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Abbreviations: AA, amino acid; APE, atom percent excess; AP, atom percent; DAA, dispensable amino acid; DIAAS, digestible indispensable amino acid score, IAA, indispensable amino acid; IRMS, isotopic ratio mass spectrometry; FAO, Food and Agriculture Organization of the United Nations; UHPLC, ultra-high performance liquid chromatography.

Abstract

Measurement of ileal amino acids (AA) bioavailability is recommended to evaluate protein quality. A dual isotope tracer method, based on plasma isotopic enrichment ratios, has been proposed to determine true digestibility in humans. In a pilot study, we aimed to evaluate whether this method could be implemented in rats to determine AA bioavailability based on isotopic enrichment ratios measured in cecal digesta or plasma samples. Goat milk proteins were intrinsically labeled with ^{15}N and ^{2}H. Wistar rats were fed a meal containing the doubly-labeled goat whey proteins and a tracer dose of ^{13}C-spirulina. Blood samples were collected 0, 1 h and 3 h after meal ingestion from the tail vein. The rats were euthanized 4 h (n = 6) or 6 h (n = 6) after meal to collect plasma and intestinal contents. True orocecal protein digestibility and AA bioavailability were assessed by means of ^{15}N and ^{2}H enrichment in cecum content and compared with absorption indexes determined at the plasma or cecum level using isotopic ratios. Plasma kinetics of isotopic enrichment could not be completed due to the limited quantity of plasma obtained with sequential blood collection. However, the absorption indexes determined from cecal ^{15}N or $^{2}H/^{13}C$ ratios gave coherent values with true orocecal AA bioavailability. This dual isotope approach with measurements of isotopic ratios in digestive content could be an interesting strategy to determine true AA bioavailability in ileal digesta of rats.

Introduction

In 2013, the Food and Agriculture Organization of the United Nations (FAO) published their recommendation to use the digestible indispensable amino acid score (DIAAS) as the preferred index for protein quality (FAO 2013). The DIAAS is derived from indispensable amino acid (IAA) profiles and individual IAA ileal bioavailability. As stated in the FAO report, ileal bioavailability must be determined, in order of preference, in humans then in pigs, and alternatively, in rats. Direct measurements of ileal amino acid (AA) bioavailability based on their ileal disappearance can be performed in healthy humans equipped with a naso-ileal tube (though the procedure is invasive and thus cannot be used as a routine method) or in pigs equipped with an ileal T-cannula. For rats, in contrast to humans and pigs, there is no standardized direct method to determine ileal AA disappearance because rats cannot be equipped with a cannula to continuously collect intestinal digesta. Although a method has been proposed (Rutherfurd and Moughan 2003), it is based on the ingestion of repeated meals containing a non-absorbable marker and a single ileal sampling after euthanization of the rat, leading to several uncertainties.

The FAO has proposed a minimally invasive but indirect method to measure AA bioavailability in humans (FAO 2014). This dual isotope tracer method is based on measurement of plasma isotopic enrichment of individual AA in subjects having received a test protein intrinsically labeled with ^{2}H or ^{15}N and a reference protein intrinsically labeled with ^{13}C. ^{15}N-labeling has often been used to determine digestibility and metabolic utilization of animal or plant proteins in humans, pigs or rodents (Deglaire et al. 2009; Gaudichon et al. 1999; Mariotti et al. 1999; Oberli et al. 2016). For the digestibility dual isotope method, ^{2}H-labeled proteins have been recommended because in contrast to ^{15}N, there are few exchanges of deuterium through transamination processes.

Since it is minimally invasive, this dual isotope tracer method has been developed to determine AA bioavailability in humans. However, its use may be of interest to overcome the drawbacks of the classical methods actually used to assess AA bioavailability in preclinical studies, in rat model. The present study is a pilot study that aimed (i) to explore the use of a dual isotope tracer method in rats to determine proxy of AA bioavailability at the plasma and cecal level, and (ii) to compare performance of both isotopes, ^{15}N and ^{2}H. For this purpose, goat milk was labeled with ^{15}N and ^{2}H, and the milk

whey proteins were extracted and purified. In the first step, we used direct measurement in digesta to determine the protein digestibility and AA orocecal bioavailability of ^{15}N- and ^{2}H-labeled goat whey proteins as well as that of ^{13}C-spirulina, used as the reference protein in the dual isotope approach. In the second step, the dual isotope approach was applied to assess the AA cecal and plasma absorption indexes of goat whey proteins, and results were compared to the direct measurements of bioavailability.

