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Presence of Unabsorbed Free Amino Acids at the End of the Small Intestine Indicates the Potential for an Increase in Amino Acid Uptake in Humans and Pigs

Nikkie van der Wielen [1,2,*], Sonja de Vries [1], Walter J. Gerrits [1], Kim Lammers-Jannink [1], Paul J. Moughan [3], Marco Mensink [2], Wouter Hendriks [1]

[1] *Animal Nutrition Group, Wageningen University and Research, Wageningen, the Netherlands;* [2] *Division of Human Nutrition, Wageningen University and Research, Wageningen, the Netherlands;* [3] *Riddet Institute, Massey University, Palmerston North, New Zealand*

A B S T R A C T

Background: Unabsorbed free amino acids (AAs) at the end of the small intestine result in a potential preventable nutritional loss.
Objectives: This study aimed to quantify free AAs in terminal ileal digesta of both humans and pigs to investigate its relevance for the nutritional value of food proteins.
Methods: Two studies with three diets were performed: a human study—ileal digesta from eight adult ileostomates were collected over 9 h after ingestion of a single meal unsupplemented or supplemented with 30 g zein or whey; pig study—12 cannulated pigs were fed for 7 d with a diet containing whey or zein or no-protein diet, and ileal digesta were collected on the last 2 d. Digesta were analyzed for total and 13 free AAs. True ileal digestibility (TID) of AAs was compared with and without free AAs.
Results: All terminal ileal digesta samples contained free AAs. The TID of AAs in whey was 97% ± 2.4% (mean ± SD) in human ileostomates and 97% ± 1.9% in growing pigs. If the analyzed free AAs would have been absorbed, TID of whey would increase by 0.4%-units in humans and 0.1%-units in pigs. The TID of AAs in zein was 70% ± 16.4% in humans and 77% ± 20.6% in pigs and would increase by 2.3%-units and 3.5%-units, respectively, if the analyzed free AAs would have been fully absorbed. The largest difference was observed for threonine from zein: if free threonine was absorbed, the TID would increase by 6.6%-units in both species ($P < 0.05$).
Conclusions: Free AAs are present at the end of the small intestine and can potentially have a nutritionally relevant effect for poorly digestible protein sources, whereas the effect is negligible for highly digestible protein sources. This result provides insight into the room for improvement of a protein's nutritional value if all free AAs are to be absorbed. *J Nutr* 2023;xx:xx–xx.
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Keywords: free amino acids, true ileal digestibility, amino acid absorption, amino acid bioavailability, protein quality

Introduction

Evaluation of the quantity and quality of proteins in food products is crucial to determine whether diets meet the nutritional requirements of a host [1]. To be of use for the host, ingested protein first needs to be digested and absorbed in the gastrointestinal tract, so that its amino acids (AAs) become available to the body. AA bioavailability is often approximated using the relevant parameter AA digestibility, that is, the net disappearance of ingested AAs from the digestive tract [2]. The latter is preferably measured at the end of the small intestine, the so-called apparent ileal digestibility (AID) of AAs. By correcting for the endogenous proteins found in the digesta, the true ileal digestibility (TID) of AA can be approximated. In the field of human nutrition, a sole correction for basal endogenous losses associated with the quantity of dry matter (DM) intake, is often applied. However, in the field of animal nutrition correction for both basal and ingredient specific endogenous losses is needed when using the term TID. In this field, the term standardized ileal digestibility (SID) is applied when correcting the AID for basal

Abbreviations used: AA, amino acid; AID, apparent ileal digestibility; DIAAS, digestible indispensable amino acid score; DM, dry matter; SID, standardized ileal digestibility; TID, true ileal digestibility.

* Correspondence author. *E-mail address:* nikkie.vanderwielen@wur.nl (N. van der Wielen).

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endogenous losses [3]. The TID, or SID, values are critical for the protein quality evaluation of foods according to the recommended digestible indispensable amino acid score (DIAAS) [1].

It is often assumed that TID is mainly influenced by protein breakdown in the gastrointestinal tract and that all breakdown products, such as free AAs, are readily absorbed by the small intestine. However, ~12% of lysine present in ileal digesta of pigs was found to be in a free AA form [4]. The free AAs are still present in the lumen at the end of the intestine while potentially these AAs could have been absorbed. The unabsorbed free AAs will flow to the large intestine, resulting in a nutritional loss [5]. Therefore, any effort to improve the absorption of free AAs would improve the digestibility and quality of ingested proteins. The measures of TID, although they accurately represent the disappearance of AA from the gastrointestinal tract, provide no information about the form of the unabsorbed AAs.

As there are limited data to estimate the size of improved digestibility with complete absorption of free AA, we aimed to quantify free AAs in terminal ileal digesta of both humans and pigs and investigate their relevance for the nutritional value of food proteins. For this purpose, we performed SID assays in human ileostomates and ileal cannulated pigs, similar to that described by Moughan et al. [6], Deglaire et al. [7], and Hodgkinson et al. [8]. Growing pigs were considered to be a suitable model for human digestive physiology, with an observed similar protein digestibility [7,9]. Moreover, the growing pig is the recommended model animal for protein quality evaluation of human foods [2]. A protein-free diet and two diets containing either whey protein isolate or the maize isolate zein as protein source were formulated. These protein isolates were selected because they differ in digestibility, with whey protein isolate being close to 100% digestible in humans and pigs [6,10] and zein being <60% digestible [11].

Methods

Protein sources

In both experiments, the protein sources were of the same batch to minimize variation between measurements. Whey protein isolate (Whey Protein Isolate 894), hereafter referred to as whey, was obtained from Fonterra and zein (#W555025) from Sigma-Aldrich. The AA composition is given in Table 1.

