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Protein Ingredient Quality within Infant Formulas Impacts Plasma Amino Acid Concentrations in Neonatal Minipiglets

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A B S T R A C T

Background: Infant formulas (IFs), the only adequate substitute to human milk, are complex matrices that require numerous ingredients and processing steps that may impact protein digestion and subsequent amino acid (AA) absorption.
Objectives: The objective was to understand the impact of the protein ingredient quality within IFs on postprandial plasma AA profiles.
Methods: Four isonitrogenous and isocaloric IFs were produced at a semi-industrial scale using whey proteins from different origins (cheese compared with ideal whey) and denaturation levels (IF-A, -B, -C), and caseins with different supramolecular organizations (IF-C, -D). Ten Yucatan minipiglets (12- to 27-d-old) were used as a human infant model and received each IF for 3 d according to a Williams Latin square followed by a 2-d wash-out period. Jugular plasma was regularly sampled from 10 min preprandial to 4 h postprandial on the third day to measure free AAs, urea, insulin, and glucose concentrations. Data were statistically analyzed using a mixed linear model with diet (IFs), time, and sex as fixed factors and piglet as random factor.
Results: IFs made with cheese whey (IF-A and -B) elicited significantly higher plasma total and essential AA concentrations than IFs made with ideal whey (IF-C and -D), regardless of the pre- and postprandial times. Most of the differences observed postprandially were explained by AA homeostasis modifications. IFs based on cheese whey induced an increased plasma concentration of Thr due to both a higher Thr content in these IFs and a Thr-limiting degrading capability in piglets. The use of a nonmicellar casein ingredient led to reduced plasma content of AA catabolism markers (IF-D compared with IF-C).
Conclusions: Overall, our results highlight the importance of the protein ingredient quality (composition and structure) within IFs on neonatal plasma AA profiles, which may further impact infant protein metabolism.

Keywords: infant formula, protein ingredient quality, piglet model, amino acid kinetics, protein metabolism

Introduction

Exclusive breastfeeding during the first 6 mo of life is considered the best early nutrition, providing multiple benefits to the growing infant [1,2]. When mothers are unable or unwilling to breastfeed, infant formulas (IFs) are the most adequate alternative covering the infant nutritional needs. However, the health effects of IFs and human milk (HM) still differ, indicating that IF biomimicry needs to be improved [3]. Although IF lipids have been largely investigated [4–8], the interest in IF proteins is more recent [9–12]. In most cases, IF proteins originate from skimmed bovine milk mixed with bovine whey proteins (WPs) to a casein:WP ratio of 40:60, such as in mature HM. Beyond this similar casein:WP ratio, some differences in terms of protein profile and structure exist between HM and IF. Efforts have been made during the last decades to reduce protein content to prevent excessive early weight gain and reduce the risk of developing obesity later in childhood [13,14]. However, the protein structures between IFs and HM, but also among IFs, differ not only due to a different protein profile but also due to different

Abbreviations: AA, amino acid; BCAA, branched-chain amino acid; BW, body weight; Citr, citrulline; EAA, essential amino acid; GMP, glycomacropeptide; HM, human milk; IF, infant formula; NEAA, nonessential amino acid; Orn, ornithine; TAA, total amino acid; WP, whey protein; 3-Mhis, 3-methylhistidine.

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processing routes. Particularly, bovine WPs used for IF production can be obtained either as a by-product of cheese making (cheese whey) or less frequently after microfiltration of skimmed milk (ideal whey). The processing routes undergone by dairy protein ingredients within IFs have been recently shown to impact not only the WP profile and structure [12,15] but also their subsequent protein digestion [12,16]. In cheese whey, a major difference is the presence of glycomacropeptide (GMP) from κ-casein, released by the action of chymosin during cheese manufacturing, which largely contributes to the protein fraction (20%–25% total protein) [17]. Unlike WPs, GMP has the disadvantage of an unbalanced amino acid (AA) profile with neither aromatic AAs (Phe, Tyr, Trp) nor Cys [18].

Due to their different protein nature and structure, HM and IF protein digestion and AA absorption rates are likely to differ and thus to result in different postprandial plasma AA kinetics, which may further play an important signaling role in AA metabolism orientation and tissue protein synthesis [19–22]. The HM plasma AA appearance pattern could be considered the optimal target to be mimicked by IFs. To our knowledge, only one study has been conducted in preterm infants to compare the plasma AA appearance of HM with that of IF, in presumed isonitrogenous conditions. The results showed that the postprandial concentration of plasma essential AAs (EAAs) was ~18% higher in infants fed IF than those fed HM, suggesting a modulating role of IF protein nature and structure on AA absorption [23].

Different parameters can modulate plasma AA kinetics, with a major factor being the gastric emptying rate as demonstrated by the concept of slow compared with fast proteins [24]. Caseins are slowly emptied from the stomach due to their acidic coagulation, whereas WPs are released more quickly from the stomach because they remain partly soluble [20,25].

Recent studies of the casein fraction of IFs showed that the mineralization of caseins modulates their supramolecular organization, which in turn has an impact on their gastric behavior [10,26]. In other dairy matrices, it was confirmed that caseins, depending on their supramolecular organization, i.e., micellar compared with nonmicellar, can present a different gastric coagulation behavior [10], thus affecting their gastric emptying rate and subsequently their postprandial AA kinetics [27,28]. The plasma AA appearance can also be partly impacted by the digestion rate, which can differ according to the IF protein structure, and particularly the WP structure, which can be modified by heat treatments. It has been reported in previous in vitro digestion studies that the proteolysis rate of native and heat-denatured WPs differ within model solution or model IF [9,29–32]. In vivo, postprandial plasma AA concentrations have been compared between neonatal piglets fed native or denatured WP solution [25] and in rodents fed diets including 40% IF containing native or heat-denatured WPs [16]. The authors showed that native WPs resulted in higher plasma EAA content in the first 60 min [25] and higher plasma TAA content [16] than denatured WPs. However, none of these studies has investigated in vivo the impact of the protein quality within a complex matrix such as commercial IF on the postprandial plasma AA appearance and with such IF as the sole source of meal. We have previously reported that the IF structure was impacted by the quality of both WP (cheese whey compared with ideal whey) and casein (micellar compared with nonmicellar) ingredients, which in turn modulated the in vitro proteolysis kinetics [12]. However, whether protein ingredient quality (composition and structure) within IFs has consequences on in vivo postprandial plasma AA appearance rate and concentration remains unknown.

The aim of the present study was thus to evaluate the impact of the protein ingredient quality (composition and structure) within IFs on postprandial plasma AA pattern and concentrations using Yucatan minipiglets as a model for the human infant. Protein structure and composition were modulated using different ingredient origins of WPs (cheese whey compared with ideal whey) and caseins (micellar compared with nonmicellar). We hypothesized that the IF protein profile and microstructure that modulated in vitro protein hydrolysis susceptibility [12] would affect the in vivo postprandial plasma AAs.