Methods

^{15}N and ^{2}H intrinsic labeling of goat milk protein

Goat milk was ^{15}N and ^{2}H-labeled using a five-day labeling protocol at the INRAE (French National Institute for Agricultural Research and Environment) experimental unit of Thiverval-Grignon. Four goats received orally 5 g of ^{15}N ammonium sulfate (99%, Eurisotop, Saint-Aubin, France) for four consecutive days to reach a target enrichment of about 1 atom percent (AP) at the isotopic plateau, based on our previous milk labeling assays (Lacroix et al. 2006a; Mahe et al. 1994). In the absence of any previous experiments on ^{2}H-labeling of milk, the goats received either 80 or 160 mL of deuterated water ($^{2}H_2O$, 98%, Eurisotop, Saint-Aubin, France) for one or three consecutive days in drinking water (**Supplemental Table 1**). Milk was collected daily throughout the protocol to determine the ^{15}N and ^{2}H enrichment patterns. The milk with the highest ^{2}H enrichment was then processed at the LRGP (UMR CNRS 7274, Université de Lorraine, Nancy, France) to extract proteins. Milk was defatted and whey proteins were purified by diafiltration.

Rats and experimental design

This animal study was conducted in compliance with the EU directive 2010/63/EU for animal experiments and approved by the Ethics committee in animal experiments of INRAE (n° 17-20, Jouy-en-Josas, France) and the French Ministry of Higher Education and Research (APAFIS#11921-2017091818236657). Twelve adult, male Wistar Rcc:Han rats initially weighing 300 g were purchased from Harlan Laboratories and used in the study. Rats were housed individually under controlled conditions (12-h light/dark cycle, lights off at 10:00, constant room temperature of 22°C) in cages with

wire bottoms to prevent coprophagia. Environmental enrichment included plastic tubes in the cage and the rats were manipulated every week day for body weight evaluation and health check. They were adapted over one week to the animal facility conditions. Rats were fed an AIN modified standard milk protein diet that provided 14.5% of energy from proteins (**Table 1**). It was available *ad libitum* from 12:00 to 18:00. At 10:00, they received a small 4 g calibrated meal of this diet, which was consumed in less than 15 minutes. This specific dietary intake pattern made it possible to include the labeled test protein in the calibrated meal, as previously described (Lacroix et al. 2006a). Criteria for exclusion of rats of the study were body weight decrease of ≥ 20% and refusal to eat the calibrated meal. No rats were excluded from the study. After 10 days of adaptation, 0.48 g of ^{15}N and ^{2}H-labeled goat milk proteins were included in the calibrated meal (Table 1). A tracer dose of 5 mg of ^{13}C-spirulina (97%, Eurisotop, Saint-Aubin, France) was incorporated in the meal. Approximately 1 to 1.5 mL of blood was also collected with a heparinized syringe from the tail vein of rats in the fasting state, and 1 h and 3 h after meal intake. Six rats were euthanized 4 h after the test meal, and six rats were euthanized 6 h after test meal ingestion, all with pentobarbital sodium anesthesia (100 mg/kg intraperitoneally). Allocation of the rats in the 2 groups was done alternatively and this was a not blinded experiment. The timing was chosen to collect the digesta 6 h after meal ingestion, allowing a compromise between complete digestion and minimal duration of cecal fermentation. In addition, this provided enough plasma samples at 4 h after meal ingestion for analyses of isotopic enrichment in individual plasma AA in order to test the dual isotope tracer method for the determination of a proxy of AA bioavailability. Isotopic enrichment is at the highest level in rat plasma 4 h after meal ingestion (Morens et al. 2000). At euthanasia, heparin was injected into the abdominal cavity, and approximately 10 mL of blood was withdrawn from the abdominal cavity following rupture of both the caudal vena cava and the aorta. Blood was ice-stored in individual glass tubes and centrifuged (20 min, 3000 g, 4°C). The plasma was stored at -80°C for subsequent analyses. Gastrointestinal segments were identified as stomach, small intestine, ileum (defined as the last 10 cm before the cecum), cecum and colon. Feces were also collected. Luminal contents of these segments were rinsed with NaCl solution (9‰), collected entirely and stored at -20°C.

