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Nutrient Requirements and Optimal Nutrition

Ileal Digestibility of Nitrogen and Amino Acids in Human Milk and an Infant Formula as Determined in Neonatal Minipiglets

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A B S T R A C T

Background: Infant formula (IF) has to provide at least the same amount of amino acids (AAs) as human milk (HM). AA digestibility in HM and IF was not studied extensively, with no data available for tryptophan digestibility.
Objectives: The present study aimed to measure the true ileal digestibility (TID) of total nitrogen and AAs in HM and IF to estimate AA bioavailability using Yucatan mini-piglets as an infant model.
Methods: Twenty-four 19-day-old piglets (males and females) received either HM or IF for 6 days or a protein-free diet for 3 days, with cobalt-EDTA as an indigestible marker. Diets were fed hourly over 6 h before euthanasia and digesta collection. Total N, AA, and marker contents in diets and digesta were measured to determine the TID. Unidimensional statistical analyses were conducted.
Results: Dietary N content was not different between HM and IF, while true protein was lower in HM (−4 g/L) due to a 7-fold higher non-protein N content in HM. The TID of total N was lower ($P < 0.001$) for HM (91.3 ± 1.24%) than for IF (98.0 ± 0.810%), while the TID of amino acid nitrogen (AAN) was not different (average of 97.4 ± 0.655%, $P = 0.272$). HM and IF had similar ($P > 0.05$) TID for most of the AAs including tryptophan (96.7 ± 0.950%, $P = 0.079$), except for some AAs (lysine, phenylalanine, threonine, valine, alanine, proline, and serine), with small significant difference ($P < 0.05$). The first limiting AA was the aromatic AAs, and the digestible indispensable AA score (DIAAS) was higher for HM ($DIAAS_{HM} = 101$) than for IF ($DIAAS_{IF} = 83$).
Conclusion: HM, compared to IF, had a lower TID for total N only, whereas the TID of AAN and most AAs, including Trp, was high and similar. A larger proportion of non-protein N is transferred to the microbiota with HM, which is of physiological relevance, although this fraction is poorly considered for IF manufacturing.

Keywords: human milk, infant nutrition, bioavailability, multiple hydrolysis, true digestibility, digestible indispensable AA score, tryptophan

Introduction

The true ileal digestibility (TID) of amino acids (AAs) in a dietary protein is recognized as a key determinant of its nutritional quality [1,2], and unlike for fecal AA digestibility, the residual dietary AA profile is not modified by colonic microbial fermentation [1,3,4]. The TID of protein and AAs has been studied in vivo in human milk (HM) and infant formula (IF) in a very limited number of studies [5,6], with no information available regarding tryptophan digestibility, as highlighted by a FAO expert consultation [2]. Such information, in addition to essential AA composition, is crucial to better characterize the nutritional quality of dietary proteins in IF as compared to HM [5]. Whereas IFs are formulated to mimic the HM aminogram, their protein profile differs notably in terms of protein abundance and nature [7,8]. HM has a high content of α-lactalbumin (0.25 g/100 mL of mature HM) and lactoferrin (0.15 g/100 mL of mature HM), which are proteins that have been reported as partially resistant to gastrointestinal digestion, while standard IF such as the one used in the present study is rich in β-lactoglobulin

Abbreviations: AA, Amino acid; DIAAS, Digestible indispensable amino acid score; HM, Human milk; HMO, Human milk oligosaccharide; IF, Infant formula; NPN, Non-protein nitrogen; PF, Protein-free; TID, True ileal digestibility.

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(0.29 g/100 mL of IF), a missing protein in HM [9–11]. Moreover, due to the heat treatment during IF manufacturing coupled with a high lactose content, IF proteins are partially denatured and aggregated, such as those reported for the present IF (58% of whey protein denaturation extent [12]), and can be engaged in Maillard reaction products (11 mg N^{ε}-carboxymethyl lysine/100 g of crude proteins in the present IF powder [12]) unlike that for HM proteins [13]. Such differences may impact the AA bioavailability of IF, particularly that of lysine [14,15].

The latest FAO protein quality index is the Digestible Indispensable Amino Acid Score (DIAAS) [2], which takes into account AA composition and the TID of each AA, unlike in the previous protein quality index (protein digestibility–corrected AA score), which was based on fecal N digestibility [2]. To determine the DIAAS of milk proteins, an accurate evaluation of the AA content in the dietary protein and ileal digesta is required. As AAs (notably serine, threonine, tryptophan, tyrosine, and valine) are not completely stable under the hydrolysis conditions required to determine their content in the protein source [4,16,17], multiple hydrolysis of the samples can be performed to evaluate the rate at which AAs are released from a protein and further degraded during hydrolysis, allowing for an overall estimation of the losses occurring during hydrolysis [4,5,17]. This procedure provides more accurate compositional data than a standard 24-h hydrolysis.

Our hypothesis was that due to different protein nature and structure in HM and IF, the TID of nitrogen (N) and AA may differ between these diets. Thus, the purpose of the present study was to accurately determine the AA composition of HM, a bovine milk–based IF, and TID of total N and all AAs in both diets. This was done after correction for AA losses occurring during the chemical hydrolysis of the samples using a least-squares nonlinear regression model applied to data obtained after multiple hydrolysis times. The Yucatan minipiglet model was used to determine the N- and AA-TID, and was assumed to be a valid model of infant digestive physiology [18–20]. The endogenous basal losses were determined using a protein-free (PF) group such as that previously reported in human nutrition (FAO 2014), allowing the TID calculation from the apparent digestibility.

Methods

Human milk collection

HM collection was approved by the Institutional Review Board of South Mediterranean V (19.12.12.65653) and was carried out in 2 stages. First, 22 frozen milk samples (range of lactation period: 0.3–5.6 months post-delivery) were donated by the milk bank of the Rennes University Hospital Centre. These samples were heat-treated using a Holder pasteurizer (62.5 °C, 30 min), pooled and stored at −20 °C until distribution to the piglets as described [21]. Second, fresh HM samples were collected from healthy volunteers (n = 15–20 mothers within each experimental block, 0.8–2.7 months post-delivery) in a sterile infant bottle, pooled in sterile bottles, and stored at 4 °C until distribution to the piglets the day after the collection.

Animal design

The present protocol was approved by the ethics committee of CREEA (Rennes Committee of Ethics in Animal Experimentation) and that of the French Ministry of Higher Education and Research (approval number: 2020020610329770). Procedures were designed and conducted in agreement with the current ethical standards of the European and French guidelines. Animals were observed daily and weighed every 3 days throughout the study to monitor their welfare. No medication or antibiotic treatments were delivered.