Methods

Ingredients and diets

Full-fat bovine milk powder, standard skimmed milk powder, and milk protein concentrate powder were provided by SODIAAL (Nutribio). They were characterized by a crude protein content of 26.5%, 35.5%, and 60% and a casein:WP ratio of 80:20, 80:20, and 90:10, respectively. A nonmicellar casein ingredient was also provided by SODIAAL (Eurosérum) with a crude protein content of 26% and a casein:WP ratio of 65:35 (Table 1). Three commercial WP powders were purchased from dairy companies. Their

TABLE 1
Characteristics of the casein-containing ingredients used in the IFs

Item	Units	Skimmed milk	Milk protein concentrate	Nonmicellar casein ingredient
Crude protein[1]	g/kg of powder	350	602	259
True protein[1]	% of crude protein	92.6	94.2	94.6
Casein:WP ratio[2]	% of true protein	80:20	90:10	65:35
Casein organization[3]		Mainly micellar	Mainly micellar	Nonmicellar only
Lipids[4]	g/kg of powder	5.0	15.6	8.7
Lactose[4]	g/kg of powder	520	240	662
Ash[4]	% of crude protein	80.0	87.0	45.3
Corresponding IF		A, B, and C	D	D

Abbreviations: IF, infant formula; RP-HPLC, reverse phase high-performance liquid chromatography; WP, whey protein.
[1] Determined by the Kjeldahl method with an N-to-protein conversion factor of 6.38.
[2] Determined by RP-HPLC.
[3] Determined by the ultracentrifugation method.
[4] Externalized analysis.

main characteristics are presented in Table 2, and their processing route has been described previously [12]. A piglet vitamin and mineral premix was purchased from Mg2Mix (Piglet premix Step 1, Mg2Mix). Lactose, minerals, and vitamins used in IFs were provided by SODIAAL (Nutribio). Nondairy fat (a blend of palm, sunflower, oleic sunflower, and rapeseed oils with lecithin as an emulsifier and ascorbyl palmitate and a blend of tocopherols as antioxidants) were purchased from SODECO.

A transition diet was made by rehydrating a powder mix consisting of half full-fat bovine milk powder and the other half of a blend of the 4 experimental IFs (-A, -B, -C, and -D), enriched with vitamins and minerals (0.5 g/100 g of dry matter, Piglet premix Step 1 Mg2Mix), with ultrapure water at a level of 22% dry matter. The transition diet feeding corresponded to 15 g protein/kg body weight (BW)/d.

The 4 experimental IFs were formulated to be isonitrogenous and isocaloric and were produced at semi-industrial scale (200 kg per IF). IF-A, -B, and -C contained skimmed milk powder as a source of caseins (micellar form), while IF-D contained a mix of nonmicellar caseins (75% of total caseins) and milk protein concentrate powder (micellar caseins representing 25% of total caseins). Regarding WPs, IF-A was based on demineralized cheese whey powder, IF-B on demineralized cheese WP concentrate powder, and IF-C and -D on demineralized ideal whey powder. IF-A and -B contained some GMP (WPs from cheese whey), whereas IF-C and -D did not contain GMP (WPs from an ideal whey).

The experimental diets were obtained by rehydrating each IF with ultrapure water at a level of 22% dry matter. Piglets were fed in a trough, 6 times per day from 8:00 to 22:30. At each meal, piglets were fed 60 mL of diet/kg BW to receive 8 g protein/kg BW/d. BW was assessed twice a week throughout the study to adjust their feed intake. Nutritional characteristics of the 4 rehydrated IFs are given in Table 3.

TABLE 2
Characteristics of the whey protein ingredients used in the IFs

Item	Units	Demineralized whey	Demineralized whey protein concentrate	Demineralized whey
Lactoserum origin		Cheese whey	Cheese whey	Ideal whey
Crude protein[1]	g/kg of powder	115.5 ± 0.1	777.2 ± 4.7	118.4 ± 0.3
True protein[1]	% of crude protein	85.6 ± 0.0	94.0 ± 0.6	92.3 ± 0.0
BLG[2]	% of protein nitrogen	59	53	64
ALA[2]	% of protein nitrogen	12	10	14
Glycomacropeptide		Presence	Presence	Absence
Tryptophan[3]	% of crude protein	1.7	1.7	2.2
BLG denaturation[4]	% of total BLG	22	0	1.7
ALA denaturation[4]	% of total ALA	59	0	22
Blocked lysine[5]	% of total lysine	19	13	14
Corresponding IF		A	B	C and D

Abbreviations: ALA, α-lactalbumin; BLG, β-lactoglobulin; IF, infant formula; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate.
[1] Determined by the Kjeldahl method with an N-to-protein conversion factor of 6.38.
[2] Determined by RP-HPLC.
[3] Externalized measurements made by HPLC.
[4] Calculated by the ratio of native BLG or ALA (native PAGE) and total BLG or ALA (SDS-PAGE).
[5] Determined by a fast and nondestructive fluorescence technique developed by SODIAAL (Eurosérum) using a model developed on Amaltheys (Spectralys Innovation).

TABLE 3
Biochemical composition (± SD) of the 4 IFs after rehydration to 22% dry matter

Per 100 mL of rehydrated IF					
Item	Unit	IF-A	IF-B	IF-C	IF-D
WP ingredient		Cheese whey	Cheese whey concentrate	Ideal whey	Ideal whey
Casein ingredient		Skimmed milk	Skimmed milk	Skimmed milk	Caseinate and milk protein concentrate
Crude protein[1]	g	2.5 ± 0.10	2.4 ± 0.02	2.4 ± 0.02	2.4 ± 0.02
True protein[1]	g	2.2 ± 0.10	2.2 ± 0.02	2.3 ± 0.02	2.2 ± 0.02
Casein:WP	%	40:60	40:60	40:60	40:60
Denaturation[2]	% of total WPs	44.3 ± 0.01	35.9 ± 0.20	30.9 ± 0.20	29.3 ± 0.10
Lipids[3]	g	6.3 ± 0.1	6.1 ± 0.1	5.6 ± 0.1	5.6 ± 0.1
Lactose	g	12.5	13.4	12.1	12.1
Ash	g	0.06	0.06	0.05	0.05
Energy	kcal	114	121	109	108
	kJ	476	505	456	454

Abbreviations: IF, infant formula; SD, standard deviation; WP, whey protein.
[1] Determined by the Kjeldahl method with 6.38 as the N-to-protein conversion factor.
[2] Denaturation level (%) $= 100 - \left(\frac{\text{Soluble proteins at pH 4.6}}{\text{Total whey proteins}} \times 100\right)$.
[3] Determined by the Teicher method.

The experimental IFs displayed different microstructures [12], with IF-A having a star-shaped microstructure with protein aggregates at the interface of casein micelles, themselves adsorbed at the fat droplet surface. IF-D presented some large aggregates of lipids and proteins, with some rearrangements of nonmicellar caseins. IF-B and -C had intermediate microstructures with a mix of caseins and WPs in the soluble phase and adsorbed at the interface of fat droplets.

