The Journal of Nutrition 155 (2025) 1398–1407

THE JOURNAL OF NUTRITION

journal homepage: https://jn.nutrition.org/

Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Repeatability of Pig True Ileal Amino Acid Digestibility Coefficients: Among and Within Laboratories

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A B S T R A C T

Background: When determining true ileal amino acid (AA) digestibility values in the growing pig to calculate digestible indispensable AA score, there are aspects of the protocol, both genetic and environmental, that vary between laboratories.

Objectives: This study aimed to determine true ileal AA digestibility in 9 foods in each of 3 laboratories (Australasia, Europe, and North America) to determine interlaboratory variability. In each laboratory, 3 foods were also evaluated twice to determine intralaboratory variation.

Methods: Each laboratory followed a standardized protocol to determine true ileal AA digestibility for each food. Growing pigs received each food for 7 d and digesta were collected via a cannula at the terminal ileum on days 6 and 7. True ileal AA digestibility coefficients were determined for each food at each laboratory to evaluate interlaboratory variability. Three foods were evaluated twice in different cohorts of pigs at each laboratory (intralaboratory variation).

Results: There was no statistically significant effect ($P > 0.05$) of the laboratory on the true ileal AA digestibility coefficients for 8 of the foods. Differences in AA digestibility were found for wheat bread. The mean coefficient of variation (CV) between laboratories for digestibility of the indispensable AAs was 5.0% with an overall mean for all AAs of 5.5%. For intralaboratory variability, there were no statistically significant differences ($P > 0.05$) in AA digestibility for any food with mean CVs for each AA within each of the 3 laboratories for the indispensable AAs of 1.3%, 1.1%, and 0.9%.

Conclusions: True ileal AA digestibility values determined in the growing pig for the same foods in different laboratories (interlaboratory variation) do not vary greatly. When a single food was evaluated a second time in the same laboratory, little variation among digestibility values was found.

Keywords: pig, true ileal AA digestibility, AAs, DIAAS, protein quality

Introduction

The use of digestible indispensable amino acid score (DIAAS) to evaluate the protein quality of foods and food ingredients was recommended by an FAO of the United Nations Expert Consultation in 2013 [1]. To calculate DIAAS, it is necessary to have data on the true ileal digestibility of the amino acids (AAs) in the food or food ingredient.

Ideally, true ileal AA digestibility would be determined directly in humans but this requires the collection of digesta from the terminal ileum, which is not straightforward. There are 2 options for collecting ileal digesta in humans. One is the use of naso-ileal intubation [2], involving inserting a small caliber tube through the nose, down the back of the throat, and along the digestive tract to the terminal ileum. The food or ingredient is consumed, and digesta aspirated through the tube. An alternative method involves the cooperation of ileostomates, people who have had their terminal ileum exteriorized because of medical problems involving their large intestine. The food or ingredient is consumed, and digesta collected [3]. However, it is not practically feasible to use either of these methods to determine true ileal AA digestibility for the large numbers of foods

Abbreviations: AA, amino acid; CV, coefficient of variation; DIAAS, digestible indispensable amino acid score; DM, dry matter; IAA, indispensable amino acids.

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https://doi.org/10.1016/j.tjnut.2025.03.013

Received 13 November 2024; Received in revised form 5 March 2025; Accepted 12 March 2025; Available online 17 March 2025

and ingredients that require evaluation. Because of this, an animal model for protein digestion in the adult human is required, and the growing pig has been suggested as a suitable model.

The digestion of proteins (AA digestibility) has been compared between the growing pig and adult humans and shown to be similar [2, 4]. In a more recent and comprehensive study, Hodgkinson et al. [5] determined true ileal AA digestibility values in 7 foods, ranging in protein digestibility, in the adult ileostomate and ileal cannulated growing pig. No statistically significant differences in true ileal AA digestibility were found between the 2 species, and the regression equation between the human (y) and pig (x) for digestibility of the indispensable AAs (those used to calculate DIAAS) was $y = 1.001\times -0.008$; close to $y = x$. Thus, the pig can be considered to be a valid model for the human for true ileal AA digestibility.

A standardized protocol for the management of pig studies to determine true ileal AA digestibility has been developed [6]. Whereas a lot of experimental details can be controlled, there will always be aspects of a protocol that will differ between laboratories. These may reflect environmental differences, differences in animal genetics and the pigs' microbiota, and small procedural differences. It is, therefore, important to determine whether different laboratories obtain similar results for true ileal AA digestibility when evaluating the same food following the same standardized protocol, and that in each laboratory digestibility results are consistent over time.

An objective was to evaluate the consistency of the determination of pig true ileal AA digestibility coefficients when determined in different laboratories (a laboratory in each of Australasia, Europe, and North America) for the same foods (interlaboratory variability). A second objective was to assess the intralaboratory reproducibility of the standardized pig study protocol in each of the 3 participating laboratories by redetermining the true ileal AA digestibility for 3 of the protein sources in each laboratory.

Methods

The Animal Research: Reporting of in vivo Experiments (ARRIVE) reporting guidelines were used to describe the study [7]. The studies were carried out in 3 laboratories: the Riddet Institute (New Zealand), the University of Illinois (United States), and Wageningen University and Research (the Netherlands).

Approval was obtained from the appropriate Animal Ethics Committee in each institution before the studies began, as follows: Riddet Institute: Massey University Animal Ethics Committee, protocol number 16/121; University of Illinois: Animal Care and Use Committee protocol number 16113; Wageningen UR: the study was authorized by the Dutch Council on Animal Experiments and experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (AVD104002015326).