The protocol was detailed in Charton et al. [21]. Briefly, eighteen 10 ± 1 day-old Yucatan piglets (10 females and 8 males) were randomly assigned to an experimental diet (HM or IF) according to their gender, litter, and body weight after an adaptation period of 8 ± 2 days (adaptation diet based on a full fat bovine milk powder enriched with vitamins and minerals). This experiment was conducted over 3 independent blocks ($n_{block\ 1} = 8$, $n_{block\ 2} = 6$, $n_{block\ 3} = 4$). The HM group was fed the pasteurized HM pool during the first 5 days and the fresh HM pool on the last day of the experiment (n = 9), while the IF group was fed a standard bovine milk–based IF (IF powder [12] rehydrated with ultrapure water to 115 g DM/L, n = 9) for 6 days. In addition, an independent block was conducted with six 10 day-old Yucatan piglets (3 females and 3 males), which were bottle-fed with rubber teats a PF liquid diet for 3 days after an adaptation period. The PF piglets were individually housed under identical conditions to those of the IF- and HM-fed piglets. The PF diet was produced within our laboratory after rehydration of dry ingredients and mixing with an oil mix as described previously [5] (**Supplemental Table**) using an ultra-thurax homogenizer, and finally homogenized using a two-stage lab-scale homogenizer (200 bar, 75 bar). The liquid was stored at 4 °C between formulation and piglet feeding.

The experimental diets (HM, IF, and PF) were supplemented with undigestible and unabsorbable dietary markers (ytterbium trichloride and cobalt-EDTA) at a level of 0.3% of dry matter (wt/wt). Only cobalt-EDTA was used for the determination of the AA and N flows due to inconsistent data observed with ytterbium. All the diets were supplemented with liquid vanilla (3.00 g/L of diet) to encourage food intake. Over the entire experimental period, the HM- and IF-fed piglets had similar food intakes (average of 255 ± 7.73 g·kg $BW^{-1}\cdot d^{-1}$) and similar weight gain (53.6 ± 5.00 g/day), such as those reported previously [21].

Sample collection

On the last experimental day, the piglets were fed liquid diets hourly over 6 times before being euthanized 30 min after the last meal by electrical stunning immediately followed by exsanguination. Gastrointestinal contents were collected along the digestive tract [stomach, proximal (first 2.5 m) and median jejunum (2.36 ± 0.117 m), ileum (60 cm before ileocecal junction), caecum and proximal colon (first-third of colon)] and mixed with 50 µL of protease inhibitor/mL of digesta [Pepstatin A (P5318, Sigma Aldrich) solution at 0.5 mg/mL of methanol for gastric digesta or Pefabloc (76307, Sigma Aldrich) at 0.1 mmol/mL of water for the intestinal digesta]. Digesta and diets were homogenized using an ultra-thurax homogenizer and stored at −20 °C until being freeze-dried. Freeze-dried samples (gut contents and diet samples) were then stored at room temperature in a desiccator under vacuum until analysis.

Sample characterization

Unless otherwise mentioned, analyses were made on freeze-dried samples.

Nitrogen content

Non-protein N (NPN) was determined in a pool of 3 representable liquid samples of fresh HM diet and in 1 representative liquid sample of IF. NPN was determined using the micro–Kjeldahl method (Kjeltec 8400 Foss system) after protein precipitation by trichloroacetic acid (12% wt/vol) and filtration on N-free Whatman Filter Paper (grade 40). The total N was measured on the same pooled samples using the micro–Kjeldahl method to allow for the determination of the proportion of NPN (g/100 g of total N) in HM and IF further used for true protein determination. Analyses were made in duplicates.

The total N content of chyme, digesta, and diet sample was measured using the Dumas method as described previously [21]. A N-to-protein factor conversion of 6.38 was used for the crude protein content in the diets [22]. The true protein content of HM and IF was calculated by subtracting the NPN from the total N and multiplying by 6.38.

Amino acid analysis

The diets and some digesta samples were subjected to acid or alkaline hydrolysis. Tryptophan was analyzed in each diet and proximal jejunum, ileal, and colonic digestas after 16 h of basic hydrolysis following basic hydrolysis as detailed elsewhere [21]. Due to the limited amount of ileal digesta collected, sulfur AAs (cysteine and methionine) were quantified only in diets after performic acid oxidation, followed by acid hydrolysis. Other AAs were analyzed in each diet and ileal digesta after acid hydrolysis. Acid hydrolysis was performed by mixing precisely weighed samples with 1 mL of 6 N hydrochloric acid in screw-cap glass tubes sealed under nitrogen and hydrolyzed at 110 °C for 24 h. Afterward, a 5 mM Norleucine solution was used as an internal standard and added to each cooled tube just after the hydrolysis step. Tubes were dried using an acid resistant under vacuum drier (Genevac EZ-2plus, Genevac Ltd) prior to rehydration with lithium buffer (pH 2.2), filtration on 0.45 μm, and injection on a Biochrom 30 + AA analyzer (Biochrom Ltd) with ninhydrin as a postcolumn reaction system (cation exchange chromatography). Analyses were conducted in duplicates.

AA recovery after hydrolysis

Multiple hydrolysis times (2, 5, 7, 16, 20, and 30 h for tryptophan and 2, 6, 16, 19, 24, 56, or 120 h for the other AAs) were performed as described above in the representative samples of diets (fresh HM and IF) and IF-fed piglet digesta (median jejunum: pool of 3 median jejunal digesta; ileum: pool of 4 ileal digesta; colon: 1 colonic digesta) samples in order to determine the rate of liberation (*h*, proportion of the amount of AA remaining in protein form) and destruction (*l*, proportion of the amount of AA in free form during hydrolysis) of each AA during hydrolysis [5,6,17]. These parameters and the protein bound (A_0) of each matrix were estimated using least-squares nonlinear regression (Excel solver function) and the compartmental model (equation 1) [5,6]. *B(t)* corresponds to the AA content released at the time *t*. B_0 corresponds to the free AA content prior to hydrolysis and was determined in the samples of diets and intestinal digesta analyzed using multiple hydrolysis. Free AA was determined after protein precipitation (sulfosalicylic acid solution including Norleucine) at 0 °C for 1 h followed by centrifugation (10000 *g*, 5 min) and supernatant filtration on a 0.45 μm membrane using the Biochrom 30+ analyzer with ninhydrin as the postcolumn reaction system.

$$B(t) = \frac{A_0 h(e^{-lt} - e^{-ht})}{h - l} + B_0(e^{-lt}) \quad (1)$$

Corrected AA content of the samples

The determined AA content at 16 h of hydrolysis for tryptophan and that at 24 h for the other AAs along with the parameters (*h* and *l*) determined using multiple hydrolysis on the diets or IF digesta were used to calculate the A_0 for each AA in each diet and digesta according to the following equation (equation 2):

$$A_0 = \frac{[B_{(t)} - B_0(e^{-lt})] \times (h - l)}{h(e^{-lt} - e^{-ht})} \quad (2)$$

The final AA content of each diet and digesta sample was then calculated by summing A_0 and B_0 values. These values were considered as the HM, IF, or digesta AA contents [5]. Additionally, the dietary tyrosine content was determined in duplicate in a representative sample of HM and IF after hydrolysis by 6 N hydrochloric acid containing 0.1% phenol for 24 h at 110 °C, and then analyzed as described above in the "AA analysis" part. Total ammonia was determined using the AA analysis after 24-h acid hydrolysis. Free urea and ammonia content were determined altogether with the free AA analysis.