Animals and experimental design

The experiment was conducted at INRAE experimental facilities (Pig Physiology and Phenotyping Experimental Facility, Agreement No. D35-275-32) in accordance with the current ethical standards of the European Community (Directive 2010/63/EU) and the French legislation on animal experimentation and ethics. The French Ministry of National Education, Research and Innovation and the Regional Ethics Committee in Animal Experiment of Brittany validated and approved (authorization #30985-2021041213063537) the entire procedure described in this work. Animal welfare was ensured by daily observation throughout the experiment. Fourteen healthy Yucatan mini-piglets (9 females, 5 males) were used in 2 replicates. The adaptation period started when piglets were separated from their mother at 5 ± 1 d of age and individually housed in stainless steel metabolic cages enriched with toys. Room temperature was maintained at 27 ± 2°C with a 14.5/9.5 h light/dark cycle. At 6 ± 1 d of age, piglets were surgically fitted with an indwelling silicone catheter in the jugular vein, under general anesthesia with aerosolized isoflurane mixed with oxygen and administered using a gas mask. The catheter was inserted through a collateral vein of the external jugular vein (auricular vein) and externalized through the back of the piglet neck, and then kept in a strengthened-elastic pocket kept directly above the site of externalization. The catheter was flushed every 2 d with a 0.5 to 1 mL saline solution mixed with heparin (1%).

After the adaptation period, including 4 d of postsurgical recovery, piglets received the experimental diets allocated according to a Williams Latin square. They received each experimental diet during 2 full d and for the first meal (8:00) of the third day corresponding to the blood sampling day. Each experimental period was followed by a 2-d wash-out period during which piglets were fed the transition diet (Figure 1). The day after the last blood collection, piglets were killed 80 min after the last meal by electrical stunning immediately followed by exsanguination.

Blood sampling

After overnight fasting (~10 h), piglets received a lower meal volume (30 mL diet/kg BW) than usual to ensure a full (>70%) and fast (<4 min) ingestion of the meal and thus a reliable postprandial plasma kinetics. The test meal provided 0.67 g protein/kg BW. A baseline blood sample was collected 10 min before the meal distribution, and additional blood samples were collected at 15, 30, 45, 60, 90, 120, 180, and 240 min postprandially. The total blood volume collected from each piglet was limited to 5 mL/kg BW for each 4-h kinetics, complying with ethical and metabolic concerns. After collection, blood samples were immediately transferred to EDTA tubes and stored on ice. After centrifugation (10 min, 3000 × g, 4°C), the available plasma was distributed in vials as follows: 210 µL for AA analysis at each time point, 200 µL at baseline and 15, 30, 60, and 120 min postprandially for insulin analysis, and 250 µL at baseline and 30 and 60 min postprandially for glucose and lipids analysis. Plasma samples were then stored at −80°C until analysis.

IF and plasma analysis

Total nitrogen and nonprotein nitrogen contents in IFs were determined by the Kjeldahl method. True protein content of IFs was calculated by subtracting the nonprotein nitrogen content from the total nitrogen content then multiplied by 6.38. Total AA content of the experimental diets was measured in duplicate, after a 24-h acid hydrolysis at 110°C in 6 N hydrochloric acid, as described previously [12]. The concentrations of the sulfur-containing AAs, Cys and Met, were measured in the experimental diets as methionine sulphone and cysteic acid after performic acid oxidation. The free AA contents, except Trp, were determined in the experimental diets and plasma samples after deproteinization with sulfosalicylic acid at a final concentration of 2.5% and 0.2-µm filtration. Total and free AAs were injected in the cation exchange column of a Biochrom 30 automatic AA analyzer (Biochrom Ltd) with postcolumn derivatization with ninhydrin (EZ Nin Reagent, Biochrom Ltd). The ninhydrine derivative of all AAs was detected at 570 nm (except proline at 440 nm). Trp was quantified on freshly reconstituted IFs in duplicate, using a previously described method [33], whereas it was not quantified in plasma because of the limited quantity available. Branched-chain AA (BCAA) concentration was considered as the sum of Ile, Leu, and Val concentrations. EAA concentration was the sum of 10 AA concentrations: BCAAs and Arg (considered EAAs for both infants and piglets), His, Lys, Met, Phe, and Thr. Nonessential AA (NEAA) concentration was the sum of 10 AA concentrations: Ala, Asp, Asn, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr. Finally, the concentration of total AAs (TAAs) was the sum of the 20 proteinogenic AA (EAAs and NEAAs) concentrations.

Individual free proteinogenic AAs (except Trp), nonproteinogenic AAs involved in the urea cycle (citrulline [Citr] and ornithine [Orn]) and in muscular proteolysis (3-methylhistidine [3-Mhis]), and urea concentrations were determined in plasma, concomitantly with free AAs as previously described, after dilution in pH 2.2 lithium buffer (except for baseline and

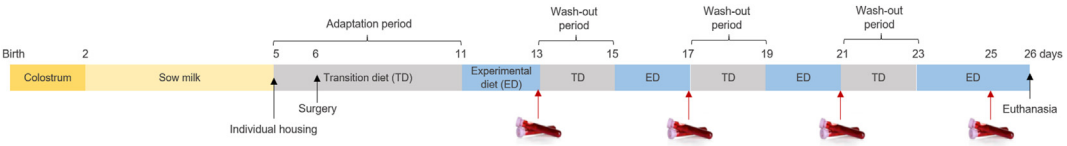

FIGURE 1. Experimental protocol.

180 and 240 min postprandial samples), deproteinization of the samples with sulfosalicylic acid at a final concentration of 2.5%, and 0.2-μm filtration.

Plasma glucose was analyzed with commercially available kits using colorimetric methods through an automatic analyzer, Konelab 20i (Thermo Fisher Scientific). Plasma concentration of insulin was measured using a radioimmunoassay kit (Insulin-CT, CisBio Bioassays) and an AIA-1800 device (Automated Immunoassay Analyzer; TOSOH Bioscience).

Insulinogenic and disposition indexes

Index of insulinoresistance (HOMA-IR) was calculated as follows (Equation 1):

$$HOMA - IR = \frac{G_0 \times I_0}{22.5} \quad (1)$$

G_0 and I_0 are basal glucose and insulin contents after a ~10 h fast.

QUICKY index was calculated (Equation 2) to determine insulin organism sensitivity as follows:

$$QUICKY = \frac{1}{\log(G_0) + \log(I_0)} \quad (2)$$

Matsuda index, another measure of insulin organism sensitivity, was calculated as follows (Equation 3):

$$Matsuda\ index = \frac{10000}{\sqrt{G_0 \times I_0 \times Glycemia_{mean} \times Insulinemia_{mean}}} \quad (3)$$

G_0 and I_0 are glucose and insulin contents after a ~ 10 h fast. $Glycemia_{mean}$ was calculated as the mean postprandial glucose content between 30 and 60 min, and $insulinemia_{mean}$ was calculated as the mean postprandial insulin content between 15, 30, 60, and 120 min.