Foods and diet preparation

Objective 1

Nine foods/ingredients were chosen, ranging in true ileal AA digestibility (mean digestibility of indispensable AAs ranged from 61% to 96%). The foods included a mixture of different "types" of protein (beans, cereals, a meat product, a dairy product, processed foods, and a purified protein). The list of the protein sources and their AA compositions is given in Table 1. A sufficient quantity of the same batch of each food for studies in the 3 laboratories was procured and shipped to each laboratory. Protocols were developed for the preparation of the foods (see Supplemental Materials) so that each of the 9 foods would be prepared and fed to the pigs similarly in each laboratory. Each food, after its preparation as described in Supplemental Materials, was combined immediately before feeding with a specific mixture of nonprotein food ingredients. This mixture was specific for each food but included purified maize starch [at a level to give a final protein concentration of 100 g/kg dry matter (DM) in each diet], sucrose, vitamins and minerals, refined vegetable oil, and purified cellulose. Titanium dioxide was also included in the nonprotein mixture to serve as an indigestible marker. The diet compositions for the test diets are given in Supplemental Table 1. Basal and protein-free diets were also prepared (Supplemental Table 2). The basal diet was fed to the pigs during the presurgery acclimatization period, during the recovery period between surgery and the assay period, and for 7 d after the feeding of the protein-free diet.

Objective 2

To establish the intralaboratory reproducibility, the repeatability of true ileal AA digestibility was assessed in each of the 3 participating laboratories. This was done by redetermining the true ileal AA digestibility and lysine availability for 3 of the protein sources (whey protein isolate, pigeon peas and sorghum) in different cohorts of cannulated pigs in each laboratory.

Pig study

The method described next was used for both objectives. In each institution, the study was carried out as described in detail by Hodgkinson et al. [6], which follows the recommendations of an FAO Working Group [8]. It was ensured that as far as possible the same methods for surgery (cannula implantation), cannula care, sampling protocols, feeding procedures, and digesta sampling procedures were used by the 3 laboratories. Humane endpoints for the studies are given in Supplemental Materials.

Female commercial breed pigs with a starting bodyweight of $\geq$30 kg (range 30–41 kg) were used. Overall, a total of 42 pigs were cannulated (see below), including extra pigs in case of any health issues. After surgery, the animals were housed individually in smooth-sided pens (minimum 1.5 $\times$ 1.5 m) with slatted floors and had toys available at all times for oral manipulation. The rooms were controlled to be 21–24°C (thermoneutral zone), with a 12-h light/dark cycle. Fresh water was freely available to all animals at all times.

Throughout the study, the daily dietary ratio for each pig was 0.08 $\times$ metabolic bodyweight ($kg^{0.75}$) calculated on a DM basis. The daily ration was given in 2 equal meals 9 h apart (08:00 and 17:00). Pigs were weighed weekly and the daily ration of each pig was adjusted according to the bodyweight of the pig.

After the pigs were adapted to the environment and basal diet for $\geq$8 d, a titanium T-cannula was surgically inserted into the end of the small intestine (terminal ileum) of each pig following the procedure described in detail by Hodgkinson et al. [6]. The

TABLE 1
Determined protein and AA composition of the protein sources (mg/g DM).

	Black beans[1]	Bovine collagen[2]	Chickpeas	Pigeon peas[3]	Sorghum	Toasted wheat bread[4]	Wheat bran[5]	Whey protein isolate[6]	Zein[7]
Protein[8]	265	1000	223	238	96	135	142	880	938
Histidine	6.7	7.8	5.4	8.7	2.2	2.5	3.2	13.2	12.8
Isoleucine	10.4	15.3	9.3	10.7	3.8	4.0	4.0	58.9	38.2
Leucine	18.6	30.1	16.7	19.1	12.7	7.8	8.0	88.8	193.1
Reactive lysine	13.3	38.3	11.6	17.4	1.8	1.6	2.1	81.4	—
Total lysine	16.3	37.6	13.5	18.1	2.3	2.4	3.5	82.2	—
Methionine	2.7	9.1	3.3	2.1	1.9	1.8	2.0	20.5	17.1
Phenylalanine	13.5	20.4	12.9	13.1	5.1	5.5	5.2	25.4	65.7
Threonine	10.6	17.0	7.7	9.8	3.2	3.3	4.1	66.0	29.9
Tryptophan	3.0	2.5	2.4	2.3	1.3	1.3	2.2	13.4	1.7
Valine	12.4	24.3	10.1	12.7	4.9	4.9	5.9	54.3	38.9
Alanine	9.8	87.6	8.7	10.9	3.9	3.4	3.5	45.4	102.5
Arginine	15.3	83.3	17.6	20.5	6.6	4.2	5.5	17.5	17.4
Aspartic acid	27.6	58.2	24.2	29.1	19.8	4.8	7.3	92.7	60.9
Cysteine	2.3	—	2.5	2.5	1.8	2.4	2.3	18.4	7.1
Glutamic acid	34.1	94.6	31.4	41.4	19.8	37.8	28.4	161.4	270.5
Serine	9.8	29.2	10.6	13.1	4.4	5.5	5.8	42.6	57.7
Tyrosine	8.5	8.2	6.7	8.8	4.2	3.8	3.9	24.2	50.7

Abbreviations: AA, amino acid; DM, dry matter.
[1] Harvest North, Henshall, Canada.
[2] Dat-Schaub, Poland.
[3] Davis Food Ingredients, New Zealand.
[4] See Supplementary Material.
[5] Kellogg's All Bran (Aust.) Pty. Ltd.
[6] Fonterra, New Zealand.
[7] Sigma, United States.
[8] Determined nitrogen × 6.25.

pigs were then given a minimum of 8 d to recover from surgery before starting the assay phase.