Analytical procedures

Total ^{15}N and ^{2}H enrichments

Milk proteins and gastrointestinal samples were freeze-dried prior to analysis. Nitrogen percentage and ^{15}N enrichment in protein isolate, digestive samples and meal were determined using an isotopic ratio mass spectrometer (IRMS; Isoprime, GV Instrument, Manchester, UK) coupled with an elemental analyser (EA; Vario Micro, Elementar, Lyon, France).

Hydrogen percentage and ^{2}H enrichment in milk protein isolate, digestive samples and meal were determined using a high temperature conversion elemental analyser (ThermoFisher Scientific) coupled to IRMS (Delta V Plus, ThermoFisher Scientific).

^{15}N, ^{13}C and ^{2}H individual amino acid enrichments

For isotopic measurements in plasmatic individual AA, analytical development was necessary to establish the protocol of AA extraction using the lowest volume of plasma without compromising the precision of isotopic measurements. Minimal volumes of plasma samples were 2 mL for ^{15}N and ^{2}H analyses and 0.5 mL for ^{13}C analyses. Consequently, an insufficient quantity of plasma was obtained from tail blood sampling. In addition, an insufficient quantity of plasma remained for ^{2}H analysis after analysis of ^{13}C and ^{15}N AA enrichment in plasma for rats euthanized at 4 h and 6 h after meal intake.

Briefly, AA from whey proteins and cecal samples hydrolysates and plasma samples were purified and analyzed as *N(O)*-ethoxycarbonyl ethyl ester derivatives by an Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an IRMS (Isoprime, GV Instrument, Manchester, UK) *via* the GC5 Isoprime interface (for details see Supplemental methods).

Amino acid concentrations

For AA concentration analysis, 10 mg of lyophilized gastrointestinal or meal samples were hydrolyzed using 6 M HCl at 110°C for 24 h with norvaline as an internal standard. The AA analysis was performed using the AccQ-Tag Ultra method (Waters Corporation, Milford, MA) as previously described (Tessier et al. 2020b).

Determination of protein digestibility and amino acid bioavailability

Protein digestibility of AA bioavailability refers to the ratio between the amount of protein or AA absorbed by the small intestine and the amount ingested. It is usually evaluated by determining the dietary protein or AA that are not absorbed in the small intestine and are recovered in the ileal digesta. In our model, orocecal protein digestibility or AA bioavailability was used as a proxy for oroileal protein digestibility or AA bioavailability to have enough digestive content for isotopic enrichment analyses (Lacroix et al. 2006b; Oberli et al. 2016; Tessier et al. 2020b). We assumed that 6 h after meal ingestion, a majority of dietary protein or AA not absorbed in the small intestine accumulated in the cecum and that microbial fermentation was minimal. Dietary protein or AA still in the stomach or small intestine were not taken into account in the digestibility/bioavailability calculations. Indeed, we assumed that the quantity of dietary protein or AA in those compartments 6 h after meal ingestion was very low and would be mainly digested and absorbed, especially for a highly digestible protein such as whey.

Protein digestibility

The dietary nitrogen or hydrogen ($N_{diet\ x}$ or $H_{diet\ x}$ in mmol) not absorbed in the intestinal tract and recovered in digesta of the compartment 'x' (ileum, cecum, colon or feces) of rats euthanized 6 h after meal ingestion was calculated as follows:

$$N_{diet\,x} = N_{tot} \times \frac{APE_s}{APE_m} \quad \text{or} \quad H_{diet\,x} = H_{tot} \times \frac{APE_s}{APE_m} \qquad \textit{[Formula 1]}$$

where N_{tot} or H_{tot} is the total amount of nitrogen or hydrogen in the sample (calculated from its nitrogen or hydrogen percentage and its weight, in mmol), and APE_s and APE_m are the ^{15}N or ^{2}H enrichment excess in the samples and in the meal ($APE_{s\ or\ m}$ = atom $\%_{s\ or\ m}$ - enrichment of digesta samples of rats fed a standard diet with unlabeled protein under comparable experimental conditions, from (Guillin et al. 2020)).