An insulinogenic index was calculated as follows (Equation 4):

$$Insulinogenic\ index = \frac{I_{30\ min} - I_{preprandial}}{G_{30\ min} - G_{preprandial}} \quad (4)$$

Disposition index was calculated as follows (Equation 5):

$$Disposition\ index = Matsuda\ index \times Insulinogenic\ index \quad (5)$$

Data analysis

All statistical analyses were conducted using R software (version 4.2.1). Plasma concentrations were analyzed using a mixed linear model (lmerTest package) with diet, time, and sex as fixed factors and piglet as random factor. The interaction between diet and time was included in the model. Residual normality and variance homogeneity of all variables were tested using Kolmogorov–Smirnov test (nortest package) and Levene test (lawstat package), respectively. Differences were considered as statistically significant when the P value was strictly <0.05. Data are presented as adjusted means (least-square means) with their SEMs.

Results

Protein and AA composition of IFs

The true protein content was similar among the 4 IFs (Table 3). Concentrations of TAAs, EAAs, NEAAs, and BCAAs were similar among IFs (Figure 2). Some differences were

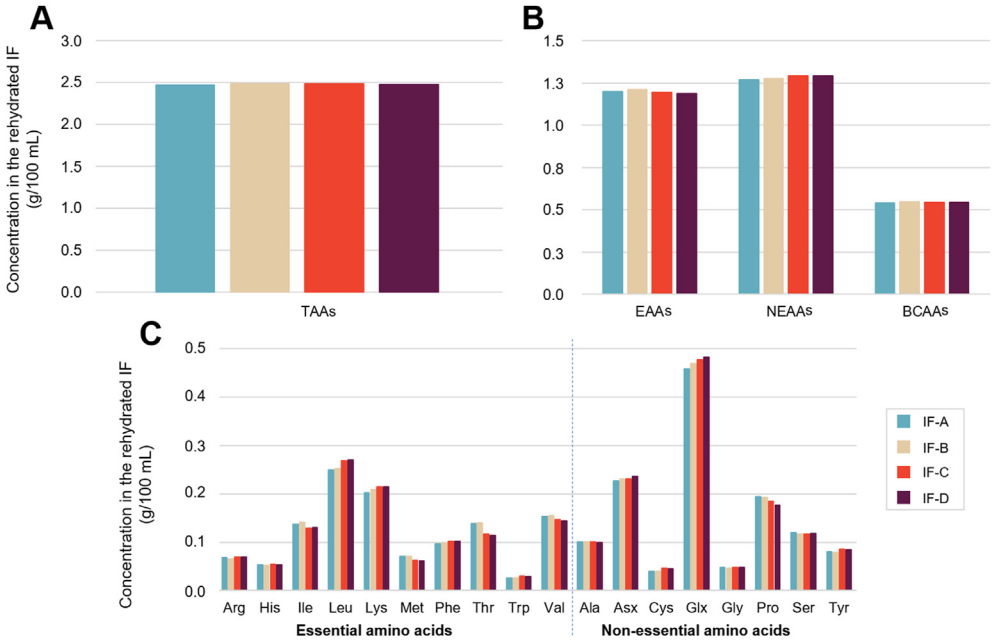

FIGURE 2. Concentrations of total (A), essential, nonessential, and branched-chain (B) and individual (C) AAs in rehydrated IFs. ($n = 2$). Asx (sum of Asn and Asp); BCAA, branched-chain amino acids (sum of Ile, Leu, Val); EAA, essential amino acids (sum of Arg, His, Ile, Leu, Lys, Met Phe, Thr, Trp, Val); Glx (sum of Gln and Glu); IF, infant formula; NEAA, nonessential amino acids (sum of Ala, Asx, Cys, Glx, Gly, Pro, Ser, Tyr); TAA, total amino acids.

observed for a few individual AAs. Thr and Met content was higher in IF-A and IF-B than in IF-C and IF-D (+19% to +23% for Thr, +12% to +15% for Met). Pro content was higher in IF-A and IF-B than in IF-D (+9%). Ile content was higher in IF-B than in IF-C and IF-D (+10%). In contrast, lower content of Cys was observed in IF-A and IF-B than in IF-C (−15%) and IF-D (−11%), as well as lower Trp content in IF-A and -B than in IF-C (−16%) and -D (−9%), likely due to the lower α-lactalbumin contents than those in IF-C and -D. Consequently, the molar Trp to large neutral AA ratios were lower for IF-A and IF-B (both 1:37) than for IF-C and IF-D (1:31 and 1:33, respectively).

Animal growth and blood sampling

During the entire experimental period, piglets remained healthy and kept growing, with an average daily BW gain of 72 ± 17 g. Four piglets were excluded during the experiment due to either inoperable catheter ($n = 1$) or digestive issues ($n = 3$). Of the 10 piglets remaining, when temporary catheter dysfunction occurred during blood sampling or when <70% of the test meal was consumed within 4 min, the corresponding blood sampling kinetics was excluded from analysis. As a result, 31 blood sampling kinetics were used for data analysis, partitioned into 8 kinetics for IF-A ($n = 4$ males, $n = 4$ females), 7 kinetics for IF-B ($n = 2$ males, $n = 5$ females), 7 kinetics for IF-C ($n = 3$ males, $n = 4$ females) and 9 kinetics for IF-D ($n = 3$ males, $n = 6$ females). The average piglet age at the blood sampling time was not significantly different between IFs, as well as the average proportion of meal ingested before blood collection (Supplemental Table 1).

Plasma proteinogenic AA kinetics

Plasma TAA concentrations increased during the first 30 min after meal ingestion (+28%, +22%, +26%, and +29% for IF-A, -B, -C, and -D, respectively, compared with the preprandial time or time 0) (Figure 3), mainly due to the increase of plasma EAA concentrations (+45%, +42%, +49%, and +56% for IF-A, -B, -C, and -D, respectively), and especially that of BCAA concentrations (+63%, +53%, +63%, and +67% for IF-A, -B, -C, and -D, respectively) (Figure 3). During the entire sampling period, the kinetics of plasma TAAs, EAAs, and BCAAs significantly differed among IFs both pre- and postprandially. Plasma TAA concentrations were significantly higher for IF-A and IF-B than for IF-C (+17% on average) and IF-D (+12% on average), at all the sampling times including at the preprandial time. It was mainly due to the plasma EAA concentrations, which were higher for IF-A and IF-B than for IF-C (+20%) and IF-D (+26%). The plasma BCAA concentrations of IF-A and IF-B were also significantly higher than that of IF-D postprandially (+12% and +16%, respectively), but without any difference preprandially. The plasma BCAA concentration of IF-C did not significantly differ from that of the 3 other IFs (Figure 3). Regarding plasma NEAAs, both IF-A and IF-B presented a higher plasma concentration than IF-C along the entire collection period including at the preprandial time (+16% and +22%, respectively), while only IF-B significantly differed from IF-D (+12%). A sex effect was observed only for plasma EAAs (Figure 3), and especially for the plasma contents of Met ($P = 0.04$) and Thr ($P < 0.0001$), higher in males than in females (Figure 4).