Each food was received by pigs within the weight range of 34–100 kg with 6 pigs receiving each experimental diet ($n = 6$) as per FAO guidelines [8]. Each pig was an experimental unit. Test cycles for each test food had a 7-d duration. Pigs were allotted to their test cycles according to an incomplete Latin square (Youden square) with diets and periods comprising the rows and the columns of the squares, respectively. No blinding of diets occurred as the diets were easily visually identified.

The initial 5 d of each test were the adaptation period to the diet. Digesta were collected from the cannula for 9 h on days 6 and 7 of each test cycle starting immediately after the first meal of the day, via small plastic bags attached to the cannula barrel using an elastic band. Bags were replaced whenever filled with digesta, and at least once every 30 min, and the digesta were immediately frozen (–20°C).

Each pig also received a protein-free diet for 7 d to allow for the correction of endogenous (nondietary) AA excretions on an individual animal basis. The pig was its own control for the correction of endogenous AAs. Each pig received the protein-free diet after receiving half of the test diets for that pig. The feeding of the protein-free diet and subsequent collection of ileal digesta were carried out in the same manner as for the test diets. After the protein-free diet, the basal diet was fed to the pigs for a period of 7 d, before beginning the following test cycle, to minimize carry-over effects from the protein-free diet. At the completion of the study, pigs either continued on to further studies or were killed.

Chemical analysis

Digesta were thawed but maintained at <4°C. After pooling and mixing the digesta, a subsample of the digesta from each pig and diet was collected and freeze-dried. The test foods, test diets, and digesta samples were sampled using standard sampling procedures. Chemical analyses were carried out at each laboratory for their samples with the exception of reactive lysine, for which all of the analyses were carried out at the Riddet Institute.

The following chemical analyses were carried out: DM according to the method described by the Association of Official Analytical Chemists (AOAC) [9]; titanium following the method of Short et al. [10]. The AA contents of the test foods, test diets, and ileal digesta samples were determined using the methods described by Rutherfurd et al. [11,12] involving a 24-h acid hydrolysis. Performic acid oxidation before acid hydrolysis was also carried out to determine the methionine and cysteine contents of the samples. The tryptophan contents of test foods, test diets, and ileal digesta samples were determined using alkaline hydrolysis [13]. The weight of each AA was calculated using free AA molecular weights and no correction was made for potential destruction/further release of AAs during the 24-h hydrolysis. The reactive (structurally altered) lysine content of the food and digesta samples was determined as described by Moughan and Rutherfurd [14].

Calculations

Values for basal gut endogenous AA were those determined for each pig fed the protein-free diet and were used to calculate true ileal digestibility coefficients on an individual basis.

Equations *1* and *2* were used to calculate the true ileal AA digestibility coefficients for the foods.

$$\text{Ileal AA}(\mu g / g\ \text{DMI}) = \frac{\text{Concentration of AA in digesta}(\mu g/g\ \text{DM}) \times \text{Diet Ti concentration}\ (\mu g/g\ \text{DM})}{\text{Digesta Ti concentration}\ (\mu g/g\ \text{DM})} \quad (1)$$

True ileal AA digestibilities were then calculated:

$$\text{True digestibility} = \frac{\text{Dietary AA}\ (\mu g/g\ \text{DMI}) - (\text{Ileal AA}\ (\mu g/g\ \text{DMI}) - \text{Endogenous AA}\ (\mu g/g\ \text{DMI}))}{\text{Dietary AA}\ (\mu g/g\ \text{DMI})} \quad (2)$$

Statistical analyses

Statistical analyses were performed using the statistical software SAS (SAS/STAT version 9.4). The normal distribution and homogeneity of variance were evaluated with the use of the Operational Data Store (ODS) graphics.

Objective 1

To statistically compare results, a two-way Analysis of Variance (ANOVA) model was used to determine the effects of laboratory ($n = 3$), diet ($n = 9$), and their interaction. The coefficient of variation (CV) was calculated for the true ileal digestibility of each AA for each food between laboratories. The CV was calculated as the SD of the mean AA digestibility value determined in each laboratory for the food divided by the mean of the values across the laboratories. The CV for each AA was then averaged across foods.

Objective 2

For the intralaboratory reproducibility, an independent paired *t* test was performed to compare the differences in digestibility for each AA in each food determined within the same laboratory but over 2 times. When variances of the treatments were unequal, the *P* value reported was obtained with the Satterthwaite separate variance *t* test. The CV was calculated as described previously for each AA in each food for each laboratory and the values averaged for each AA.