Indigestible marker

The cobalt content was measured in diet and digesta samples using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on an ICAP-TQ from Thermo Scientific equipped with collision cell technology (Platform AEM2, University of Rennes 1/Biochemistry Laboratory, University Rennes Hospital). Samples were precisely weighed and mineralized by ultrapure concentrated nitric acid (69%, Fisher Chemical, Optima Grade) in Teflon PFA–lined digestion vessels. Mineralization was carried out at 180 °C in a microwave oven device (Mars 6, CEM). The remaining volume was centrifuged at 3488 *g* for 10 min at room temperature. Supernatants were diluted at 1:100 in ultrapure water before filtration on a 0.2 μm PES membrane. The source of plasma was argon (Messer) with a high degree of purity (>99.99%). The collision/reaction cell used was pressurized with helium (Messer). Rhodium (Fisher Scientific) was used as an internal standard. The calibration range preparation was carried out using a multielement calibrator solution (SCP Science Plasma Cal).

Total N and AA digestibility calculation

Total N and tryptophan digestibility values were determined in proximal jejunal, ileal, and colonic digestas. The digestibility of the other AAs was determined in terminal ileal digesta only. Total N (including NPN) and AA flows were determined using equation 3, while the endogenous total N or AA flows were

calculated using equation 4 (units are in g/100 g of fresh dry matter):

$$Total\ N\ or\ AA\ flow = [Total\ N]_{digesta}\ or\ [AA]_{digesta} \times \frac{[Cobalt]_{diet}}{[Cobalt]_{digesta}} \quad (3)$$

$$Endogenous\ total\ N\ or\ AA\ flow = [Total\ N]_{PF\ digesta}\ or\ [AA]_{PF\ digesta} \times \frac{[Cobalt]_{PF\ diet}}{[Cobalt]_{PF\ digesta}} \quad (4)$$

The apparent and true digestibilities were then calculated using equations 5 and 6, respectively, as described White by the FAO Expert Working Group (2014) [23]:

$$Total\ N\ or\ AA\ apparent\ digestibility\ (\%) = 100\ \times \frac{([Total\ N]_{diet}\ or\ [AA]_{diet} - Total\ N\ or\ AA\ flow)}{[Total\ N]_{diet}\ or\ [AA]_{diet}} \quad (5)$$

$$Total\ N\ or\ AA\ true\ digestibility\ (\%) = 100 \times \frac{[Total\ N]_{diet}\ or\ [AA]_{diet} - (Total\ N\ or\ AA\ flow - Endogenous\ Total\ N\ or\ AA\ flow)}{[Total\ N]_{diet}\ or\ [AA]_{diet}} \quad (6)$$

DIAAS

DIAAS was calculated according to the FAO protocol [2] using the published pattern of HM as the reference protein for infants (0–6 months) and using the following equation (equation 7):

$$DIAAS\ (\%) = Min\left(\frac{mg\ indispensable\ AA\ per\ g\ of\ true\ dietary\ protein \times \%\ True\ digestibility\ of\ the\ AA}{mg\ of\ the\ AA\ per\ g\ of\ reference\ protein}\right) \times 100 \quad (7)$$

Statistical analysis

All statistical analyses were performed using the R software, version 3.6.2 [24]. A linear model was used to test the significance of diet and block on the dietary nutritional composition of HM and IF. Prior to performing the statistical analysis on digestibility data, samples from 2 HM-fed piglets were considered as outliers according to the Grubbs test ("OUTLIERS" package Komsta, 2011) and were removed from the dataset. The final dataset included data for 6 HM- and 9 IF-fed piglets. A linear model was used to test the diet, block, and gender effects on the percentage of endogenous N to total N and apparent and true digestibility of total N and tryptophan for each intestinal site. A similar model was used for analyzing the diet, block, and gender effects on the ileal digestibility of other AAs studied. When block or gender effects were non-significant ($P > 0.1$), these effects were removed from the linear model. Models were accepted when the normality (Shapiro–Wilk test) and homoscedasticity (Levene's tests) of the residuals were non-significant ($P > 0.01$). When one of the previous conditions was not fulfilled, linear models were applied using transformed data (natural logarithmic transformation). Even though normality and homoscedasticity were still not verified, the diet effect was assessed using a non-parametric test (Wilcoxon's test). The effect was considered as statistically significant for $P \leq 0.05$.

Unless otherwise mentioned, the results are expressed as mean ± SEM.

Results

Diet composition

The crude protein content was not different between fresh HM and IF ($P = 0.403$, Table 1). The NPN content was 7-fold

TABLE 1
Crude and true protein, non-protein nitrogen, amino acids nitrogen, and lipid content in fresh human milk (n = 3) and infant formula (n = 3).

g/L diet	HM	IF	*P*-value
Freeze-dried matter	12.8 ± 0.397	11.7 ± 0.178	0.073
Crude protein[1]	13.7 ± 0.694	14.4 ± 0.231	0.403
Non-protein N	4.22 ± 0.00501	0.631 ± 0.000398	<0.001
True protein[2]	9.45 ± 0.480	13.7 ± 0.221	0.001
Amino acid N[3]	1.40 ± 0.0788	1.77 ± 0.00472	0.009
Lipid	27.9 ±0.370	31.5 ± 0.629	0.008

Data are presented as mean ± SEM.
Abbreviations: HM, fresh human milk; IF, infant formula.
[1] Crude protein = total nitrogen × 6.38.
[2] True protein = (total nitrogen − non-protein nitrogen) × 6.38.
[3] Amino acid nitrogen calculated by summing the AA amounts.

higher in HM, resulting in a lower true protein and amino acid nitrogen (AAN) content in HM ($P = 0.001$ and $P = 0.009$, respectively). The lipid content also differed between diets with a slightly lower concentration in HM.