TABLE 1
Amino acid composition[1] of protein sources (grams per kilogram of dry matter)

	Zein	Whey
Ala	96.8	52.4
Arg	16.5	19.8
Asp	53.8	106.0
Cys	7.8	19.4
Glu	236.6	179.1
Gly	10.1	15.1
His	12.6	17.3
Ile	41.4	69.3
Leu	201.8	101.8
Lys	0.4	92.7
Met	15.9	20.8
Phe	68.0	28.2
Pro	98.2	67.0
Ser	52.3	50.3
Thr	28.3	78.3
Trp	1.2	15.1
Tyr	51.6	26.7
Val	37.4	59.7
Total[2]	1030.2	995.1

[1] Analyzed in duplicate.
[2] Amino acid content was reported using their weight in free form, which is one water molecule heavier than AAs in a peptide chain. For this reason, the sum of all AAs can become >1000 g/kg.

Human study

The experimental protocol was approved by the medical ethical committee of Wageningen University (registered at clinicaltrials.gov as NCT04207372). The experiment generally followed standard procedures for AA digestibility determinations in humans [6]. The study was conducted from May 2018 to January 2019.

Subjects

Males and females with an ileostomy were recruited through advertisement by Hospital de Gelderse Vallei (Ede, Netherlands) and the Dutch Ostomy Association. All potential subjects were informed about the study and signed an informed consent form. Subjects were included for participation if they were aged between 18 and 60 y with a normally functioning, well-established ileostomy, and an otherwise good general health. Most of the subjects had undergone total colectomy for peristaltic problems in the colon, colon cancer, or inflammatory bowel disease. Subjects were included when they reported that not >20 cm of their small intestine was removed during surgery. Subjects were excluded if they used antibiotics or other medications that impair small intestinal digestion and absorption or if they presented with having renal impairment, coeliac disease, or diabetes. Other exclusion criteria were pregnancy, breastfeeding in 12 mo before the study, diet or weight loss regimen, taking protein supplements, or consumption of high quantities of alcohol during the study period. Body weight and height were measured at the study facilities. Two of the subjects dropped out during the study owing to personal reasons or the inability to ingest the meal; these subjects were replaced by two other participants. Eight subjects, five men and three women, completed the entire study protocol. These subjects, aged 41 ± 13 y, were 1.81 ± 0.08 m tall, with a body weight of 85.0 ± 11.9 kg and BMI of 25.9 ± 3.28 kg/m^2.

Experimental procedure

The participants visited the research facilities of Wageningen University and Research on 6 d, where each participant received the protein-free or whey- or zein-containing meals each on two occasions. These test days were separated by ≥3 d. On each test day, participants arrived at the research facilities in the morning after a fasting period of ≥10 h. Each subject was attached with a new ostomy bag and subsequently consumed one of the three test meals within 30 min. One of the three test meals were provided to the participant. During the subsequent 9 h collection period, the ostomy bags were emptied every 2–3 h. In this collection period, participants were permitted to drink coffee, tea, water, and soft drinks without proteins. Moreover, subjects were allowed to read, watch TV, work, or walk around during the 9 h period. Between the different test days, participants consumed their normal diet.

On the test days, the digesta collected every 2–3 h was immediately frozen at −20°C. The digesta samples collected from each participant over the two d they received the same protein source were thawed at <4°C and pooled to obtain one representative digesta sample per participant per protein source. Digesta samples were freeze-dried and ground (Retsch ZM200 centrifugal mill; at 12000 RPM with a 1-mm sieve) before chemical analyses.

Test meals

The three test meals in this study consisted of a protein-free cookie combined with a drink that was either unsupplemented or supplemented with whey or zein. The 160-g protein-free cookie was baked at 190°C for 40–50 min and contained 66.4 g Waxy Maize Starch (Body&Fit), 45.76 g margarine (Albert Heijn), 37.12 g sucrose (Van Gilse), 8 g powdered cellulose (VITACEL), 1.12 g baking powder (Dr. Oetker), 0.8 g ground ginger (Conimex), and 0.8 g celite (Advanced Minerals). The drink contained 500 g fruit smoothie (Innocent magnificent mango) and 5 g polyethylene glycol (Macrogol 4000; Dulcosoft) and was supplemented with 30 g of each protein source or with no supplement for the protein-free diet. The protein sources and drink mixed with cookie were freeze-dried and ground for chemical analysis. The total AA content was 29.8 g for whey-containing, 30.9 g for zein-containing, and 1.9 g for protein-free meals (Supplemental Table 1).

Pig study

The pig study was authorized by the Dutch Council on Animal Experiments (CCD), and experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (AVD104002015326). The animal experiment was conducted at Wageningen University and Research from November 2017 to January 2018.

In brief, the digestibility of whey or zein were determined as part of a larger study in which 10 protein sources and a protein-free diet were tested during eight periods according to an incomplete 10 × 8 Youden square design, using 12 pigs and 4 pigs as spare pigs. This resulted in seven observations for zein and 12 observations for whey, as repeatability of the assay was tested for whey, thereby going beyond the recommended five pigs per protein source needed to determine TID [2]. The protein-free diet was included to determine endogenous losses. It was provided in the fifth experimental week, halfway through the trial. After this protein-free diet, 1 wk of basal diet was provided for wash out purposes before the start of the next experimental week. All procedures were similar to those previously reported for DIAAS assessment [8]. Sixteen gilts (Topigs20; Landrace × Large white) were fitted with an ileal t-cannula, after an acclimatization period of 13–14 days. Detailed surgical procedures and housing conditions are specified in **Supplemental Methods**.

