101.0
Cysteine	0.678	152.2	1.2	17.2
Glutamic acid	0.967	1828.2	15.1	203.3
Glycine	0.924	228.2	1.9	25.2
Proline	0.912	858.9	7.1	95.3
Serine	0.887	458.4	3.8	50.7
Tyrosine	0.980	403.8	3.3	45.1

[1]Overall mean values from Table 4.
[2]Correction of values in Table 3 (after correction for time of hydrolysis effect) for TIAAD.
[3]DM, dry matter; when no dry matter content of breast milk was given in the original publication an average of the collected data ($n = 6$) was used.
[4]TP, true protein = N × 6.38 × 0.75.

TABLE 6 Absorbed amino acid composition of human milk based on the present work (Table 5) compared with reference values from FAO (2).[1]

Amino acid	Calculated values[3]	FAO
Histidine	31	21
Isoleucine	61	55
Leucine	115	96
Lysine	80	69
Methionine + cysteine	35	33
Phenylalanine + tyrosine	87	94
Threonine	51	44
Tryptophan	22	17
Valine	61	55

Amino acid values are mg/g TP[2], as calculated by FAO (2).
[1]FAO (2) recommendations are not corrected for true ileal amino acid digestibility.
[2]TP = N × 6.38 × 0.75.
[3]Values based on published literature with correction for the effects of hydrolysis time and true ileal amino acid digestibility.

the concentration of each amino acid determined using a 20 to 24 h hydrolysis period and that determined using multiple hydrolyses (estimated amino acid concentration in the milk) for each amino acid in each study was averaged to calculate average correction factors. These correction factors were used to correct the total amino acid concentration in human milk (reported in Table 2; based on 20 to 24 h hydrolysis) to that if multiple hydrolysis had been used for each individual published study, and the mean results are reported in Table 3. In the publication by Charton et al. (44) data corresponding to 24 h hydrolysis were not included, but data were provided (A. Deglaire, personal communication) to allow the correction to be made.

For most of the amino acids the effect of correction was small but for histidine, phenylalanine, threonine, tryptophan, cysteine, serine and tyrosine the differences were considered to be practically important (correction factor ≥ 2%). For leucine, isoleucine and valine the determined correction factors were smaller than expected, though the hydrolysis behaviour is likely to vary with the substrate being analysed. The amino acid affected by hydrolysis to the greatest extent was threonine, which is known to be sensitive to oxidation (6).

3.3 Correction for the true ileal amino acid digestibility

True ileal amino acid digestibility coefficients for human milk determined using the piglet as a model for the human infant were reported in three studies (13, 44, 45). For each amino acid, digestibility coefficients were averaged across the three studies (Table 4) and applied to the data presented in Table 3. The overall mean true ileal amino acid digestibility coefficients and mean amounts of true ileal digestible amino acids are presented in Table 5.

For most of the amino acids, digestibility was high, but for some amino acids, notably cysteine and threonine, true ileal amino acid digestibility was much lower than for the other amino acids. The digestibility of threonine was consistently lower across the three studies, but only one of the studies provided digestibility data for cysteine. Mavromichalis et al. (50) reported high amino acid digestibility for sow's milk (95–100%), but also found relatively low true ileal digestibility for cysteine and threonine (84%). Although the suckling piglet is a well-accepted animal model for protein digestion in the human infant, there may be differences in digestion (e.g., differences in gut microbial populations), and this should be borne in mind in interpreting the results.

3.4 Comparison with FAO recommendations

The amounts of true ileal digestible amino acids determined from the literature, corrected for multiple hydrolysis time and true ileal digestibility are presented in Table 6 along with the current FAO reference values. The FAO (2) Expert Consultation recommended the amino acid content of breast milk as the current best estimate of amino acid requirements for infants, and gave a recommended amino acid profile for mature human milk based on the deliberations of the FAO/WHO/UNU (1) Expert Consultation. FAO (2) accepted the appropriateness of correcting the total amino acid contents for amino acid digestibility, but did not make the correction at that time as only one published set of values for digestibility was available. The FAO (2) recommended values also relate to only a few older studies on the amino acid composition of human milk (37, 38, 51).

It is apparent from the values listed in Table 6 that the estimates of amino acid requirements for the human baby as determined after correcting published values for the amino acid content of mature human milk for the effects of hydrolysis time and true ileal amino acid digestibility, are quite different from the most recent FAO recommendations (2). The values presented here and based on the published literature up until 2023, and corrected for the estimated effect of hydrolysis time during amino acid analysis and for true ileal amino acid digestibility, led to higher concentrations (more than 16%) in breast milk for leucine, lysine and threonine and considerably higher values (greater than 30%) for histidine and tryptophan. All of these amino acids play critical roles in infant growth and development (5). A potential limitation of the present data is that although they are based on multiple studies, the hydrolysis correction factors and amino acid digestibility estimates are based on three studies only, and more investigation of these important aspects is

required. In addition to the effects of the correction for hydrolysis time and amino acid digestibility, potential differences due to factors such as population (ethnic and nutritional differences), methodology related to milk collection, and advances in amino acid analysis are all undoubtedly important. Nevertheless, the corrected values shown in Table 6 are put forward as the currently most accurate estimates for the absorbed amino acid composition of human breast milk. It would seem appropriate to reassess the FAO international recommendations.

Author contributions

PM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AD: Writing – original draft, Writing – review & editing. YY: Writing – original draft, Writing – review & editing. PW: Writing – original draft, Writing – review & editing. WXJL: Data curation, Resources, Writing – original draft, Writing – review & editing. NS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SD: Writing - review & editing. IS: Writing - review and editing. SH: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

YY, SD, and IS were employed by Inner Mongolia Dairy Technology Research Institute Co. Ltd. PW was employed by Oceania Dairy Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Plant-based diets–impacts of consumption of little or no animal-source foods on human health

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The world, in 2024, faces both climate and biodiversity crises, and the food system does contribute significantly to these crises. For some, the solution is simple - intakes of animal source foods (ASFs) should be considerably reduced, and consumption of plant-source foods (PSFs) should be greatly increased. Advocates for such a dietary transformation express confidence that plant-based diets will not only benefit planetary health, but will provide nutrient adequacy for all, and will also result in considerable protection from chronic non-communicable diseases (NCDs). However, as described in this perspective, the dramatic reductions in ASFs, entailed by many plant-based diets, will worsen already prevalent micronutrient and protein deficiencies. The protections provided by plant-based diets against NCDs appear to be more strongly associated with reduced intakes of calories and salt, and increased intakes of fruit, vegetables, nuts and whole grains, rather than with reduced intakes of ASFs. Any possible absolute adverse effects of red and processed meat consumption on NCDs are very small and uncertain. Other ASFs either appear to have no impact on NCDs (poultry meat and eggs), or are associated with protections against obesity, cardiovascular events, brain disorders and some cancers (seafood and dairy). Rigorous randomized controlled trials of all newly proposed environmentally-protective plant-based diets are required, so as to provide clear-cut evidence of micronutrient and protein adequacy, with or without, supplementation, fortification and/or biofortification. In the meantime, dietary guidelines should advise moderating excessive consumption, rather than substantially limiting or excluding ASFs from the human diet.

KEYWORDS

animal-source foods, plant-source foods, plant-based diets, micronutrients, adequacy, non-communcable diseases

Introduction

Humans have been omnivorous rather than herbivorous for a long time (1). About 3 million years ago, a period of climate change resulted in a decline of heavily forested lands, an expansion of drier grasslands and semi-forested regions, lessor availability of digestible plant source foods (PSFs), and greater availability of foods from grazing animals. Dietary divergence of hominins from other apes, toward animal source foods (ASFs), was followed by the physiological and metabolic adaptations that culminated in modern humans. With consumption of nutrient-rich, cooked, readily digested and absorbed ASFs, neither

voluminous fermentation chambers, such as a rumen or cecum, nor an extensive colon, were required, gastrointestinal tract length and absorptive surface area could be greatly reduced, and brain size and complexity greatly increased (2).

However, the world in 2024, now faces both climate and biodiversity crises. Food production and consumption, and in particular livestock farming and consumption of its products, do contribute to these crises. The food system is currently estimated to be responsible for about one third of total greenhouse gas emissions (3), and the conversion of natural ecosystems to agricultural land has been reported to be the largest threat to species extinction (4). Hence there is indeed a need to transform our food system so that all have access to healthy diets, while at the same time safeguarding the planet's health. The details of how that is best achieved is the subject of considerable debate – how much change should come from each domain of the food system – how much change should come from food production, processing, distribution, retailing, consumption and waste management?

For some, the solution to this challenge is simple, the human diet should revert back to being based on PSFs. It has been proposed that intakes of ASFs, particularly ruminant products, red meat and dairy foods, should either be considerably reduced, or totally excluded from the human diet (5–7). Advocates for such a dietary transformation express confidence that such plant based diets will not only benefit planetary health, but will provide nutrient adequacy for all, and will also result in considerable protection from chronic non-communicable diseases (cancers, diabetes mellitus, heart attacks and strokes).

In this article, the reliability of the claims of plant-based diets, with very reduced intakes of ASFs, for nutritional adequacy, and for protection against chronic disease events, is examined. Additionally, the impact and consequences of influential, but inaccurate, published metrics and recommendations, remaining uncorrected, are considered.

Plant based diets – impacts of little or no ASFs on nutritional adequacy

In 2019 the EAT-Lancet Commission on Food, Planet and Health published their planetary health reference diet (5). This was probably the first attempt to balance human dietary and planetary environmental needs to generate widespread interest among nutritional and environmental scientists, health professionals, policy makers and the general public (8, 9). The paper made headlines across the world, and on social media, content connected to the report have had more than 1 million shares in over 200 countries (8). According to Altmetric, the report is among the top 20 most discussed science papers across all academia (9) – it has been cited by 5,593 scientific papers and 798 policy documents in the 5 years since publication.

The EAT-Lancet Commission's planetary health diet is not a compulsory vegan diet – it does allow low quantities of red or processed meats and eggs to be consumed, and can include moderate amounts of seafood and poultry. However the diet largely consists of vegetables, fruits, whole grains, legumes, nuts and unsaturated plant oils – in total, only 13% of calories in the diet are from ASFs. Despite this low content of ASFs, the EAT-Lancet Commission were confident that the diet would meet all nutritional requirements of both adults and children older than 2 years. This confidence was surprising for a number of reasons.

Firstly, Beal and colleagues have clearly demonstrated that, as the percentage of energy coming from ASFs in national food supplies decreases, the prevalence of micronutrient inadequacy increases exponentially (10, 11). This particularly pertains to nutrients and micronutrients found in higher quantities, and in more bioavailable forms in ASFs, such as vitamins A, B_{12}, and D, key minerals including calcium, iodine, iron, phosphorus and zinc, long-chain polyunsaturated omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and essential amino acids. Overall, Beal and colleagues concluded that an average of 35% of calories from ASFs is required to provide a nutritionally adequate diet for populations (10, 11).

A recently published systematic literature review of the subject has found clear-cut evidence that dietary changes aiming to reduce environmental impacts result in lower intakes and status of a wide range of micronutrients of public health concern (12). Most of the 56 studies included in this review suggested that folate intake would increase with plant-based diets, but intakes of zinc, calcium, iodine and vitamins A, B_{12} and D would all decrease. The review also reported that total intake of iron would increase, but that might not result in improved iron status due to the lower bioavailability of iron from PSFs.

The review relied primarily on observational and modeling studies – of the 56 included studies, 10 were dietary intake studies, 45 were dietary modeling studies, and only one was a randomized controlled trial with biomarker data. Pellinen et al. studied the effects of partly replacing animal proteins with plant proteins on vitamin B_{12}, vitamin C, folate, iodine, iron and zinc, intakes and statuses in healthy adults (13). One hundred and 36 volunteers were randomly allocated to consume diets with 70% animal-source protein/30% plant-source protein, 50% animal-source protein/50% plant-source protein or 30% animal-source protein/70% plant-source protein, for 12 weeks. Key findings included that decreasing animal-source protein, even to the 50% level, led to important declines in the intakes and statuses of vitamin B_{12} and iodine. Zinc intake also decreased, but, due to the lack of an appropriate biomarker, zinc status was not evaluated. There were no differences in vitamin C intake nor status among the diet groups. While iron and folate intakes increased with greater consumption of PSFs, no significant differences in biomarker levels were observed. The authors concluded that longer duration trials, with biomarker data, in a range of healthy populations, were mandated to further study the effects of plant-based diets on the status of a wide range on nutrients, and particularly on iron status.

It is good that one of the EAT-Lancet Commissioners, Professor Jessica Fanzo, has recently confirmed that their first version of a planetary health diet would result in significant essential micronutrient shortfalls (14). In a paper published in Lancet Planetary Health in 2023, it was acknowledged that insufficient attention had been paid to the latest evidence on recommended nutrient intakes, to the greater bioavailability of iron and zinc from ASFs, and to the presence of anti-nutrients in many of the protein-rich PSFs. In the absence of micronutrient supplementation, in order to achieve micronutrient adequacy, it appears that intakes of ASFs, in such a flexitarian diet, would have to be doubled, accounting for at least 27% of calories, and intakes of PSFs, rich in phytates and polyphenols, such as whole grains, pulses and nuts, would need to be considerably reduced (14).

Plant-based diets – impacts of little or no ASFs on chronic non-communicable diseases

In 2019, the EAT-Lancet Commission also expressed confidence that widespread uptake of their recommended diet would reduce the incidence of non-communicable diseases (NCDs) and overall mortality - they estimated that approximately 11 million premature deaths among adults could be avoided annually through global adoption of the diet (5). However, these estimates have not been universally confirmed in further modeling and observational studies.

Zagmutt and colleagues were the first to question these estimates of avoided mortalities – they identified flaws in the assumptions and methods used, and their corrected analysis suggested that any mortality reduction effect of the EAT-Lancet diet was no greater than the impact of energy consumption changes that would prevent under-weight, over-weight and obesity alone (15, 16).

Adherence to the EAT-Lancet reference diet was reported to be inversely associated with all-cause mortality in three reports, the United Kingdom Biobank Study (17), the Malmo Diet and Cancer Study (18), and in three prospective United States cohorts (Nurses' Health Study I and II, and Health Professionals Follow-up Study) (19). It is noteworthy that the food groups contributing most strongly and consistently to the protection from mortality were increased intakes of PSFs rather than reduced intakes of ASFs – the top three food groups were fruits, vegetables and whole grains in the Swedish study (18), and added unsaturated fats, whole grains, and nuts in the United States study (19). A number of possible limitations were acknowledged by the authors of these three reports (17–19). Firstly, all cohorts were from high income countries. Secondly, those most adherent to the EAT-Lancet diet were also those most likely to follow a healthy lifestyle, and therefore residual confounding was highly likely to operate, and possibly explain some or all of the observed associations. Finally, adherence to the EAT-Lancet diet of even the most adherent subgroups was relatively low. The mean Planetary Health Diet Index score for the top decile in the United States-based cohorts was only 94 points out of a possible 140 points (19). Similarly, the dietary index of the quintile with highest adherence of the Swedish cohort ranged from 23 to 35 points out of a possible 42 points (18). In the United Kingdom Biobank study, the high adherence group did score 8 to 11 points out of a possible 11 points. However, due to lack of information in the United Kingdom Biobank questionnaire, adherence to three food groups (tubers, legumes and nuts) could not be assessed. Furthermore, this high adherence group accounted for less than 5% of the total cohort (17). Hence, the impact of strict adherence to the EAT-Lancet diet was, in reality, not tested in any of these three analyses.

By contrast, strict adherence to the EAT-Lancet reference diet was reported to provide no additional protection from mortality in the Oxford component of the European Prospective Investigation into Cancer and Nutrition study (20), the Prospective NutriNet-Santé Cohort study (21), and the Prospective Urban Rural Epidemiology (PURE) study (22). Interestingly, while adherence to the EAT-Lancet diet was not shown to be protective in the PURE study, adherence to the PURE healthy eating pattern was shown to be advantageous - each quintile higher PURE diet score was associated with a 9% (95% confidence intervals; 7–11) lower risk of death, and a 6% (3–8) lower risk of a major cardiovascular disease event (22). Rather than focusing on potentially disadvantageous foods, the PURE diet score is based on intakes of six protective foods, fruit, vegetables, nuts, legumes, fish and dairy (mainly whole-fat) (22). Hence, a key difference between the two diets is the guidance on ASFs. Intakes of meat (poultry, red and processed), dairy, fish and eggs should all be limited according to the EAT-Lancet diet (5). However, recent reviews have concluded that there is no additional risk of NCDs associated with consumption of poultry meat and eggs (23). Furthermore, based on evidence from cohort studies, metanalyses and biomarker studies of the protective effects of regular fish and dairy consumption against total mortality, cardiovascular disease, cognitive dysfunction, obesity and some cancers (7, 24–27), the PURE healthy eating pattern advises 2 to 3 servings of fish weekly, and 2 servings of dairy daily. An evaluation of the PURE diet score with and without each of the 6 food components confirmed that all 6 components, including the two ASFs, seafood and dairy, contributed to the observed protective associations. A further analysis of the PURE data found that inclusion of unprocessed red meat in the PURE score had no material effect on risk – hence, the PURE investigators did not advise any limitation to this food. This is in agreement with the conclusions of the comprehensive series of systematic reviews and guideline published in Annals of Internal Medicine in 2019 (28–34). The NutriRECs Consortium reported that the possible absolute effects of red and processed meat consumption on all-cause mortality are very small – reducing intakes of unprocessed red meat and processed meat by 3 servings weekly could prevent 8(0-15) and 9 (5–15) deaths per 1,000 persons, respectively, over 11 years (34). The consortium also, importantly, judged the certainty of evidence for this protection, as low or very low, and concluded that red and processed meat avoidance were not priority targets for improved human health (34).

The EAT-Lancet Commission relied on data and analyses from the Global Burden of Disease (GBD) 2017 Risk Factor Study (35) for their estimates of avoided mortalities achievable through global adoption of their diet. This GBD 2017 study reported that 11 million deaths (22% of all adult deaths), and 255 million disability adjusted life years (DALYs) (15% of all adult DALYs), were attributable to 15 dietary risk factors. High intake of sodium (3 million deaths and 70 million DALYs), and low intakes of whole grains (3 million deaths and 82 million DALYs), fruits (2 million deaths and 65 million DALYs), nuts and seeds (2 million deaths and 50 million DALYs), and vegetables (1.5 million deaths and 34 million DALYs) were the leading dietary risk factors. It is noteworthy that higher intakes of ASFs were estimated to be associated with protection against NCD events (seafood and dairy), or to have relatively small adverse impacts (unprocessed red meat: 25 thousand deaths and 1.3 million DALYs. processed meats: 0.1 million deaths and 3.6 million DALYs).

Using the above described GBD 2017 point estimates, the EAT-Lancet authors identified reduced intakes of salt, and increased intakes of whole grains, fruits, nuts and vegetables, as the main contributors to the putative planetary health diet's protective effects. However, as previously highlighted by many leading nutritional epidemiologists, almost all nutritional variables are highly correlated with each other, and also with other lifestyle patterns (36, 37). The risk associations of excess salt consumption, and low intakes of whole grains, fruits, vegetables and nuts with disease burdens are neither independent, nor necessarily causal effects. Individuals with high intakes of calories, salt and ultraprocessed foods, are frequently the same individuals who rarely consume fruits, vegetables or oily fish,

and who are also more likely to smoke and to take little exercise. Hence the GBD 2017 Diet Collaborators' statement that dietary risks were responsible for 22% of all deaths and 15% of all DALYs among adults in 2017, very probably represents extensive residual confounding. Furthermore the use of causal language ("attributable to" and "responsible for") by the GBD collaborators, when reporting on epidemiological associations, does not appear in accordance with good scientific principles (34, 36, 37).