AA content in HM and IF diets and in intestinal digesta

The AA concentrations in HM and IF were determined after applying correction factors derived from the multiple hydrolyses method [5,17]. This method allowed for the determination of instantaneous loss (*l*) and hydrolysis rates (*h*) using the least-square non-linear regression model. Three distinct types of profiles were observed (Figure). First, 3 AAs (isoleucine, methionine, and valine), except for valine in the ileal digesta, were released slowly from their matrix and only slightly degraded during hydrolysis, with 24 h as an optimal hydrolysis time (mean *h* rate: 0.206 ± 0.016; mean *l* rate: 0.0000; Table 2). Second, most AAs (histidine, leucine, lysine, phenylalanine, alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, and proline) were completely released from their matrix before the standard hydrolysis time of 24 h (on average at 13 ± 1 h) and they remained relatively stable across longer hydrolysis times (mean *h* rate: 0.541 ± 0.031; mean *l* rate: 0.0000; Table 2). Finally, some AAs (threonine, tryptophan, serine, tyrosine, and valine in ileal digesta only) were rapidly released from their matrix but also rapidly degraded (mean *h* rate: 0.607 ± 0.060; mean *l* rate: 0.0032 ± 0.0006; Table 2). This was particularly true for tyrosine in the diets and for valine in the ileal digesta, where a shorter hydrolysis time of 6 h would result in the maximum value. Thus, correction based on multiple hydrolysis correction factors resulted in the estimated tyrosine value being 13% higher than the measured value at 24 h for diets, whereas for valine, a decrease of 12% was observed on average for ileal digesta. For serine, the impact on the measured value was 4%. For tryptophan, the lower hydrolysis time (16 h) limited the degradation of this AA. For the remaining AAs, the correction was 1.3% ± 0.2%.

The predicted (multiple hydrolyses model) concentration of each AA, expressed in milligrams of AA per liter of diet, was lower in HM than in the IF ($P \leq 0.05$, Table 3), except for arginine, cysteine, glycine, proline, and tyrosine, which were not different ($P > 0.05$). Regarding the AA profile (AA content expressed per 100 g of true protein), most of the AA contents were higher ($P \leq 0.05$) in HM than in IF except for lysine, which was less concentrated in HM. For methionine, alanine, aspartic

TABLE 2
Loss rate (*l*, proportion of the amount of AA in free form during hydrolysis) and hydrolysis rate (*h*, proportion of the amount of AA remaining in protein form) measured using multiple hydrolysis times and application of a non-linear regression model for freeze-dried human milk and infant formula powder rehydrated with ultrapure water to 115 g DM/L and freeze-dried ileal digesta (IF).

Amino acid	*l*, Loss rate			*h*, Hydrolysis rate		
	HM	IF	Ileal digesta	HM	IF	Ileal digesta
Essential AA						
Histidine	0.0000	0.0000	0.0002	0.391	0.348	0.407
Isoleucine	0.0000	0.0000	0.0000	0.229	0.181	0.209
Leucine	0.0000	0.0000	0.0000	0.432	0.444	0.385
Lysine	0.0000	0.0000	0.0000	0.425	0.400	0.572
Methionine[1]	0.0000	0.0000	—	0.284	0.192	—
Phenylalanine	0.0000	0.0000	0.0000	0.508	0.349	0.392
Threonine	0.0010	0.0015	0.0014	0.324	0.325	0.427
Tryptophan	0.0022	0.0029	0.0034	0.596	0.535	0.549
Valine	0.0000	0.0000	0.0057	0.196	0.153	0.826
Non-essential AA						
Alanine	0.0000	0.0000	0.0001	0.491	0.479	0.759
Arginine	0.0007	0.0009	0.0004	0.490	0.343	0.425
Aspartic acid[2]	0.0000	0.0000	0.0003	0.694	0.600	0.715
Cysteine[3]	0.0001	0.0004	—	0.987	0.925	—
Glutamic acid[4]	0.0000	0.0000	0.0000	0.519	0.500	0.501
Glycine	0.0000	0.0000	0.0000	0.772	0.555	0.933
Proline	0.0000	0.0000	0.0000	0.562	0.433	0.579
Serine	0.0023	0.0027	0.0031	0.596	0.500	0.722
Tyrosine	0.0070	0.0067	0.0011	1.07	0.873	0.550

Abbreviations: AA, amino acid; HM, fresh human milk; IF, infant formula powder rehydrated with ultrapure water to 115 g DM/L.
[1] Detected as methionine sulfone.
[2] Asparagine + aspartate.
[3] Detected as cysteic acid.
[4] Glutamate + glutamine.

TABLE 3
Amino acid concentration[1] of fresh human milk (n = 3) and infant formula powder rehydrated with ultrapure water to 115 g DM/ L diet (n = 3).

	(mg/L diet)			(g/100 g true protein[2])		
	HM	IF	*P* value	HM	IF	*P* value
Essential amino acids						
Histidine	264 ± 12.5	305 ± 5.25	0.041	2.80 ± 0.0308	2.22 ± 0.00280	<0.001
Isoleucine	620 ± 32.3	767 ± 8.36	0.012	6.57 ± 0.0246	5.59 ± 0.149	0.003
Leucine	1140 ± 58.3	1546 ± 7.85	0.002	12.1 ± 0.0626	11.3 ± 0.230	0.029
Lysine	765 ± 43.2	1211 ± 0.498	<0.001	8.09 ± 0.145	8.83 ± 0.140	0.021
Methionine[3]	285 ± 28.5	475 ± 58.1	0.043	3.02 ± 0.263	3.45 ± 0.380	0.404
Phenylalanine	404 ± 23.3	551 ± 9.16	0.004	4.27 ± 0.0323	4.02 ± 0.0828	0.044
Threonine	522 ± 31.9	658 ± 12.1	0.016	5.52 ± 0.112	4.80 ± 0.165	0.023
Tryptophan	204 ± 11.8	264 ± 5.31	0.010	2.16 ± 0.0144	1.93 ± 0.0280	0.002
Valine	673 ± 32.1	876 ± 10.0	0.004	7.12 ± 0.117	6.39 ± 0.176	0.025
Non-essential amino acids						
Alanine	4169 ± 26.9	568 ± 6.52	0.005	4.40 ± 0.122	4.14 ± 0.112	0.200
Arginine	361 ± 29.3	361 ± 4.84	0.983	3.81 ± 0.157	2.63 ± 0.0520	0.002
Aspartic acid[4]	1012 ± 61.7	1360 ± 23.8	0.006	10.7 ± 0.192	9.92 ± 0.327	0.108
Cysteine[5]	229 ± 12.4	259 ± 19.9	0.274	2.42 ± 0.0295	1.88 ± 0.118	0.011
Glutamic acid[6]	1956 ± 75.9	2564 ± 32.9	0.002	20.7 ± 0.404	18.7 ± 0.468	0.031
Glycine	260 ± 19.8	258 ± 9.44	0.915	2.75 ± 0.127	1.88 ± 0.0990	0.006
Proline	1056 ± 58.1	969 ± 9.34	0.214	11.2 ± 0.196	7.06 ± 0.135	<0.001
Serine	490 ± 28.1	652 ± 10.4	0.006	5.18 ± 0.0958	4.76 ± 0.137	0.065
Tyrosine[7]	358 ± 59.0	406 ± 43.0	0.116	3.75 ± 0.421	2.96 ± 0.307	0.275
Tyrosine[8]	525 ± 11.4	580 ± 17.2	0.118	5.56 ± 0.0694	3.92 ± 0.103	0.019
Other nitrogen content						
Total ammonia	398 ± 22.1	254 ± 5.17	0.003	4.22 ± 0.189	1.85 ± 0.0679	<0.001
Free ammonia	4.39 ± 1.00	1.20 ± 0.0403	0.033	0.0464 ± 0.0104	0.00884 ± 0.000294	0.023
Urea	342 ± 7.60	38.6 ± 0.0917	<0.001	3.62 ± 0.0806	0.28 ± 0.000668	<0.001