The true orocecal digestibility refers to the proportion of ingested protein that is breakdown into absorbable constituents and absorbed by the small intestine between oral intake and cecum, taking into account basal and specific gut endogenous losses. It was calculated based on the dietary nitrogen or

hydrogen not absorbed and recovered in the ileum and cecum 6 h after meal ingestion as calculated by formula 1. It was calculated according to formula 2:

$$\text{Orocecal digestibility (\%)}_{\text{N}} = 100 \times \frac{\text{N}_{\text{ing}} - (\text{N}_{\text{diet ileum}} + \text{N}_{\text{diet cecum}})}{\text{N}_{\text{ing}}}$$

or

$$\text{Orocecal digestibility (\%)}_{\text{H}} = 100 \times \frac{\text{H}_{\text{ing}} - (\text{H}_{\text{diet ileum}} + \text{H}_{\text{diet cecum}})}{\text{H}_{\text{ing}}} \qquad \textit{[Formula 2]}$$

where N_{ing} or H_{ing} is the total amount of ingested nitrogen or hydrogen (in mmol), N or $H_{diet\ ileum}$ and N or $H_{diet\ cecum}$ are the amounts of dietary nitrogen or hydrogen (in mmol) recovered in the ileum and the cecum, respectively, 6 h after the test meal. The true whey protein orofecal digestibility was calculated according to the same formula as true whey protein orocecal digestibility, except that dietary nitrogen or hydrogen recovered in the colon and feces was taken into account.

Individual amino acid bioavailability

Individual AA orocecal bioavailability of whey protein (^{15}N and ^{2}H) and ^{13}C-spirulina was determined. The amount of dietary AA ($AA_{diet\ x}$, in mmol) not absorbed and recovered in digesta of the compartment 'x' (ileum, cecum) of rats euthanized 6 h after meal ingestion was determined for each AA according to formula 3:

$$\text{AA}_{\text{diet x}} = \text{AA}_{\text{tot}} \times \frac{\text{APE}_{\text{s}}}{\text{APE}_{\text{m}}} \qquad \textit{[Formula 3]}$$

where AA_{tot} is the amount of individual AA in the sample (calculated from its AA content and its weight, in mmol), and APE_s and APE_m are the ^{15}N, ^{13}C or ^{2}H enrichment excess of the AA in the sample and meal, respectively ($APE_{s\ or\ m}$ = atom $\%_{s\ or\ m}$ - enrichment of the samples of rats fed a standard diet without labeled protein from (Guillin et al. 2020)).

The true individual AA orocecal bioavailability was calculated based on the dietary AA recovered in the cecum 6 h after meal ingestion as calculated by formula 3 and according to the following formula 4:

$$\text{AA orocecal bioavailability (\%)} = 100 \times \frac{\text{AA}_{\text{ing}} - \text{AA}_{\text{diet cecum}}}{\text{AA}_{\text{ing}}} \qquad \textit{[Formula 4]}$$

where AA_{ing} is the total amount of ingested AA (in mmol) and $AA_{diet\ cecum}$ is the amount of dietary AA (in mmol) not absorbed and recovered in the cecum.

The principle of the dual isotope tracer method was to estimate the cecal dietary AA from whey not absorbed 6 h after meal ingestion, relative to not absorbed cecal dietary AA from ^{13}C-spirulina, and to calculate digestive AA absorption index as a proxy of AA orocecal bioavailability, using either ^{15}N or ^{2}H. The percentage of dietary AA from spirulina not absorbed and found in the cecum corresponds to 100 - orocecal AA bioavailability of spirulina calculated by formula 4. This value was corrected by the ratio of ^{15}N or ^{2}H/^{13}C AA enrichment in cecum on ^{15}N or ^{2}H/^{13}C AA enrichment in meal. Then, the digestive AA absorption index was calculated according to formula 5:

$$\text{Digestive 15N AA absorption index} = 100 - \left[(100 - \text{AA bioav. spi}) \times \frac{\frac{15N}{13C}\text{cecum}}{\frac{15N}{13C}\text{meal}}\right]$$

And

$$\text{Digestive 2H AA absorption index} = 100 - \left[(100 - \text{AA bioav. spi}) \times \frac{\frac{2H}{13C}\text{cecum}}{\frac{2H}{13C}\text{meal}}\right]$$

[Formula 5]

where ^{15}N/^{13}C or ^{2}H/^{13}C cecum and meal are the enrichment ratios of the AA in cecal samples and meal, respectively, and AA bioav. spi is the AA bioavailability of the ^{13}C spirulina tracer dose as calculated by formula 4.