The dangers of disregarding best practice in nutritional epidemiology (34, 36, 37), by using low-or very low-certainty evidence, in the development of guidelines, or in the calculation of global health metrics, is illustrated by the very different GBD risk estimates for unprocessed red meat, included in the GBD 2017, GBD 2019 and Burden of Proof (BoP) 2022 studies (35, 38, 39). In the 2017 estimates, based on associations with colorectal cancer and diabetes mellitus, the GBD Risk Factor Collaborators stated that diets high in unprocessed red meat were responsible for 25 thousand deaths and 1.3 million DALYs, globally (35). However, in 2019, the GBD Collaborators reported finding sufficient evidence supporting additional causal relationships of red meat intake with ischaemic heart disease, breast cancer, hemorrhagic stroke, ischaemic stroke and subarachnoid hemorrhage (38). Thus, they estimated that 896 thousand deaths and 23.9 million DALYs were attributable to unprocessed red meat consumption. This represented 36-fold and 18-fold increases over the GBD 2017 estimates for deaths and DALYs, respectively. The evidence for the 2019 estimates came from in-house, newly conducted, systematic reviews and meta-regressions - these had not been peer-reviewed nor published, and no assessments of certainty had been conducted. Many among the scientific community questioned the reliability of these dramatically changed estimates, and, rightly, requested publication of PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) compliant reports of the newly conducted systematic reviews (40–43).

These questions and requests eventually led to the publication of the BoP study of the health effects associated with unprocessed red meat consumption, in Nature Medicine in October 2022, by the GBD Collaborators (39). The relative risk curves and the conclusions of the BoP 2022 Study are very different from those of the GBD 2019 Risk Factors Study (38) - only the association between unprocessed red meat and colorectal cancer retained statistical significance. Even that relationship is doubtful, as statistical significance was only achieved after application of a monotonic constraint which resulted in an up to four-fold inflation of risk (44). In any case, the overall conclusion of the paper was similar to those of both the PURE study and the NutriRECS Consortium, namely that there is no or only very weak evidence that unprocessed red meat consumption is associated with any increased risk of NCDs.

Consequences of delayed or non-correction of inaccurate metrics concerning ASFs and plant-based diets

The GBD collaborators have publically acknowledged that their 2019 risk estimates of unprocessed red meat for NCD events were erroneously greatly inflated (39, 45, 46). However, despite requests to the GBD authors, and to The Lancet's editorial team and ombudsperson, no corrections have been applied to the published paper, and the 2019 risk estimates remain unchanged on the GBD website (47). Additionally, to date, the GBD collaborators have only published systematic reviews for the risk estimates associated with unprocessed red meat and with vegetable consumption (39, 48). No PRISMA compliant reports of the other 13 dietary risk factors have been published. Hence, considerable doubt remains over the accuracy of these GBD 2019 risk estimates.

Despite these important limitations, the GBD 2019 Risk Factors Study continues to be extensively cited. As can be seen in Table 1, the paper has been cited 3,651 times in the past 4 years. Among these publications, 233 have specifically commented on levels of red or processed meat consumption and/or its associated risks. At least 25 publications, in a wide range of national and international journals, have utilized the GBD 2019 Risk Factors Study's theoretical minimum risk exposure level (TMREL) value of zero, and/or their relative risk curves, as the primary evidence for adverse outcomes being associated with, or caused by red or processed meat consumption (49–73). It is of concern that the monthly rate of such publications, using these erroneous estimates, continues to climb.

Two of these publications, the 2022 and 2023 Reports of the Lancet Countdown on Health and Climate Change (58, 68) used both the TMRELs of the GBD 2019 Risk Factors Study, and the optimal intakes of the EAT-Lancet Reference Diet, as evidence for their model assumptions concerning diet and health co-benefits. The headline findings of these two reports were similar - 11.5 million deaths were attributed to imbalanced diets, of which approximately 8 million deaths were associated with insufficient consumption of plant-based foods and 2 million deaths were associated with excessive consumption of dairy, red and processed meats. The reports' estimates of 600,000 excessive deaths due to dairy consumption are particularly questionable – the authors assumed that the optimal intake for milk and dairy was zero to 250 mL per day, and stated that daily intakes above 250 mL contributed to overweight and obesity, and thereby caused approximately 600,000 cancer, cardiovascular or diabetic deaths annually. The authors appeared to ignore or disregard the already referenced evidence of two or more daily helpings of full-fat dairy (500–900 mL/day) being associated with protection against overweight, obesity and diabetes mellitus, colorectal and breast cancer, cardiovascular events and total mortality (7, 25–27).

The reports from the EAT-Lancet Commission and the GBD Risk Factors Collaborators also appear to continue to influence food policy decisions and international dietary guidelines. Figure 1 illustrates the quantities of ASFs recommended by a number of recently published international and national guidelines for healthy and sustainable diets (7, 74, 75). Only the German Nutrition Society (74) recommends two servings of dairy per day (Figure 1A, panel). The maximum dairy intakes recommended by either the World Health Organization (WHO/Europe) (7) or the World Wildlife Fund (75) is one serving per day (≤ 250 ml/day). It is noteworthy that the WHO/Europe diet impact assessment tool uses the same models to evaluate human health impacts as the above described reports of the Lancet Countdown on Health and Climate Change Commission (58, 68) Figure 1B panel illustrates that the total amounts of meat, seafood and eggs, recommended by World Health Organization, the World Wildlife Fund and the German Nutrition Society, are less than a third of the total required for micronutrient adequacy according to Beal and colleagues (14). Indeed the

TABLE 1 Summary of the numbers of publications that have cited the GBD 2019 Risk Factors Study (November 2020 – Febuary 2024) over the past 4 years, and of the key findings of the 25 publications which have utilized the theoretical minimum risk exposure levels and/or the relative risk curves of GBD Risk Factors Study 2019 as primary evidence for adverse outcomes being associated with, or caused by, red or processed meat consumption.

Year	Total number of citations (number of citations/month)	Total number of citing publications mentioning levels of red or processed meat consumption and/or associated risks (number of citations/month)	Publications which utilized the theoretical minimum risk exposure levels and/or the relative risk curves of GBD Risk Factors Study 2019 as primary evidence for adverse outcomes being associated with, or caused by, red or processed meat consumption			
			First author	Title	Journal	Headline/Key Finding
2024	333 (167)	27 (14)	Hong et al. (49)	Global burden of diabetes mellitus from 1990 to 2019 attributable to dietary factors: An analysis of the Global Burden of Disease Study 2019	Diabetes, Obesity and Metabolism	The three largest dietary contributors to the burden of diabetes mellitus were high intake of red meat, high intake of processed meat, and low intake of fruit.
			Moreno et al. (50)	The burden of cardiovascular disease attributable to dietary risk factors in Australia between 1990 and 2019	PLoS ONE	Although the burden of diet-related CVD has decreased significantly in the Australian population over the past 30 years, diets low in wholegrains and high in red meat continue to contribute significantly to the overall CVD burden. Future nutrition programs and policies should target these dietary risk factors.
			Liu et al. (51)	Colorectal cancer's burden attributable to a diet high in processed meat in the Belt and Road Initiative countries	World Journal Gastrointestinal Oncology	The burden of colorectal cancer in relation to the consumption of a diet high in processed meat threatens public health.
2023	1,576 (131)	97 (8)	Yan et al. (52)	Global burden of ischemic heart disease associated with high red and processed meat consumption: an analysis of 204 countries and territories between 1990 and 2019	BMC Public Health	Implementing targeted policies and interventions is required to reduce the burden of IHD caused by a high intake of red and processed meat.
			Liang et al. (53)	Distributions and Trends of the Global Burden of Colorectal Cancer Attributable to Dietary Risk Factors over the Past 30 Years	Nutrients	To alleviate colorectal cancer burdens, it is recommended to elevate the intake of whole grains, milk, calcium, and fiber while reducing consumption of red and processed meats.
			Sharma et al. (54)	Temporal patterns of breast cancer incidence, mortality, disability-adjusted life years and risk factors in 12 South American Countries, 1990–2019: an examination using estimates from the global burden of disease 2019 study	Breast Cancer Research and Treatment	Alcohol use, diet high in red meat and smoking contributed the maximum DALYs in most countries in 2019.

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TABLE 1 (Continued)

Year	Total number of citations (number of citations/month)	Total number of citing publications mentioning levels of red or processed meat consumption and/or associated risks (number of citations/month)	Publications which utilized the theoretical minimum risk exposure levels and/or the relative risk curves of GBD Risk Factors Study 2019 as primary evidence for adverse outcomes being associated with, or caused by, red or processed meat consumption			
			First author	Title	Journal	Headline/Key Finding
			Li et al. (55)	Burden of early-onset colorectal cancer along with attributable risk factors from 1990 to 2019: a comparative study between China and other G20 countries	BMC Public Health	In China, the five leading risk factors, for both sexes, were diet low in milk [18.54% (95% UI: 12.71–24.07)], diet low in calcium [15.06% (95% UI: 10.70–20.03)], alcohol use [12.16% (95% UI: 8.87–15.64)], smoking [9.08% (95% UI: 3.39–14.11)], and diet high in red meat [9.08% (95% UI: 3.39–14.11)] in 2019.
			Forray et al. (56)	The Global Burden of Type 2 Diabetes Attributable to Dietary Risks: Insights from the Global Burden of Disease Study 2019	Nutrients	The results show that in 2019, 26.07% of T2DM mortality and 27.08% of T2DM DALYs were attributable to poor diets, particularly those low in fruits and high in red and processed meats.
			Wu et al. (57)	The Global Burden of Disease Attributable to Diet High in Red Meat in 204 Countries and Territories, 1999–2019: An updated Analysis of the Global Burden of Disease Study	Molecular Nutrition and Food Research	Globally, since 1999, deaths and DALYs caused by diets high in red meat have steadily increased.
			Romanello et al. (58)	The 2023 report of the Lancet Countdown on health and climate change: the imperative for a health-centred response in a world facing irreversible harms	The Lancet	Headline finding: In 2020, 7.8 million deaths were associated with insufficient consumption of nutritious plant-based foods and 1.9 million deaths were associated with excessive consumption of dairy, and red and processed meat.
			Mubarik et al. (59)	Breast cancer epidemiology and sociodemographic differences in BRICS-plus countries from 1990 to 2019: An age period cohort analysis	SSM - Population Health	High body mass index, high fasting plasma glucose, and a diet high in red meat contributed to the highest death and DALYs rates in most BRICS-plus nations in 2019.
			Zhang et al. (60)	Global Burden of Cardiovascular Disease from 1990 to 2019 Attributable to Dietary Factors	Journal of Nutrition	High socio-demographic index regions had the highest population attributable fractions for cardiovascular disease mortality and DALYs associated with high red and processed meat intake
			O'Hearn et al. (61)	Incident type 2 diabetes attributable to suboptimal diet in 184 countries	Nature Medicine	Largest type 2 diabetes burdens were attributable to insufficient whole-grain intake (26.1% (25.0–27.1%)), excess refined rice and wheat intake (24.6% (22.3–27.2%)) and excess processed meat intake (20.3% (18.3–23.5%))

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TABLE 1 (Continued)

Year	Total number of citations (number of citations/month)	Total number of citing publications mentioning levels of red or processed meat consumption and/or associated risks (number of citations/month)	Publications which utilized the theoretical minimum risk exposure levels and/or the relative risk curves of GBD Risk Factors Study 2019 as primary evidence for adverse outcomes being associated with, or caused by, red or processed meat consumption			
			First author	Title	Journal	Headline/Key Finding
			Lv et al. (62)	Trend of disease burden and risk factors of breast cancer in developing countries and territories, from 1990 to 2019: Results from the Global Burden of Disease Study 2019	Frontiers in Public Health	Percentage changes in deaths from the seven risk factors in low-to middle-socio-demographic index regions increased significantly over time across all age groups. However, a diet with high red meat and high body mass index accounted for the most considerable increase in the magnitude.
			Wang et al. (63)	Trends of burden on ischemic heart disease and related risk factors among residents in Jiangsu Province, 1990–2019	Chinese Journal of Disease Control and Prevention	From 1990 to 2019, DALYs attributed to ambient particulate matter pollution (ARC = 1.29%), high body-mass index (ARC = 1. 76%), diet high in red meat (ARC = 0. 36%), diet high in processed meat (ARC = 0. 32%), and alcohol use (ARC = 4. 19%) exhibited the greatest increase.
2022	1,241 (103)	84 (7)	Liu et al. (64)	Worldwide burden attributable to diet high in red meat from 1990 to 2019	Archives of Medical Science	In 2019, a diet high in red meat was responsible for 0.9 million (95% UI 0.5 to 1.3 million) deaths and 23.9 million (95% UI 15.6 to 32.0 million) DALYs worldwide. From 1990 to 2019, the total deaths and DALYs attributable to a diet high in red meat increased by over 50%. Increasing consumption of red meat remains a global challenge, especially in the low-middle and middle SDI countries.
			Chen et al. (65)	Stroke mortality attributable to high red meat intake in China and South Korea: An age–period–cohort and joinpoint analysis	Frontiers in Nutrition	Controlling the intake of red meat may be a cost-effective strategy to reduce stroke mortality risk and the corresponding disease burden, especially for Chinese male individuals.
			Zhao et al. (66)	Epidemiological trends of female breast and gynecologic cancers in adolescents and young adults in China from 1990 to 2019: Results from the Global Burden of Disease Study 2019	Frontiers in Oncology	Of the deaths and DALYs, diet high in red meat was the greatest contributor to breast cancer, while a high body mass index was the greatest contributor to cervical, ovarian, and uterine cancers. A non-red meat diet, and the control of body weight could reduce female breast and gynecologic cancers burden in China.
			Li et al. (67)	Thirty-year changes in disability adjusted life years for colorectal cancer in China: a screening perspective analysis	Chinese Journal of Endemiology	Compared with 1990, the colorectal cancer -caused DALYs in China increased by 181.5% in 2019. Factors with the largest increase in the attributable percentage were high body mass index (151.1%), diet high in red meat (86.4%) and diet high in processed meat (78.8%).

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TABLE 1 (Continued)

Year	Total number of citations (number of citations/month)	Total number of citing publications mentioning levels of red or processed meat consumption and/or associated risks (number of citations/month)	Publications which utilized the theoretical minimum risk exposure levels and/or the relative risk curves of GBD Risk Factors Study 2019 as primary evidence for adverse outcomes being associated with, or caused by, red or processed meat consumption			
			First author	Title	Journal	Headline/Key Finding
			Romanello et al. (68)	The 2022 report of the Lancet Countdown on health and climate change: health at the mercy of fossil fuels	The Lancet	Headline finding: in 2019, 1·9 million deaths were associated with excessive consumption of dairy, and red and processed meat.
			Chen et al. (69)	Long-Time Trend of Colorectal Cancer Mortality Attributable to High Processed Meat Intake in China and a Bayesian Projection from 2020 to 2030: A Model-Based Study	International Journal of Environmental Research and Public Health	Colorectal cancer death attributable to high processed meat intake is still high in China, and elderly males were at higher risk. Gradually decreasing the intake of processed meat could be an effective way to reduce colorectal cancer mortality.
			Wu et al. (70)	The burden of stroke attributable to risk factors and their trends from 1990 to 2019 in China	Chinese Journal of Disease Control and Prevention	From 1990 to 2019, the DALYs of ischemic stroke and intracerebral hemorrhage attributable to ambient particulate matter pollution, high BMI, alcohol use and diet high in red meat significantly increased by 410.46, 320.48, 277.03, 245.41 and 168.93%, 132.07, 60.01, 84.58%, respectively.
			Machado et al. (71)	Burden of non-communicable diseases attributable to dietary risks in Brazil, 1990–2019: an analysis of the Global Burden of Disease Study 2019	Revista da Sociedade Brasileira de Medicina Tropical	Diet high in red meat and sodium, and low in whole grains were the three main risk factors contributing to the burden of NCDs both in 1990 and 2019.
2021	501 (42)	25 (2)	Chung et al. (72)	Global red and processed meat trade and non-communicable diseases	BMJ Global Health	Results show that global increases in red and processed meat trade contributed to the abrupt increase of diet-related NCDs
			Romanello et al. (73)	The 2021 report of the Lancet Countdown on health and climate change: code red for a healthy future	The Lancet	Headline finding: between 2017 and 2018, estimated deaths due to excess red meat consumption rose by 1·8% to 842,000.

ARC, annual change rate. BMI, body mass index. BRICS, Brazil, Russia, India, China, and South Africa. CVD, cardiovascular disease. DALY, disability adjusted life-year. IHD, ischaemic heart disease. NCD, non-communicable disease. SDI, socio-demographic index. T2DM, type 2 diabetes mellitus. UI, uncertainty interval.