Results

Objective 1

The mean true ileal digestibilities for each AA in each food determined in each of the 3 laboratories are presented in Tables 2–4. The overall mean (determined over the 3 laboratories) true ileal AA digestibilities for each AA in each food are also shown. No statistically significant interactions ($P > 0.05$) were found between laboratory and food for true ileal AA digestibility. For 8 of the 9 foods evaluated, there was no effect of the laboratory on the true ileal AA digestibility coefficients. For wheat bread, however, for 7 of the 18 AAs, there was a significant difference ($P < 0.05$) between laboratories, with 1 laboratory having slightly lower digestibility coefficients than the other 2 laboratories for 6 AAs and another for 1 AA (Table 3). The pooled SEM values are also presented in Tables 2–4 as an indication of the level of variability. For most of the foods, the values can be considered to be low; except for collagen and zein, the mean of the pooled SEM for all AAs was <0.03. More variability was found for collagen and zein (mean pooled SEM for all AAs of 0.06 and 0.06 for collagen and zein, respectively).

The CVs for true ileal AA digestibility values for each AA across the foods and laboratories are presented in Table 5. The average CV for the indispensable AAs was 5.0% with an overall average of 5.5%. The indispensable AA with the greatest variation was tryptophan (13.6%) with isoleucine having the lowest variation (2.8%).

Objective 2

The mean true ileal digestibility AA values determined by each laboratory for each of the 3 foods are given in Tables 6–8. One pig from laboratory 2 was excluded when receiving the sorghum diet because of a very low diet intake. There were no statistically significant differences ($P > 0.05$) within each laboratory between the 2 times that the true ileal AA digestibility was determined for each of the 3 foods.

The CV for true ileal AA digestibility for each laboratory calculated for each AA across the 3 foods (Table 9) was low, with the mean CV for the indispensable AAs being 1.5%, 1.2%, and 1.3% for each laboratory, respectively, with mean values over all for the AAs of 1.3%, 1.1%, and 0.9% for the 3 laboratories.

Discussion

The uptake of DIAAS for the protein quality evaluation of foods and food ingredients is increasing and DIAAS is expected to supersede protein digestibility amino acid score as the method of choice for regulatory purposes [15]. True ileal AA digestibility values, needed to calculate DIAAS, cannot be determined routinely in humans, and, therefore, an animal model is needed. An animal assay also has the advantage of allowing more standardization than with human assays, with lower expected variability around the determined mean digestibility value. The growing pig is expected to provide a suitable model for protein digestion in adult humans, based on close similarities in the anatomy and physiology of the respective digestive tracts from the mouth to the terminal ileum [16,17]. Furthermore, the

TABLE 2
Mean results obtained from each laboratory, overall mean values, and pooled SEM for pig true ileal amino acid digestibility coefficients for 3 protein sources determined in 3 laboratories and probability of a difference between laboratories for each protein source (n =6).

	Black beans				SEM	Bovine collagen				SEM	Chickpeas				SEM
	Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean	
Histidine	0.74	0.76	0.76	0.75	0.017	0.81	0.84	0.88	0.84	0.050	0.84	0.82	0.87	0.84	0.019
Isoleucine	0.79	0.81	0.78	0.79	0.016	0.88	0.90	0.92	0.90	0.043	0.84	0.83	0.85	0.84	0.017
Leucine	0.80	0.82	0.79	0.80	0.018	0.85	0.89	0.91	0.88	0.037	0.85	0.85	0.85	0.85	0.017
Reactive lysine	0.83	0.84	0.85	0.84	0.037	0.87	0.93	0.89	0.90	0.016	0.90	0.88	0.90	0.89	0.034
Methionine	0.77	0.78	0.78	0.78	0.033	0.88	0.92	0.90	0.90	0.025	0.87	0.85	0.88	0.87	0.016
Phenylalanine	0.81	0.79	0.80	0.80	0.017	0.89	0.82	0.92	0.88	0.037	0.90	0.87	0.89	0.88	0.012
Threonine	0.71	0.75	0.69	0.72	0.028	0.79	0.81	0.87	0.82	0.061	0.80	0.69	0.79	0.76	0.063
Tryptophan	0.73	0.69	0.81	0.74	0.048	—	—	—	—	0.335	0.82	0.75	0.86	0.81	0.030
Valine	0.75	0.78	0.73	0.76	0.024	0.87	0.90	0.91	0.89	0.045	0.83	0.80	0.82	0.82	0.027
Mean IAA	0.77	0.78	0.78	0.78	0.026	0.81	0.82	0.89	0.83	0.072	0.85	0.82	0.86	0.84	0.026
Total lysine	0.79	0.84	0.73	0.79	0.029	0.87	0.90	0.93	0.90	0.021	0.88	0.87	0.92	0.89	0.034
Alanine	0.71	0.77	0.77	0.75	0.029	0.91	0.93	0.92	0.92	0.019	0.80	0.77	0.85	0.81	0.029
Arginine	0.83	0.86	0.94	0.87	0.018	0.94	0.94	0.96	0.94	0.023	0.93	0.92	0.98	0.94	0.012
Aspartic acid	0.65	0.70	0.71	0.68	0.017	0.61	0.75	0.67	0.68	0.056	0.68	0.74	0.78	0.73	0.029
Cysteine	0.31	0.47	0.29	0.36	0.057	—	—	—	—	0.080	0.70	0.71	0.63	0.68	0.038
Glutamic acid	0.82	0.84	0.83	0.83	0.014	0.85	0.92	0.91	0.89	0.026	0.87	0.86	0.89	0.87	0.018
Serine	0.75	0.78	0.76	0.76	0.021	0.78	0.82	0.83	0.81	0.047	0.83	0.78	0.87	0.83	0.027
Tyrosine	0.80	0.82	0.79	0.80	0.019	0.77	0.84	0.86	0.82	0.067	0.87	0.86	0.87	0.87	0.018
Overall mean[1]	0.74	0.77	0.76	0.75	0.026	0.77	0.83	0.85	0.82	0.060	0.83	0.81	0.85	0.83	0.025
P[2]				NS					NS					NS	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.
[2] P Probability; NS No significant differences ($P > 0.05$) found between laboratories for this food.