Data are presented as mean ± SEM.
Abbreviations: HM, fresh human milk; IF, infant formula.
[1] Predicted using a non-linear regression model applied to multiple hydrolysis times.
[2] True protein = (total nitrogen − non-protein nitrogen) × 6.38.
[3] Detected as methionine sulfone.
[4] Asparagine + aspartate.
[5] Detected as cysteic acid.
[6] Glutamate + glutamine.
[7] Tyrosine content determined using multiple hydrolysis times.
[8] Tyrosine content determined after 24-h acid hydrolysis containing 0.1% phenol.

acid, serine, and tyrosine, the content did not differ ($P > 0.05$, Table 3). The dietary tyrosine content determined after acid hydrolysis with phenol was higher than that predicted by multiple hydrolyses (+47% and +43% for HM and IF, respectively). This content was used for the DIAAS determination.

Endogenous N and AA flow along the digestive tract

The endogenous flows of AAs and N determined in the ileal digesta of 6 piglets fed a PF diet for 3 days are presented in Table 4. The proportion of endogenous N to total N was different ($P < 0.04$) between IF- and HM-fed piglets from the stomach to the caecum, but not in the colon ($P = 0.328$, Table 5).

Apparent and true ileal digestibility of total N, AAN, and AAs

Apparent and true digestibilities of total N were lower for HM in the median jejunum, ileum, and caecum ($P < 0.015$, Table 6) and were not different in the proximal jejunum ($P = 0.153$) and the colon ($P = 0.634$).

In the ileum, the apparent digestibility of total N was 10% lower for HM, while the apparent digestibility of AAN was slightly but still lower in HM (−4%; $P = 0.006$; Table 7). The N-TID was lower in HM (−7.4%; Table 7) unlike that of AAN, which was not statistically different between groups ($P > 0.05$), with a mean value of 97.4% ± 0.655% (Table 7).

Regarding the individual AAs, the apparent ileal digestibility was not different for histidine, lysine, arginine, glutamic acid, and glycine, unlike that for the other AAs where digestibility was lower for HM (Table 7). The TID of lysine was slightly but higher for HM (+2.7%). In contrast, the TID of phenylalanine, threonine, valine, alanine, proline, and serine was lower for HM than for IF (−2.7% to −7.7% in HM; Table 7). No difference was observed for the other AAs studied, including those of tryptophan (Table 7). In the proximal jejunum and the colon, the apparent and true digestibility of tryptophan were not different between the HM and IF diets (Table 7).

DIAAS measurement

The protein reference pattern used for the DIAAS calculation was the HM reference, as described in the FAO report [2]. The DIAAS measured for both diets corresponded to the digestible score for the aromatic AAs (phenylalanine + tyrosine) (Table 8).

TABLE 4
Endogenous flows of total N, amino acid N, and individual amino acids at the terminal ileum[1] from piglets (n = 6) that were fed a protein-free diet.

	Mean endogenous flow[2] (μg/g of FDM intake)
Total N	2080 ± 167
Amino acid N	848 ± 119
Essential AA	
Histidine	163 ± 11.4
Isoleucine	194 ± 20.7
Leucine	345 ± 36.1
Lysine	244 ± 27.6
Phenylalanine	208 ± 22.5
Threonine	520 ± 68.8
Tryptophan[3]	725 ± 267
Valine	340 ± 46.3
Non-essential AA	
Alanine	251 ± 25.9
Arginine	207 ± 27.8
Aspartic acid[4]	472 ± 48.3
Glutamic acid[5]	547 ± 60.7
Glycine	1340 ± 299
Proline	387 ± 45.7
Serine	348 ± 43.9
Tyrosine	132 ± 19.3

Data are presented as mean ± SEM.
Abbreviations: AA, amino acid; FDM, Freeze-dried matter; N, nitrogen.
[1] Terminal ileum: 80 cm before ileocecal valve.
[2] AA content corrected using multiple hydrolyses.
[3] Tryptophan content was measured after 16 h of alkaline hydrolysis.
[4] Asparagine + aspartate.
[5] Glutamate + glutamine.

Discussion

The present study demonstrates that the AAN-TID was similar and high in both infant diets unlike that for total N and some AAs, which was lower in HM ($P < 0.001$). This is the first time that the tryptophan-TID was determined *in vivo* in HM-fed piglets and was shown to be high and similar between HM and IF. The multiple hydrolyses, rarely used in previous studies, allowed for a more accurate AA determination.

Diet composition

The pools of fresh HM, collected from at least 15 mothers per block and used on the last experimental day, limited the potential inter-individual variability in composition. The similar crude protein contents of HM and IF were in the upper range of the values previously reported for mature HM (1.2 g/100 mL [5,6,25–29]) or for current commercial IFs (1.2–1.4 g/100 mL over 5 commercial brands), respectively. In contrast, the true protein content differed between diets and were in the lower range of those previously reported [8,26,28,30–32]. This can be explained for HM by the somewhat higher proportion of NPN than that reported in the literature (30% in the present HM compared with 20%–25% in the literature [27,33]). This NPN fraction consisted of urea, for which the contribution to NPN (24.3% ± 1.7%) was somewhat lower than that previously reported (30%–50% [34–36]). Other components that contributed to this NPN fraction in HM were free AA (8.6%), HM oligosaccharides (HMO) N (9.1%), and free ammonia (0.5%; data not shown), in agreement with previous data [36]. In IF, the present NPN proportion was in the range of previously published data with a lower urea contribution in IF (18.2% ± 0.3%) than in HM but also than that previously reported for IF (27%–65% of the NPN fraction) [27,33]. This can be explained by the different manufacturing processes used for the dairy ingredients, particularly regarding whey proteins, which were the ideal serum for the present IF, although cheese serum is more commonly used for IF (5%–16% NPN, [27,37,38]) in addition to variability induced by the milk origin (e.g., season, breed, and organic label [38]). Differences in true protein determination methods can also explain the differences observed [26,28].