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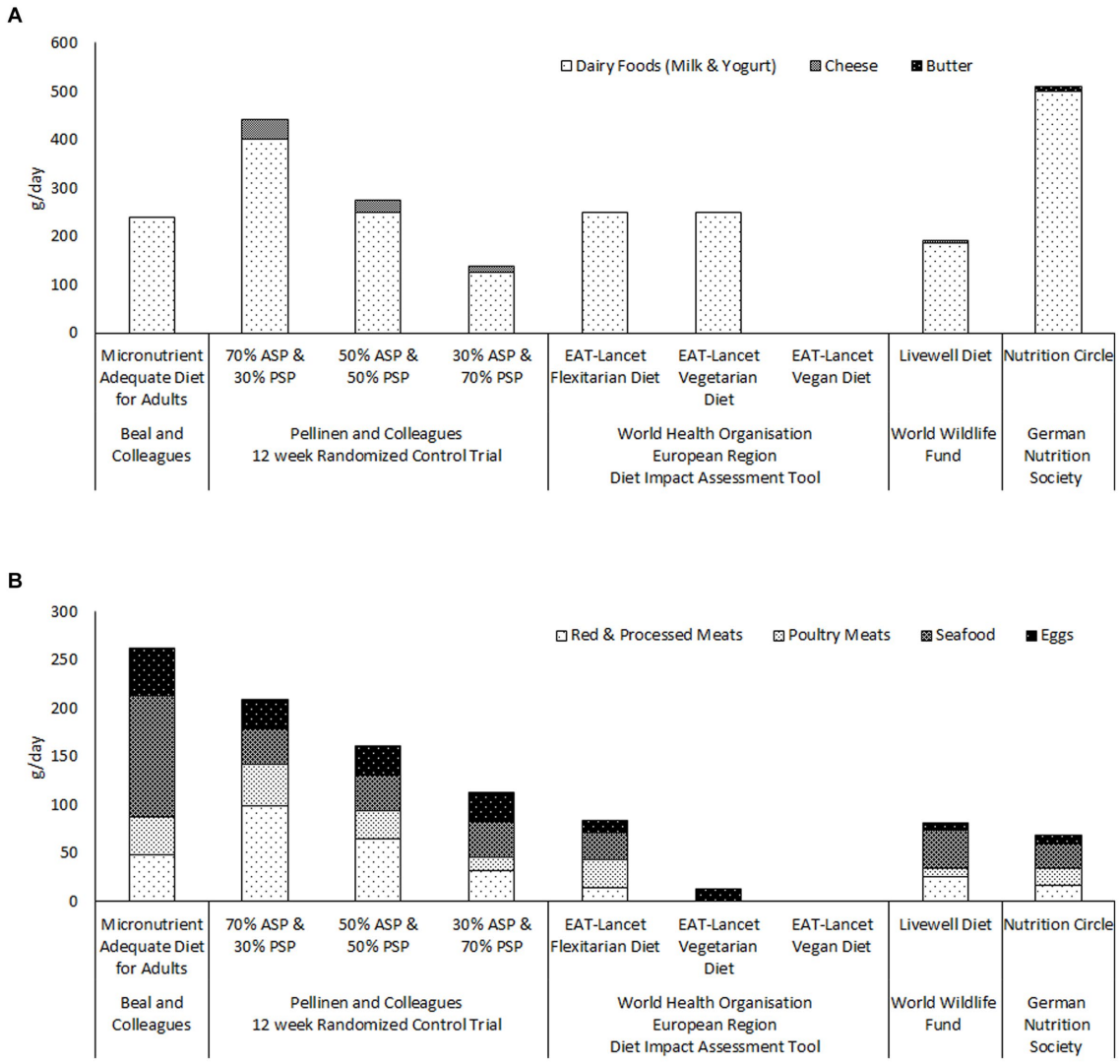

FIGURE 1
Comparison of the quantities of ASFs recommended by recently published guidelines for healthy and sustainable diets; the World Health Organisation European Region's Flexitarian, Vegetarian and Vegan diets (7); the World Wildlife Fund's Livewell diet (75); and the German Nutrition Society's Nutritional Circle (74), with the quantities included in Beal and colleagues' Micronutrient Adequate Diet for Adults (14), and in the three food groups of Pellinen and colleagues' randomised controlled trial (13). Panel **(A)** illustrates the quantities of dairy foods recommended by each of the diets. Panel **(B)** illustrates the quantities of meats, seafood and eggs recommended by each of the diets. ASP; animal-source protein. PSP; plant-source protein.

quantities of meat, seafood and eggs recommended by the five diets, from these three bodies, are all less than those consumed by the 30% animal-source protein group of Pellinen and et al. randomized controlled trial (13). It is difficult to see how any of these diets could provide either protein or micronutrient adequacy at the population level.

Concluding comments

It is clear that any evidence that moderate consumption of ASFs is detrimental to human health, is weak and uncertain. The relationship between red meat and disease burden, like those of calories and salt with disease burden, is most likely U-shaped. Excess red and processed meat consumption (>4 portions or 500 g/week) may be associated with very small increases in morbidity and mortality (low certainty evidence). Insufficient meat consumption (<2 portions/week) is associated with very large increases in anemia, stunted childhood growth and cognition, osteoporosis and sarcopenia (high certainty evidence). Poultry meat and eggs appear to have no impact on NCDs, while consumption of dairy and seafood not only protects against key deficiencies, these foods also likely protect against obesity, cardiovascular events, brain disorders and some cancers.

It is also clear that the dramatic reductions in ASFs, advised by many plant-based diets, will worsen already prevalent micronutrient and protein deficiencies worldwide. This will have particular impact in low and middle income countries, and on vulnerable groups, including women, children and the elderly. These were the conclusions of Ty Beal's recent editorial in the American Journal of Clinical

Nutrition (76). I agree with his calls for; moderating excessive consumption, rather than substantially limiting or excluding ASFs from the human diet; and further research into the roles that supplementation, fortification and biofortification can play in achieving healthy sustainable diets for all. Furthermore, it is of considerable importance that rigorous randomized controlled trials of all newly proposed environmentally protective diets are conducted. These trials should include validated biomarkers of nutrient status, and should assess levels of supplementation and/or fortification, that would be required so as to ensure micronutrient and protein adequacy.

Finally, scientists, policy-makers and all involved in the food system should be extremely wary of reports, guidelines or global health estimates that are not rigorously and transparently evidence-based. A wide range of sustainably produced, nutrient-rich, animal-and plant-sourced foods, in appropriate evidence-based quantities, should continue to be included in national and international guidelines for healthy diets. Further research, finances and effort should be directed toward objective and reliable measurements and improvements in sustainability of each component of the food system; production; processing; distribution; retailing; consumption; and waste management.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

Author contributions

AS: Writing – original draft, Writing – review & editing.

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Conflict of interest

Alice Stanton, was a part-time employee of Devenish Nutrition (2017–2023); and currently owns stock in Devenish Nutrition, an agri-technology company specializing in sustainable food solutions.

Publisher's note

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Global protein sustainability and the United Nations, through to the 2030 agenda

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Organizations and initiatives concerned with food security and nutrition have long positioned protein, together with dietary energy, as the keystone for life itself. Indeed, the word protein, derived from the Greek *proteios*, means 'of primary importance'. There is a long history of attention to, and controversies over, proteins in UN processes, beginning in the 1930s and continuing to this day. The importance of protein for agriculture, health, food security and nutrition is reflected in the data collected and presented in the statistical databases of the Food and Agriculture Organization (FAOSTAT), available per commodity, per country and over an extensive time series. Protein features directly and indirectly in all 17 Sustainable Development Goals (SDG), which constitute the United Nations 2030 Agenda. Most directly involved is SDG 2. The short title for SDG 2 is 'zero hunger'. The long title offers more detail: end hunger, achieve food security and improved nutrition and promote sustainable agriculture.

KEYWORDS

protein, United Nations, policy, nutrition, FAOSTAT, Food and Agriculture Organization of the United Nations

Introduction

Historical overview of protein and the UN

International cooperation and collaboration in nutrition began in earnest in 1936 when the League of Nations set up a technical committee to establish recommended levels of protein intake (1). In 1945, with the creation of the United Nations (UN) and soon thereafter its specialized technical agencies, attention to protein continued. Several of the UN's specialized agencies concerned themselves with dietary protein, but the two with the longest history of dealing specifically with proteins are the Food and Agriculture Organization of the United Nations (FAO) (2), and the World Health Organization (WHO) (3). Of major concern was the 'protein gap' related to both production and consumption (4). In one way or another, the theoretical protein gap and its remedies feature directly and peripherally in goals, targets, policies, research, interventions, and more, to this very day.

In 1948–1950, FAO established and convened meetings of the Standing Advisory Committee (5) to address the most pressing nutrition problems, with protein and dietary energy at the top of the list. From the late 1940s, there was a series of meetings and several technical reports on protein (2, 3), as it was commonly agreed that a major nutrition problem was a lack of sufficient protein in the diets of young children, known as kwashiorkor from the Ga language of Ghana (6). The First Joint FAO/WHO Expert Committee on Nutrition noted that "one of the most widespread nutritional disorders in tropical and sub-tropical areas is the

syndrome at present ill-defined and known by various names such as kwashiorkor" (7).

In 1971, the UN itself in the body of the General Assembly (UNGA) devoted a full segment of its meeting to protein resources (8, 9). The membership put forward a set of 16 resolutions as "Essential elements of the Strategy Statement on Action to Avert the Protein Crisis in the Developing Countries."

Viewed from a 21st century vantage point, some of these protein-related resolutions succeeded, while others remain intransigent 50+ years later and feature in the goals and targets of the 2030 Agenda. Table 1 shows a subset of the resolutions mapped to comparable SDGs, along with comments on the success, failures, and consequences over the timeframe. Among the resolutions identified as successful are some that ironically also contribute to our sustainability crises, with specific examples. On several occasions since then, the UNGA has returned its focus to protein, mainly in the context of livestock, climate change and consumption of animal source proteins (10).

In the 1972 meeting of the UN Protein Advisory Group (9), Hugues Gounelle de Pontanel states the conclusion that, "*Every doctor, nutritionist or political leader concerned with the problem of world hunger has now concluded that the major problem is one of protein malnutrition.*"

But not everyone agreed, and one of the most spectacular controversies in nutrition – the Great Protein Fiasco—became public soon thereafter (11) (see below). Nevertheless, from the 1970s to the present, FAO and WHO, and occasionally with the International Atomic Energy Agency and United Nations University, conducted many more meetings and expert consultations leading to protein-related reports, policies and recommendations, and necessary reviews and revisions as nutrition science and data availability increased and improved over time. Topics included protein requirements, production issues, measurement/methods, composition/quality, and consumption. Table 2 provides a list.

Assessment and implications

The great protein fiasco

The UN reports and their recommendations were not without controversy (11). World protein supply has long been estimated using production data from FAO and national statistical agencies, consumption data made mainly with proxy measures from FAO food balances (i.e., protein available for human consumption) and disappearance data, which is then analyzed against requirements (without considering inequalities in accessing protein in the population). As such, there seemed to be a shortfall which was called the "protein gap." Consideration was also given to protein quality measurements and calculations, further defining the gap, as vegetable and other non-animal-source proteins were of poorer quality than animal source proteins. It was concluded by scientists and policy-makers alike that the protein gap would only widen unless alternative or unconventional sources of high-quality protein could be found. Leaf protein concentrate, insects and single cell organisms (12) were then included in nutrition research and development programmes around the world. The Protein Advisory Group, a UN agency, had been established in 1955 to advise on the "safety and suitability" of these new protein-rich foods.

However, in 1974, Donald McLaren, professor at the American University in Beirut, published a paper in The Lancet titled: "The great protein fiasco" (11), proposing that dietary energy should be the focus of attention, and that would bring about adequacy across the nutrient spectrum, protein included. A year after McLaren's paper appeared in The Lancet, John Waterlow and Philip Payne from the London School of Hygiene and Tropical Medicine published an analysis of diets of children in developing countries (13). Their analysis revealed that protein deficiency was rare, and when it occurred it was caused by a simple lack of food, rather than the low-protein content of food. In a 2011 interview, reflecting on his life and career, McLaren described the belief in the protein gap as "one of the greatest errors committed in the name of nutrition science in the past half-century" (14). Was it, though? Debates continue, with the overriding view that there is a nutrition crisis in the world, with protein as a feature, and it is related to both production and consumption.

UN's 2030 agenda

SDG 2, the hunger goal, has five targets, 2.1–2.5, with an additional three added (2.A, 2.B, 2.C); each target has one or more indicator(s). The following section presents data from the FAO Statistical Databases (FAOSTAT), some of which correspond to SDG indicators, showing trend analyses and projections into the future. The data on protein available for human consumption used in this study are derived from the latest series of Food Balance Sheets (FBS) based on a new methodology (15) and from the new dataset of nutrient conversion factors (16), both developed by FAO. Data expressed in units '*per capita* per day' reflect availability within a country/region or special group and are used as a convenient but crude proxy for consumption.

Figure 1 shows that dietary protein available for human consumption at the global level has increased by 7 % since 2010, from around 85 to more than 90 g/capita/day, despite slight decreases in Africa and Oceania. Europe has the highest dietary protein supply (112 g/capita/day) in 2021, followed by the Americas (104 g/capita/day), Oceania (102 g/capita/day), Asia (92 g/capita/day) and Africa (66 g/capita/day). Protein quality is not considered in this metric.

Both animal and vegetal foods supply dietary protein, with animal source foods providing higher quality protein than vegetal foods (based on quantity and balance in the amino acid composition). As shown in Figure 2, the proportion supplied by each source of protein varies with the income level of the country (17). In 2021, in high-income countries, 63 percent of the protein (amounting at 71.2 g/capita/day) is supplied from animal sources. In low-income countries it was only 18 percent (amounting at 10.9 g/capita/day). Controversies abound regarding the conflicting issues surrounding livestock production and consumption – nutritional equity, or lack thereof which is illustrated with these data, plus human health and environmental sustainability risks and benefits, to name but a few.

Focusing on special groups, in 2021 the percentage of protein from animal sources in small islands developing states was 43 percent, equivalent to 32.4 g/capita/day. On the contrary, that year, the net food importing developing countries obtained 27

TABLE 1 Comparing the 1971 United Nations General Assembly (UNGA) 26th session recommendations on protein resources (1) with relevant/comparable Sustainable Development Goal (SDG) targets (2).

UNGA 26th Protein Session, 1971	SDG targets	Notes/comments
Make every effort to increase the production of food crops, particularly through the exploitation of new high-yield varieties, bearing in mind the special need for an expanded production of protein-rich pulses and oilseeds;	2.3 "Double the agricultural productivity and incomes of small-scale food producers, in particular women, indigenous peoples, family farmers, pastoralists and fishers…."	New varieties and high yield are the UNGA focus, as the Green Revolution is seen as a great agriculture success story. The specific focus on pulses and oilseeds is particularly noteworthy. Implicit is encouragement for the movement away from too-heavy reliance on animal source foods, which is a hallmark of many sustainability recommendations (3). Unfortunately, the SDGs make no mention of pulses or other high protein plant source foods.
Encourage accelerated and expanded research designed to improve the nutritive value of cereal proteins through genetic engineering;	2.5 "Maintain the genetic diversity of seeds, cultivated plants and…their related wild species …."	UNGA, at the time exposed to primarily only to the benefits of the Green Revolution, does not consider biodiversity (genetic diversity) in its recommendations. The SDGs, 40 years later and therefore mindful of the negative consequences, focus instead on conserving biodiversity.
Encourage accelerated and expanded research designed to develop high-yielding pulses, legumes and oilseed crops;	2.4 "Ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems, …."	As above, UNGA valued food crops engineered for high yield to the exclusion of many other considerations. Explicit in the complete text for SDG 2.4 is "the need to improve ecosystems, that strengthen capacity for adaptation to climate change, extreme weather, drought, flooding and other disasters and that progressively improve land and soil quality."
Encourage the increased production of animal proteins, particularly through research on increasing forage yields and production;	2.5 "Maintain the genetic diversity of… farmed and domesticated animals and their related wild species…and promote access to and fair and equitable sharing of benefits arising from the utilization of genetic resources and associated traditional knowledge …."	UNGA references animal production for human nutrition, whereas the SDGs avoid livestock-related targets for both production and consumption. Nevertheless, animals are included in the 'no hunger' goal, but seemingly for their function as ecosystems services.
Make every effort to prevent an unnecessary loss of protein-containing foods in field, storage, transport and home	12.3 "Halve *per capita* global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses…"	Unacceptably high levels of food losses and waste remain an enduring problem and appear with equal consideration in both the SDGs and UNGA recommendations.
Encourage increased production from marine and freshwater fishery resources	14.4 "Effectively regulate harvesting and end overfishing, illegal, unreported and unregulated fishing and destructive fishing practices and implement science-based management plans, in order to restore fish stocks in the shortest time feasible, at least to levels that can produce maximum sustainable yield as determined by their biological characteristics…"	It was obviously not foreseen in the UNGA recommendation that production from fishery resources would quadruple over the next 50 years to become unsustainable.
Conduct informational and educational campaigns related to protein production and consumption	12.8 "Ensure that people everywhere have the relevant information and awareness for sustainable development and lifestyles in harmony with nature…"	
Improve protein utilization through the control and prevention of infectious diseases	2.2 "End all forms of malnutrition, including achieving, by 2025, the internationally agreed targets on stunting and wasting in children under 5 years of age …."	Given that stunting in particular is often a manifestation of insufficient quantity or poor quality protein, SDG 2.2 is well aligned to the UNGA recommendation.
Review and improve policies, legislation and regulations regarding all aspects of food and protein production, processing and marketing so as to remove unnecessary obstacles and encourage appropriate activities	2.b "Correct and prevent trade restrictions and distortions in world agricultural markets, …." 2.c "Adopt measures to ensure the proper functioning of food commodity markets and their derivatives …."	The UNGA recommendation and SDG 2b, 2c are well aligned, acknowledging the obvious technological and regulatory developments over time.

(Continued)

TABLE 1 (Continued)

UNGA 26th Protein Session, 1971	SDG targets	Notes/comments
Give special attention to the protein needs of vulnerable groups; and Initiate intervention programmes aimed at ensuring that vulnerable groups will receive the most appropriate type and a sufficient quantity of food by the most effective means	2.1 "End hunger and ensure access by all people, in particular the poor and people in vulnerable situations, including infants, to safe, nutritious and sufficient food all year round…"	Considering that 40+ years after UNGA 1971, and equally 40 years before, SDG 2.1 has been the most basic enduring problem for humankind, with hunger representing the poignant denial of the most basic of human rights.
Recognize the role of economic development and social modernization in solving the protein problem.	12.8 "Ensure that people everywhere have the relevant information and awareness for sustainable development and lifestyles in harmony with nature…"	One of the greatest shifts in thinking from the time of the UNGA recommendations to the time of the SDGs is the recognition that 'economic development and social modernization' is often in conflict with 'harmony with nature'. Throughout the SDG, harmony with nature takes priority.

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2. United Nations. The UN Sustainable Development Goals. New York; 2015.
3. Jones R, Vogliano C, Burlingame B. Sustainable diets and food-based dietary guidelines. In: Burlingame B, Dernini S, editors. Sustainable diets: linking nutrition and food systems. CABI; 2018. p. 158–71.

TABLE 2 Examples of protein reports from United Nations (UN) agencies, 1936 to the present.

1936: Report on the Physiological Bases of Nutrition. The Health Committee of the League of Nations, Geneva.
1949. Report of the First Joint FAO/WHO Expert Committee on Nutrition. Geneva, 24–28 October. Geneva.
1957: Protein requirements: report of the FAO Committee. FAO Nutritional Series No. 16, 1957
1963: Protein requirements - Report of a Joint FAO/WHO Expert Group. FAO Nutrition Meeting Report Series No. 37, 1964 and WHO Technical Report Series No. 301, 1964.
1970: Amino-Acid content of foods and biological data on proteins. FAO food and nutrition series. Rome, Italy: FAO.
1973: Energy and protein requirements: Report of a joint FAO/WHO *ad hoc* expert committee. Rome: FAO Nutrition Meetings Report Series No. 52. Geneva: WHO Technical Report Series No. 522.
1974: 'The Great Protein Fiasco'; McLaren calls the protein gap theory "one of the greatest errors committed in the name of nutrition science (11)."
1981: Joint FAO/WHO/UNU Expert Consultation on Energy and Protein Requirements
1985: WHO/World Health Organization/Food and Agriculture Organization/ United Nations University (1985) Energy and protein requirements Report of a Joint FAO/WHO/UNU Expert Consultation. WHO Technical Report Series, No. 724. Geneva: WHO.
1991: Protein quality evaluation. Report of Joint FAO/WHO Expert Consultation, FAO Food and Nutrition Paper 51.
2007: Protein and Amino Acid Requirements in Human Nutrition Report of a Joint WHO/FAO/UNU Expert Consultation. WHO Technical Report Series No. 935
2013: Dietary protein quality evaluation in human nutrition: Report of an FAO Expert Consultation, FAO Food and Nutrition Paper 92.
2019: Nitrogen and protein content measurement and nitrogen to protein conversion factors for dairy and soy protein based foods: a systematic review and modeling analysis. WHO and FAO.

percent of protein from animal sources equivalent to 18.4 g/capita/day (Figure 3).