For comparison, the plasma absorption index of each AA was also calculated with formula 6 in rats euthanized 4 h after meal ingestion as it is understood to be the peak of isotopic enrichment following a meal (Morens et al. 2000):

$$\text{Plasma AA absorption index} = \text{AA bioav. spi} \times \frac{\frac{15N}{13C}\text{plasma}}{\frac{15N}{13C}\text{meal}} \qquad \textit{[Formula 6]}$$

where ^{15}N/^{13}C plasma and meal are the enrichment ratios of the AA in plasma and meal, respectively, and AA bioav. spi is the AA bioavailability of the ^{13}C spirulina tracer dose as calculated by formula 4.

206

207 **Statistical analysis**

208 A power calculation was performed to determine the sample size required to detect significant
209 differences between AA bioavailability and absorption index with a statistical power of 80% and α
210 level set at 0.05. Since the dual isotope tracer method used for AA bioavailability has not been applied
211 in rats previously and this was a pilot study, we estimated that a difference of less than 5% between
212 AA bioavailability and absorption index was acceptable. According to former studies, interindividual
213 variability in AA bioavailability measured at caecal level with comparable isotopic method in rats was
214 around 1% (Tessier et al. 2020a; Tessier et al. 2020b). In Humans, interindividual variability in AA
215 bioavailability measured with the dual isotope method was around 3.5% (Calderon de la Barca et al.
216 2021; Kashyap et al. 2019b; Kashyap et al. 2018). Hence, a sample size of six animals per group has
217 been determined (G*Power 3.1).

218 The results are presented as means and their standard deviations (SD). Comparisons between
219 bioavailability assessed by ^{15}N and ^{2}H enrichment, or bioavailability and digestive or plasma
220 absorption indexes, were performed using a paired t-test with Prism 6.04 (Graph Pad Software Inc.).
221 Normality of data was tested with Shapiro-Wilk test. Significance was considered to be reached at $P <$
222 0.05.

223

224 **Results**

225 **^{15}N and ^{2}H labeling of milk proteins and amino acids**

226 The ^{15}N enrichment gradually increased during the first three days of ^{15}N ammonium sulfate
227 administration, reaching a plateau of 1.00 ± 0.07 AP (**Fig. 1A**). Regardless of the $^{2}H_2O$ dose, three-day
228 administration (goats 2 and 4) enabled a greater ^{2}H enrichment than one day (goats 1 and 3), reaching
229 0.032 AP and 0.051 AP with 80 mL (goat 3) and 160 mL (goat 4), respectively (**Fig. 1B**). In contrast to
230 ^{15}N, the ^{2}H isotopic plateau was not reached after the three-day administration.

231 The ^{15}N enrichment was determined in 14 AA and increased from day 1 to day 3 to a plateau of
232 0.94 ± 0.11 AP, on average. The ^{15}N enrichment was uniform across individual AA, varying from 0.87
233 ± 0.05 AP for threonine to 1.06 ± 0.07 AP for serine (except for histidine, 0.61 ± 0.03 AP) (**Fig. 2A**).

The ^{2}H enrichment was determined in 14 AA (**Fig. 2B**). The highest dose of deuterated water combined with three-day administration enabled greater ^{2}H enrichment in AA on the last day of the protocol (goat 4, day 5, **Fig. 3A**). In this goat, the ^{2}H enrichment in individual AA gradually increased during and after $^{2}H_2O$ administration (day 1 to day 5, **Fig. 3B**). However, labeling covered a wider range across AA, varying from 0.04 AP for phenylalanine to 0.27 AP for alanine (Fig. 2B). The highest enrichments were observed in dispensable AA (alanine, proline, glutamate, glycine; Fig. 2B). The milk from the last day of collection from goat 4 was selected and processed for extraction and purification of whey proteins used in the *in vivo* study.

Whey protein digestibility evaluated by ^{15}N and ^{2}H

We determined the amount of dietary nitrogen and hydrogen, expressed as the percentage of nitrogen or hydrogen from whey proteins ingested (originating from labeled goat milk) and that remained in the gastrointestinal tract 6 h after the meal (**Fig. 4**). Most of the dietary nitrogen or hydrogen non-absorbed was found in the cecum. The comparison of dietary nitrogen and hydrogen remaining in the stomach, ileum or cecum revealed no differences, whereas in the small intestine and colon, recovery of dietary hydrogen was significantly reduced compared to nitrogen ($P < 0.001$ and $P = 0.02$, respectively).