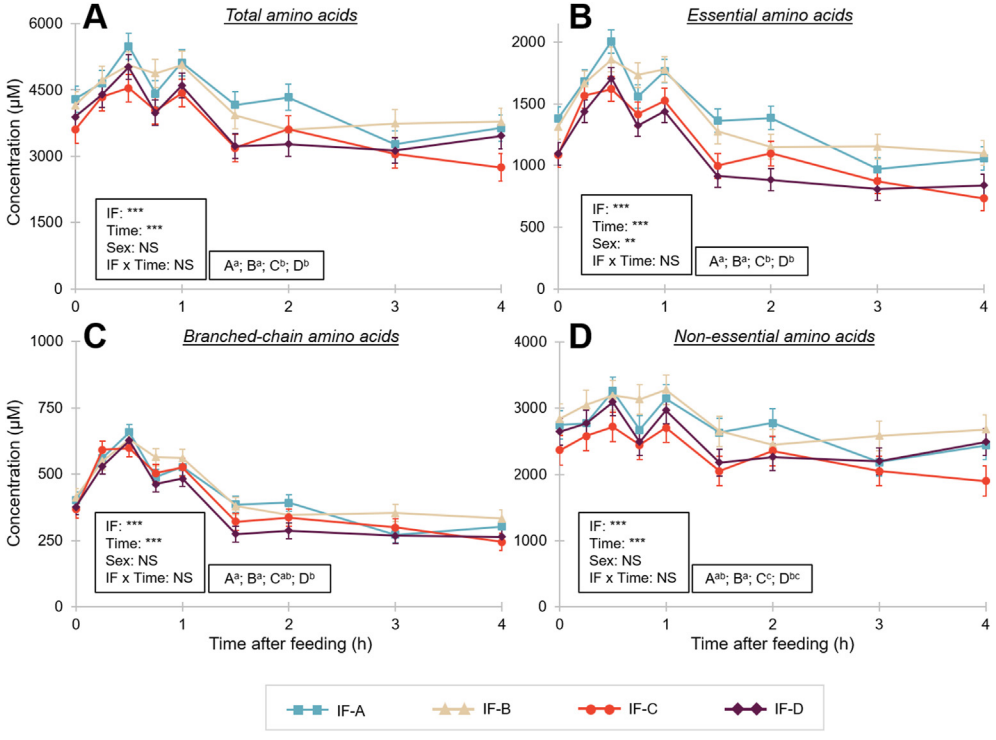

FIGURE 3. Time course of plasma concentrations of (A) total amino acids, (B) essential amino acids, (C) branched-chain amino acids, and (D) and nonessential amino acids from baseline to 4 h postprandially in piglets fed IF-A, -B, -C, and -D. Means ± standard error, with $n = 8$ for IF-A, $n = 7$ for IF-B, $n = 7$ for IF-C, and $n = 9$ for IF-D. Statistical significance is indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $P > 0.05$ (nonsignificant [NS]). IF, infant formula; A, B, C, and D for IF-A, IF-B, IF-C, and IF-D. Branched-chain amino acids is the sum of Ile, Leu, and Val; essential amino acids is the sum of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val; nonessential amino acids is the sum of Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr; total amino acids is the sum of all 20 proteinogenic amino acids. ^{a,b,c} groups without a common letter differ significantly ($P < 0.05$).

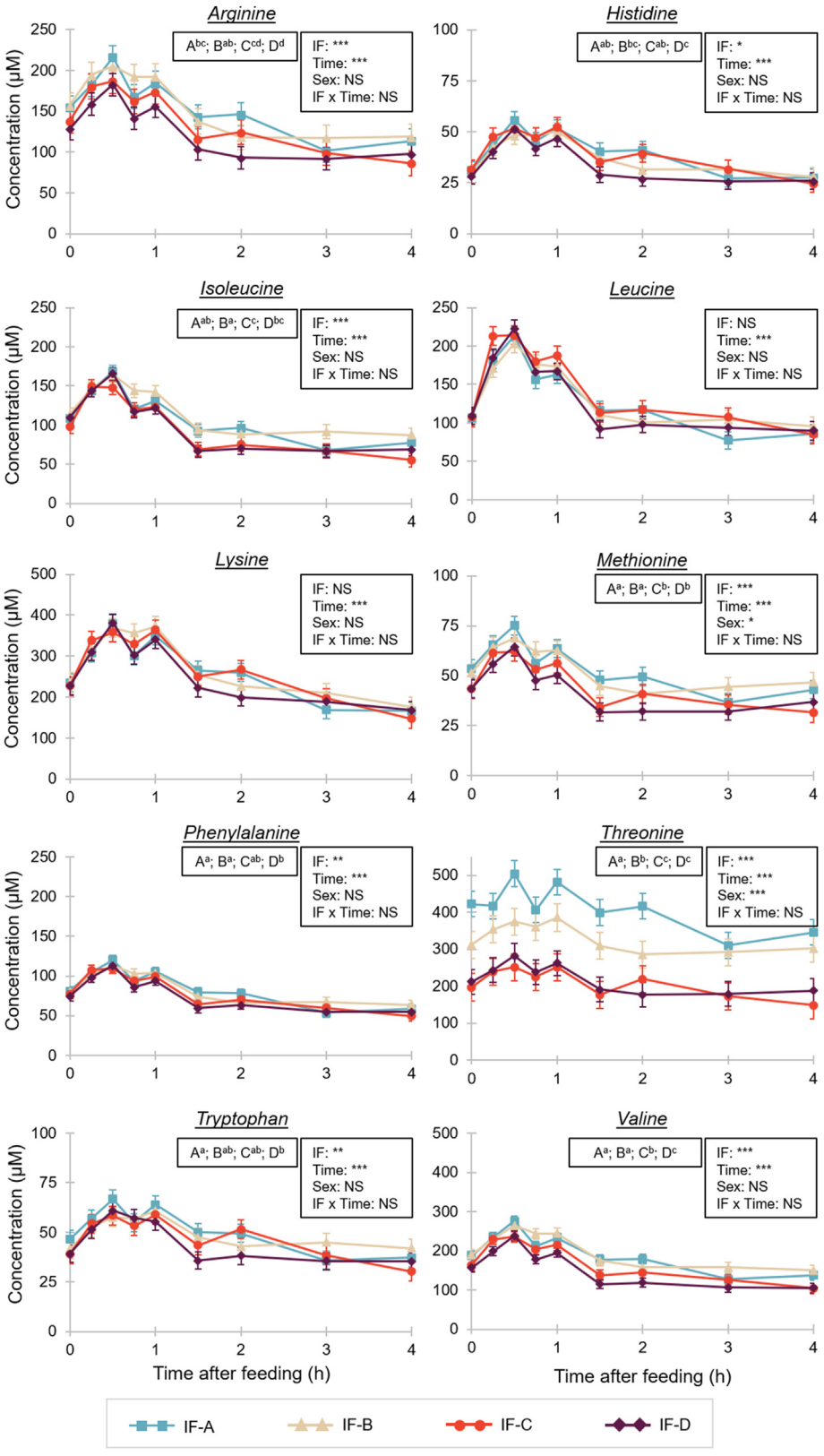

FIGURE 4. Time course of plasma concentrations of essential amino acids from baseline to 4 h postprandially in piglets fed IF-A, -B, -C, and -D. Means ± standard error, with $n = 8$ for IF-A, $n = 7$ for IF-B, $n = 7$ for IF-C, and $n = 9$ for IF-D. Statistical significance indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $P > 0.05$ (nonsignificant [NS]). IF, infant formula; A, B, C, and D for IF-A, IF-B, IF-C, and IF-D. ^{a,b,c,d} groups without a common letter differ significantly ($P < 0.05$).