TABLE 3
Mean results obtained from each laboratory, overall mean values, and pooled SEM for pig true ileal amino acid digestibility coefficients for 3 protein sources determined in 3 laboratories and probability of a difference between laboratories for each protein source.

	Pigeon peas				SEM	Sorghum				SEM	Wheat bread				SEM
	Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean	
n	12	12	12			12	11	12			6	6	6		
Histidine	0.92	0.87	0.87	0.88	0.013	0.85	0.87	0.91	0.88	0.024	0.93	0.90	0.96	0.93	0.035
Isoleucine	0.90	0.90	0.86	0.89	0.012	0.89	0.89	0.88	0.89	0.023	0.92	0.89	0.95	0.92	0.011
Leucine	0.90	0.90	0.85	0.88	0.012	0.91	0.91	0.88	0.90	0.018	0.94	0.91	0.96	0.94	0.011
Reactive lysine	0.94	0.94	0.92	0.93	0.023	0.90	0.82	0.93	0.88	0.027	0.74	0.62	0.83	0.73*	0.021
Methionine	0.86	0.88	0.82	0.85	0.020	0.93	0.92	0.95	0.93	0.015	0.95	0.92	0.96	0.95	0.011
Phenylalanine	0.91	0.88	0.87	0.89	0.012	0.91	0.87	0.89	0.89	0.023	0.95	0.92	0.96	0.94	0.026
Threonine	0.85	0.84	0.76	0.82	0.023	0.82	0.78	0.84	0.82	0.040	0.86	0.79	0.93	0.86	0.027
Tryptophan	0.81	0.78	0.80	0.80	0.028	0.90	0.82	0.92	0.88	0.033	0.92	0.84	0.97	0.91*	0.016
Valine	0.89	0.88	0.81	0.86	0.016	0.88	0.87	0.87	0.87	0.028	0.91	0.88	0.95	0.91	0.017
Mean IAA	0.89	0.88	0.84	0.87	0.018	0.89	0.86	0.90	0.88	0.026	0.90	0.85	0.94	0.90	0.019
Total lysine	0.92	0.93	0.84	0.90	0.024	0.88	0.84	1.00	0.86	0.031	0.73	0.75	0.99	0.82*	0.093
Alanine	0.85	0.87	0.84	0.85	0.017	0.90	0.90	0.91	0.91	0.020	0.89	0.74	0.96	0.86*	0.030
Arginine	0.95	0.95	0.98	0.96	0.007	0.91	0.90	1.07	0.96	0.026	0.91	0.79	1.05	0.92*	0.063
Aspartic acid	0.87	0.86	0.87	0.87	0.011	0.86	0.87	0.91	0.88	0.027	0.81	0.74	0.92	0.82*	0.025
Cysteine	0.73	0.73	0.59	0.68	0.030	0.80	0.82	0.84	0.82	0.025	0.91	0.88	0.93	0.91	0.011
Glutamic Acid	0.92	0.92	0.90	0.91	0.011	0.90	0.91	0.89	0.90	0.019	0.97	0.96	0.98	0.97	0.004
Serine	0.90	0.85	0.85	0.87	0.017	0.85	0.81	0.90	0.85	0.029	0.91	0.84	0.98	0.91*	0.015
Tyrosine	0.89	0.89	0.85	0.88	0.013	0.90	0.91	0.84	0.88	0.029	0.94	0.95	0.95	0.95	0.025
Overall mean[1]	0.88	0.87	0.84	0.86	0.017	0.88	0.87	0.90	0.88	0.025	0.90	0.85	0.95	0.90	0.022
P[2]				NS					NS					$P<0.05$	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.
[2] *P* denotes probability; NS, no significant differences ($P > 0.05$) found between laboratories for this food. Mean values followed by a * were statistically different between laboratories.

TABLE 4
Mean results obtained from each laboratory, overall mean values, and pooled SEM for pig true ileal amino acid digestibility coefficients for 3 protein sources determined in 3 laboratories, and probability of a difference between laboratories for each protein source.