Procedure for experimental period

After recovery from the surgery, eight experimental diets were provided consecutively; each experimental diet was provided for 7 d, with the last 2 d being used for digesta collection for 9 h after the morning meal. Digesta were collected in small plastic bags attached to the cannulas, which were replaced when full or at least every 30 min and immediately frozen at −20°C. The samples collected on the two consecutive days were thawed at <4°C and pooled to obtain 1 representative digesta sample. Digesta samples were freeze-dried and ground (Retsch ZM200 centrifugal mill; at 12000 RPM with a 1-mm sieve) before chemical analyses. At the completion of the study, pigs were killed, and placement of the cannulas was checked.

Body weight at surgery was 35.4 ± 3.60 kg and at autopsy 80.0 ± 10.64 kg. The pigs were healthy during the experimental trials, and cannulas had minimal leakage. In some pigs, there was formation of fibrous tissue around the edges of the cannula; however, the opening of the cannula was never affected. One pig experienced excessive cannula leakage and was killed. This pig was replaced by a spare pig.

Diets

Feed was provided in two equal meals, at 7.00 and 16.00. Feeding level was set at 0.08 kg/body weight$^{0.75}$ and adjusted weekly. Diets (Supplemental Tables 2 and 3) were formulated according to guidelines for TID determination [8]. These diets contained 100 g/kg of DM crude protein and 4 g/kg of titanium dioxide. To prevent deficiencies of feeding zein protein isolate that is low in lysine and tryptophan, these AAs were included according to NRC recommendations [12] on days 1–5 of each period, before digestibility measurements. Furthermore, the digestibility coefficients of these 2 AAs were not determined.

Chemical analyses

For the human study, the AA composition of each meal was determined after defatting procedures [13]. All digesta material, protein sources, and pig diets did not require defatting procedures, and their AA composition was determined directly. Procedures according to ISO13904 [14] and ISO13903 [15] were followed to determine AA composition. In brief, for cysteine and methionine determination, the samples were oxidized before hydrolysis. For all other AAs, no oxidation step was required before hydrolysis. The AAs were separated by ion exchange chromatography and determined by reaction with ninhydrin, using photometric detection at 570 nm and 440 nm for proline. Tryptophan was determined by reversed-phase C18 HPLC with fluorescence detection. These chemical analyses were executed in duplicate, and when the coefficient of variation was >5%, analyses were repeated.

The free AA content was determined according to ISO13903 [15]. In brief, free AAs were extracted with diluted hydrochloric acid and proteins removed with sulfosalicylic acid precipitation. The filtered solution was pH adjusted to 2.2 and subsequently subjected to ion exchange chromatography, before detection of each AA. Peaks of Cys, His, and Trp could not be properly integrated, and therefore, these AAs were not quantified in their free form. Aspartic acid and glutamic acid were excluded because these AAs could not be compared in free and bound form owing to effects of hydrolysis.

The pig diets and ileal digesta were analyzed for Ti content. Ti was hydrolyzed with concentrated sulfuric acid in the presence of a copper catalyst at 420°C, with a subsequent addition of peroxide. The colored complex was analyzed by spectrophotometric determination at 408 nm.

Data analysis

For the human study, total collection within the 9-h collection period was assumed; therefore, apparent and true AA digestibility were determined using the following equations:

$$\text{AID (\%)} = [(\text{AA intake} - \text{AA output})/\text{AA intake}] \times 100\%$$

$$\text{TID (\%)} = \{[\text{AA intake} - (\text{AA output} - \text{endogenous output})]/\text{AA intake}\} \times 100\%$$

where AA output is the total content of a particular AA in the digesta over the 9 h period (in grams) and AA intake is the total content of a particular AA in the meal (in grams). Endogenous output is the total content of a particular AA in the digesta over the 9-h period (in grams) after ingestion of the protein-free meal in the same subject.

For the pig study, Ti was used as an indigestible marker, and apparent and true AA digestibility were determined using the following equations [3]:

$$\text{AID (\%)} = [1 - (AA_{digesta}/AA_{diet}) \times (M_{diet}/M_{digesta})] \times 100\%$$

where $AA_{digesta}$ and AA_{diet} represented the AA concentrations (in grams per kilogram) in digesta and diet on DM basis, respectively, and M_{diet} and $M_{digesta}$ represented the marker concentrations (in grams per kilogram) in diet and digesta on DM basis, respectively.

The protein-free diet was used to estimate endogenous losses for each pig (Supplemental Table 4) as follows:.

$$\text{Endogenous losses} = AA_{digesta} \times (M_{diet}/M_{digesta})$$

where the basal endogenous losses of an AA (in grams per kilogram of DM intake) are calculated with $AA_{digesta}$, representing the concentration of that AA in the ileal digesta (in grams per kilogram of DM), and M_{diet} and $M_{digesta}$ representing markers concentrations in diet and digesta, respectively (in grams per kilogram of DM).

The TID was calculated using the following equation:

$$\text{TID (\%)} = \text{Apparent digestibility} + [(\text{endogenous losses/AAdiet}) \times 100\%]$$

To calculate the potential physiological effect of free AAs as if they would have been fully absorbed, the free AA concentration was subtracted from the AA concentration in digesta and the abovementioned formulas were re-applied. To calculate the proportion of AAs in free form in ileal digesta, the concentration of the free AAs in ileal digesta (in grams per kilogram of DM) was divided by the concentration of total AAs in the same sample of ileal digesta (in grams per kilogram of DM) for that particular AA.