The true orofecal digestibility of whey proteins was comparable between both isotopes, with values of 97.7 ± 0.4% and 97.8 ± 0.2%, respectively ($P > 0.05$). Similarly, the true orocecal digestibility of whey proteins was comparable (98.2 ± 0.4% for dietary nitrogen and 97.9 ± 0.1% for hydrogen, $P > 0.05$).

Individual amino acid bioavailability of ^{15}N and ^{2}H goat whey and ^{13}C spirulina

True orocecal bioavailability of individual spirulina (**Table 2**) and whey protein (**Table 3**) AA were determined from ^{15}N and ^{2}H or ^{13}C enrichment in meal and cecum content collected 6 h after meal ingestion. For ^{13}C-spirulina, true orocecal bioavailability values of AA varied from 71.3 ± 5.0% for serine to 96.4 ± 0.4% for glutamate, with an average AA bioavailability of 90.2 ± 1.5% (Table 2). With ^{15}N, true orocecal bioavailability of whey AA (Table 3) varied from 95.4 ± 2.1% for glycine to

99.3 ± 0.1% for proline, with an average AA bioavailability of 98.3 ± 0.3%. When assessed with ^{2}H, true orocecal bioavailability of nearly all whey individual AA (except alanine and glycine) were significantly lower than when assessed with ^{15}N (paired *t*-test, $P < 0.001$ to $P = 0.002$ depending on the AA). Hence, true orocecal bioavailability of whey AA determined from ^{2}H enrichment varied from 91.3 ± 1.0% for threonine to 99.0 ± 0.1% for proline, with an average AA bioavailability of 95.7 ± 0.5%.

Digestive and plasma absorption indexes of whey amino acids determined with a dual isotope tracer method

Based on the cecal AA bioavailability values of ^{13}C-spirulina as well as the ^{15}N or ^{2}H and ^{13}C enrichments of individual AA in cecal samples collected 6 h after meal intake, we calculated the digestive absorption indexes obtained from the ratio method of the dual isotope approach (Table 3). They were compared to the true orocecal bioavailability values obtained with the standard direct method. Using ^{15}N, the values obtained with the ratio method were close to the bioavailability values, with no significant difference for mean AA bioavailability. The highest discrepancies were observed for serine, with an underestimation of the digestive absorption index of 1.9%, and for glycine with a difference of 4.3%. Although statistical differences were observed for several other AA, they were relatively small (mean of 0.2%). Higher discrepancies were found with ^{2}H. The digestive absorption indexes underestimated the values for serine (3.4%) and glycine (5.3%) but overestimated the values for tyrosine (2.8%) and threonine (3.6%). As a result, the ^{2}H digestive absorption index significantly overestimated the mean AA bioavailability by 1.3%. Nevertheless, the differences between orocecal bioavailability and digestive absorption ratio with ^{2}H were physiologically small. Comparison of ^{15}N and ^{2}H revealed that digestive absorption indexes with ^{2}H were significantly lower than ^{15}N for nearly all AA, similarly as for directly assessed orocecal bioavailability. Overall, values obtained with the ratio method were consistent, especially with ^{15}N and when considering the IAA.

In addition, based on the true cecal AA bioavailability values of ^{13}C-spirulina and the ^{15}N and ^{13}C enrichments of free AA in plasma, the plasma absorption indexes were also calculated according to a dual isotope tracer method. We were not able to determine the enrichment kinetics in plasma due

to insufficient plasma collected with repeated blood sampling at 0, 1 and 3 h after meal intake. Hence, we calculated the ^{15}N-to-^{13}C enrichment ratio in plasma obtained at euthanasia, 4 h after meal intake. However, all values (except for threonine) were inconsistent with the values obtained with the standard method (**Supplemental Table 2**), and several values were incoherent and higher than 100%. The inter-individual variability was extremely high. No data were available for the plasma absorption index determined with ^{2}H owing to insufficient quantity of plasma available.

Discussion

This study assessed the feasibility of a dual isotope tracer method to measure AA bioavailability in rats. The true bioavailability of whey proteins (by means of ^{15}N and ^{2}H) and spirulina (^{13}C) AA was directly determined at the cecal level. In parallel, the AA enrichment ratios of the dual isotope method were applied at the cecal and plasma levels. Whereas results of this dual isotope tracer method applied in plasma was inconclusive, the digestive absorption indexes determined from the cecal ratios ^{15}N/^{13}C and ^{2}H/^{13}C gave coherent values with true orocecal AA bioavailability. The use of isotopic ratios measured at the digestive level appears to be a possible approach to determine digestive AA losses and consequently, true AA bioavailability in rats.