Figure 4 shows the top five providers of protein from crops and livestock, in 2021. Wheat flour was the main source of protein in the world, and in Africa, Asia and Europe, while it was chicken meat in the Americas and in Oceania. Among animal food, chicken meat is the main source of protein everywhere except Europe and Asia, where pig meat is the main source. Other relevant sources are cattle meat in the Americas, chicken meat in Asia and pig meat in Oceania. Raw milk of cattle was within the top five main sources in all regions except Africa, where four out of the five main sources were cereals. Raw milk of cattle + meat was within the top five providers of protein in all the regions.

As presented in Figure 5, since 1961, in the world, the production of milk (from all livestock including the amount used to feed them) and meat (in terms of dressed carcass weight, excluding offal and slaughter fats) have increased (16); however, at different paces. While the production of meat has increased five-fold since 1961, that of milk increased only by a factor of 2.7. In *per capita* terms (18), the global production of meat has almost doubled since 1961, while that of milk remained fairly constant.

Discussion and recommendations

Presented here is a brief review of UN-led evidence-based initiatives on protein, along production and consumption data from FAOSTAT, the combination of which forms the foundation of policies and programmes, and indeed, the part of the SDG monitoring. But the current activities and data are only small pieces for a bigger puzzle requiring integration of many sectors and disciplines. Looking back at some of the recommendations from the 1971 UNGA meeting (8), it should have been predictable that 'increased production of animal proteins, particularly through research on increasing forage yields and production' could lead to environmental degradation and biodiversity loss; or that 'increased production from marine and freshwater fishery resources' could lead to crises in the capture fisheries sector with over-fishing, and extreme pollution from the farmed fish sector. Similarly, what are the consequences of our current trajectory for protein production, consumption, and research?

FAOSTAT provides useful data on consumption and production for national and global assessments, and monitoring trends over time, but more granularity and greater disaggregation would improve the

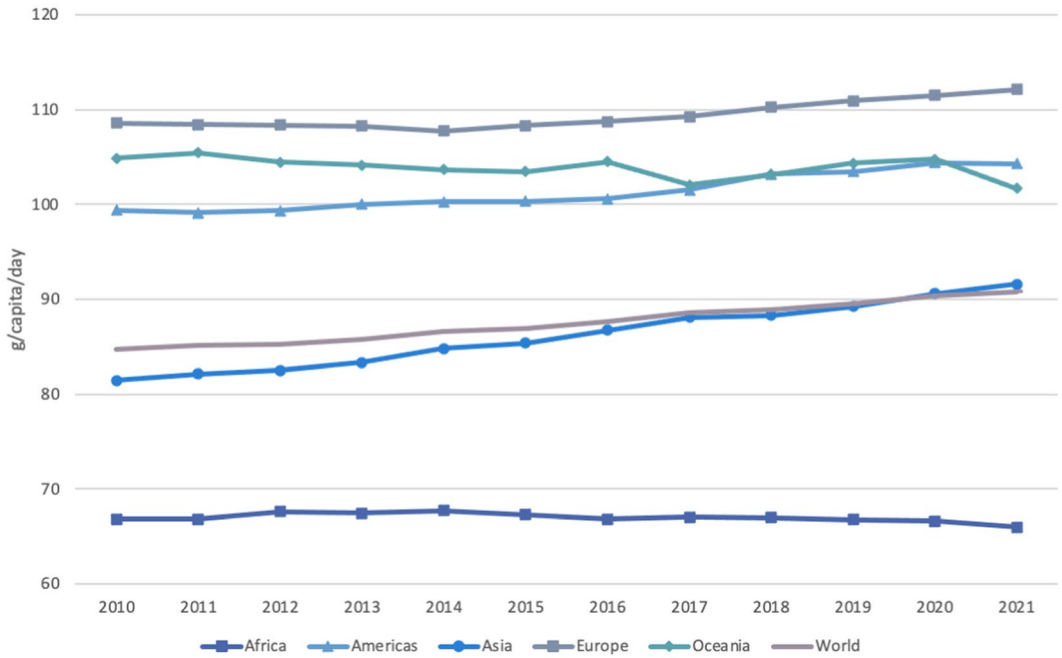

FIGURE 1
Total dietary protein supply by region and in the world between 2010 and 2021, in g/capita/day. Reproduced from FAO. 2023. FAOSTAT. Food Balances. Food Balances (2010-), licensed under CC BY 4.0.

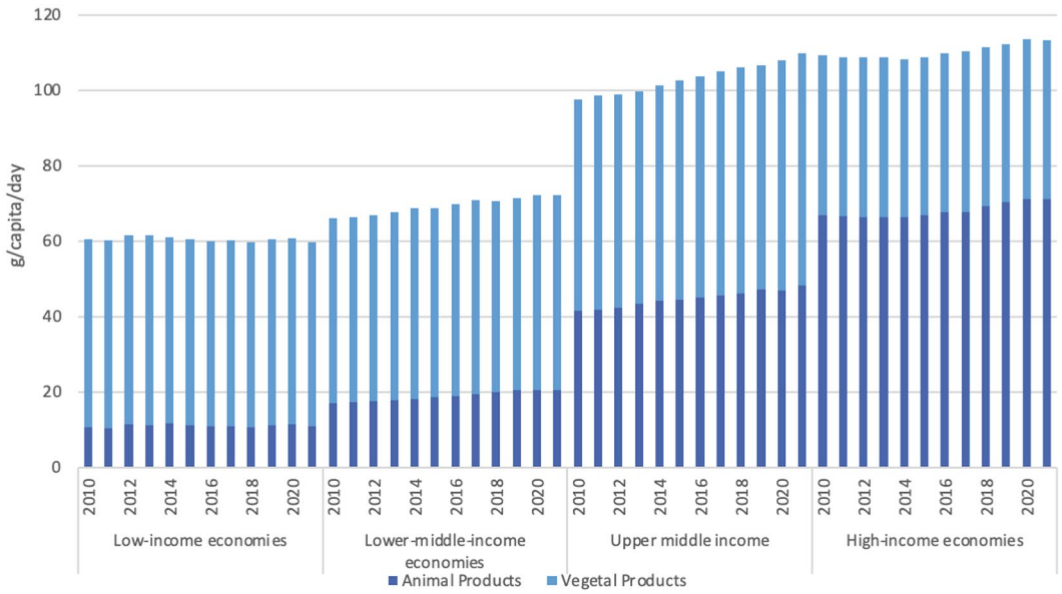

FIGURE 2
Dietary protein supply from animal and vegetal sources by income economy, in g/capita/day. Reproduced from FAO. 2023. FAOSTAT. Food Balances. Food Balances (2010-), licensed under CC BY 4.0.

value of these data sets for achieving goals and targets related to human nutrition generally, and protein specifically. It is clear, despite all the efforts and initiatives focussing on human nutrition, that the world is not on track for meeting the 2030 Agenda. In the time remaining, different forms of knowledge, including traditional knowledge from the millennia of lived science of indigenous peoples, need to be given greater attention, and more transdisciplinary and multisectoral collaborations need to be marshaled for sustainable

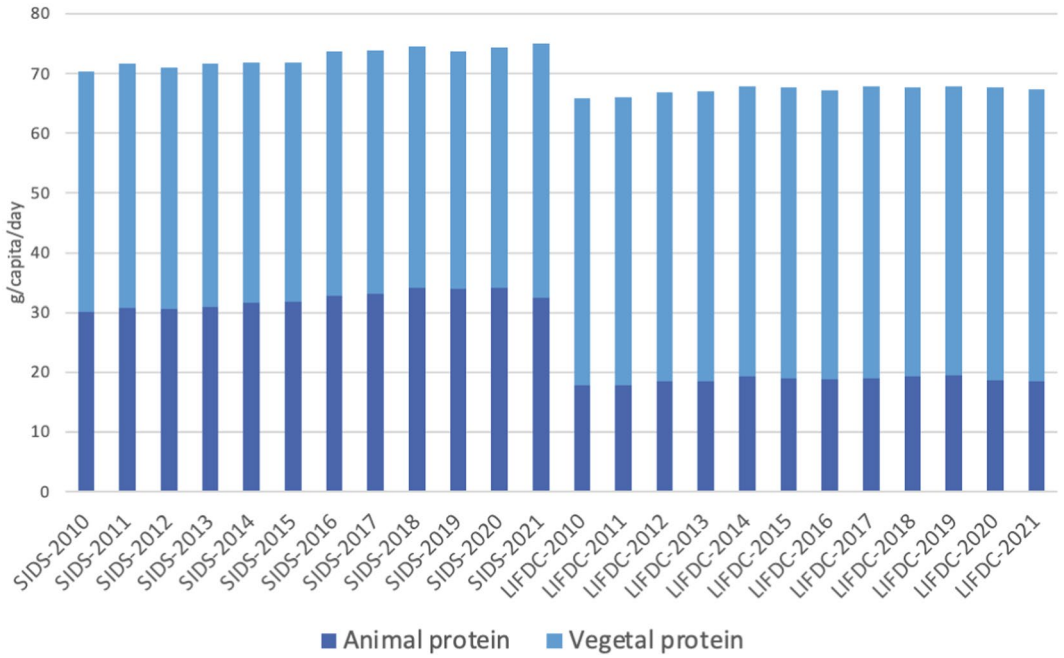

FIGURE 3
Dietary protein supply from animal and vegetal sources for special groups, in g/capita/day. Notes: SIDS, Small Island Developing States; NFIDC, Net Food Importing Developing Countries. Reproduced from FAO. 2023b. FAOSTAT. Food Balances. Food Balances (2010-). November 2023, licensed under CC BY 4.0.

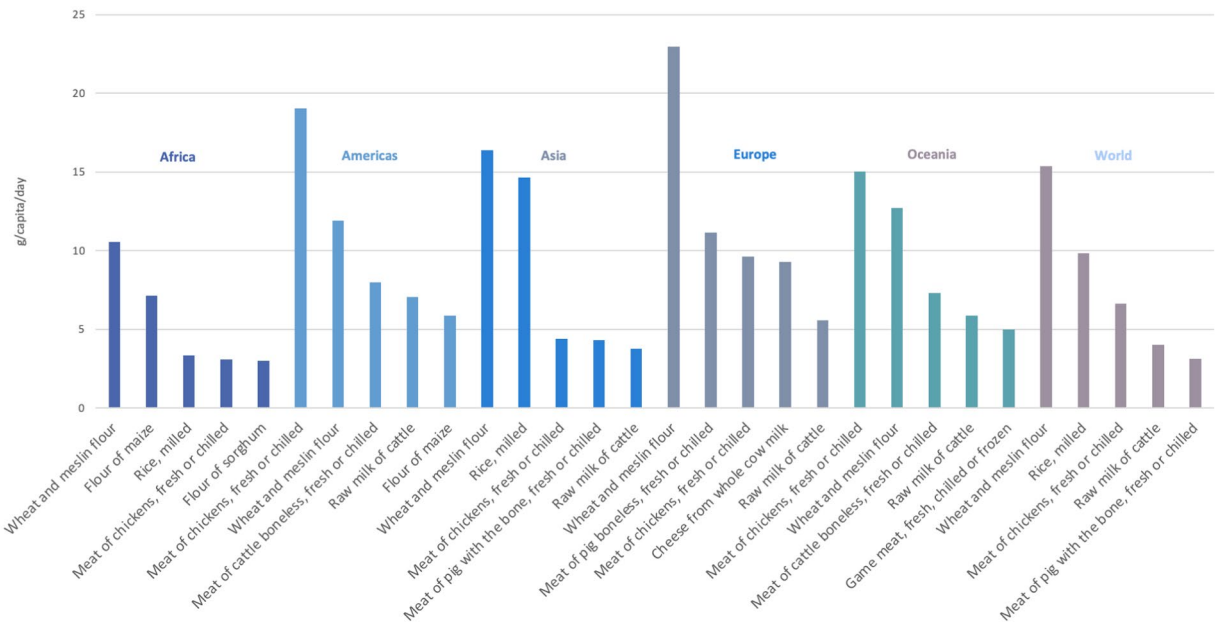

FIGURE 4
Top five main food sources of dietary protein by region and in the world, in 2021, in g/capita/day. Reproduced from FAO. 2023. FAOSTAT. Food Balances. Supply Utilization Accounts (2010-) [on the internet], licensed under CC BY 4.0. Population and Employment. Annual Population [on the internet], licensed under CC BY 4.0.

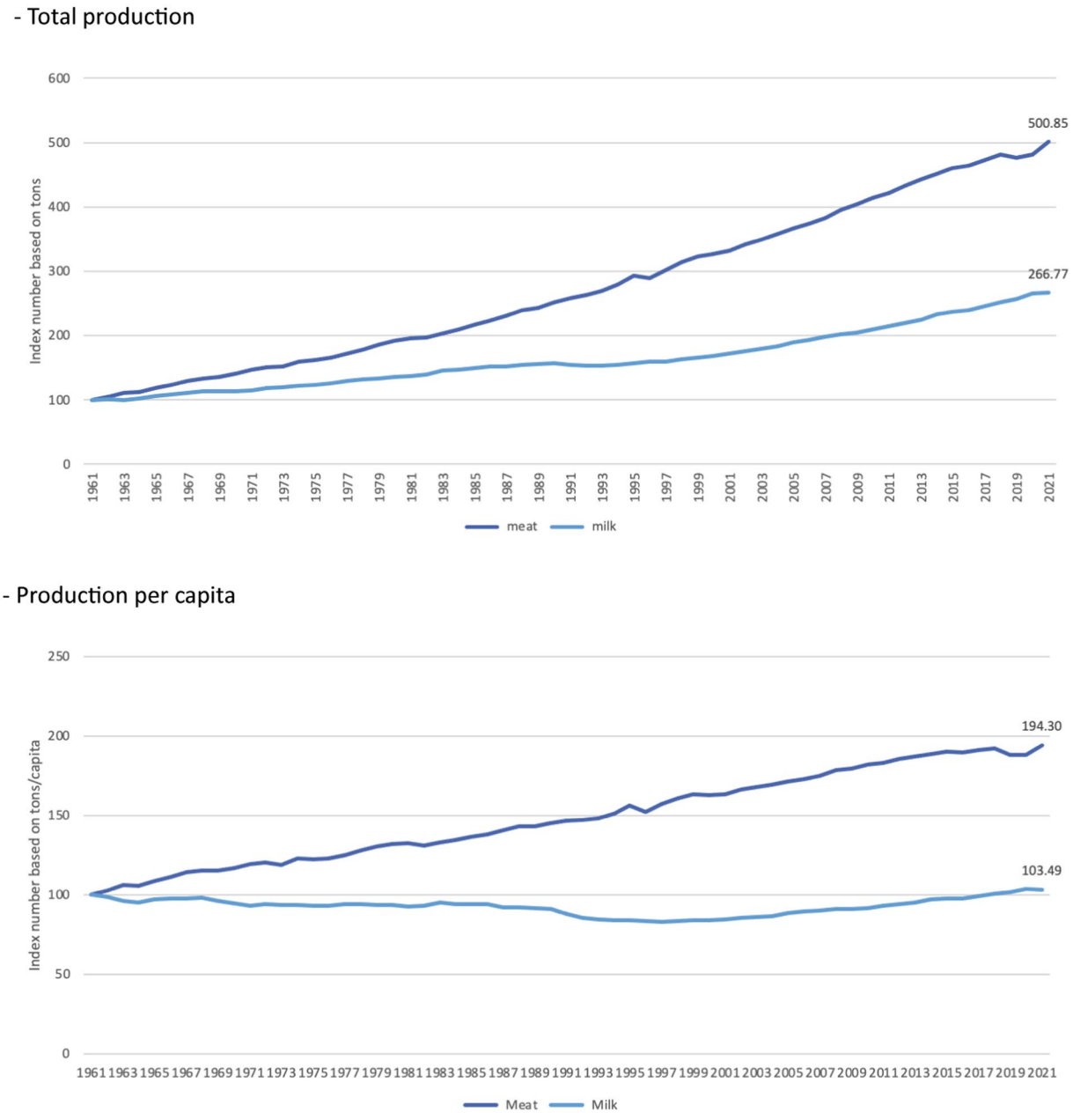

FIGURE 5
Index numbers of the production of meat and milk in the world between 1961 and 2021 (1961 = 100). Note: Production of raw milk (from all livestock including the amount used to feed them); Production of meat (red and white meat from commercial and farm slaughter) is given in terms of dressed carcass weight, excluding offal and slaughter fats. Reproduced from FAO. 2023. FAOSTAT. Production. Crops and livestock products [on the internet], licensed under CC BY 4.0.

development to be a reality. This includes the realm of protein, resolving and/or avoiding another protein fiasco.

Author contributions

BB: Conceptualization, Writing – original draft, Writing – review & editing, Formal analysis. AM: Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. CC: Formal analysis, Methodology, Writing – original draft, Writing – review & editing.

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Protein quality malnutrition

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Protein quality refers to the evaluation of a food or a diet based on its amino acid composition, protein digestibility, and protein bioavailability. When these parameters are specified, either through direct measurement or estimation, the amino acids provided by the diet are compared to those required by a healthy individual, and based on this comparison, an adequacy ratio or score is assigned. Two widely used protein quality scoring systems are the protein digestibility-corrected amino acid score (PDCAAS) and the digestible indispensable amino acid score (DIAAS), neither of which account for the dietary source of the protein. In malnourished children, metabolic adaptations reduce the endogenous availability of amino acids and increase the demand for protein synthesis. These increased amino acid requirements are primarily driven by the presence of acute infection and the need for tissue accretion. This review examines two large clinical feeding trials involving moderately malnourished children, where dietary protein quality was carefully measured. The finding s suggest that protein quality scores alone do not reliably predict weight gain or recovery in these children and that consuming milk protein provides distinct advantages over vegetable-based proteins.

KEYWORDS

protein quality, protein metabolism, malnutrition, PDCAAS, DIAAS

Introduction

Protein quality refers to the evaluation of a food or a diet based on its amino acid composition, protein digestibility, and protein bioavailability (1). Amino acid composition refers to the amounts of each amino acid present in the food. There are 20 different amino acids, 9 of which are classified as essential because they cannot be synthesized by the human body, while the remaining 11 are non-essential and can be synthesized in limited quantities.

Since the interconversion of one amino acid to another is quite limited, essential amino acids function as independent, essential nutrients (2). Digestibility refers to the ability of the human digestive tract to denature proteins and enzymatically or chemically break them into smaller peptides or individual amino acids. Animal-based proteins are typically more digestible, while plant-based proteins may form aggregates that resist digestion (3).

Bioavailability refers to the extent to which digested amino acids and small peptides are absorbed in a form that supports protein synthesis. Food processing and cooking generally enhance both the digestion and bioavailability of dietary protein. Inadequate protein quality, or protein quality malnutrition, occurs when a diet chronically fails to deliver enough amino acids to the systemic circulation to sustain the wide range of physiological functions needed for optimal health.

Two widely used protein quality scoring systems are the protein digestibility-corrected amino acid score (PDCAAS) and the more recently developed digestible indispensable amino acid score (DIAAS) (4, 5). The DIAAS provides a more accurate assessment of the three components of protein quality: amino acid composition, digestibility, and

bioavailability. Both scoring systems identify the limiting amino acid in a given food or diet, which restricts the potential for protein synthesis.

This approach assumes that the amino acid requirements are similar to those of a healthy individual. Studies involving animals raised for meat have validated this assumption by demonstrating that the addition of the limiting amino acid results in significant gains in lean body mass in growing animals (6). Due to this robust evidence, most animals raised for meat in the current times are fed diets that are fortified with up to five additional amino acids to improve the protein quality score. However, a key limitation of both scoring systems is the inaccurate assumption that all consumers share uniform physiological states.