Statistical analysis

Data were processed using Microsoft Excel (Microsoft Office 365 ProPlus) and IBM SPSS statistics (version 25), and all data are presented as means ± SD. The proportion of AA in ileal digesta that are in free form was analyzed per species using linear mixed models procedures with maximum likelihood with diet as a fixed factor and subject (pig or person) as a repeated factor with a compound symmetry covariance structure. Least-square means were compared using Bonferroni adjustments for multiple comparisons. Residual normality and variance homogeneity were evaluated using Shapiro-Wilk test and visual inspection. When residuals did not meet normality assumption, data were log-transformed. Differences in digestibility coefficients calculated with and without free AA were tested using Wilcoxon signed-rank test. P values of <0.05 indicate significant differences.

Results

One human ileostomate and one cannulated pig showed abnormally high gut endogenous losses. Therefore, observations from these subjects were excluded from all analyses. Furthermore, the collection of ileal digesta from one pig in the first collection week failed owing to leakage from the ileal cannula; therefore, this particular observation (whey diet) was excluded from the analyses. This resulted in seven observations for all diets in the human study and six observations for zein and 10 observations for whey in the pig study. All these pigs also received the protein-free diet ($n = 12$).

The total free AA flow was 0.6 ± 0.51 and 0.7 ± 0.41 g/kg of DM intake after ingestion of a protein-free meal for human ileostomates and cannulated pigs, respectively (Supplemental Table 5). After ingestion of a whey-containing meal, the total free AA flow was 0.8 ± 0.57 and 0.8 ± 0.35 g/kg of DM intake for human ileostomates and cannulated pigs, respectively, whereas this was more than three times higher after ingestion of a zein-containing meal: 2.4 ± 1.16 and 3.3 ± 3.52 g/kg of DM intake for human ileostomates and cannulated pigs, respectively.

Apparent ileal digestibility

The AID of zein in humans was 59% ± 16.4% for all AAs, whereas in pigs, the AID was 63% ± 22.4% for all AAs. By adjusting for free AA, the AID of zein increased by 3.1%-units for the 13 AAs in human ileostomates and for pigs by 5.0%-units (Table 2). The largest effects were observed for threonine and glycine, whereas the smallest effects were observed for methionine in both species. For zein, AID of glycine was negative in pigs, meaning that the glycine that remained in the digesta was more than ingested, most likely owing to an effect of the endogenous losses.

The AID of whey in humans was 87% ± 3.9% overall AAs, whereas in pigs, the AID was 90% ± 2.8% for all AAs. For the AAs that could be analyzed for free AA content, adjusting for the free AA content in the AID calculation increased the AID significantly (Table 3). In human ileostomates, the AID of whey increased by 1.3%-units for the 13 AAs and for pigs by 1.3%-units. The largest effects were observed for glycine in both species.

TID

The overall TID of AAs in zein was 70% ± 16.4% in humans, whereas in pigs, the overall TID was 77% ± 20.6%. Adjusting the TID for free AA increased the TID of all AAs in humans (Table 4). However, for the pigs, TID of glycine and proline did not significantly increase if the free glycine and proline were assumed to be absorbed. In human ileostomates, the TID of zein increased by 2.3%-units for the 13 AAs if the free AAs would have been absorbed and for pigs by 3.5%-units. The largest difference in TID of zein was observed for threonine. If the free threonine would have been absorbed, this would increase threonine TID by 6.6%-units in both species. For both species, the

TABLE 2
Apparent ileal digestibility (AID) of zein, calculated with and without adjustment for free amino acids (AAs), in human ileostomates and cannulated growing pigs

	Human ($n = 7$)			Pig ($n = 6$)		
	AID (%)	AID adjusted for free AA (%)[1]	Difference (%-units)[2]	AID (%)	AID adjusted for free AA (%)[1]	Difference (%-units)[2]
Ala	66 ± 16.0	68 ± 15.2	2 ± 1.2*	77 ± 19.4	80 ± 16.3	3 ± 3.1*
Arg	61 ± 14.6	63 ± 13.6	3 ± 1.8*	49 ± 28.2	56 ± 22.1	7 ± 6.3*
Asp	63 ± 15.0	—	—	68 ± 18.0	—	—
Cys	47 ± 17.5	—	—	64 ± 13.1	—	—
Glu	67 ± 16.6	—	—	79 ± 18.2	—	—
Gly	33 ± 24.8	39 ± 23.0	6 ± 3.4*	−30 ± 68.6	−11 ± 52.1	19 ± 17.2*
His	56 ± 10.8	—	—	63 ± 13.7	—	—
Ile	62 ± 16.7	65 ± 15.6	3 ± 1.4*	73 ± 18.8	77 ± 15.1	4 ± 3.9*
Leu	66 ± 16.5	68 ± 15.6	2 ± 1.2*	79 ± 18.8	82 ± 15.3	3 ± 3.8*
Met	64 ± 15.2	65 ± 14.8	1 ± 0.6*	80 ± 15.8	81 ± 14.6	1 ± 1.3*
Phe	61 ± 17.6	63 ± 16.8	2 ± 1.2*	73 ± 19.3	75 ± 16.5	3 ± 2.9*
Pro	65 ± 15.4	67 ± 14.7	3 ± 1.1*	49 ± 40.9	54 ± 36.6	5 ± 5.3*
Ser	60 ± 19.1	62 ± 18.5	2 ± 0.9*	70 ± 20.9	72 ± 18.7	2 ± 2.3*
Thr	55 ± 17.6	63 ± 14.6	8 ± 4.4*	60 ± 17.0	68 ± 8.7	7 ± 8.6*
Tyr	63 ± 16.6	66 ± 15.7	3 ± 1.4*	77 ± 17.9	80 ± 15.1	3 ± 2.9*
Val	60 ± 16.0	63 ± 15.0	3 ± 1.4*	72 ± 17.9	76 ± 14.2	4 ± 3.8*

Values are given as mean ± SDs.
[1] It was assumed that free AAs are absorbable; thus, digestibility was adjusted to reflect the situation where the free AAs were completely absorbed before reaching the terminal ileum.
[2] Difference between digestibility values calculated with and without adjustment for free AAs using Wilcoxon signed-rank test: *$P < 0.05$.

proportion of free threonine was significantly higher in ileal digesta after ingestion of zein (Tables 5 and 6).