Goat milk protein was intrinsically and uniformly labeled. Classically, ^{15}N or ^{13}C intrinsic labeling of protein has been used to assess the digestive and metabolic fate of dietary proteins (Boirie et al. 1997; Daenzer et al. 2001; Gaudichon et al. 1999; Mahe et al. 1994). Intrinsically ^{15}N-labeled milk can be easily obtained by adding ^{15}N ammonium sulfate to the diet of lactating ruminants (Gaudichon et al. 1999), as in the present study where proteins were uniformly labeled at a level of approximately 1 AP. Recently, ^{2}H-labeled proteins were used to determine true ileal digestibility of IAA using a minimally invasive dual isotope tracer method (Devi et al. 2020; Shivakumar et al. 2019; Kashyap et al. 2019a; Kashyap et al. 2018; Devi et al. 2018). Regular watering with deuterated water during plant growth was used to obtain rice, finger millet, mung bean and chick pea ^{2}H-labeled proteins (Shivakumar et al. 2019; Devi et al. 2018). Feeding animals with a mixture of [U-^{2}H]-labeled crystalline AA mix enabled production of ^{2}H-labeled hen eggs and meat (Kashyap et al. 2019a;

318 Kashyap et al. 2018). In the present study, we labeled goat milk proteins by orally-administered
319 deuterated water to goats. The ^{2}H-labeling, however, was variable among AA and relatively low from
320 0.04 to 0.15 AP, which represents 2.5 to 17 times the natural abundance. Higher doses (for instance,
321 200 mL instead of 160 mL) or a longer duration of deuterated water administration (for instance, five
322 days instead of three days) would certainly have resulted in higher enrichments.
323 Deuterium is preferred in human dual isotope studies because, in contrast to ^{15}N, it is minimally
324 affected by transamination (Devi et al. 2018). However, deuterium enrichment in AA may be affected
325 by other factors, such as addition of water ($^{1}H_2O$) during hydrolysis or exchange between ^{2}H and ^{1}H
326 during reactions of intermediary metabolism. By doubly-labeling milk protein, we were able to
327 compare the performance of both tracers. Lower ^{2}H enrichment was found in the colon and small
328 intestine; this can be ascribed to the dilution of ^{2}H in other non-enriched compounds, thus reducing the
329 enrichment to below the threshold of analytical detection for some rats. No difference was observed in
330 fecal or cecal protein digestibility determined with ^{15}N and ^{2}H. In contrast, small but significant
331 differences, ranging from 0.2% to 6.6%, were detected regarding orocecal bioavailability of individual
332 AA. There was systematic underestimation with ^{2}H compared to ^{15}N. This is in line with a previous
333 study in which we reported consistent values between both isotopes but with variations ranging from
334 0.5 to 3%, depending on the AA (Tessier et al. 2020b). In the present study, it seems that the highest
335 ^{2}H-enriched AA in milk presented the smaller differences in bioavailability when compared with ^{15}N.
336 It is thus possible that the level of enrichment in ^{2}H of few AA (phenylalanine and serine) was not
337 sufficient to trace them efficiently in the gastrointestinal tract, and thereby to accurately determine
338 their bioavailability under our experimental and analytical conditions. Regardless of the isotopes, the
339 recycling of the ^{15}N or ^{2}H in the digestive enzymes may result in an overestimation of endogenous
340 losses. However, the resulting underestimation of bioavailability is relatively small and has been
341 estimated to be about 1% (Deglaire et al. 2020).
342
343 We explored implementation of a dual isotope approach in rats. Such an approach was
344 conducted in the view of proposing a novel method to determine AA bioavailability at the ileal level in
345 rats. AA ileal bioavailability of protein sources should be preferably determined in humans, but if this