Anatomy, physiology, and biochemistry of protein metabolism

The small intestine is divided into two anatomic sections: the duodenum/jejunum, which contains fewer microbes, and the ileum, which hosts a large and highly active microbial population. Amino acid digestion in the duodenum/jejunum is primarily enzymatic, with absorption occurring via distinct transmembrane transport complexes. In the ileum, mammalian enzymatic digestion continues, but microbes may both catabolize and synthesize certain amino acids. Microbial amino acids may also be absorbed. While the ileum is not the primary site of amino acid absorption, it can serve as an important source of essential amino acids that are not directly derived from the diet.

Protein synthesis and degradation are continuous and dynamic processes in the human body. Unlike other nutrients, amino acids are not stored in reservoirs, but the constant degradation of proteins provides a steady source of amino acids for new protein synthesis. During periods of increased protein demand, such as in response to acute infection, muscle proteins undergo proteolysis to supply the necessary amino acids for enhanced protein synthesis.

Dietary amino acid requirements include both the amino acids necessary for maintaining basic cellular functions and those required to meet special physiological demands. In low-resource settings, children often experience three particular physiological conditions that significantly affect their dietary protein needs: concurrent acute infection, chronic illness, and the demand of growth and development (7). During an acute infection, amino acid requirements typically increase by approximately 50% to support the body's needs (8).

During growth, the amino acid requirements increase by 50–100%, as more amino acids are required to generate new tissue compared to the amount needed for maintaining existing tissue (9). The most common chronic illnesses associated with increased dietary amino acid requirements are those characterized by excessive inflammation, such as HIV or tuberculosis. These infections are estimated to raise amino acid requirements by 10–20% (10). Notably, new tissue accretion cannot occur simultaneously with the body's response to an acute infection, as these processes compete for amino acids. Some nutritionists have speculated that chronic inflammation of the small bowel, commonly known as environmental enteric dysfunction (EED), might impair amino acid absorption. However, recent isotopic studies indicate that amino acid absorption is not compromised in cases of EED (11).

Malnutrition and protein metabolism

The metabolic response to malnutrition involves a reduction in protein kinetics, which helps conserve amino acids and other nutrients that are in limited supply. However, this adaptation also decreases the body's capacity to respond to acute infection, thereby increasing the risk of poorer outcomes. This was evident in malnourished Malawian children approximately 20 years ago (12–17). As shown in Figure 1A, well-nourished children with infection exhibit higher rates of whole-body protein synthesis than wasted children with infection, even when both groups are fed an isonitrogenous and isoenergetic diet. Figure 1B shows that amino acid oxidation is also elevated in well-nourished children. In malnourished children, the metabolic protein kinetic response is blunted, as evidenced by similar rates of whole-body protein synthesis in wasted children with and without infection.

The milk and egg white + tryptophan diets were both isonitrogenous and isoenergetic, although the egg white + tryptophan diet had a higher protein quality score. As shown in Figure 1C, whole-body protein synthesis is comparable between the two diets and increases with higher protein intake. Figure 1D demonstrates that the diet with the higher protein quality score resulted in a lower rate of amino acid oxidation, as the dietary amino acids more closely matched the body's response to acute infection. These metabolic studies support the practice of providing malnourished children with food aid products with high protein quality scores and generous amounts of animal-based protein.

Clinical trial evidence regarding protein quality

Two trials involving food aid products in malnourished children, where protein quality was controlled, suggest that the food source of dietary protein—beyond just its quality—may also play a role in determining clinical outcomes. Both trials were conducted among Malawian children with moderate wasting. These two trials used peanut-based ready-to-use supplementary foods (RUSFs). The results from the first trial are shown in Figure 2A, where similar RUSFs were made with either soy protein or whey protein (18). The formulations were adjusted to achieve similar DIAAS of approximately 0.73. In order to achieve this score, the soy formulation included 17.1 g of the total protein, while the whey formulation included 11.4 g of the total protein. All other nutrients were provided in similar quantities. Rates of recovery and weight gain were higher in the children who received the whey formulation.

Figure 2B shows the results comparing two RUSFs containing different types of dairy protein: whey or milk (19). The DIAAS of the whey RUSF was 0.63 and that of the milk RUSF was 0.95. This study was unique in that the DIAAS was measured using a growing pig model, which requires feeding the test diet for 1 week. Other determinations of the DIAAS were made by calculations from previous experiments. Rates of recovery rate and weight gain were identical among the 2,200 study children. These two studies suggest that in malnourished children, the source of the protein is a determinant of clinical outcomes, irrespective of the protein quality score. Dairy protein, particularly milk protein, appears to confer a benefit.

It has been established that the DIAAS correlates well with *in vivo* measures of protein utilization in adult humans. However, the measurement of growth may not be sensitive enough to detect such differences. Furthermore, the amino acid score for a healthy child may

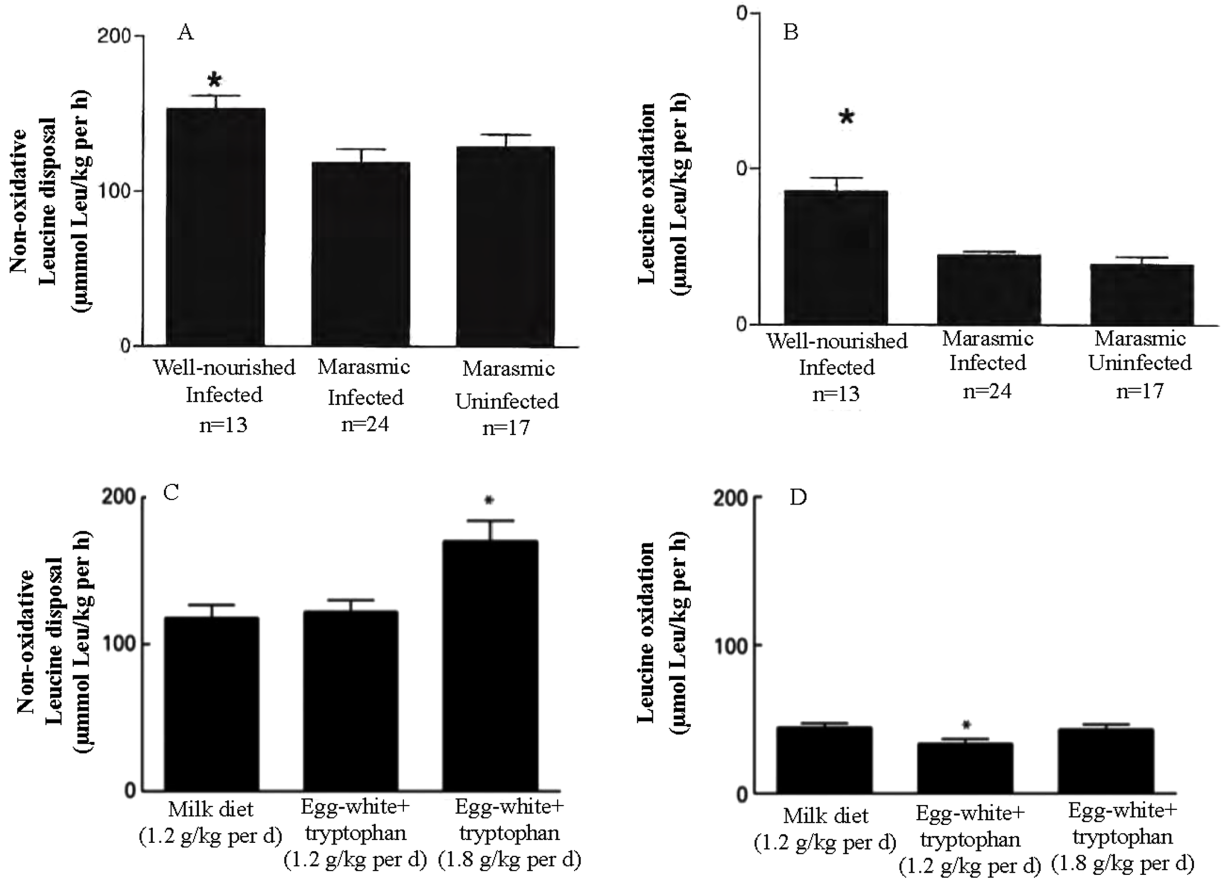

FIGURE 1
(A, B) Represent all malnourished children. **(C, D)** Compare protein kinetics in children receiving one or two differing amounts and types of protein.

not correspond well with that of a malnourished child, and dietary factors other than absorbed amino acids may affect recovery.

Discussion

Studies on animals provide strong evidence for the importance of the protein quality score in a homogenous population—one that is healthy and free from acute infections or varying degrees of wasting. These studies focus on a specific outcome: the accretion of lean body mass. Therefore, the assumption regarding the amino acid requirements of the reference population, which serves as the denominator of the protein quality score, is more likely to be valid.

In contrast, among moderately wasted children, there is more dynamic variation in the nature and duration of intercurrent infectious illnesses, as well as in the degree of catch-up growth required for recovery. These variations affect each child's amino acid requirement, making the determination of the DIAAS less precise. Empirical protein kinetic data are needed to more accurately determine a protein quality score for this population.

One of the goals of developing protein quality scores is to compare highly diverse foods and diets and predict their effects on human nutrition. Data from moderately wasted children suggest that milk protein is superior to whey protein, which is superior to soy protein. Milk protein contains much more casein than whey, and casein is known to be a source of bioactive peptides (20–22).

These peptides are created by the hydrolysis of casein during ingestion, rather than existing as distinct minor components of milk. There are over 700 casein-derived bioactive peptides. Their activities include anti-inflammatory, antioxidant, and antimicrobial effects. These peptides exert their effects through specific receptors in the gut. The presence of both casein and whey proteins in all mammalian milk suggests that they have been conserved through evolution to provide benefits to immature, growing mammals. The potential benefits of these bioactive milk peptides for malnourished children should be considered when evaluating the source of dietary protein, in addition to the protein quality score of the diet.

Additionally, the DIAAS allows for additivity and complementarity of dietary proteins in a meal. Lower-quality proteins, or proteins with limiting IAAs, can be complemented by higher-quality proteins, or proteins in which the limiting amino acid is present in excess, resulting in an overall higher protein quality meal. This property has driven an agenda advocating plant-based proteins as alternatives to animal-based proteins, largely due to concerns regarding the environmental impact

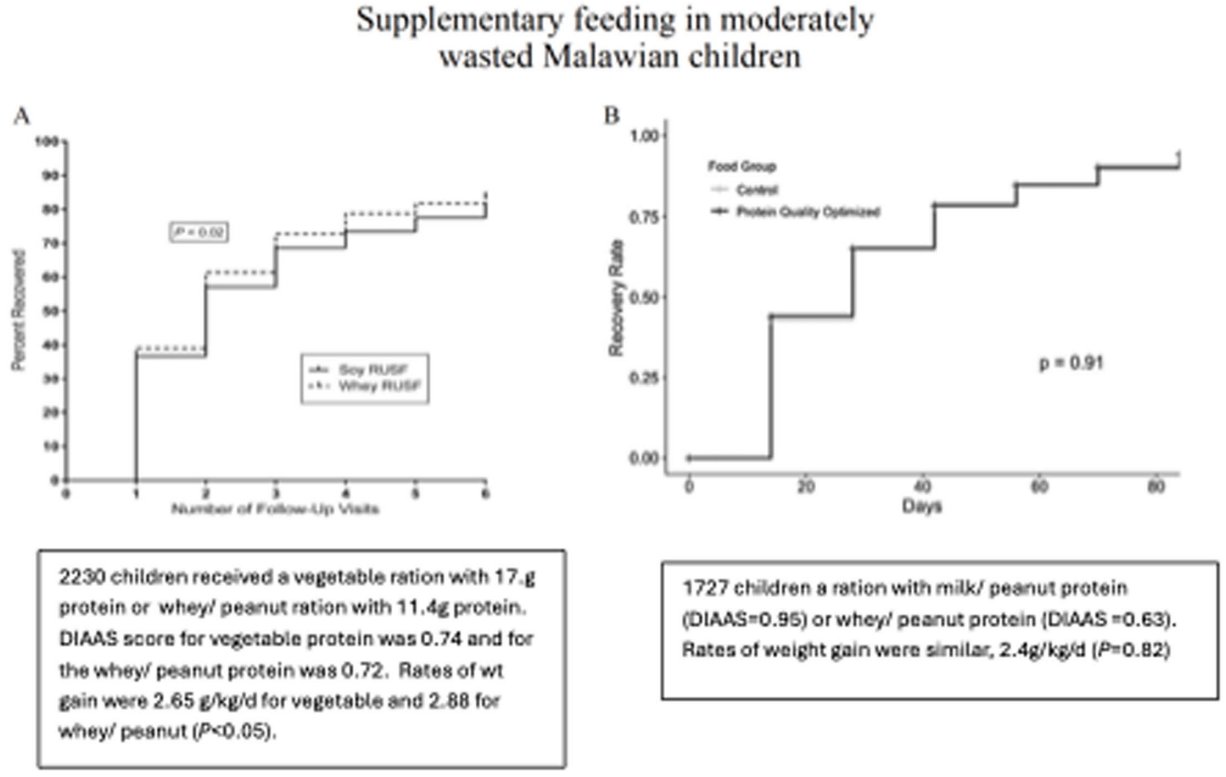

FIGURE 2
In panel **A**, 2220 children received a daily ration that contained 17.1 g vegetable protein or 11.4g whey/ peanut protein. DIASS score for vegetable protein 0.74 and 0.72 for the whey/ peanut. Rates of wt gain 2.88 g/kg/d whey vs 2.65 ($P< 0.05$). In panel **B**, 1727 children received a ration peanut/ milk protein (DIAAS=0.95) or peanut/ whey protein (DIAAS=0.63). The rates of weight gain were 2.44 g/kg/d and 2.40 g/kg/d.

of animal protein production. However, as demonstrated above, such considerations should be approached with caution in populations with high levels of malnutrition, where both protein quality and protein source may be critical to preventing protein quality malnutrition.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MM: Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. DW: Data curation, Project administration, Visualization, Writing – original draft, Writing – review & editing. KM: Conceptualization, Formal analysis, Project administration, Writing – original draft, Writing – review & editing.

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Consideration of the role of protein quality in determining dietary protein recommendations

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The quality of a dietary protein refers to its ability to provide the EAAs necessary to meet dietary requirements. There are 9 dietary amino acids that cannot be metabolically produced in the body and therefore must be consumed as part of the diet to avoid adverse metabolic consequences. These *essential amino acids* (EAAs) serve a variety of roles in the body. The amount and profile of the dietary EAAs relative to the individual EAA requirements and the digestibility of the dietary protein are the key factors that determine its quality. Currently the Digestible Indispensable Amino Acid Score (DIAAS) is the best available approach to quantifying protein quality. The most prominent metabolic role of dietary EAAs is to stimulate protein synthesis by serving as signals to activate molecular mechanisms responsible for the initiation of protein synthesis and, most importantly, to provide the necessary precursors for the synthesis of complete proteins. Current dietary recommendations generally do not consider protein quality. Accounting for protein quality in dietary patterns can be accomplished while staying within established ranges for dietary protein consumption. Poor protein quality can be compensated for to some extent by eating more low-quality protein, but to be effective ("complementary") the limiting EAA must differ between the low-quality protein and the base diet to which it is being supplemented. Adding a high-quality protein to a dietary pattern based on low-quality protein is more effective in meeting EAA goals than increasing the amount of low-quality protein, even if the low-quality proteins are complementary. Further, reliance entirely on low-quality protein food sources, particularly in circumstances that may benefit from a level of dietary EAAs greater than minimal requirements, is likely to include excessive caloric consumption. While protein consumption in high-income nations is generally perceived to be adequate or even excessive, assessment of dietary patterns indicates that a significant percentage of individuals may fall short of meeting optimal levels of EAA consumption, especially in circumstances such as aging in which the optimal EAA consumption is greater than basal values for healthy young individuals. The case is made that protein quality is an important consideration in meeting EAA requirements.

KEYWORDS

protein quality, essential amino acid, dietary requirements, dietary protein, protein scoring

Introduction

Dietary protein has been recognized for more than 100 years as vital for growth, health, and even survival (1). Amino acids are the building blocks of dietary protein, and it is the amino acids absorbed from digested dietary protein that serve the various metabolic roles. Dietary amino acids serve as precursors for the synthesis of neurotransmitters, nucleotides, and a variety of other important products. Dietary amino acids also support multiple aspects of immune function, and influence satiety. Most prominently, dietary amino acids serve as precursors for the synthesis of new proteins in the body. There are thousands of different proteins in the body, all with specific functions. Proteins comprise about two-thirds of the mass of the body that is not water. Each protein is distinguished by the unique amount and profile of amino acids of which it is composed. All proteins in the body are in a constant state of turnover, meaning continuous breakdown and synthesis (2). Protein turnover enables a replenishing of older, less functional proteins with new, better-functioning proteins (3). Most adults are in a steady state in which the synthesis of proteins over the course of the day balances breakdown.

Protein turnover proceeds continuously throughout the day and night, regardless of whether amino acids from dietary protein are being absorbed. The amount of time throughout the day that dietary amino acids are being absorbed varies according to patterns of consumption. Eating patterns vary in different cultures. In the United States, it is common to eat discrete meals (usually three) per day containing dietary protein, but most protein is often consumed in the evening meal. The consumption of discrete meals results in periods of 3–6 h each throughout the day during which amino acids are absorbed (post-prandial state), depending on the composition of the meal. Regardless of the pattern of consumption of dietary protein there are periods when dietary amino acids are not being absorbed (post-absorptive state). The post-absorptive state is characterized by a net breakdown of body proteins due to the rate of protein breakdown exceeding the rate of protein synthesis. Although the amino acids released by protein breakdown can serve as precursors for the synthesis of new proteins, the availability of certain amino acids from protein breakdown is insufficient to allow protein synthesis to balance the rate of breakdown in the post-absorptive state because of the irreversible oxidation of those amino acids and the inability of the body to replace them metabolically. Also, amino acids are lost directly from the body via routes such as the gastrointestinal tract and skin. The amino acid components of body protein that cannot be synthesized in the body are called the dietary *essential amino acids* (EAAs). The necessity of including EAAs in the diet has been recognized for close to 100 years (4). The EAAs for human nutrition are histidine, leucine, lysine, isoleucine, methionine, phenylalanine, threonine, tryptophan, and valine. There are an additional 11 dietary dispensable amino acids that are also components of body proteins but can be produced in the body. The extent of oxidation of each EAA released in the process of protein breakdown largely defines its dietary requirement. Consumption of at least that amount of each EAA is necessary to maintain protein balance over the course of the day.

The post-absorptive state generally lasts for a matter of hours, but it is possible for humans to survive for a month or more without dietary protein consumption (5). Protein turnover occurs in all tissues and organs in the body and sustained negative protein balance in certain tissues and organs, such a skin, heart, brain, etc. is not compatible with life. In this circumstance skeletal muscle serves as a "reservoir" of amino acids for the tissues and organs with a high priority to maintain protein balance. The net breakdown of muscle protein and release of amino acids into plasma in the absence of dietary protein intake enables sufficient availability of EAAs to maintain protein balance in the other tissues and organs in the body.