	Wheat bran				SEM	Whey protein isolate				SEM	Zein				SEM
	Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean		Laboratory 1	Laboratory 2	Laboratory 3	Mean	
	6	6	6			12	12	12			6	6	6		
Histidine	0.71	0.67	0.72	0.70	0.035	0.98	0.98	0.95	0.97	0.008	0.77	0.84	0.71	0.77	0.052
Isoleucine	0.72	0.73	0.70	0.72	0.011	0.98	0.98	0.94	0.97	0.004	0.74	0.83	0.68	0.75	0.048
Leucine	0.75	0.74	0.72	0.74	0.011	0.96	0.99	0.96	0.97	0.007	0.79	0.86	0.69	0.78	0.052
Reactive lysine	0.66	0.56	0.79	0.67	0.021	0.98	0.98	0.97	0.98	0.003	–	–	–	–	–
Methionine	0.76	0.80	0.72	0.76	0.011	0.97	0.99	0.96	0.97	0.007	0.77	0.87	0.70	0.78	0.052
Phenylalanine	0.79	0.75	0.75	0.76	0.026	0.91	0.95	0.95	0.93	0.028	0.77	0.81	0.68	0.75	0.050
Threonine	0.61	0.62	0.55	0.59	0.027	0.89	0.93	0.87	0.90	0.018	0.71	0.78	0.73	0.74	0.050
Tryptophan	0.64	0.67	0.70	0.67	0.016	0.97	0.99	0.96	0.97	0.009	–	–	–	–	–
Valine	0.69	0.70	0.65	0.68	0.017	0.95	0.97	0.91	0.94	0.011	0.76	0.84	0.71	0.77	0.051
Mean IAA	0.70	0.69	0.70	0.70	0.019	0.96	0.97	0.94	0.96	0.011	0.76	0.83	0.70	0.71	0.051
Total lysine	0.50	0.50	0.49	0.50	0.093	0.98	0.98	0.95	0.97	0.011	–	–	–	–	–
Alanine	0.65	0.65	0.64	0.65	0.030	0.95	0.96	0.87	0.93	0.008	0.79	0.78	0.69	0.75	0.064
Arginine	0.79	0.77	0.84	0.80	0.063	0.95	0.95	0.99	0.96	0.034	0.70	0.65	0.80	0.72	0.114
Aspartic acid	0.45	0.52	0.49	0.48	0.025	0.96	0.98	0.95	0.96	0.006	0.74	0.77	0.72	0.75	0.063
Cysteine	0.56	0.59	0.45	0.53	0.011	0.97	0.99	0.93	0.96	0.008	0.65	0.83	0.68	0.72	0.058
Glutamic Acid	0.86	0.85	0.85	0.85	0.004	0.96	0.97	0.91	0.95	0.004	0.79	0.79	0.69	0.76	0.060
Serine	0.68	0.68	0.67	0.68	0.015	0.90	0.93	0.81	0.88	0.013	0.77	0.74	0.73	0.74	0.071
Tyrosine	0.74	0.76	0.70	0.73	0.025	0.94	0.99	0.93	0.95	0.014	0.78	0.85	0.69	0.77	0.051
Overall mean[1]	0.69	0.69	0.68	0.69	0.022	0.95	0.97	0.93	0.95	0.011	0.75	0.80	0.71	0.73	0.060
P[2]				NS					NS					NS	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.
[2] *P* denotes probability; NS, no significant differences ($P > 0.05$) found between laboratories for this food.

TABLE 5
Mean coefficient of variation (CV, %) of true ileal amino acid digestibility values for each amino acid across 3 laboratories for 9 foods (interlaboratory variation).

	CV
Amino acid	Across sites
Histidine	3.8
Isoleucine	2.8
Leucine	3.1
Reactive lysine	5.8
Methionine	3.2
Phenylalanine	3.3
Threonine	5.4
Tryptophan	13.6
Valine	3.6
Mean indispensable AAs[1]	5.0
Total lysine	13.7
Alanine	4.3
Arginine	6.0
Aspartic acid	5.5
Cysteine	16.4
Glutamic acid	2.5
Serine	4.1
Tyrosine	3.7
Mean overall[1]	5.5

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: AA, amino acid.
[1] Reactive lysine is included in overall mean, but total lysine is not.

growing pig has been validated in direct pig-to-human protein and AA digestibility comparisons [5]. The growing pig was used here as a model for protein digestion in the adult human, as recommended by FAO [8].

The calculation of DIAAS requires determining the true ileal AA digestibility of the food or ingredient and such work would be conducted in multiple laboratories around the world. Although most aspects of the method used can be standardized between laboratories, there are always specific aspects that will differ. Such differences may arise due to the use of different breeds and strains of pigs and the microbiome within the pigs, small variations in procedure, and potentially uncontrolled environmental differences. Thus, it is important to evaluate any differences in ileal AA digestibility that may arise because of such factors being operative in individual laboratories. To do this, the same foods were evaluated here for ileal digestibility in laboratories operating in different parts of the world. The laboratories involved in this evaluation were located in Australasia, Europe, and North America and each laboratory routinely determines the true ileal AA digestibility of foods and food ingredients using the cannulated growing pig. Because the same foods (from the same batches) were analyzed in each laboratory, 3 different sites were considered sufficient to test the consistency of results.

It is important, when determining true ileal AA digestibility coefficients for DIAAS calculation that the pigs receive the foods as closely as possible to the manner they would be consumed by humans. To ensure consistency, therefore, the preparation of the foods (e.g. cooking when required) and diet preparations in this study were standardized between laboratories.

As shown in Tables 2–4, there was no statistically significant difference in the true ileal AA digestibility values between laboratories for any food other than wheat bread (objective 1). The coefficients of variation based on variance estimates between laboratories were low for the AAs used to calculate DIAAS, ranging from 2.8% for isoleucine to 13.6% for tryptophan. There are many interlaboratory testing programs that evaluate differences between laboratories for AA analysis (only AA analysis, not including an animal study), and from these studies, typical CVs range from 4% to 20% [18]. Thus, the interlaboratory CVs found in the present study fall within the lowest part of this range and can thus be considered as acceptable, supporting the robustness of the standardized method to determine true ileal AA digestibility. The variability between the laboratories (indicated by pooled SEM) can also be considered to be low, with the highest variability found for bovine collagen [0.072 and 0.060 for the mean of indispensable amino acids (IAAs) and overall mean digestibility]. The pooled SEM values were lowest for

TABLE 6
Mean true ileal amino acid digestibility coefficients and pooled SEM for pigeon peas determined twice in 3 laboratories with the growing pig ($n = 6$).