The TID of AAs in whey in humans was 97% ± 2.4% for all AAs, whereas in pigs, the TID was 97% ± 1.9% for all AAs. Adjusting the TID for free AAs increased the TID significantly for all measured AAs except for arginine, methionine, and tyrosine for humans and except arginine, glycine, phenylalanine, proline, and tyrosine for pigs (Table 7). In human ileostomates, the TID increased by 0.4%-units for the 13 AAs and for pigs by 0.1%-units.

TABLE 3
Apparent ileal digestibility (AID) of whey, calculated with and without adjustment for free amino acids (AAs), in human ileostomates and cannulated pigs

	Human ($n = 7$)				Pig ($n = 10$)			
	AID (%)	AID adjusted for free AAs (%)[1]	Difference (%-units)[2]	P^3	AID (%)	AID adjusted for free AAs (%)[1]	Difference (%-units)[2]	P^3
Ala	90 ± 2.8	92 ± 2.3	1 ± 0.9	0.018	91 ± 2.1	92 ± 1.6	1 ± 0.6	0.005
Arg	85 ± 4.9	86 ± 4.0	1 ± 1.6	0.027	84 ± 7.0	86 ± 5.6	2 ± 1.7	0.005
Asp	90 ± 3.2	—	—	—	93 ± 1.5	—	—	—
Cys	86 ± 4.4	—	—	—	94 ± 1.2	—	—	—
Glu	93 ± 2.3	—	—	—	94 ± 0.7	—	—	—
Gly	64 ± 11.7	68 ± 9.9	4 ± 2.7	0.018	62 ± 16.2	69 ± 13.9	7 ± 3.1	0.005
His	79 ± 5.5	—	—	—	89 ± 2.0	—	—	—
Ile	94 ± 1.7	95 ± 1.5	1 ± 0.3	0.017	96 ± 0.6	96 ± 0.6	0 ± 0.1	0.005
Leu	94 ± 1.8	95 ± 1.4	1 ± 0.5	0.016	96 ± 0.9	96 ± 0.9	0 ± 0.1	0.005
Lys	94 ± 1.9	95 ± 1.4	1 ± 0.7	0.018	96 ± 0.8	96 ± 0.9	0 ± 0.2	0.005
Met	94 ± 1.8	94 ± 1.7	0 ± 0.2	0.041	96 ± 1.5	96 ± 1.6	1 ± 0.2	0.005
Phe	80 ± 5.5	81 ± 5.1	1 ± 1.0	0.018	88 ± 2.7	88 ± 2.7	1 ± 0.1	0.005
Pro	87 ± 3.9	88 ± 3.4	1 ± 0.7	0.018	80 ± 22.7	82 ± 20.6	2 ± 2.3	0.005
Ser	84 ± 4.4	85 ± 4.1	1 ± 0.4	0.017	87 ± 2.1	88 ± 2.1	1 ± 0.1	0.005
Thr	85 ± 3.9	86 ± 3.6	1 ± 0.6	0.018	89 ± 2.5	90 ± 2.4	0 ± 0.2	0.005
Trp	84 ± 5.3	—	—	—	93 ± 1.6	—	—	—
Tyr	86 ± 4.6	87 ± 3.9	1 ± 1.1	0.018	91 ± 2.1	92 ± 2.2	1 ± 0.2	0.005
Val	89 ± 3.2	90 ± 2.8	1 ± 0.7	0.018	92 ± 1.1	93 ± 1.1	1 ± 0.1	0.005

Values are given as mean ± SDs.
[1] It was assumed that free AAs are absorbable; thus, digestibility was adjusted to reflect the situation where the free AAs were completely absorbed before reaching the terminal ileum.
[2] Difference between digestibility values calculated with and without adjustment for free AAs using Wilcoxon signed-rank test: *$P < 0.05$.

TABLE 4
True ileal digestibility (TID) of zein, calculated with and without adjustment for free amino acids (AAs), in human ileostomates and cannulated pigs

	Human ($n = 7$)			Pig ($n = 6$)		
	TID (%)	TID adjusted for free AAs (%)[1]	Difference (%-units)[2]	TID (%)	TID adjusted for free AAs (%)[1]	Difference (%-units)[2]
Ala	69 ± 16.0	71 ± 15.2	2 ± 1.0*	80 ± 19.5	82 ± 16.4	3 ± 3.2*
Arg	76 ± 14.8	78 ± 13.9	1 ± 1.1*	64 ± 26.6	69 ± 20.9	5 ± 6.0*
Asp	75 ± 14.8	—	—	77 ± 18.6	—	—
Cys	71 ± 17.0	—	—	75 ± 14.1	—	—
Glu	70 ± 16.7	—	—	81 ± 18.4	—	—
Gly	72 ± 23.4	74 ± 22.4	3 ± 2.0*	26 ± 66.6	34 ± 49.2	8 ± 17.8
His	79 ± 10.5	—	—	75 ± 15.7	—	—
Ile	69 ± 16.6	71 ± 15.5	2 ± 1.2*	77 ± 19.2	80 ± 15.4	3 ± 4.0*
Leu	69 ± 16.6	70 ± 15.6	2 ± 1.1*	81 ± 19.0	84 ± 15.4	3 ± 3.8*
Met	70 ± 15.0	70 ± 14.7	1 ± 0.6*	83 ± 16.5	84 ± 15.4	1 ± 1.2*
Phe	69 ± 17.7	71 ± 16.9	2 ± 1.0*	75 ± 19.4	78 ± 16.6	2 ± 2.9*
Pro	70 ± 15.2	72 ± 14.5	2 ± 0.8*	64 ± 33.9	67 ± 29.4	2 ± 5.5
Ser	68 ± 19.2	70 ± 18.6	1 ± 0.8*	74 ± 21.2	76 ± 18.9	2 ± 2.3*
Thr	72 ± 17.6	78 ± 15.1	7 ± 4.0*	70 ± 17.7	76 ± 9.5	7 ± 8.6*
Tyr	69 ± 16.6	71 ± 15.8	2 ± 1.1*	80 ± 18.1	82 ± 15.2	3 ± 2.9*
Val	71 ± 16.1	73 ± 15.1	2 ± 1.1*	78 ± 18.5	81 ± 14.7	3 ± 3.8*