While consumption of dietary protein promotes protein synthesis throughout the body, stimulation of *muscle* protein synthesis in the post-prandial state to replenish protein lost in the post-absorptive state is a primary metabolic role of dietary protein. Skeletal muscle protein metabolism is not only central to maintaining protein homeostasis throughout the body, but muscle serves a variety of other roles. The importance of maintaining muscle mass and function in relation to physical activity is well-known (6). Less well appreciated, skeletal muscle protein turnover plays an important role in maintaining energy balance, as both muscle protein synthesis and breakdown require energy in the form of ATP (7). The difficulty in maintaining weight loss after caloric restriction weight loss is related in part to the extent of loss of muscle mass (8). Maintaining the metabolic function of muscle is central to avoiding metabolic syndrome and type 2 diabetes, since muscle is the primary site of glucose clearance from plasma (7). Muscle contraction puts torque on bone that is essential for bone strength (9). These multiple and varied roles of skeletal muscle are important for all individuals, and especially for vulnerable populations such as the elderly, and must be supported by adequate EAA consumption.

The quality of a dietary protein can be described as its ability to provide the EAAs necessary to maintain protein balance in the body by stimulating protein synthesis. Evaluation of the importance of protein quality therefore requires consideration of the role of EAAs in stimulating protein synthesis (10), and the factors that determine the effectiveness of the dietary protein in delivering the necessary EAAs to the tissues and organs of the body. These factors include the amount and profile of EAAs in a dietary protein relative to nutritional requirements, and the digestibility of the protein. Fundamental issues related to the importance of protein quality include the scoring of protein quality, the relation between the true ileal digestibility of dietary EAAs and the stimulation of protein synthesis, the mechanism of stimulation of protein synthesis by EAAs, the accuracy of EAA requirements that are targeted in assessing protein quality, whether consideration of protein quality can be incorporated into diet planning while staying within established nutritional recommended ranges for dietary protein consumption, the effect of physiological and metabolic circumstances on optimal EAA consumption the significance of non-protein components of protein food sources, if poor protein quality can reasonably be compensated for by eating more protein, and the relevance of the quality of dietary protein in high-income nations. We will briefly discuss these issues in relation to the Digestible Indispensable Amino Acid Score (DIAAS) to quantify protein quality.

Scoring protein quality: a case for DIAAS

A variety of approaches have been used to describe the quality of a dietary protein. Some approaches have been based entirely on digestibility. These include oro-ileal true amino acid digestibility, total (fecal) crude protein digestibility and a dual isotope tracer method that compares circulating amino acids from an intrinsically labeled

test protein with a reference protein labeled differently with known digestibility (11). Biological value (BV) is based on measures of nitrogen digestibility and urinary nitrogen excretion, whereas net protein utilization (NPU) is based on nitrogen intake and urinary nitrogen excretion. The Protein Efficiency Ratio (PER) reflects the physiological response to the dietary protein and is based on the ratio of weight gain to protein consumed by the test group as compared to the control (most commonly casein) over time (12). The first efforts to consider EAAs as individual nutrients and to assign a numerical value to the quality of a dietary protein involved chemical scores such as the Protein Digestibility Corrected Amino Acid Score (PDCAAS). PDCAAS is based on the fecal digestibility of crude protein and the content and profile of EAAs (11). The Digestible indispensable Amino Acid Score (DIAAS) was published in 2013 as a more accurate estimation of the factors comprising PDCAAS (13). The underlying concept of protein quality as quantified by both PDCAAS and DIAAS is that the amino acids in a dietary protein must be digested and absorbed to have metabolic value, and that the amount and profile of the absorbed EAAs should be in line with the dietary requirements for each EAA. The improved accuracy of DIAAS as compared to PDCAAS derives from the use of true ileal digestibility of each essential amino acid (EAA) in the dietary protein rather than the fecal digestibility of crude protein, and DIAAS (but not PDCAAS) is not truncated in the case of high-quality proteins. DIAAS also specifically accounts for the availability of lysine in processed foods. While DIAAS most conventionally applies to single dietary proteins, the DIAAS of a complete diet can also be calculated (13). Thus, DIAAS is the most accurate method currently available to provide a basis for dietary recommendations for protein consumption to account for quantitative differences in dietary protein quality. The validity of DIAAS for this purpose has two principal aspects: the use of true ileal amino acid digestibility and the role of EAAs in controlling protein synthesis in the body. We have previously analyzed the validity of the factors comprising DIAAS in depth (14). One possible shortcoming of DIAAS is that the value is expressed in terms of the percent of dietary EAA requirements met when the EAR for protein is consumed. Using the EAR value is useful on a population basis, but it may be more appropriate to express the percent of requirements met when the RDA for protein is consumed when determining dietary recommendations on an individual basis.

Use of true ileal amino acid digestibility (TID) in quantifying protein quality

It is self-evident that for a dietary protein to have metabolic value it must be digested and the amino acids absorbed. Thus, there is little argument that an accurate scoring of protein quality should take account of digestibility. There are two basic approaches to determining protein digestibility directly: fecal or ileal digestibility. Digestibility determined at the ileal level is fundamentally superior to determining digestibility at the fecal level since there is little absorption of amino acids in the large intestine and there is an abundance of microflora that digests and utilizes undigested protein, peptides or amino acids exiting the small intestine (15, 16). In addition, amino acids can also be synthesized and microbial degradation products absorbed in the large intestine (17). The catabolism and synthesis of amino acids by the microflora in the large intestine confounds fecal measurements of protein or amino acid digestibility and will usually result in the over-estimation of the true digestibility of the EAAs in the test protein. Further, the amino acid composition of fecal protein bears no necessary resemblance to the undigested dietary protein leaving the ileum. Accounting for digestibility at the end of the small intestine (ileal digestibility), as is done with calculation of DIAAS, overcomes the problems of interpreting fecal digestibility data.

Use of true ileal amino acid digestibility (TID) in quantifying protein quality is important because TID can vary across amino acids, even within the same protein source. For example, TID of dietary proteins in India was found to differ by more than 20% across the dietary EAAs for many foods and food ingredients examined (18). Even for highly digestible protein sources the range in true ileal amino acid digestibility within a protein source can be significant (18). TID generally varies more in plant-based dietary proteins than animal proteins. For example, TID of EAAs in beef sirloin ranges from 98–100%, while the corresponding measurements in boiled potato protein range from 56% (tryptophan) to 83% (lysine) (19). Failure to take account of true ileal digestibility in this example would result in not only an overestimation of the quality of potato protein but would change the limiting amino acid from histidine to lysine. This is not to imply that all plant proteins have low and variable digestibility. For example, amino acid digestibility is relatively high for soy isolate, but in general animal proteins have higher and less variable digestibility. The main concerns with ileal measurement of amino acid digestibility include how well digesta samples reflect the total digesta, if the contribution of the non-dietary EAAs derived from digestion of digestive enzymes and other intestinal proteins has been accurately accounted for, and whether any effects that small intestinal bacteria may have on digestibility is considered. The primary factor limiting the use of TID in scoring protein quality is that values have not been determined in some dietary proteins A major effort to determine TID in a wide range of dietary proteins is under way and completion of that work will enable a broader application of TID in scoring protein quality.

EAAs and protein synthesis

In addition to TID, accurate scoring of protein quality must account for the amount and profile of the EAAs in a test protein relative to the corresponding values in the reference protein, and the accuracy of EAA requirements on which the amino acid scoring pattern of the reference protein is based (14). The mechanisms responsible for how EAAs regulate protein synthesis are thus central to understanding the basis for DIAAS. Further, accounting for how EAAs regulate protein synthesis is important in determining the adequacy of protein consumption in a variety of physiological states.

Dietary EAAs are primarily responsible for the stimulation of protein synthesis in the post-prandial state. Consumption of a relatively small dose of only EAAs in the profile of beef protein stimulates muscle protein synthesis (MPS) as much as a mixture of the same amount of EAAs plus additional dispensable amino acids (DAAs) that can be produced in the body (10). When only EAAs are consumed the DAAs that are also required for the synthesis of new proteins can be derived from reutilization of endogenous

DAAs released by protein breakdown or synthesized in the body, often from simple nitrogenous precursors. In contrast to the stimulatory effect of EAAs on protein synthesis, ingestion of a mixture of DAAs in the profile found in whey protein failed to stimulate MPS (20). Further, the magnitude of increase in whole-body protein synthesis appears to be directly related to the amount of EAAs in a dietary protein, provided that high-quality proteins with high digestibility are considered (21, 22) (Figure 1). Dietary proteins with low digestibility would not yield the same relation between the amount consumed and the stimulation of protein synthesis.

While the EAAs are primarily responsible for the stimulation of protein synthesis, the dietary DAAs may also play a role. The importance of dietary DAAs in maintaining N balance was documented in the early studies of amino acid metabolism. The efficiency of utilization of the EAAs as assessed by N-balance was shown to be enhanced by the amount of DAAs given concurrently (23). The exact amounts of either total or individual dietary DAAs that are necessary to maximize the effectiveness of dietary EAAs have not been determined. Agricultural science literature indicates that the ideal composition of feed for the maximum growth and muscle development of farm animals consists of approximately two-thirds amino N in the form of EAAs (24), but comparable data for humans are not available. Thus, although there is some (uncertain) need for DAA intake, it is most likely that the prevalence of DAAs and other nitrogenous compounds in dietary protein is more than adequate to provide ample DAAs when sufficient protein is ingested to meet EAA requirements. Dietary protein ranges between 30 and 50% EAAs, which means that the contribution of DAAs to amino acid composition of proteins is likely more than adequate to meet requirements if the animal literature can be extrapolated to human diets. Further, normal dietary consumption of DAAs is sufficient to support protein synthesis resulting from ingestion of a relatively small amount of free EAAs (25, 26).

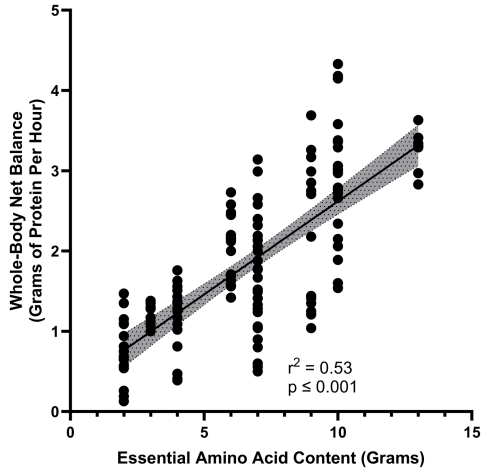

FIGURE 1
Relationship between increase in essential amino acid content of a protein source and the gain in whole-body protein balance (represented as grams per hour) (14, 15).

Mechanisms of stimulation of protein synthesis by EAAs

Measures of protein quality must be consistent with the mechanisms whereby EAAs stimulate protein synthesis. Much of what we know about EAAs and protein synthesis in humans comes from studies of muscle protein synthesis (MPS). The mechanisms whereby EAAs affect protein synthesis in general and MPS specifically fall into two general categories: transcription and translation. The transcription of messenger RNA (mRNA) from DNA results from activation of the relevant genes. Activation of genes is reflected in the number of specific mRNAs in the cell on which the assembly of new proteins occurs. Several studies have used mRNA content of specific proteins as an index of the rate of synthesis of those proteins, but there is generally a poor correlation between mRNA content and MPS (27). Consequently, it is likely that in most circumstances, mRNA content is not rate limiting for MPS.

The translational control of protein synthesis by EAA availability has been recognized since 1958 (28). Translation involves the sequential bonding of amino acids in the order dictated by the mRNA code. Free intracellular amino acids are bound to specific transfer RNAs (tRNAs) inside the cell that have codons of three nucleotides that correspond to the codons on the mRNA for specific amino acids. Charged tRNA molecules sequentially transfer the attached amino acids to the sites on the mRNA dictated by the mRNA code. Translational elongation can only proceed to completion if adequate amounts of all required amino acid precursors are available. A relative deficiency of any EAA will make that EAA limiting. Lack of availability of the limiting EAA will cause the termination of translational elongation of protein synthesis before the process is complete, and the partially synthesized protein being degraded.

Translation of the mRNA is initiated by a complex process which consists of several linked stages that are mediated by eukaryotic initiation factors (eIFs). The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of the activation of downstream eIFs that are mediators of MPS initiation. Translational initiation of the protein synthetic process can be stimulated by an increased availability of EAAs, and leucine in particular is a potential regulator of mTORC1 (29, 30). The activation of mTORC1 by leucine seems to be especially important in anabolic-resistant states such as aging (31). When older individuals were given a mixture of EAAs in the profile of whey protein the net anabolic response of muscle protein increased only about half of the amount of the response to the same mixture of EAAs in younger individuals (32). Decreased responsiveness of MPS to nutritional stimulation is termed *anabolic resistance*. When comparable older individuals consumed a different mixture of the same amount of EAAs in which leucine comprised approximately 35% of the total mixture, the anabolic response doubled but the enhanced mixture had no greater effect in younger individuals than the profile of EAAs in whey protein (33). The potential role of leucine in triggering the initiation of protein synthesis highlights the importance of considering protein quality in designing dietary plans, particularly in circumstances such as aging (34). The concentration of leucine in plasma must increase approximately 3- fold to activate mTORC1 (35), which translates to consumption of approximately 2.5–3 g of leucine. A relatively large proportion of a dietary protein must be comprised of leucine to achieve that level of intake. Circumstances benefitting from a high leucine intake generally means reliance on animal

proteins, which generally contain greater amounts of leucine than plant-based dietary proteins (36), although there are some specific plant proteins that contain relatively high amounts of leucine.

The prevalence of all the EAAs, and perhaps specific EAAs such as leucine, is thus an important aspect of protein quality. In addition, the amount of the limiting EAA in a dietary protein determines the amount of body protein that can be synthesized.

Use of DIAAS to evaluate protein quality of a meal

DIAAS was developed for the comparison of the quality of dietary proteins, with a particular focus on regulatory issues. For this reason, DIAAS is normalized for the amount of the test protein. As such, DIAAS is not directly relevant to the protein quality of a meal or dietary patern. DIAAS can be calculated for mixtures of proteins as occurs in a meal, but the DIAAS of different proteins in a mixture is not additive because the DIAAS of individual proteins in a meal may be based on different limiting amino acids. Rather, the digestible amounts of each amino acid in a meal are additive. As referred to in FAO Dietary Protein Quality Evaluation in Human Nutrition 13, each EAA in a meal should be treated as an individual nutrient. Thus, the amount of each EAA in the meal, corrected for TID of that amino acid, is compared to the amino acid scoring pattern to determine how well each EAA meets dietary requirements. Whereas the amino acid scoring patterns for DIAAS conventionally match the individual EAA requirements as promulgated by the FAO, alternative scoring patterns to determine the adequacy of each EAA in a meal can be used to better match specific circumstances, such as aging, exercise, etc. Better defining appropriate amino acid scoring patterns for different circumstances should be a high research priority.

Required vs. flexible protein and EAA consumption

Accounting for protein quality in formulating dietary guidelines could impact current recommendations for protein intake. If a diet is comprised of predominantly low-quality proteins, a level of protein consumption greater than the RDA of protein could potentially be necessary to meet all EAA requirements. It is reasonable to evaluate if it is possible to increase dietary protein consumption above the RDA and stay within recommended dietary guidelines. The Dietary Reference Intakes published by the US Institute of Medicine cites the RDA for protein as 0.8 g high-quality protein /kg/day, and the RDA for carbohydrate as 130 g /day (37). There is no RDA for fat intake, but the adequate intake (AI) of linoleic acid is given as 17 and 12 g/day for men and women, respectively, and the AI for linolenic acid is 1.5 and 1.1 g/day for men and women, respectively (37). For a representative 30-year-old adult man weighing 80 kg these recommendations correspond to 256 kcal/day of protein, 520 kcal/day of carbohydrate, and 166 kcal/day of fat, for a total of 942 Kcal/day. The total energy requirement for such a man is dependent not only on body weight but also height, sex, and activity level. An average daily energy expenditure for the representative man is approximately 3,000 kcal/day, and the corresponding value is approximately 2,500 kcal for a representative woman (37). These recommendations indicate that the required amounts of protein, carbohydrate and fat constitute as little as 30–40% of the total caloric requirement to maintain energy balance. The remaining 60–70% of energy consumption could be considered to be discretionary. While it would be reasonable for part of the discretionary energy consumption to be in the form of dietary carbohydrates and fat, it is equally reasonable that dietary protein consumed at a rate greater than the RDA would comprise at least a component of the discretionary energy intake. Increasing dietary protein intake above the RDA is consistent with the Acceptable Macronutrient Distribution Range (AMDR) also published in the Dietary Reference Intakes (37). The AMDR for protein ranges from 10 to 35% of total energy intake; the RDA for protein accounts for approximately 10% of energy intake. These data indicate that an increase in dietary protein consumption well above the RDA to accommodate a greater need for EAAs can be accomplished while staying within current guidelines for dietary protein consumption.

A potential problem with dietary protein constituting as much as 30–35% of protein is whether all nutrient requirements can be met, particularly with a relatively low caloric content. We performed a modeling exercise in which two single-day menus were created, each consistent with the USDA food group serving recommendations for a (relatively low) 2000-kcal healthy U.S.-style eating pattern (38). We found a diet with 30% of energy derived from protein can be achieved without compromising food group serving intake recommendations for fruits, vegetables, grains, including whole grains, and dairy foods, meeting all nutrient requirements (38). A variety of sources of high-quality protein food sources, including fish, poultry, milk, and cheese in addition to meat were used in the diets. It was necessary to rely on these sources of protein in the meal plans, as the protein density relative to total calories in plant-based protein sources alone is generally low. A meal plan relying on plant-based protein sources alone could only be used in a meal plan targeting a higher caloric intake (38). The necessity of relying on animal proteins in this modeling exercise implies an importance of protein quality in not only providing an optimal level of EAAs, but also meeting all other nutrient requirements. It is possible, and potentially desirable, to increase intake of plant-based proteins, but protein quality needs to be carefully considered. High-quality plant proteins (eg, soy) and animal proteins play an important role in maintaining overall dietary protein quality.

Physiological circumstances benefitting from increased protein and EAA consumption

The preceding discussion makes clear that current estimates of requirements for dietary EAAs are minimal values and suggest that there is room within traditional nutritional recommendations for a level of protein intake that exceeds the RDA to optimize EAA consumption. It is therefore relevant to evaluate if different physiological circumstances increase the optimal level of protein and EAA consumption significantly above the RDAs. It is of further interest if the optimal profile of EAAs may differ in various physiological circumstances from the profile of the FAO reference protein, which is predicated on basal EAA requirements in young, healthy individuals.