	Laboratory 1		SEM	Laboratory 2		SEM	Laboratory 3		SEM
Histidine	0.93	0.90	0.011	0.88	0.85	0.013	0.87	0.87	0.013
Isoleucine	0.91	0.88	0.012	0.90	0.89	0.009	0.86	0.86	0.013
Leucine	0.91	0.89	0.014	0.91	0.89	0.010	0.85	0.86	0.014
Reactive lysine	0.95	0.93	0.009	0.94	0.94	0.007	0.93	0.91	0.038
Methionine	0.89	0.83	0.021	0.88	0.89	0.018	0.84	0.81	0.019
Phenylalanine	0.93	0.90	0.012	0.89	0.88	0.011	0.87	0.87	0.012
Threonine	0.87	0.83	0.018	0.85	0.83	0.018	0.76	0.76	0.031
Tryptophan	0.82	0.80	0.025	0.80	0.76	0.025	0.80	0.80	0.034
Valine	0.90	0.87	0.013	0.89	0.87	0.012	0.80	0.82	0.021
Mean IAA	0.90	0.87		0.88	0.87		0.84	0.84	
Total lysine	0.93	0.91	0.011	0.93	0.93	0.009	0.82	0.86	0.038
Alanine	0.87	0.84	0.018	0.87	0.87	0.016	0.84	0.83	0.015
Arginine	0.96	0.94	0.007	0.95	0.94	0.008	0.98	0.97	0.007
Aspartic acid	0.88	0.86	0.011	0.85	0.87	0.011	0.87	0.87	0.011
Cysteine	0.76	0.71	0.024	0.76	0.69	0.028	0.59	0.59	0.037
Glutamic acid	0.93	0.91	0.010	0.92	0.92	0.012	0.90	0.90	0.011
Serine	0.91	0.89	0.011	0.84	0.86	0.015	0.84	0.86	0.023
Tyrosine	0.91	0.88	0.011	0.91	0.88	0.012	0.84	0.86	0.016
Overall mean[1]	0.90	0.87		0.88	0.87		0.84	0.84	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.

TABLE 7
Mean true ileal amino acid digestibility coefficients and pooled SEM for sorghum determined twice in 3 laboratories with the growing pig.

	Laboratory 1		SEM	Laboratory 2		SEM	Laboratory 3		SEM
n	6	6		5	6		6	6	
Histidine	0.86	0.85	0.014	0.85	0.89	0.025	0.92	0.90	0.031
Isoleucine	0.90	0.88	0.014	0.88	0.89	0.017	0.89	0.88	0.033
Leucine	0.91	0.91	0.011	0.90	0.92	0.011	0.88	0.88	0.027
Reactive lysine	0.92	0.88	0.023	0.83	0.82	0.041	0.96	0.90	0.003
Methionine	0.93	0.92	0.013	0.91	0.94	0.011	0.95	0.94	0.019
Phenylalanine	0.91	0.91	0.011	0.85	0.89	0.022	0.89	0.88	0.031
Threonine	0.83	0.81	0.020	0.77	0.80	0.035	0.85	0.84	0.057
Tryptophan	0.89	0.91	0.031	0.81	0.83	0.031	0.95	0.88	0.037
Valine	0.89	0.87	0.013	0.87	0.88	0.018	0.88	0.87	0.043
Mean IAA	0.89	0.88		0.85	0.87		0.91	0.88	
Total lysine	0.90	0.86	0.027	0.83	0.85	0.046	1.00	1.00	0.003
Alanine	0.90	0.90	0.013	0.89	0.91	0.015	0.92	0.91	0.029
Arginine	0.92	0.91	0.013	0.89	0.90	0.024	1.09	1.05	0.037
Aspartic acid	0.87	0.86	0.017	0.87	0.86	0.025	0.93	0.89	0.036
Cysteine	0.80	0.79	0.014	0.81	0.83	0.021	0.85	0.83	0.035
Glutamic acid	0.90	0.90	0.013	0.89	0.92	0.014	0.90	0.89	0.026
Serine	0.86	0.85	0.015	0.80	0.82	0.028	0.90	0.89	0.039
Tyrosine	0.90	0.89	0.013	0.90	0.92	0.018	0.85	0.83	0.044
Overall mean[1]	0.89	0.88		0.86	0.88		0.91	0.89	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.

TABLE 8
Mean true ileal amino acid digestibility coefficients and pooled SEM for whey protein isolate determined twice in 3 laboratories with the growing pig ($n = 6$).