Regarding the AA profile, that of the present IF was in line with the regulations [8], whereas that of HM was in the upper range of values previously published [5,25,39,40] and could be explained by the differences in methodology. When expressed as a proportion of true protein, most essential AAs were present in greater proportion in the HM protein than in IF, indicating that

TABLE 5
Endogenous N flow and percentage of endogenous N to total N along the digestive tract of HM- and IF-fed piglets.

Digestive tract site	Mean endogenous N flow (g/100 g of FDM intake)	% Endogenous N		
		HM[3]	IF	*P* value
Stomach	0.262 ± 0.0749[1]	10.9 ± 0.704	12.4 ± 0.231[4]	0.023
Proximal jejunum	0.519 ± 0.0185[1]	47.9 ± 7.35	34.0 ± 2.06[4]	0.039
Median jejunum	0.377 ± 0.0491[2]	77.9 ± 4.37	104 ± 6.08[4]	0.005
Terminal ileum	0.201 ± 0.0133[2]	59.1 ± 3.70	87.3 ± 5.98[5]	0.002
Caecum	0.425 ± 0.0973[1]	87.2 ± 10.8	119 ± 7.12[5]	0.007
Colon	0.579 ± 0.0631[2]	120 ± 14.9	125 ± 6.30[4]	0.328

Abbreviations: FDM, Freeze-dried matter; HM, fresh human milk; IF, infant formula powder rehydrated with ultrapure water to 115 g DM/L; N, nitrogen;
PF, protein-free diet.
Data are presented as mean ± SEM.
[1] $n_{PF\text{-}fed\ piglets} = 5$ (limited sample amount).
[2] $n_{PF\text{-}fed\ piglets} = 6$.
[3] $n_{HM\text{-}fed\ piglets} = 7$ (outliers' piglets).
[4] $n_{IF\text{-}fed\ piglets} = 9$.
[5] $n_{IF\text{-}fed\ piglets} = 8$ (limited sample amount).

TABLE 6
Apparent and true digestibility values[1] of total N and Trp[2] throughout the intestine.

Site	Protein content	Apparent digestibility (%)			True digestibility (%)		
		HM	IF	*P* value	HM	IF	*P* value
Proximal jejunum	Total N	23.6 ± 1.22	20.4 ± 4.40	0.186	53.8 ± 1.22	48.0 ± 4.50	0.153
	Tryptophan	29.6 ± 2.34	35.5 ± 3.56	0.218	36.2 ± 2.32	42.8 ± 3.57	0.167
Median jejunum	Total N	70.5 ± 1.87	80.7 ± 1.08	<0.001	93.0 ± 1.90	100 ± 1.08	<0.001
Caecum	Total N	71.1 ± 2.58	80.9 ± 1.11	0.003	90.8 ± 2.72	98.1 ± 1.11	0.013
Colon	Total N	66.7 ± 6.67	75.5 ± 1.37	0.125	101 ± 6.90	106 ± 1.37	0.634
	Tryptophan	78.9 ± 6.66	84.5 ± 0.902	0.955	85.5 ± 6.71	101 ± 4.29	0.281

Abbreviations: AAs, amino acids; HM, fresh human milk; IF, infant formula; N, nitrogen.
[1] Data represents the mean ± SEM of 7 HM-fed piglets and 9 IF-fed piglets.
[2] Tryptophan concentration was determined after 16 h basic hydrolysis and corrected by multiple hydrolysis correction factors prior to digestibility determination.

TABLE 7
Apparent and true ileal digestibility values of total N[1], AAN[2], and AAs.[1,3]

AA	Apparent digestibility (%)			True digestibility (%)		
	HM	IF	*P* value	HM	IF	*P* value
Total N	79.3 ± 1.13	87.6 ± 0.867	<0.001	91.3 ± 1.24	98.0 ± 0.866	<0.001
AAN[2]	88.8 ± 0.876	92.3 ± 0.641	0.006	96.7 ± 0.961	98.0 ± 0.663	0.272
Essential AAs						
Histidine	90.1 ± 1.01	92.4 ± 0.543	0.061	97.9 ± 1.05	97.8 ± 0.698	0.536
Isoleucine	92.4 ± 0.645	94.5 ± 0.455	0.018	96.3 ± 0.681	97.6 ± 0.467	0.174
Leucine	94.3 ± 0.664	96.0 ± 0.385	0.046	98.2 ± 0.697	98.5 ± 0.395	0.584
Lysine	94.3 ± 0.724	93.7 ± 0.500	0.475	98.4 ± 0.762	95.8 ± 0.550	0.021
Phenylalanine	89.7 ± 0.799	94.5 ± 0.536	<0.001	96.3 ± 0.864	98.7 ± 0.570	0.021
Threonine	76.5 ± 1.58	87.2 ± 0.793	<0.001	89.2 ± 1.76	96.0 ± 0.845	<0.001
Tryptophan	89.1 ± 0.871	93.0 ± 0.543	0.001	95.5 ± 0.907	97.5 ± 0.563	0.079
Valine	86.8 ± 1.05	93.3 ± 0.602	<0.001	93.1 ± 1.10	97.0 ± 0.774	0.002
Non-essential AAs						
Alanine	85.4 ± 1.25	91.8 ± 0.835	<0.001	93.1 ± 1.31	96.7 ± 0.842	0.026
Arginine	89.2 ± 1.42	90.3 ± 0.987	0.545	96.5 ± 1.52	96.6 ± 1.00	0.828
Aspartic acid[4]	89.0 ± 0.913	93.0 ± 0.507	0.002	94.9 ± 0.980	97.1 ± 0.520	0.058
Glutamic acid[5]	94.1 ± 0.642	95.3 ± 0.383	0.117	97.6 ± 0.653	97.7 ± 0.384	0.740
Glycine	54.8 ± 3.27	63.2 ± 5.46	0.228	120 ± 4.01	121 ± 6.46	0.709
Proline	89.4 ± 0.689	92.9 ± 0.475	<0.001	94.1 ± 0.711	97.6 ± 0.486	0.001
Serine	84.8 ± 1.36	90.8 ± 0.625	<0.001	93.8 ± 1.45	96.8 ± 0.645	0.003
Tyrosine	90.7 ± 0.931	94.1 ± 0.714	0.012	95.9 ± 1.02	97.7 ± 0.658	0.105