Values are given as mean ± SDs.
[1] It was assumed that free AAs are absorbable; thus, digestibility was adjusted to reflect the situation where the free AAs were completely absorbed before reaching the terminal ileum.
[2] Difference between digestibility values calculated with and without adjustment for free AAs using Wilcoxon signed-rank test: *$P < 0.05$.

Discussion

This study aimed to investigate the free AA content of digesta from the terminal ileum of adult humans and growing pigs using a range of diets and to show the potential relevance of these free AAs for the nutritional value of food proteins. If the detected free AAs in the ileal digesta would had been absorbed, this would result in an increase in protein digestibility. Differences in TID, by considering the free AAs or not, seems to be rather irrelevant for proteins with a very high digestibility, as shown for whey protein isolate where only a minor effect was observed, despite similar proportion of free AAs relative to total AAs. However, for protein sources that are lower in digestibility, relevant differences in the TID values are expected, as shown for zein where TID values could be ≤6.6%-units higher. This observation provides a valuable perspective on the room for improvement of the nutritional value of a protein meal when all free AAs would have been absorbed, and future work is needed to investigate whether these findings can be extrapolated to other high-digestible and low-digestible dietary proteins.

Our experiments in both humans and pigs showed that the free AAs contributed to ~7%–12% to the total AAs losses at the end of the small intestine. These proportions are similar to the 5%–12% free AA nitrogen relative to total nitrogen reported previously in human ileostomates that ingested a very low nitrogen diet, soy bean diet, or high fiber diet [16]. Moreover, our free lysine proportion, which ranged from 5% to 12.6%, was close to the reported 13% of total lysine in ileal digesta of

TABLE 5
Proportion of free amino acids (AAs) relative to total AAs (%) in ileal digesta obtained from growing pigs with ileal T-cannula after ingestion of meals containing zein, whey, or a protein-free meal

Proportion AAs in free form (%)				*P*		
	Protein-free meal ($n = 12$)	Whey ($n = 10$)	Zein ($n = 6$)	PF vs. whey	PF vs. zein	Whey vs. zein
Ala	15 ± 6.3	14 ± 2.6	11 ± 3.5	1	0.196	0.496
Arg	12 ± 7.4	10 ± 5.9	12 ± 4.8	1	1	0.514
Gly	19 ± 9.9	18 ± 3.8	13 ± 5.1	0.574	0.999	0.131
Ile	8 ± 4.5	10 ± 2.8	12 ± 4.1	0.965	0.092	0.513
Leu	8 ± 4.0	9 ± 3.2	12 ± 4.8	0.723	0.200	1
Lys	5 ± 5.2	10 ± 5.3	11 ± 6.2	0.120	0.024	0.946
Met	5 ± 5.2	16 ± 9.4	5 ± 2.7	0.125	0.559	0.005
Phe	7 ± 4.7	5 ± 1.8	8 ± 3.6	0.820	1	0.326
Pro	20 ± 6.4	10 ± 5.8	9 ± 4.0	0.007	0.001	0.518
Ser	7 ± 2.2	5 ± 1.5	6 ± 2.7	0.112	1	0.124
Thr	8 ± 2.5	5 ± 1.7	15 ± 10.6	0.013	0.010	<0.001
Tyr	8 ± 3.9	10 ± 4.5	10 ± 3.0	0.817	0.408	1
Val	8 ± 3.6	8 ± 1.7	12 ± 4.0	1	0.016	0.038
Total AAs	13 ± 4.8	9 ± 1.7	10 ± 4.1	0.048	0.172	1
Total indispensable AAs	7 ± 3.7	7 ± 2.0	11 ± 4.8	1	0.126	0.132

Values are given as mean ± SDs. PF = protein free.

TABLE 6
Proportion of free amino acids (AAs) relative to total AAs (%) in ileal digesta obtained from human ileostomates after ingestion of meals containing zein, whey, or a protein-free meal