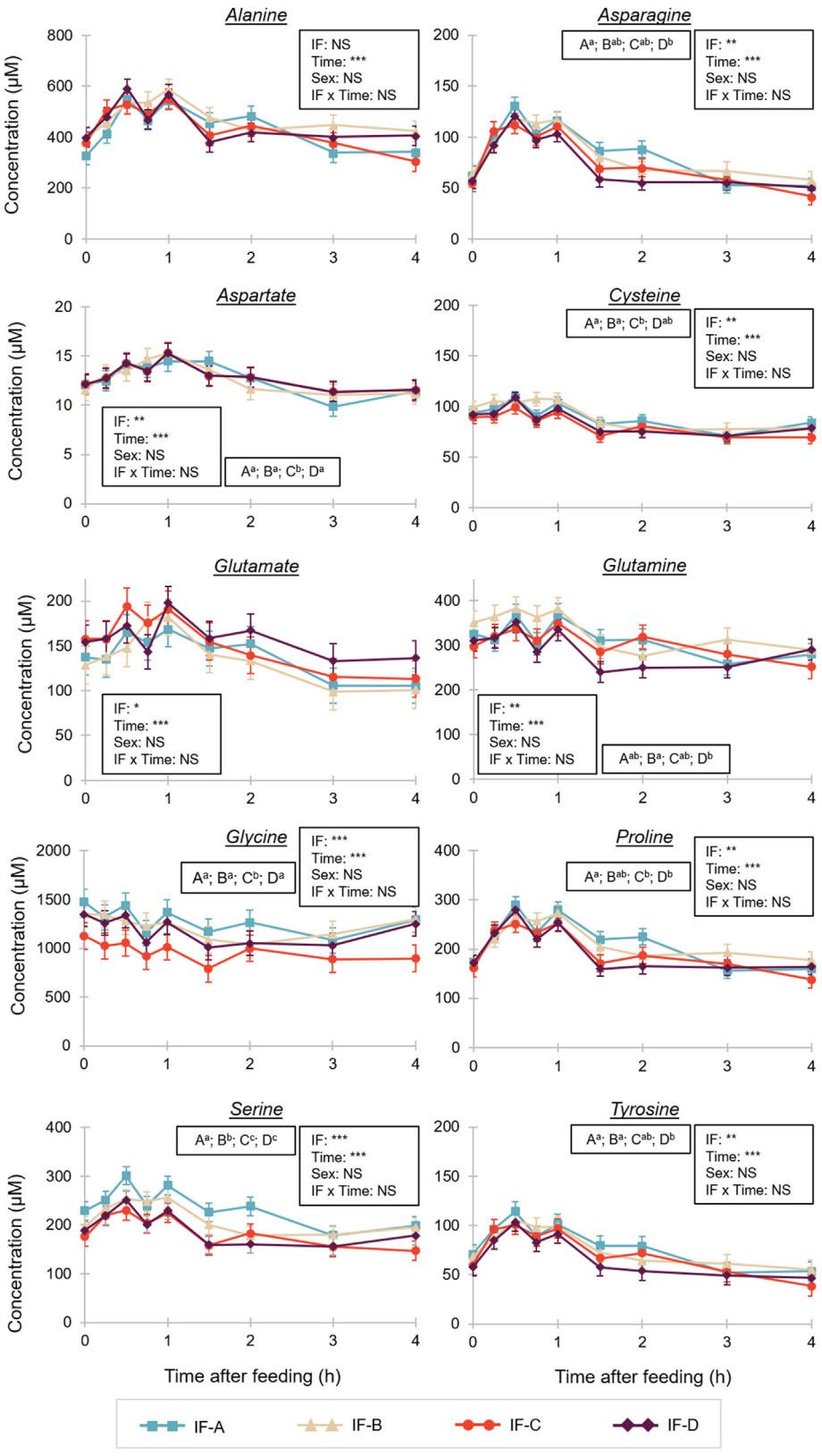

FIGURE 5. Time course of plasma concentrations of nonessential amino acids from baseline to 4 h postprandially in piglets fed IF-A, -B, -C, and -D. Means ± standard error, with $n = 8$ for IF-A, $n = 7$ for IF-B, $n = 7$ for IF-C, and $n = 9$ for IF-D. Statistical significance indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $P > 0.05$ (nonsignificant [NS]). IF, infant formula; A, B, C, and D for IF-A, IF-B, IF-C, and IF-D. ^{a,b,c} groups without a common letter differ significantly ($P < 0.05$).

Taken individually, the plasma concentration of EAAs (Figure 4) and NEAAs (Figure 5) peaked between 30 and 60 min postprandially, except plasma Gly concentration, which decreased over time. All individual plasma EAA (except Leu and Lys) and NEAA (except Ala) concentrations significantly differed among IFs, being significantly higher for IF-A than for IF-C and IF-D. The individual plasma AA concentrations in IF-A did not significantly differ from those in IF-B, except for Thr (+25% in IF-A) and Ser (+11% in IF-A). Individual IF-A plasma AA concentrations were higher than those in IF-C, with average differences between diets along the entire collection time varying from ~100% difference for Thr, 30% for Gly and Ser, and 17%–10% for Met, Val, Ile, Asp, and Pro. Individual IF-A plasma AA concentrations were also higher than those in IF-D, with average differences along the entire collection time varying from ~90% difference for Thr to 25% for Met, Val, Arg, and Ser and 18% to 9% for Tyr, Asn, His, Trp, Phe, Ile, and Pro. The plasma AA concentrations of IF-B were higher than those of IF-C with average differences along the entire collection time varying from 60% difference for Thr to 30% for Gly and 19%–10% for Ile, Met, Val, Ser, Arg, Asp, and Cys. Similarly, IF-B plasma concentrations were higher than those of IF-D, with average differences of 50% for Thr, 28%–23% for Val, Arg, and Met, and 16%–11% for Ile, Gln, Tyr, Phe, and Ser. Finally, the few differences in individual plasma AA concentrations between IF-C and IF-D were in His and Val (+14% and +10% in IF-C than in IF-D, respectively), and for Asp and Gly (−11% and −18% in IF-C than in IF-D, respectively).