Data are presented as mean ± SEM.
Abbreviations: AA, amino acid; AAN, amino acid nitrogen; HM, fresh human milk; IF, infant formula; N, nitrogen.
[1] Total N data represent the mean of 7 HM-fed piglets and 9 IF-fed piglets for total nitrogen and tryptophan analysis. AA data represent the mean of 7 HM-fed piglets and 8 IF-fed piglets due to a low amount of ileal content available for the AA assay of 1 IF-fed pig.
[2] AAN was calculated by summing the amount of N supplied by all the AAs. The digestibility of AAN was then calculated as described in the Materials and Methods section.
[3] AA concentrations were determined on freeze-dried samples after 24 h acid hydrolysis except for tryptophan, which was determined after 16 h basic hydrolysis. All AA concentrations were corrected by multiple hydrolysis correction factors prior to digestibility determination.
[4] Asparagine + aspartate.
[5] Glutamate + glutamine.

bovine proteins, even after readjustment of the caseins-to-whey protein ratio, do not entirely mimic human proteins and that the true protein content needs to be higher in IF than in HM to cover the essential AA needs of an infant. Nevertheless, the tryptophan and leucine contents were higher in the present IF than that previously reported in other IFs [6,41–43], likely due to the whey protein origin, which is obtained by bovine milk microfiltration (ideal serum) and not from cheese serum such as that for most commercial IFs.

Multiple hydrolyses

Multiple hydrolyses allowed accurate determination of the AA values and highlighted different AA behavior associated with the hydrolysis for serine and threonine [4,5,17,44] and with the matrix for tyrosine and valine. Nevertheless, the impact of the correction factor was small for most AAs (average mean difference: +1.14%), except for serine (average mean difference: +3.53%), threonine (average mean difference: +2.13%),

TABLE 8
Digestible Indispensable Amino Acid Score (DIAAS) of HM and IF

	DIAA ratio[1]	
Amino acid	HM	IF
Histidine	130	103
Isoleucine	115	99
Leucine	123	116
Lysine	115	122
Phenylalanine + tyrosine[2]	101	83
Threonine	112	105
Tryptophan	122	111
Valine	121	113
Cysteine + methionine[3]	165	162
DIAAS	**101**	**83**

Abbreviations: DIAAS, Digestible Indispensable Amino Acid Score; HM, fresh human milk; IF, infant formula.

[1] HM amino acid profile presented in the FAO report published in 2013 used as protein reference pattern [2]. The content of AA expressed in mg/g of true protein using 6.38 as the protein conversion factor and the non-protein nitrogen (NPN) fraction measured as described above in the Methods section (31% N for HM and 4% N for IF).

[2] Tyrosine content was determined using 24 h acid hydrolysis containing 0.1% phenol.

[3] AA content was determined as cystic acid and methionine sulphone. The digestibility of methionine and cysteine was assumed to be 100% as it was not determined in the present study.

tryptophan (average mean difference: +3.77%), and tyrosine (average mean difference: +9.74%), which were degraded during hydrolysis, such as that previously reported [4,44,45]. Although multiple hydrolyses aims to correct for hydrolytic losses, tyrosine degradation was mainly due to its halogenation into chlorotyrosine that can occur during HCl hydrolysis [45,46]. Phenol addition during acid hydrolysis [46] prevented tyrosine degradation leading to values closer to those reported in the literature [5,25,39] without any effect on the other AAs. Thus, this value was used for the calculation of the digestible AA score of tyrosine. These multiple hydrolyses also highlighted that 24 h of hydrolysis at 110 °C, such as those usually performed [4,45], is a good compromise to the determined content of AAs slowly or rapidly released during hydrolysis.

Protein digestibility using Yucatan piglets as an infant model

The TID of AAs or N was obtained after correction for ileal endogenous N or AA losses determined in PF-fed piglets, which were consistent with previous data [5,47–49]. Such digestibility is also called as standardized digestibility in animal nutrition [3] unlike human nutrition [23,50]. Along the digestive tract, as expected, the proportion of endogenous to total N increased from the stomach up to the colon where it reached a value above 100%, suggesting an overestimation of the colonic endogenous N losses in PF-fed piglets as compared to HM- or IF-fed piglets, which is potentially due to a different microbial activity. Feeding a PF diet is usually recognized as somewhat underestimating the ileal N losses, only allowing for the estimation of the basal endogenous losses, without considering any variation linked to the diet composition [48,49,51,52]. The present diets, with no fiber or anti-nutritional factors, are expected to have a minimal enhancing impact on the basal endogenous losses. The determination of the real ileal digestibility with ^{15}N-labeled dietary proteins would have given more accurate value; however, such labeling is not possible in human lactating mothers. Thus, the PF diet remains the best alternative for evaluating the endogenous losses in the present study, similar to those indicated by the FAO Expert Working Group [23].

The present study has demonstrated that TID of total N was lower than that of AAN solely for HM, in line with previous data [5], whereas the TID of AAN was similar between diets. This can be attributed to the undigestible and/or unabsorbable fraction of NPN in HM, such as for urea or to a lower extent for HMO glucosamine [33–36,53], which are thus transferred to the colon where they can carry out some physiological function such as bifidogenic properties [34,36,54–56]. Such an NPN fraction is present in low quantity in IFs. Due to its potential impact on the host microbiota, such a fraction, particularly urea in addition to HMO, should be further considered for IF formulation.

The present TID of most AAs were in the same range as those previously reported [5,6], except for glycine in HM and IF. Although glycine's apparent digestibility was in line with previous data [6], its TID was overestimated (>100%) due to an overestimation of the endogenous glycine loss in PF-fed piglets, which is potentially linked to an enhanced bile salt production and subsequently to an enhanced deconjugation of the glycocholate bile salt in the terminal ileum of PF-fed piglets [48]. Whereas the TID of most AAs did not differ between HM and IF, the TID of lysine was higher ($P = 0.021$) in HM than in IF, although the extent of the difference was small (+2.6%). This may be related to the glycation of the lysine residue within the Maillard reaction products formed during IF processing (~10 mg of N^{ε}-carboxymethyl lysine/100 g of crude proteins in the present IF powder [12]) that can induce some reduction in lysine bioavailability [57,58]. The determination of reactive lysine [15] could have provided a more accurate digestibility value.