Proportion AAs in free form (%)				*P*		
	Protein-free meal ($n = 7$)	Whey ($n = 7$)	Zein ($n = 7$)	PF vs. whey	PF vs. zein	Whey vs. zein
Ala	11 ± 6.1	12 ± 5.9	8 ± 2.9	1	0.050	0.016
Arg	7 ± 4.8	8 ± 6.7	6 ± 3.6	1	1	1
Gly	8 ± 3.4	11 ± 4.8	9 ± 3.7	0.239	1	0.099
Ile	7 ± 3.9	9 ± 3.5	7 ± 2.4	0.082	1	0.232
Leu	7 ± 4.5	10 ± 5.5	6 ± 2.3	1	0.024	0.005
Lys	8 ± 5.7	13 ± 6.1	12 ± 5.4	0.033	0.083	1
Met	1 ± 0.8	2 ± 2.2	3 ± 1.5	0.39	0.069	0.759
Phe	3 ± 2.3	5 ± 3.8	6 ± 2.3	0.024	<0.001	0.180
Pro	11 ± 4.9	10 ± 3.2	8 ± 2.2	1	0.026	0.084
Ser	4 ± 2.2	5 ± 1.7	4 ± 1.6	0.250	1	0.914
Thr	7 ± 3.9	6 ± 2.9	17 ± 7.0	0.702	<0.001	<0.001
Tyr	7 ± 3.4	10 ± 4.5	7 ± 2.7	0.104	1	0.121
Val	8 ± 4.6	9 ± 3.9	8 ± 2.9	0.221	1	0.566
Total AAs	7 ± 3.8	9 ± 4.0	7 ± 2.7	0.467	1	0.395
Total indispensable AAs	7 ± 4.0	8 ± 4.1	7 ± 2.9	0.240	0.614	1

Values are given as mean ± SDs. PF = protein free

growing pigs on a protein-free or protein-containing diets [4]. As only few studies report free AA contents of ileal digesta, we also compared our results with those of studies that reported nitrogen content with different molecular sizes. Approximately 25%–46% of ileal nitrogen in pigs was present in peptides smaller than 1 kDa [18] and ~20% of soluble proteinaceous material in ileal digesta of pigs fed with a protein-free diet was smaller than 1 kDa [19]. Combining this information with the fact that ~26% of small, sulfosalicylic acid soluble, nitrogen was in the form of free AAs in aspirated ileal digesta of humans [20] would give an estimated free AA content of 5%–12% of total nitrogen. These values from literature and our own data are higher than the 2% free AAs in pigs fed with a protein-free diet that was reported by Moughan and Schuttert [21]. The reason for the discrepancy in the results may lie in methodological differences. Hulshof et al. [4] discussed that the thawing process for mixing and subsampling may have caused spontaneous hydrolysis or the utilization of sulfosalicylic acid may partially hydrolyze peptides [4]. Moreover, differences in digesta collection interval may have resulted in proteolytic activity after sampling. Although, in our human study, this interval was 2–3 h compared with the 30-min interval in pigs, similar proportions of free AAs were observed.

TABLE 7
True ileal digestibility (TID) of whey, calculated with and without adjustment for free amino acids (AAs), in human ileostomates and cannulated pigs

	Human ($n = 7$)			Pig ($n = 10$)		
	TID (%)	TID adjusted for free AAs (%)[1]	Difference (%-units)[2]	TID (%)	TID adjusted for free AAs (%)[1]	Difference (%-units)[2]
Ala	97 ± 2.3	97 ± 2.1	0 ± 0.3*	95 ± 1.8	96 ± 1.6	1 ± 0.5*
Arg	99 ± 3.9	99 ± 3.8	0 ± 0.5	95 ± 7.1	95 ± 6.0	0 ± 1.7
Asp	97 ± 2.1	—	—	97 ± 1.8	—	—
Cys	97 ± 3.3	—	—	98 ± 1.4	—	—
Glu	97 ± 1.8	—	—	97 ± 0.7	—	—
Gly	92 ± 9.7	94 ± 8.7	2 ± 1.9*	94 ± 17.7	94 ± 13.8	0 ± 7.6
His	96 ± 4.4	—	—	98 ± 2.7	—	—
Ile	98 ± 1.4	99 ± 1.3	0 ± 0.1*	98 ± 0.7	98 ± 0.6	0 ± 0.2*
Leu	99 ± 1.5	99 ± 1.4	0 ± 0.2*	98 ± 1.0	99 ± 1.0	0 ± 0.2*
Lys	98 ± 1.4	99 ± 1.2	0 ± 0.3*	98 ± 0.9	98 ± 0.9	0 ± 0.2*
Met	99 ± 2.1	99 ± 2.1	0 ± 0.1`	98 ± 1.6	99 ± 1.8	0 ± 0.2*
Phe	97 ± 6.2	97 ± 6.1	0 ± 0.5*	94 ± 2.7	94 ± 2.9	0 ± 0.4
Pro	95 ± 2.7	95 ± 2.5	0 ± 0.4*	97 ± 21.1	96 ± 19.9	−1 ± 1.9`
Ser	93 ± 3.8	94 ± 3.7	0 ± 0.2*	92 ± 2.1	92 ± 2.1	0 ± 0.2*
Thr	92 ± 3.3	92 ± 3.3	0 ± 0.2*	93 ± 2.3	93 ± 2.3	0 ± 0.2*
Trp	98 ± 3.9	—	—	98 ± 1.4	—	—
Tyr	98 ± 3.6	99 ± 3.4	0 ± 0.5`	98 ± 2.4	98 ± 2.4	0 ± 0.4`
Val	96 ± 2.4	96 ± 2.2	0 ± 0.2*	96 ± 1.1	96 ± 1.0	0 ± 0.2*

Values are given as mean ± SDs.
[1] It was assumed that free AAs are absorbable; thus, digestibility was adjusted to reflect the situation where the free AAs were completely absorbed before reaching the terminal ileum.
[2] Difference between digestibility values calculated with and without adjustment for free AAs using Wilcoxon signed-rank test: *$P < 0.05$; `$P < 0.1$.