Plasma nonproteinogenic AA and urea kinetics

There was a significant effect of time on plasma Citr, Orn, and 3-Mhis concentrations, but not on that of urea (Figure 6). Plasma 3-Mhis and urea concentrations were significantly lower in IF-A than in IF-B (−13% and −11%, respectively). IF-A also induced lower plasma urea than IF-C (−15%) and higher plasma Citr and Orn than IF-D (+30% and +11%, respectively). Plasma Citr, Orn and 3-Mhis concentrations of IF-B were significantly higher than those of IF-D (+31%, +19%, and +11%, respectively). IF-C induced significantly higher plasma Citr, Orn, and urea concentrations than IF-D (+24%, +15%, and +13%, respectively).

Plasma glucose and insulin kinetics

There was no significant effect of time and IF on plasma glucose concentration. Plasma insulin concentration significantly increased during the first 15 min postprandially for the 4 IFs with a decrease afterward, except for IF-B insulin concentration, which did not change after 15 min postprandially. However, no significant effect of IF was observed on plasma insulin concentration (Figure 7). No significant effect of IF was observed on the HOMA-IR, QUICKY, Matsuda, insulinogenic, and disposition indexes (Supplemental Figure 1).

Discussion

The present study clearly demonstrated that the protein ingredient quality (composition and structure) within IFs

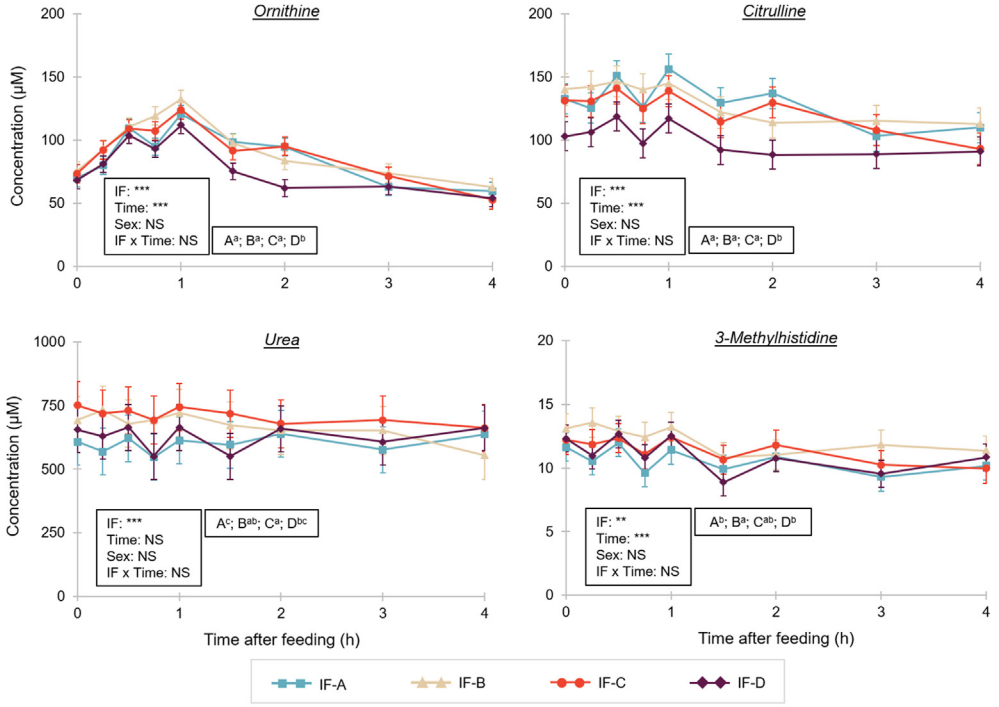

FIGURE 6. Time course of plasma concentrations of ornithine, citrulline, urea, and 3-methylhistidine from baseline to 4 h postprandially in piglets fed IF-A, -B, -C, and -D. Means ± standard error, with $n = 8$ for IF-A, $n = 7$ for IF-B, $n = 7$ for IF-C, and $n = 9$ for IF-D. Statistical significance indicated as ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and $P > 0.05$ (nonsignificant [NS]). IF, infant formula; A, B, C, and D for IF-A, IF-B, IF-C, and IF-D. ^{a,b,c} groups without a common letter differ significantly ($P < 0.05$).

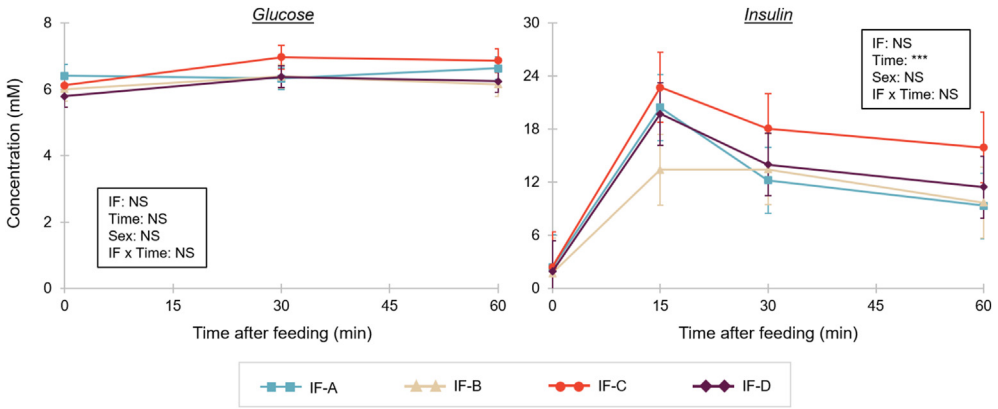

FIGURE 7. Time course of plasma concentrations of glucose and insulin from baseline to 1 h postprandially in piglets fed IF-A, -B, -C, and -D. Means ± standard error, with $n = 8$ for IF-A, $n = 7$ for IF-B, $n = 7$ for IF-C, and $n = 9$. Statistical significance indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $P > 0.05$ (nonsignificant [NS]). IF, infant formula; A, B, C, and D for IF-A, IF-B, IF-C, and IF-D.

affected plasma AA concentrations in the neonatal minipiglet, a well-established animal model for the human infant with respect to body size, gastrointestinal physiology, and AA metabolism [34,35]. The main differences were attributed to the WP ingredient source (cheese whey in IF-A and IF-B compared with ideal whey in IF-C and IF-D, respectively). In contrast, the impact of the IF microstructure, either depending on WP denaturation level (IF-A compared with IF-C) or casein supramolecular organization (IF-C compared with IF-D), was more limited. Interestingly, the 2-d adaptation to the experimental diets influenced the AA homeostasis, thus impacting both the preprandial and postprandial AA concentrations.

The postprandial kinetics of most AAs followed a bell-shaped curve with an increase in plasma concentration during the first 30–60 min after meal ingestion, in agreement with the literature [36,37]. This may reflect, at least partly, the food transit through the upper part of the digestive tract, and particularly the gastric emptying rate, as previously reported [24]. The postprandial AA peak time and the relative peak magnitude (after removal of the baseline) was similar among IFs, potentially suggesting similar gastric emptying despite differences in protein microstructures. The decrease of most plasma EAA concentrations occurred 15 min after the postprandial plasma insulin peak (occurring at 15 min postprandially), which agrees with the role of insulin as an anabolic hormone stimulating protein synthesis and intracellular AA transport [38]. The small volume of meal ingested by piglets in the present study, as required to enable a short ingestion time, led to earlier postprandial AA peak and return to baseline concentrations (90 min after meal) than that previously reported. In previous studies, plasma AA levels peaked at 90 min postprandially after ingestion of a WP solution in piglets [25] and returned to the preprandial baseline between 120 to 180 min after meal ingestion in preterm infants [37].