Several studies have demonstrated beneficial effects of dietary protein intake greater than the RDA and that EAAs are primarily responsible for the responses. For example, increased dietary protein improves muscle mass and strength in older individuals (39), and EAAs can be credited with the beneficial response. Supplementation of the normal diet with 11 g twice per day of EAAs in older individuals improved LBM, strength and functional tests (40, 41). When low-function elderly consumed increased dietary protein in the form of whey protein (DIAAS = 0.96) for 16 weeks, muscle strength and function were significantly improved as compared to a control group given only nutritional education (42). Improvements in all aspects of physical function measured were greater when the same amount of EAAs as whey protein were provided, indicating that the EAA component of the whey protein was responsible for the improvements in physical function (42). Similarly, the loss of LBM and muscle strength that occurred with 28 days of bed rest in healthy young subjects (43, 44) as well as 10 days of bed rest in elderly individuals (45) was ameliorated by supplementation with additional dietary protein or EAAs. The results from the bed rest studies are particularly significant because all known factors other than total protein or EAA intake that might potentially affect LBM changes, including activity and other macronutrient intake, were completely controlled. Increased EAA consumption has also been shown to have beneficial effects in a variety of circumstances, including rehabilitation (46–49); stroke (50, 51); peripheral artery disease (52); renal failure (53–57) inflammation (58, 59); critical illness (60); lung cancer (61); cystic fibrosis (62); chronic obstructive pulmonary disease (63–65); wound healing (66); brain injury (67, 68); metabolic syndrome and cardiovascular risk factors (69–71); obesity (8, 72); liver fat (69, 73–75); and diabetes (76–80).

The optimal profile of dietary EAAs may also be affected by different physiological circumstances. For example, selective oxidation of leucine or the branched chain amino acids commonly occurs in stressful conditions, such as serious injury or illness, due to activation of branched chain keto-dehydrogenase (81). Aerobic exercise causes a greater increase in the oxidation of leucine as compared to the other EAAs (82). Further, some conditions involving anabolic resistance, such as aging, may stem in part from decreased activity of mTORC1. In this case a greater proportion of leucine in a dietary protein or a composition of EAAs may serve as a nutraceutical by activating mTORC1 (29, 30).

While the many studies that have reported beneficial effects of increasing EAA consumption provide a strong rationale for quantifying protein quality on the basis of the EAA profile and amount, it should be recognized that many of the above-mentioned studies used free EAA compositions to raise EAA consumption. It is unclear if a reasonable amount of even high-quality dietary protein alone can elicit the same metabolic and physiological responses. Plasma EAA concentrations increase more rapidly and to higher levels when free EAA compositions are consumed than when the same EAA are components of dietary protein (22). Further, free EAA mixtures often have little or no associated DAAs or non-protein components that may elicit different physiological responses than dietary protein food sources.

In contrast to the many studies demonstrating beneficial effects of increased protein and EAA consumption, there has never been a study to our knowledge in which the RDA for protein or EAA consumption was compared with a higher level of protein intake and the lower level of protein consumption was found to be superior.

An ideal approach to scoring protein quality would account for known effects of specific physiological circumstances on optimal EAA consumption at a group level, and even at an individual level (i.e., "*personalized nutrition*"). A recent publication describes such an approach for any situation in which the optimal amount and profile of EAAs is known (83). DIAAS can also account for altered demand for EAAs in specific circumstances by using a scoring pattern reflecting the optimal amount and profile of EAA consumption for that circumstance rather than the FAO scoring pattern based on the RDAs for the individual EAAs. However, the value of any protein quality scoring is dependent on the accuracy of the target for EAA consumption, and more data in this regard may be necessary for the successful implementation of personalized nutrition.

Current protein nutrition guidelines

Recommendations for dietary protein intake have been expressed in terms of grams of protein or nitrogen (N) per day for more than 100 years. While occasionally the proviso that recommendations apply to "good quality" protein has been included (e.g., DRIs), in general protein recommendations have not directly specified the source or quality of dietary proteins.

Evaluation of the relevance of protein quality to nutritional guidance is timely. The Dietary Guidelines for Americans (DGAs) published by the United States Department of Agriculture (USDA) have made a pronounced shift over the past 40 years away from animal protein toward plant-based protein food sources. Such a shift may have a significant impact on the overall protein quality of the diet, as animal proteins generally are higher quality (as reflected by DIAAS) than are plant-based proteins (14). Although only about 5% of the US population classifies themselves as vegetarian and about 2% classify themselves as vegan (84), there has been a progressive shift away from consumption of animal-based protein food sources in individuals who do not consider themselves to be vegetarian. For example, between 1970 and 2005 there was a 17% drop in consumption of red meat and of eggs in the U.S. (85), and the downward trend in red meat consumption has continued, in part due to perceived concerns about health and growing publicity regarding the environmental impact of the beef industry in particular (86). However, calls for reduced consumption of animal-based protein food sources have not taken account of the potential physiological implications of a significant reduction in the overall protein quality of the diet.

"Ounce equivalents" of dietary protein

The DGA's aim to create recommended dietary patterns that meet or exceed RDAs for both micro- and macronutrients. Levels of protein intake are not the primary focus of the DGAs, perhaps in part because the RDA for dietary protein can be met with almost any western diet that maintains caloric balance. However, the RDA expresses the minimal amount of dietary protein consumption necessary to avoid deficiencies in young, healthy individuals, and, as discussed above, there are many circumstances in which the optimal amount of dietary protein may be greater than the

RDA. Further, the DGAs do not currently address the issue of protein quality. DIAAS indicates that animal proteins can more readily provide the daily requirement of EAAs than plant proteins (14).

MyPlate is designed to simplify for the public the key elements of the DGAs (87). MyPlate recommends meeting protein needs by eating a variety of "ounce equivalents" of protein food sources. The DGAs state that 1 ounce (28 g) of meat is equivalent to 1 cooked egg, ¼ cup (70 g) of red kidney beans, 1 tablespoon (15 g) of peanut butter, 2 ounces (56 g) of tofu and 0.5 ounces (14 g) of mixed nuts. The labeling of these disparate protein food sources as "equivalents" implies an equal metabolic benefit should be obtained from each of the "ounce equivalents" of protein food sources, although neither the DIAAS nor the amount of EAAs provided are in fact equivalent. To determine if the different protein food sources provide the same anabolic stimulus, stable isotope tracer methodology was used to quantify the response to ingestion of each of the "ounce equivalents" (88). The changes from baseline following consumption of one of seven different protein food sources were compared to the baseline value for that individual. Consumption of ounce equivalents of animal-based protein food sources (beef sirloin, pork loin, eggs) resulted in a greater gain in whole-body net protein balance above baseline than the ounce equivalents of plant-based protein food sources (tofu, kidney beans, peanut butter, mixed nuts; $p < 0.01$). Most importantly, the magnitude of the whole-body net balance (anabolic) response was correlated with the EAA content of the protein food source ($p < 0.001$) (Figure 1). These data illustrate the limitations of dietary guidelines failing to consider protein quality.

Beyond protein quality: the significance of non-protein components of protein food sources

DIAAS quantifies the quality of a single protein or a group of proteins. Neither DIAAS nor any other measure of protein quality accounts for the non-protein components of a dietary protein food source. However, apart from nutritional supplements, dietary protein is normally consumed in a food source that contains non-protein components. Dietary carbohydrate and fat components of protein food sources may potentially affect many aspects of the physiological response, including the net gain in body protein (the anabolic response). Carbohydrate is well known to amplify the protein synthetic response to dietary protein (89). Dietary fat also increases the magnitude of response to dietary protein. For example, whole milk increases the protein synthetic response to dietary protein as compared to the same amount of protein in the form of skim milk (90). Furthermore, an acute increase in plasma fatty acids improved muscle protein synthesis despite inducing insulin resistance (91).

It is not obvious how the role of the non-protein components of protein food sources can be included in the assessment of protein quality. One approach is to normalize the anabolic response to the protein food source by the corresponding caloric value. In the example of the ounce equivalent protein food sources discussed above, the protein food sources with the highest DIAASs (beef, pork, eggs and tofu) stimulated the anabolic response with less caloric intake than those with the lower DIAASs (kidney beans, peanut butter, and mixed nuts) (Figure 2).

The potential physiological significance of the non-protein components of protein food sources can be appreciated by calculating the caloric content of the amount of each dietary protein food source that would be required to fully meet all EAA requirements (Figure 3). For lower quality proteins such as nuts and beans the entire diet would have to be comprised of only those protein food sources to avoid a positive energy balance (i.e., weight gain). These data highlight the importance of considering the total caloric content when translating DIAASs to dietary recommendations. Accounting for the non-protein components of protein food sources is particularly important during caloric restriction weight loss (CRWL). CRWL induces a negative energy balance that impairs the anabolic response to dietary protein. For that reason, an intake of at least 1.2 g protein /kg/day is necessary to maintain muscle mass during CRWL (92). To reach this goal, it is necessary to rely entirely on high quality protein food sources so that the accompanying caloric value associated with the non-protein components is minimized.

Protein quantity vs. quality

Can consumption of more of the same dietary protein food sources compensate for low protein quality? This question can be addressed by considering the *utilizable protein* in a diet pattern. The utilizable protein in a single mixed meal or the entire daily protein consumption can be calculated by multiplying the overall DIAAS (as described in ref. 13) by the amount of protein consumed. The rationale underlying this approach is that the synthesis of complete proteins from dietary EAAs requires the availability of all the EAAs, and when the demand for the limiting EAA exceeds the amount of that EAA absorbed, further protein synthesis from the non-limiting dietary EAAs cannot proceed.

To illustrate the interaction of dietary protein quantity and quality we will consider a simplified numerical example in which the daily intake of dietary protein is comprised of two proteins, with one of the proteins being low-quality protein (DIAAS = 50%) and one being high-quality (DIAAS = 100%), and to simplify the math the limiting amino acid is assumed to be the same for both proteins. We will assume that total daily protein intake is 50 g, which would correspond to a 70 kg person consuming slightly more than the EAR (0.66 g protein/kg/day), and that 25 g of each protein is consumed. The DIAAS of this combination would be 75%, and the utilizable protein is $(25\,g \times 0.5) + (25\,g \times 1.0) = 37.5\,g$, which would be less than the EAR. If consumption of the low-quality protein is increased to 50 g and the high-quality protein consumption remains at 25 g, the overall DIAAS would be reduced to 66%, but the total utilizable protein consumption would increase to 49.5 g (approximately equal to the EAR) because of the increase in total protein consumption. However, this approach would require consumption of 75 g of dietary protein, which may be difficult for some to achieve due to issues of cost, taste and convenience. These factors are important drivers of food consumption (93). Further, since low-quality proteins are usually plant based (14), increasing consumption would likely significantly increase the associated caloric content of the protein food sources of the diet. If we consider another example in which the initial parameters are the same, but the consumption of the high-quality protein is increased to 50 g while consumption of the low-quality protein is maintained at 25 g, the overall DIAAS would increase to 83%. The product of DIAAS and protein consumption would increase the utilizable protein to 62.5 g and would likely involve a smaller increase in caloric intake than when the low-quality protein consumption is

increased. Thus, increasing the quantity of both low-quality and high-quality protein can help to meet dietary EAA targets, but the increase in EAA consumption will be greater when the overall DIAAS is increased by increasing the consumption of the high-quality protein.

This simplified example illustrates that, when the limiting amino acid is the same for different dietary proteins, increasing the amount of high-quality protein consumed increases the utilizable protein more effectively than increasing the amount of low-quality protein consumed. However, in more realistic dietary patterns, proteins may be complementary, due to the limiting amino acids being different. The amount that complementary proteins improve the overall quality of the dietary protein (i.e., DIAAS) is dependent on the magnitude of the difference between the EAA content relative to the reference protein of the limiting EAAs of the two proteins. In addition, the EAA content relative to the reference protein for next-limiting EAAs in the two proteins will impact the extent to which the proteins are complementary. While specific numerical examples would be complicated, some generalizations are possible. Complementary low-quality proteins have the potential to increase the DIAAS more than with complementary high-quality proteins, because the discrepancies between the limiting EAAs are likely to be greater with low-quality proteins. High-quality proteins have DIAASs >100%, meaning that there is not a large difference between the values for the limiting EAAs (14). While combining complementary low-quality dietary proteins will increase the utilizable protein, this approach will not achieve the same increase in utilizable protein as combining a low-quality protein with a high-quality protein, particularly if the limiting EAAs in the low- and high-quality proteins differ (i.e., they are complementary). Combining low- and high-quality complementary proteins will result in a DIAAS greater than the DIAAS of the low-quality protein, but lower than the DIAAS of the high-quality protein, with corresponding impact on the amount of utilizable protein.

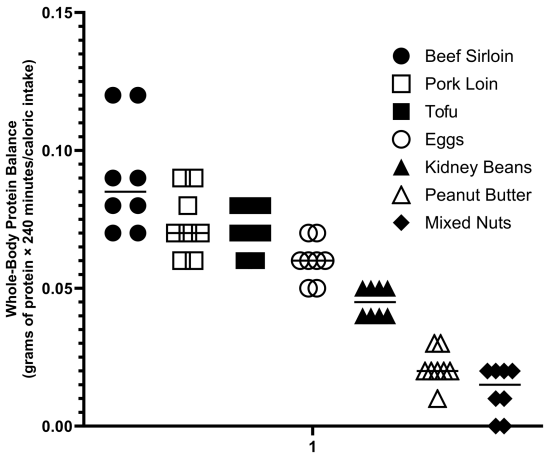

FIGURE 2
Anabolic response determined by stable isotope tracer methodology of ounce equivalents protein food sources normalized for energy content of the non-protein components (81).

Differences in the metabolic fate of EAAs

Nutritional guidelines for dietary protein have been derived from whole-body measurements, primarily N-balance or isotopic tracer methods. However, differences in tissue- and organ-specific responses may arise in response to varied protein food sources that are not evident from the whole-body responses yet have physiological significance. For example, the response of peripheral blood levels of EAAs following consumption of soy protein is limited by extensive splanchnic clearance of absorbed EAAs (94, 95). As a result, there may be minimal stimulation of muscle protein FSR by soy protein consumption (96), even though the whole-body protein net balance response (which includes splanchnic

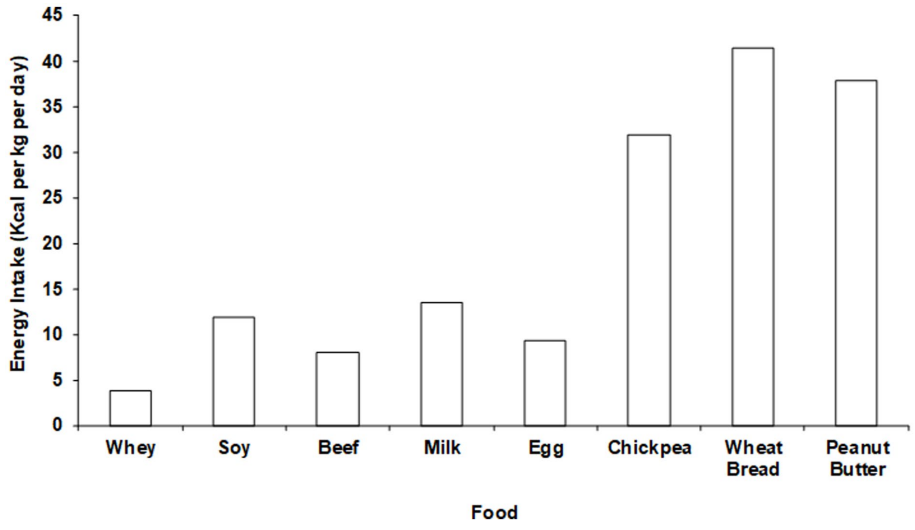

FIGURE 3
Net whole-body protein balance per calorie of intake with different "ounce equivalent" protein food sources.

uptake) is comparable to that following consumption of the same amount a different high-quality protein such as beef. Consumption of beef protein, on the other hand, results in a relatively rapid and greater total plasma EAA response than soy, with a corresponding greater stimulation of MPS (92). The differences in splanchnic and peripheral responses to dietary protein are demonstrated by the results of a recent study we performed comparing the MPS and whole- body protein responses to consumption of a 4 oz. beef patty vs. the responses to consumption of a 4 and to an 8 oz (97). "Impossible Burger" comprised of soy-based protein. The response of plasma EAA concentrations was greater following consumption of the 4 oz. beef patty than the 4 oz. Impossible Burger, which corresponded to the differences in EAA contents of the two proteins. As a result, both whole-body and MPS were significantly stimulated by the beef patty, but neither were stimulated by consumption of the 4 oz. Impossible burger (98). More relevant to the issue of differing fates of ingested EAAs, the response of plasma EAAs following consumption of 4 oz. beef burger was greater than the Impossible Burger despite the greater total EAA content of the 8 oz. Impossible Burger (corrected for the lower digestibility of soy protein) (Figure 3). As a result of greater splanchnic extraction of absorbed EAAs following soy protein consumption, MPS was stimulated to a greater extent by the 4 oz. beef patty than the 8 oz. of Impossible Burger despite equivalent increases in whole body net protein balance (98).

Is protein quality relevant in high income countries?

Average protein consumption in underdeveloped countries may be insufficient to meet all EAA requirements (99). In high-income countries there is less concern that dietary protein consumption is inadequate to provide adequate EAAs. Rather, the notion that dietary protein is "over-consumed" is commonly expressed in publications ranging from scientific to lay articles in high-income countries. However, a careful analysis of dietary protein intake in high income countries that takes protein quality into account has been lacking. This issue has recently been addressed by analyzing the implications of variations in dietary protein quality for the adequacy of dietary protein intake in the United States (100). The analysis used published FAO food supply data sets giving overall total protein intakes, as well as NHANES survey data across a well described population. Account was taken of potential differences in dietary protein quality, as quantified by DIAAS. Data were analyzed for healthy adults, as well as for specific nutritional states that may affect the optimal level of protein and EAA consumption, such as caloric restriction weight loss diets, aging, aerobic and resistance exercise training, and vegan/vegetarian diets. Protein consumption data were compared with both the EAR (0.66 g protein/kg/day) and the RDA (0.83 g protein/kg/day) for different populations. To account for protein quality, the utilizable protein intake (calculated as described above) was calculated (Figure 4).

Data from the US National Health and Nutrition Examination Surveys for 2001–2018 was used to assess the percentage of the adult population having utilizable protein intakes potentially less than recommended levels. Utilizable protein intake was calculated for DIAASs ranging from 1.0 to 0.6. An analytical sample of 44,018 (22,079 males and 21, 939 females) was used, stratified by age and gender. 11% of the adult population had estimated utilizable protein intakes below the EAR even if a DIAAS of 1.0 is assumed (i.e., all protein consumption was "high quality" protein), and the percentage increased to 20% in the 71+ age group if the DIAAS of the total protein intake was 1.0. The percentage of the population 19–50 year of age consuming protein intakes below the EAR when DIAAS was assumed to be 1.0 was higher for women than men (16% versus 5%), and the percentage increased with age (71+ years male and female = 20%). The percentage of the

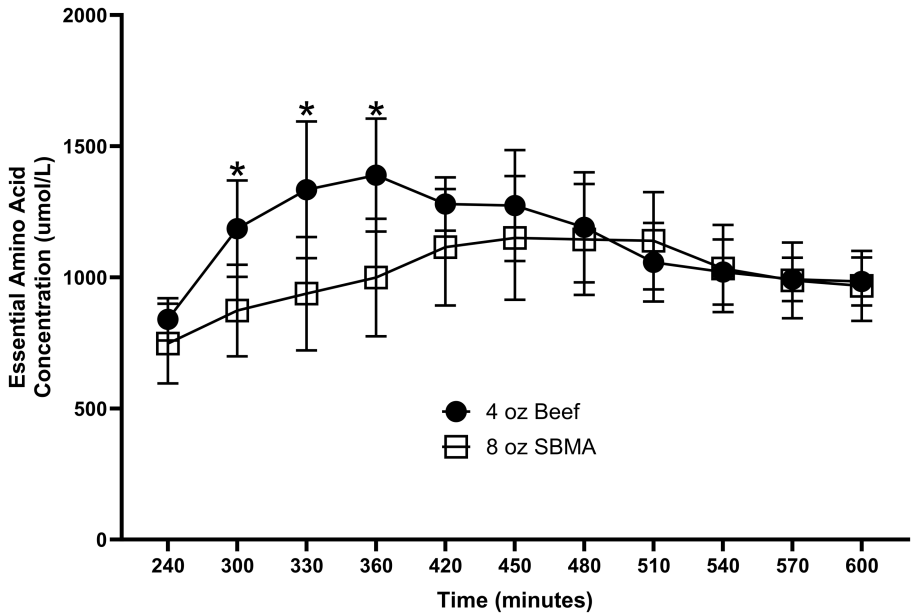

FIGURE 4
Energy requirements to meet minimal EAA requirements with different "ounce equivalent" protein food sources. Total energy requirements are approximately 35 kcal/kg/day (Adapted from (98), licensed under CC BY 4.0).

population with utilizable protein intakes potentially falling below the EAR increased considerably as DIAAS declined, with potentially 72% of the 71+ year-old population having utilizable protein intakes falling below the EAR if DIAAS was taken to be 0.6, and that number increased to 88% if compared to the RDA. While these values are theoretical, the data analysis highlights the potential importance of protein quality in meeting EAA requirements, even in a high-income country.