	Laboratory 1		SEM	Laboratory 2		SEM	Laboratory 3		SEM
Histidine	0.97	0.98	0.006	0.98	0.98	0.006	0.95	0.95	0.011
Isoleucine	0.97	0.99	0.004	0.99	0.98	0.004	0.94	0.94	0.005
Leucine	0.99	0.94	0.009	0.99	0.98	0.006	0.96	0.96	0.005
Reactive lysine	0.99	0.98	0.002	0.98	0.99	0.003	0.96	0.97	0.003
Methionine	0.99	0.96	0.008	0.99	0.99	0.005	0.96	0.96	0.008
Phenylalanine	0.98	0.84	0.046	0.97	0.93	0.011	0.96	0.93	0.010
Threonine	0.88	0.90	0.007	0.93	0.93	0.008	0.93	0.80	0.029
Tryptophan	0.97	0.97	0.007	0.99	0.98	0.009	0.97	0.96	0.011
Valine	0.94	0.95	0.004	0.97	0.96	0.007	0.95	0.88	0.017
Mean IAA	0.96	0.95		0.98	0.97		0.95	0.93	
Total lysine	0.98	0.98	0.018	0.98	0.98	0.006	0.95	0.95	0.003
Alanine	0.94	0.95	0.007	0.96	0.95	0.006	0.87	0.88	0.010
Arginine	0.94	0.97	0.019	0.97	0.92	0.018	0.96	1.01	0.052
Aspartic acid	0.96	0.96	0.006	0.98	0.97	0.005	0.94	0.95	0.007
Cysteine	0.98	0.96	0.006	1.00	0.99	0.009	0.93	0.93	0.007
Glutamic acid	0.96	0.96	0.003	0.97	0.98	0.003	0.90	0.91	0.006
Serine	0.90	0.91	0.008	0.93	0.92	0.009	0.80	0.83	0.019
Tyrosine	0.98	0.90	0.015	1.00	0.98	0.013	0.95	0.92	0.014
Overall mean[1]	0.96	0.95		0.98	0.96		0.93	0.92	

The lines that are in underline is used to differentiate from the other data lines.
Abbreviation: IAA, indispensable amino acids.
[1] Reactive lysine is included in overall mean, but total lysine is not.

whey protein isolate (WPI) and pigeon peas (0.011, 0.018 for the mean of IAAs for WPI and pigeon peas, respectively, and 0.011 and 0.017 for overall mean of AAs for WPI and pigeon peas, respectively). It should be noted that as WPI, pigeon peas, and sorghum were tested in 11 (sorghum for 1 laboratory) or 12 pigs (all other laboratories and foods) in each laboratory, this would likely decrease the pooled SEM values compared with foods tested in 6 animals per laboratory.

It was noted with wheat bread that the true ileal digestibility values for several AAs from 1 laboratory were slightly lower ($P < 0.05$) than those for the other 2 laboratories (Table 3). This may have been due to incomplete mixing of the indigestible marker titanium dioxide within the bread dough and was reflected by notable differences in the determined titanium concentration of the bread between laboratories (range 4.2–6.1 g/kg).

An additional aspect that was tested here was the intra-laboratory variation in true ileal AA digestibility. Three foods, ranging in protein digestibility (whey protein isolate, pigeon peas, and sorghum) were evaluated in each laboratory 2 times using different cohorts of pigs. Because different cohorts of pigs were used for the 2 evaluations for these 3 foods, repeating the evaluation twice was considered sufficient especially when the ethical cost of additional evaluations was considered; to evaluate

TABLE 9
Mean coefficient of variation (%) for pig true ileal AA digestibility values relating to 3 foods with each food evaluated twice in each of 3 laboratories (intralaboratory variation).

Amino acid	Laboratory 1	Laboratory 2	Laboratory 3	Mean
Histidine	1.1	1.9	0.6	1.2
Isoleucine	1.3	0.8	0.4	0.8
Leucine	1.7	1.1	0.5	1.1
Reactive lysine	1.4	0.3	2.1	1.3
Methionine	2.7	1.0	1.2	1.6
Phenylalanine	4.4	2.5	1.3	2.8
Threonine	2.0	1.6	4.2	2.6
Tryptophan	1.0	2.1	2.1	1.7
Valine	1.4	1.1	2.8	1.7
Mean indispensable AAs	1.5	1.2	1.3	1.3
Lysine	1.6	0.7	1.3	1.2
Alanine	1.2	0.9	0.6	0.9
Arginine	1.3	1.4	2.3	1.7
Aspartic acid	0.9	0.5	1.3	0.9
Cysteine	2.7	2.7	0.5	2.0
Glutamic acid	0.6	0.9	0.6	0.7
Serine	1.4	1.4	1.4	1.4
Tyrosine	3.0	1.6	1.5	2.0
Mean overall[1]	1.3	1.1	0.9	1.1

The lines that are in underline is used to differentiate from the other data lines.
Abbreviations: AA, amino acid; IAA, indispensable AAs
[1] Reactive lysine is included in overall mean, but total lysine is not.

the foods in 3 cohorts of pigs would have required an additional 18 animals overall (6 per laboratory). All of the CV values based on variance estimates within each laboratory for true ileal AA digestibility were low, with no laboratory having a mean CV for the indispensable AAs >1.5% (Table 7), and the overall mean CVs were all <1.5%. As discussed previously, typical CV values between laboratories for AA analysis (not including an animal assay) are between 4% and 20% [18].

In conclusion, true ileal AA digestibility values determined in the growing pig for the same foods in different laboratories (interlaboratory variation) do not vary greatly. When a single food was evaluated a second time in the same laboratory, very little variation among digestibility values was found. These results support the robustness of the standardized protocol using the growing pig model to determine true ileal AA digestibility for the calculation of DIAAS for application in the adult human.

Author contributions

The authors' responsibilities were as follows – PJM, WH, SMH, SdV, HS: responsible for planning the study; SMH, NS, HS, SdV, NvdW: conducted the studies; CAM, SMH: performed the statistical analysis and interpreted the data; SMH: prepared the first draft of the manuscript which was revised by PJM; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

Funding

Project Proteos, funded by a consortium of food companies and food sectors, coordinated by Global Dairy Platform, Chicago, USA.

Data availability

The data described in the manuscript will be made available on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2025.03.013.

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