It is noticeable that the magnitude of the present postprandial plasma AA responses was lower than those previously observed by Welch-Jernigan et al. [25] due to the lower nitrogen intake in the present test meal (0.67 g protein/kg BW) than that in the previous test meal (3.75 g protein/kg BW [25]). Consequently, the plasma urea concentration did not change postprandially because the low nitrogen intake would probably not require excessive AA deamination during the postprandial blood sampling period.

Differences in plasma AA concentrations were mainly observed between piglets fed IF-A and IF-B compared with IF-C and IF-D, which indicated that the WP ingredient source (cheese whey and ideal whey, respectively) was a main factor influencing the plasma proteinogenic AA profile. In contrast, the impact of WP-induced or casein-induced differences in IF microstructures on proteinogenic plasma AA concentrations seems to be less obvious. Indeed, the plasma concentrations of most AAs in piglets fed IF-B and IF-C were different although these IFs presented a similar microstructure, with a mixture of both native and denatured/aggregated WPs in the soluble phase and with caseins adsorbed at the surface of fat droplets [12]. In addition, differences in plasma concentrations between IF-C and IF-D were only observed in 4 of 20 proteinogenic AAs (Arg, Gly, His, and Val), although their microstructures were previously shown to differ with large aggregates of caseins and WPs at the interface of flocculated fat droplets in IF-D [12]. These in vivo results corroborate those previously observed in vitro for IF-C and -D, which exhibit more similar proteolysis, but largely differing from that of IF-A [12]. Nevertheless, plasma concentrations of AAs involved in the urea cycle (Arg, Citr, Orn) and of urea, indicative of protein catabolism, were lower in the IF-D group than the IF-C group. Further investigation on the consequences of casein supramolecular organization on protein metabolism is thus needed.

The protein quality of IFs impacted both preprandial and postprandial plasma AA concentrations, demonstrating for the first time that the fine protein composition and, to a limited extent, the protein structure, within IFs influenced AA homeostasis. The observed IF-induced changes in preprandial plasma AA concentrations may suggest a rapid metabolic adaptation occurring after a 2-d period of IF consumption. The effect of such short-time dietary adaptation was reported in a previous study that evaluated the metabolic profiles of breastfed and formula-fed term infants at the plasma level [36]. Formula-fed infants presented higher plasma urea, Thr, and Val concentrations than breastfed infants at baseline and after meal ingestion. Other studies evaluating the impact of IF feeding on plasma AA contents in pigs and infants were performed either without any adaptation period [23,25,37] or with a unique blood sampling time [39–45], thus not allowing the comparison of pre- and postprandial plasma contents.

The differences in individual AA contents among IFs were due to the whey origin. The presence of GMP in cheese whey (IF-A and IF-B), an Ile-, Thr-, and Pro-rich peptide, explained most of the differences with the ideal whey-based IFs (C and D). GMP is the third most abundant polypeptidic chain in cheese whey after β-lactoglobulin and α-lactalbumin, accounting for 9%–15% of total protein in standard IFs. The unbalanced AA profile of GMP with only 47% of EAAs and without any aromatic AAs (Phe, Tyr, Trp), Arg, His, and Cys, limits its incorporation in IFs [18], whereas purified WPs contain 59% of EAAs [46]. Consequently, EAAs represents only 45% of TAAs in cheese whey, whereas they account for 52% of TAAs in ideal whey. Therefore, the differences observed for plasma AAs, such as Pro and Ile, reflected differences in the dietary AA profiles of IFs.

The plasma concentrations of Thr resulted from more complex mechanisms and did not strictly reflect IF AA profiles. Plasma Thr concentration differences were significantly higher than those observed among IFs, with a 10-fold higher difference between groups in the plasma than in the IFs, with the IF-A and -B groups having greater dietary and plasma levels of Thr than the IF-C and -D groups. This may be explained by the higher Thr level in the IF-A and -B than IF-C and -D diets combined with a limited Thr degradation ability in piglets. Thr dehydrogenase, the main catalytic enzyme of Thr in pigs, has been reported to have a limited activity during the first weeks of life [47], with no modulation of the dietary intake [48,49]. It was therefore possible that the increased Thr intake with IF-A and -B diets exceeded the ability of Thr degradation, resulting in a major modification of Thr homeostasis. It has also been reported that Thr concentration in plasma, as well as that in liver and brain, is positively correlated to dietary Thr intake in piglets and low-birth-weight infants [42,44,45,48,50], with an enhanced increase in plasma Thr concentration compared to dietary Thr content. A higher Thr intake also induced higher plasma Gly concentration in the IF-A and -B groups compared to the IF-C and -D groups, corroborating previous results reported by Sarwar et al. [48]. Gly is a product of Thr catabolism, thereby related to Thr metabolism.

Based on the present results, it is difficult to conclude which IF plasma AA responses are the most biomimetic of HM plasma AA responses. A previous study reported that HM induced lower plasma AA concentrations than IF in preterm infants [23]; however, the diets were not strictly isoproteic. Furthermore, it should be kept in mind that the present postprandial plasma AA concentrations as measured in the systemic circulation result not only from the protein digestion and absorption kinetics but are also impacted by the first-pass extraction from the splanchnic tissues and by the protein turnover and AA metabolism of the whole organism, which could differ among IFs. In the same way, in vitro AA bioaccessibilities [12] cannot be directly compared to in vivo plasma AA concentrations, knowing that the in vitro model did not include any intestinal brush border enzyme or absorption phase, and did not take into account the splanchnic extraction. The use of more invasive techniques such as permanent catheters in the portal vein combined with labeled dietary proteins (e.g., ^{13}C or ^{15}N) would have allowed a more accurate study of the metabolic fate of dietary AAs.

Overall, the present study highlighted that the quality of protein ingredient, and particularly the protein and AA profiles of WPs, greatly influence plasma AA patterns at baseline and postprandial times, with major differences related to the origin of WP ingredients (cheese whey compared with ideal whey). These results illustrate, for the first time, the necessity to take into consideration the protein ingredient sources and associated protein structure and composition specificities for IF manufacture. Whether the differences observed impacted the intestinal physiology and microbiota development remain to be investigated.

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Author contributions

The authors' responsibilities were as follows – LC: designed and conducted research, analyzed data, wrote article, and had primary responsibility for final content; AB, GH: analyzed data; YLG, SG, RJ, AC: conducted research; DD, ML, ILHL, AD: designed and supervised research, wrote and reviewed article, had primary responsibility for final content; and all authors: read and approved the final manuscript.

Conflict of interest

LC and ML are employed by SODIAAL International. All other authors report no conflicts of interest.

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Data availability

Data described in the manuscript, code book, and analytic code will not be made available because they are confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.05.009.

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