The question remains: why are these free AAs not taken up by the small intestine and why are there differences between AAs? One hypothesis is that the rate of absorption by their AA transporters is a limiting factor because the rate of AA absorption is known to differ between AAs. When an equimolar mixture of 8 AAs was perfused in the human intestine, methionine showed the highest absorption rate in two studies, whereas other AAs such as threonine showed a lower absorption rate [22,23]. This fits our finding that free methionine concentrations in ileal digesta were very low or even undetectable. In addition, the absorption rate is influenced by the presence of other AAs: for example, threonine absorption showed a higher absorption rate when perfused as a single AAs than its perfusion in an AA mixture [22]. Finally, the absorption rates vary per intestinal segment. For example, the absorption rate of leucine and threonine was higher in the jejunum than that in the ileum [24]. A slow digestion rate of proteins in the small intestine may result in more free AAs becoming available in distal parts of the small intestine, having lower absorption rates and limited time until the end of the small intestine is reached. Finally, mixing may be a critical step before absorption, although to the best of our knowledge, these effects have not been studied for AA absorption.

With our study, we could not differentiate between the origins of free AAs; is it dietary or endogenous protein and is its quantity dependent on the type of meal ingested? For both species, the proportion of free threonine after ingestion of zein was ~2–3 times higher than that after ingestion of whey-containing and protein-free meal. The TID of threonine in zein is also greatly affected when including threonine in free form or not, whereas that of whey was hardly influenced. Although threonine content was ~3× lower in zein than that in whey, whey is virtually fully digested; therefore, its digesta is expected to be very similar to that for a protein-free diet. Another observation was that the concentration of free lysine after zein ingestion was higher than that with a protein-free meal. These two observations may indicate that zein increases endogenous protein losses compared with the protein-free meal, because of the following: *1*) zein contains very low concentrations of the essential AA lysine; therefore, after ingestion of zein, values similar to those on a protein-free diet were expected. However, the proportion of free lysine and the free lysine ileal flow were remarkably higher after ingestion of zein than that after ingestion of a protein-free diet, especially for pigs. *2*) Endogenous proteins are characterized by high threonine concentrations. In humans, threonine was reported to be the highest contributor to indispensable AA losses after a protein-free diet [25,26], although in our own data, phenylalanine was slightly greater. Moreover, in pigs, threonine had the highest concentration of all indispensable AAs in basal endogenous losses estimated based on different methods from 33 ileal digestibility studies [27], although in our data, leucine losses were slightly greater. The composition of endogenous losses can be affected by the contribution of individual sources of endogenous protein, and mucus loss is a significant contributor to endogenous threonine in ileal digesta [28]. More importantly, little re-absorption of endogenous threonine from mucins was shown in piglets using isotope techniques [29]. Altogether, we speculate that zein increases mucus release and that the AAs from digested mucus are not fully absorbed. This may contribute to the observed large effect when adjusting for free threonine in the TID calculation of zein. A potentially increased mucus release could be related to the poor solubility and gelation potential of zein [30]. Nevertheless, our study was not designed to distinguish between basal endogenous losses and diet-specific losses by zein.

The differences in TID values with or without free AAs also indicate the potential deviation from the in vivo situation compared with in vitro assays for digestibility because many of these assays determine digestibility, or bioaccessibility, with the assumption that all protein that is digested would also be absorbed. These in vitro assays often use degree of hydrolysis assessed with the *o*-phthalaldehyde method [31] or a size-excluding membrane [35] for their measurements. When doing this, one would expect values more similar to our TID calculated when adjusting for free AAs. Altogether, the presence of free AA at the end of the small intestine, possibly by incomplete absorption, are ignored with in vitro assays, and therefore, these lead to a potential overestimation of digestibility or DIAAS values in vivo. Apart from the free AA, it should be mentioned that most AAs are absorbed as dipeptides or tripeptides. In this study, these were not analyzed; however, it is possible that these small peptides are present in the ileal digesta, especially as a considerable amount of peptides <1 kDa were reported to be present in pig ileal digesta [16,19]. These peptides would contribute to an incomplete absorption efficiency in the small intestine and a potential discrepancy between in vivo and in vitro determined digestibility coefficients.

We compared our TID values for zein and whey protein isolate to literature. For whey protein isolate, we observed TID values >95% for many AAs similar to human ileostomates in a previous study [6] but higher values for histidine and lysine and lower values for glycine. We report slightly higher, digestibility coefficients in our human ileostomates compared with the results obtained when using naso-ileal intubation [25,36]. In addition, the digestibility coefficients obtained in pigs fed whey protein hydrolysate were generally very similar (>95%) to those reported in pigs [10]. In the human ileostomates, the TID of zein was slightly higher than the 63% reported by Calvez et al. [25], although rather high variation in the digestibility coefficients was observed in both studies. The observed TIDs in pigs were higher than those reported by Mathai et al. [37] for all AAs except threonine, which was equal, and arginine and proline, which showed lower digestibility coefficients in our study. Altogether, the digestibility coefficients in this study were very similar to those reported in literature.

Overall, we conclude that free AAs are present at the end of the small intestine in meaningful amounts and can have a nutritionally relevant effect for poorly digestible protein sources, for example, zein, although the effect is negligible for highly digestible protein sources, such as whey. We hypothesize that this may be related to specific endogenous losses. Our observed differences in AID and TID could provide a valuable perspective on the room for improvement of the nutritional value of a protein meal if all free AAs were to be absorbed. However, to reduce the quantity of free AAs that is lost to the large intestine, first, the underlying mechanism needs to be understood. Apart from this, our observations may have implications for in vitro estimations of in vivo protein digestibility for which total absorption of all free AAs in the small intestine is generally assumed.

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Author disclosures

The authors report no conflicts of interest.

Data Availability

Data described in the manuscript and analytic code will be made available on request pending application and approval.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.01.038.

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