Conclusion

Dietary protein quality, defined generally as the ability to provide to the body an optimal amount and profile of EAAs per gram protein consumed, in accord with dietary requirements, varies between proteins. EAAs cannot be synthesized in the body, and consumption of at least the RDAs for each EAA is required for optimal protein nutrition. Currently, protein quality can most accurately be quantified by the DIAAS, although DIAAS has potential shortcomings when applied to dietary planning. DIAAS is based on the EAR for protein, and individual dietary planning will most commonly be based on the RDA for protein.

When account is taken of protein quality by means of the DIAAS, utilizable dietary protein may fall below the amount needed to meet EAA requirements, even in high-income countries. Moreover, optimal EAA consumption is likely well above the minimal acceptable amount in a wide range of metabolic and physiological circumstances. In such circumstances it is important that dietary protein consumption is composed largely of high-quality proteins. Reliance on low quality proteins to meet elevated EAA recommendations will usually involve a significant increase in caloric intake due to the non-protein components of the low-quality protein food source. The next major advance in protein/amino acid nutrition will be the tailoring of dietary patterns to individual needs, predicated on the metabolic and physiological state of the individual. This progression will require better understanding of optimal levels of EAA consumption in different circumstances, coupled with use of a scoring system such as DIAAS, to quantify the utilizable protein in a dietary pattern.

Author contributions

RW: Conceptualization, Writing – original draft, Writing – review & editing. DC: Data curation, Formal analysis, Visualization, Writing – review & editing. AF: Writing – review & editing. PM: Writing – review & editing.

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Conflict of interest

Robert R. Wolfe is a founder and part owner of The Amino Company. He is an inventor of multiple patents for EAA-based dietary supplements. Arny Ferrando is a co-inventor on three patents related to Essential Amino Acid formulations.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Protein quality evaluation: FAO perspective

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United Nations agencies have a unique role in achieving the Sustainable Development Goals (SDGs) and aligned global nutrition targets by 2030. According to the latest estimates the world is moving backward in its efforts to end hunger, food insecurity and malnutrition in the presence of a more challenging and uncertain context, including climate change, war conflicts and other challenges. Shifts to plant and novel foods such as insects have been suggested to have good nutritional quality, as well as less environmental impact compared to "traditional" animal source foods. In the context of changing food systems, considering the nutritional quality of foods is essential and accurately assessing protein quality of foods is particularly important, given the large variability in amino acid composition and digestibility between dietary proteins. Indeed, protein quality estimates have the potential to inform policies and programs for actions to improve nutrition throughout the world and have been discussed during past and recent expert consultations. Recently, the Food and Agriculture Organization of the United Nations has been working with the International Atomic Energy Agency and international experts to review and update evidence and related methods on protein quality assessment and to develop a Protein Digestibility Database to aid dialog on the evaluation of protein quality and protein sufficiency in different populations.

KEYWORDS

amino acids, protein quality, digestibility, PD-CAAS, DIAAS

Introduction

With the global population projected to reach 11 billion by the year 2050, sustainably nourishing the world's population is one of the most pressing challenges we face, which is compounded by the acceleration of climate change (1). Notably, according to the 2023 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 (2), with global estimates indicating that among children under 5 years old, around 148.1 million were stunted (22.3 per cent), 45 million were wasted (6.8 per cent) and 37 million were overweight (5.6 per cent) (2). The aftermath of COVID-19 pandemic and the health impacts of climate change, including malnutrition, are increasingly clear especially in the Global South (3). The consequences of malnutrition are enormous, including avoidable ill-health and premature death, as well as significant economic and societal costs (4).

Alongside the wider international system, United Nation Agencies, including the Food and Agriculture Organization of the United Nations (FAO), have a unique role to play promoting lasting solutions to malnutrition as part of a wider sustainable food systems transformation. Better nutrition is one of the pillars of FAO's Strategic Framework (2022–2031), which articulates FAO's vision of a sustainable and food secure world for all (5) in the context of the Agenda 2030 for Sustainable Development (6). The right to adequate food established and transition toward healthy diets for national populations is of crucial importance for the enjoyment of all human rights (7) and is at the core of better nutrition, alongside better production, better environment and better life (5). In this regard, providing an adequate, sustainable and nutritious supply of nutrients,

including protein, is of critical importance and defining accurately the amount and quality of protein required to meet nutritional needs and describing appropriately the protein supplied by foods and diets is essential. United Nation Agencies, FAO, World Health Organization (WHO) and others have 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.

Dietary protein

Dietary proteins provide nitrogen (N) and amino acid (AA) and must be supplied by the diet in adequate quantity and proportion. Dietary protein account for a significant part of animal and plant tissues and microorganisms, contributes to metabolism and homeostasis and plays an essential role in human health for growth, maintenance, reproduction, and immune function (or immunity) (8).

The general dietary requirement for protein is defined as an estimated average requirement (EAR) and recommended dietary allowance (RDA). For healthy adults at maintenance, the estimated average requirement for protein based on N balance experiments is 0.66 g/kg body weight/day, and the Recommended Dietary Allowance (RDA) or Population Reference Intake (PRI) is 0.83 g/kg body weight/day (9). Recommendations are also provided for infants and children, and for women during pregnancy and lactation, by including additional components of protein needs by a factorial approach (8). However, protein consumption differs globally and particularly in low-and middle-income countries (LMICs), the amount of protein consumed is consistently lower than in high income countries (HICs), especially for proteins from animal source foods.

At the same time, alternative and novel protein market is growing, with consumers being increasingly exposed to new foods, some of which are novel propositions (plant-based meat alternatives) while others are traditional food items currently introduced to new geographic areas (e.g., tofu and species of edible insects). Cell-based production, which is the field of growing animal agricultural products directly from cell cultures, also continues to expand and has been explored as an alleged sustainable alternative to the conventional livestock agricultural system (10). 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.

There are two distinct uses of protein quality data: assessment of a diet's ability to meet human protein and AA requirements, and assessment of the protein adequacy for regulatory purposes of foods and food products sold to consumers (11). How to accurately measure protein quality has been a subject of debate among experts for many years. Proteins are made up of 20 AAs, of which nine are termed indispensable amino acids (IAA), as they are essential but cannot be synthesized in the human body (8). The quality of a protein is defined by its ability to meet age specific nitrogen and IAA requirements for growth, maintenance and specific physiological states (8). Factors affecting the protein quality of a food are the total protein content, the IAA content of the proteins in the food and the metabolic availability of the AAs.

Regulatory and policy implications of protein quality evaluation

Protein quality estimates are used to inform policies and programs for actions to improve nutrition throughout the world. They are closely tied to food composition data which serve as a critical resource, offering crucial information about the amino acid profiles and digestibility of various protein sources. Various stakeholders, including research institutions, governments and industries with varying levels of expertise, utilize this data to calculate the protein quality of individual foods and mixtures of foods. Additionally, they can be used to evaluate the protein quality of local food sources, guiding agricultural practices to promote the cultivation of high-quality protein crops, thereby improving food security and nutrition.

Regulatory bodies use these estimates to shape international food policies, food security programs and national dietary assessments. Specifically, standardized data on food protein quality in humans can inform recommendations on protein requirements, as well as compositional requirements for foods for special meals. This includes advice on appropriate amino acid complementation or supplementation to enhance the quality of traditional plant-based diets and for setting specialized nutrition standards.

Protein quality guidance also supports the development of food-based dietary guidelines (FBDGs), which provide national recommendations on foods, food groups and dietary patterns for providing required nutrients to the general public to promote overall health and prevent chronic diseases (12). These guidelines are intended to establish a basis for public food and nutrition, health and agricultural policies and nutrition education programs to foster healthy eating habits and lifestyles. They are particularly important in addressing malnutrition in vulnerable populations. Moreover, scientific advice on protein quality evaluation is relevant for the development of Codex Alimentarius food standards and guidelines including information provided on food labels, such as nutrition labeling and protein content claims. The Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) has addressed the issue of protein quality in foods and diets on several occasions. In 2019, the FAO and the WHO issued guidance on nitrogen to protein conversion factors for estimating the protein content of soy-based and milk-based ingredients used in infant formulas and follow-up formulas (13) to support the development of the Codex Standard for Follow-up Formula (CXS 156–1987) (14). Additionally, a FAO Expert Working Group provided scientific advice on Protein Quality Assessment in Follow-up Formula for Young Children and Ready to Use Therapeutic Foods (RUTF), outlining future research recommendations for different protein sources (15). This was followed by the provision of FAO supplementary guidance to members of the CCNFSDU uses on computing the Protein

Abbreviations: AA, amino acid; DIAAS, digestible indispensable amino acid score; EAR, estimated average requirement; FAO, food and agriculture Organization of the United Nations; HIC, high income countries; IAA, indispensable amino acid; IAEA, International Atomic Energy Agency; LMICs, low-and middle-income countries; PDCAAS, protein digestibility corrected amino acid score; RDA, recommended dietary allowance; SOFI, state of food security and nutrition in the world; UNU, United Nations University; WHO, World Health Organization.

Digestibility-Corrected Amino Acid Score (PDCAAS) in follow up formulas (16, 17).

Protein quality evaluation

For FAO, setting global human nutrient and energy requirements has been an important part of the organization's work since its founding, with 65 years in setting nutrient requirements, also in collaboration with WHO, and establishing guidelines on diet and nutrition. The determination of protein requirements for human nutrition was reviewed by FAO for the first time in 1955 (18) and in subsequent years with the WHO (8).

Related expert meetings on protein quality evaluation have been discussed over the past decades. In 1989, following a request by the Codex Committee on Vegetable Protein, for determining protein quality in the human diet PDCAAS was adopted by a joint FAO/WHO expert consultation as the most suitable approach for the routine evaluation of overall protein quality for humans and its adoption was recommended as an official method to assess protein quality at international level (19). In calculating PDCAAS the limiting AA score (i.e., the ratio of the first-limiting AA in a gram of target food protein to that in a reference protein or requirement value) is multiplied by protein digestibility, with the intention of assessing how well dietary protein can match the demand for AAs and allowing the prediction of dietary protein utilization (19).

However, the PDCAAS method has received criticism since its adoption. In 2002, the joint FAO/WHO/UNU Expert Consultation on Proteins and Amino Acids in Human Nutrition reviewed the validity of these criticisms recognizing that PDCAAS had several shortcomings (8). In short, PDCAAS does not assign additional nutritional value to proteins with high biological value, it overestimates the nutritional value/protein digestibility of foods that contain antinutrients, and it overestimates the protein digestibility of foods with low digestibility when supplemented with the corresponding limiting AA. The expert consultation recommended that an additional expert consultation be convened to review the validity of PDCAAS for protein quality assessment, suggest appropriate revisions to the method, or adopt a better method applicable to a wider range of human diets (8).

Recognizing limitations in PDCAAS and new research findings, in 2011, FAO convened an expert consultation to review methods for determining dietary protein quality to reflect current best practices (20). A new method for protein quality assessment, the Digestible Indispensable Amino Acid Score (DIAAS,) was proposed as a method for dietary quality assessment for regulatory purposes (20). Experts noted that ileal protein digestibility better reflects the true quantity of AAs digested and absorbed and should be used in calculating DIAAS, as well as that in dietary protein quality evaluation, dietary AA should be treated as individual nutrients and wherever possible digestible or bioavailable AA data should be given in food tables.

However, knowledge and research gaps were also noted, most importantly that there was a lack of human digestibility data available that utilized DIAAS (20). Indeed most existing AA digestibility data came from the pig model, and there was also a lack of public health impact analysis prior to the adoption of DIAAS as the standard method for protein quality assessment. At the time, a move toward DIAAS could have had significant implications for protein requirements and scientific advice for human protein nutrition. Therefore, experts recommended that further research utilizing DIAAS in human subjects was needed before this method could be adopted (20).

Following the 2011 dietary protein quality assessment in human foods, the FAO convened in 2014 an expert working group to update recent advances in protein quality assessment and to discuss the most appropriate methodologies for measuring protein digestibility and utilization in humans (21). The working group aimed to propose and agree on research protocols using both human and animal models to evaluate the ileal AA digestibility of human foods, particularly foods and diets consumed in LMICs (21). Five research protocols in use at the time or that had the potential for further development were recommended for measuring DIAAS, namely: the true ileal digestibility of AA, the use of a dual stable isotope tracer, oxidation of indicator AAs, utilization of postprandial proteins, and net postprandial protein utilization. Experts further recommended 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.

Toward the development of a database

With funding from the Public Health Agency of Canada, the FAO, in collaboration with IAEA, has initiated the development of a protein quality database. A technical meeting held in October 2022 highlighted the urgent need to create and populate this database, as sufficient data now exists (22). Developing and hosting this database falls within one of FAO's core functions, to "assemble, analyze, monitor and improve access to data and information, in areas related to FAO's mandate" (23). The FAO publishes several databases and encourages their use for statistical, scientific, and research purposes, while also offering expertise to guide countries on using the data to help strengthen evidence-based decision-making in the food and agriculture sectors a. Such a data platform is expected to significantly benefit LMICs and smaller nations that lack the technical and financial resources to collect protein quality data and may not otherwise have free access to such information.

Experts at the Joint FAO-IAEA technical meeting (22) presented available and valid models that look at ileal AA digestibility, noting that *in vitro* methods are the way forward, with recommendations for optimization and standardization. They also emphasized the need for collection of additional data to assess the effects of processing, preparation and storage on protein quality, as well as protein quality data from mixed meals, complex foods, and complementary foods. Additionally, they highlighted the need for collection of digestibility data from alternative and novel protein sources, including climate resilient crops, as well as protein quality data from foods. In the discussion on AA requirements and respective reference patterns, members agreed that there are currently no sufficient data to justify setting new requirements, however recommending the need for generating data from vulnerable population groups (with focus on infants and elderly).

Practical steps have been taken to make the database a reality. A FAO/IAEA protein quality database technical advisory group has been established consisting of field experts and secretariat members

from the main UN agencies (FAO and IAEA). This group will provide feedback, input and recommendations as needed to guide the construction of the joint FAO-IAEA database and to provide up to date information on the protein quality from food sources, according to the appropriate scoring method. Key actions include formulating and publishing calls for data to populate the database and establishing a framework for its validation that, which allow for data use across various domains. The database will ultimately be populated with peer-reviewed published data and unpublished microdata from these sources, enabling comprehensive meta-analyses to be carried out. A technical advisory group meeting is scheduled for November 2024 to advance the database construction and evaluate the necessary actions for its finalization.

Discussion

Many individuals do not have access to safe, affordable healthy diets needed to promote health and wellbeing (24) with healthy diets being of reach for more than 3.1 billion people (2). As a result, malnutrition in all its forms is a problem of global proportion, and no country is free from its effects. A healthy diet is one which promotes growth and development and prevents malnutrition. One of the nutrients most discussed in this regard is protein, as there is variability in the contribution of dietary proteins to human nutrient requirements, due to differences in AA composition and in digestibility (25).

An accessible robust database of ileal AA digestibility of individual, complex foods and diets commonly consumed in different parts of the world is needed for informed decisions regarding protein quality using DIAAS. Such a data platform is expected to also benefit LMICs and small countries that lack the technical and financial resources to collect protein quality data and may not otherwise have free access to such data.

The database will be the first of its kind, where comprehensive data on the protein content, AA composition and ileal digestibility of proteins and individual AAs in foods, collected using any accepted validated method (human, pig, rat, *in vitro*), is available free of charge. 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 post-harvest storage conditions will be covered, as well as proteins in mixed meals and in complementary foods for young children.

Research institutions, governments, and industry with various levels of skill and background knowledge would be able to use the data to calculate the protein quality of individual foods and mixtures of foods. The data would 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 would also allow assessment of complementarity of protein sources, such as combining different foods that complement one another to provide the IAAs as part of a mixed diet, or in combining such foods in food products like complementary foods; as well as on how poorly digestible proteins can be supplemented with limiting AAs in order to improve the quality of some traditional plant-based diets. Finally, following the eventual regulatory adoption of DIAAS by governments, the data can be used by food regulatory agencies to evaluate food health and nutrition claims by industry.

Moving forward and to further advance the research agenda, there is also a need to identify and stimulate the accrual of funds to support research and generate data and human and technical resources. Research should focus on the generation of protein quality data from various foods and diets in Low-and Middle-Income Countries, as well as data on climate- resilient crops to also address increasing sustainability concerns.

Data availability statement

The presenting findings are from past and recent expert working group meetings and consultations held by FAO and other UN agencies.

Author contributions

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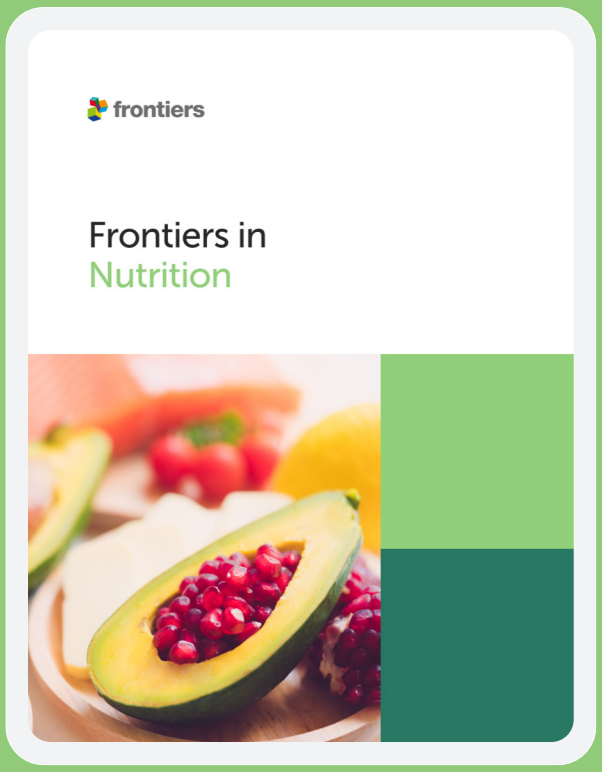

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