The TID differed the most for threonine, being lower in HM than in IF (−7.7%). Additionally, alanine, proline, serine, and valine had lower TID than the other AAs in both diets and were particularly less digestible in HM than in IF. This is likely due to the different protein nature between HM and IF, with these AAs particularly represented in immune proteins (lactoferrin and lysozyme), present in HM but as traces in bovine milk–based IF [59], which are known to be resistant to digestion especially in their native structure [60] more likely in the present HM compared to IF proteins that were partially denatured (58% denaturation [12]) during IF manufacturing.

DIAAS

The present DIAAS values were similar to those found in the literature, with the previously reported values of 100 for HM [5] and 86 for IF [6] for the same limiting AA (aromatic AAs). The present values of DIAAS, determined in regard to the true protein content of the food with a N-to-protein factor of 6.38, were slightly reduced when using a N-to-protein factor of 6.25 with the same difference between HM and IF and the same limiting AA ($DIAAS_{HM} = 96$ compared with $DIAAS_{IF} = 80$, data not shown) [22]. However, the difference between HM and IF DIAAS decreased when the

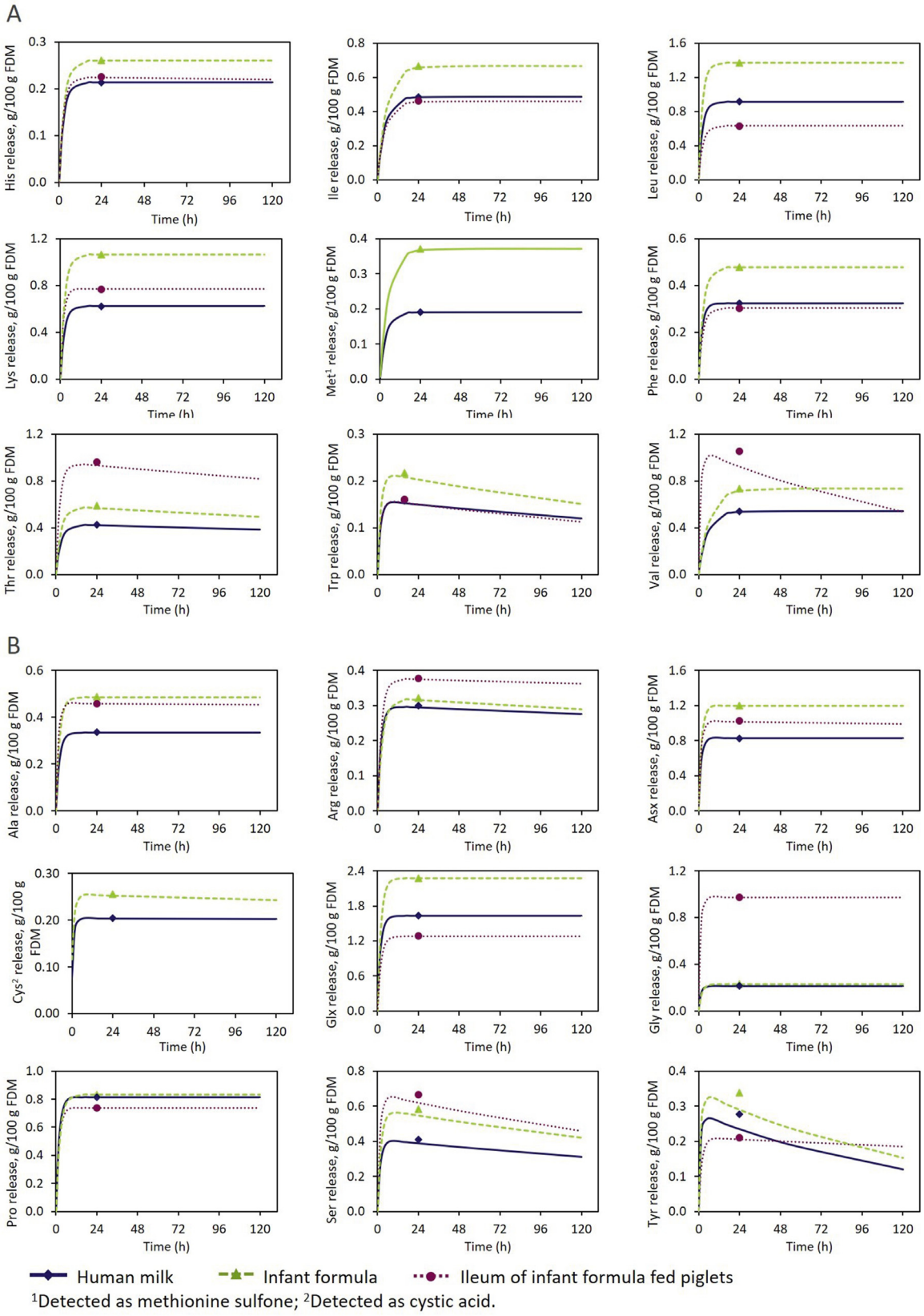

FIGURE. Modeled essential amino acid (A) and non-essential amino acid (B) release across hydrolysis times and estimated value at 24 h using the compartmental model (calculated by summing the protein bound at 24 h and free amino acid content prior hydrolysis) in human milk, infant formula and a pool of ileal digesta for infant formula-fed piglets.

AA profile of the food was expressed on the basis of the mass AA residue profile ($DIAAS_{HM} = 90$ compared with $DIAAS_{IF} = 87$, data not shown). Altogether, this highlights that the DIAAS calculation methodology requires some clarification [61].

In conclusion, the present study has demonstrated that compared with IF, HM has a lower TID solely for total N while the AAN-TID was similar. Part of the NPN fraction appears to be undigestible and unabsorbable, and hence, it is transferred to the colon. The present data indicate that the AAs within the present IF were highly digestible (>95%) and 7 AAs were even slightly more digestible than in HM, with the exception of lysine, possibly due to the Maillard reaction. However, a higher protein level in standard IF is required to compensate for the unbalanced AA profile notably for the limiting aromatic AAs. Finally, whether the differences in whey protein quality (structure and composition due to different origin) within IF would impact the present results remain to be investigated.

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Data availability

Data described in this article will not be made publicly available.

Author contribution

The authors' responsibilities were as follows – EC, IL, AD, DD, PM: contributed to the conception and design of the study; EC, IL, AD, GH, AC, YLG, PD, AB: collected the data; EC, AD: analyzed the data; EC: prepared the first draft of the manuscript; AD, IL, EC: contributed to writing of a section of the manuscript; EC, AD, IL: had primary responsibility for the final content; and all authors: read, revised, and approved the final manuscript.

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Author disclosures

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary material to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.02.025.

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