346 is not possible, animal models such as pigs or rats can be used (FAO 2013). The determination of AA
347 bioavailability by the classical method requires quantitatively evaluating the amount of digesta
348 throughout the post-prandial period. There are two options to do so. One is to collect the entirety of
349 ileal post-prandial digesta, which is not feasible using a cannula in rats. The second is to collect ileal
350 samples at a single time point after repeated meal feeding and to estimate the total recovery by using a
351 non-absorbable marker in the test meal. However, the latter has been associated with errors as accurate
352 measurement of these markers is difficult, and interactions between the markers and protein has
353 occurred (Kim et al. 2020; Prawirodigdo et al. 2019). The ileal content is also limited in rats, about 50
354 to 150 mg of dry matter, which is not sufficient to analyze current non-absorbable marker
355 concentration, AA content and AA isotopic enrichments. One option is to pool the ileal digesta of
356 several rats to obtain a larger digestive content. Another option is to use a method consisting of
357 quantitatively collecting the cecal digesta during a limited post-digestion period (5-6 h) and
358 determining cecal, instead of ileal, bioavailability (Lacroix et al. 2006b; Oberli et al. 2016; Tessier et
359 al. 2020b). In our opinion, this is a good compromise between limiting the fermentation time in the
360 cecum and having a complete digestion of the test meal. Orocecal bioavailability values of AA
361 obtained in rats are comparable with ileal data obtained in pigs and humans (Tessier et al. 2020b;
362 Deglaire et al. 2009), which suggests that orocecal bioavailability may be an adequate proxy of ileal
363 AA bioavailability. However, fermentation occurs in the cecum, and the possibility that the values of
364 cecal AA bioavailability are over- or underestimated in comparison to ileal bioavailability cannot be
365 excluded.

366 The dual isotope tracer method applied to evaluate AA bioavailability is an indirect method, as
367 absorption indexes are related to bioavailability of a reference protein. Therefore, implementing this
368 method at the ileal level may overcome drawbacks of the classical method in the rat model, such as
369 use of non-absorbable markers. In the present study, we evaluated this approach to compare the data
370 obtained with orocecal bioavailability. In humans, isotopic enrichment ratios of ^{15}N or $^{2}H/^{13}C$ are
371 evaluated in plasma in the hours following meal intake. Plasma absorption indexes are calculated with
372 the area under the curve or the mean ratio at a plateau state (FAO 2014). However, this approach
373 cannot be easily applied in rats owing to practical issues. After analytical development and

optimization, we set the minimal amount of plasma necessary to accurately evaluate isotopic enrichment in individual AA at 2 mL for ^{15}N and ^{2}H and 0.5 mL for ^{13}C. The volume of blood obtained by sequential sampling in the tail vein of rats is thus not sufficient (< 1.5 mL). We attempted to measure the enrichment ratio at the supposed plasma peak of isotopic enrichment (4 h after meal intake according to Morens et al. (Morens et al. 2000)), but it was inconclusive. To summarize, the results of plasma absorption indexes of this pilot study show that currently, the minimally invasive dual isotope tracer method which is currently used in humans and based on the plasma ratio is not appropriate in rats. Further analytical development is needed to drastically reduce the quantity of plasma used (< 0.5 mL for ^{2}H and ^{15}N) to determine isotopic enrichment and consider this dual isotope method applied in plasma in rats. The use of LC-MS/MS is a way to reduce the amount of sample needed as it was shown to be as performant as GC-C-IRMS (Borno et al. 2014; Zabielski et al. 2013). However, it is accurate for the determination of ^{13}C enrichment because the labelling pattern of U-^{13}C spirulina is known, but this is not the case for ^{2}H or ^{15}N enrichments from intrinsic labeled proteins and GC-c-IRMS has to be used (Devi et al. 2020; Devi et al. 2018; Kashyap et al. 2019b).

Based on the differential isotopic AA enrichments, we calculated the amount of AA losses from goat whey compared to the ^{13}C-AA losses from spirulina, and thus their bioavailability. The AA absorption indexes were calculated with the cecal $^{15}N/^{13}C$ and $^{2}H/^{13}C$ enrichment ratios. Comparable values of digestive absorption indexes and orocecal bioavailability were obtained for both ^{15}N and ^{2}H. With ^{15}N, the differences between digestive absorption indexes and orocecal bioavailability of AA were smaller than with ^{2}H and these differences were mainly not physiologically relevant (< 0.5%) for most AA, especially IAA. The main discrepancies were for glycine and serine for both isotopes, and for threonine for ^{2}H, but differences were not higher than 6%. The spirulina cecal bioavailability of glycine and serine was reduced compared to other AA and presented high inter-individual variabilities. This may have led to uncertainties in determination of the absorption indexes for these two AA. To summarize, despite some differences, digestive absorption indexes and true orocecal bioavailability were comparable for most AA and for both isotopes, ^{15}N and ^{2}H. A dual isotope approach in cecal digesta may thus be of interest to implement this qualitative method at the ileal level in rats, because it has the potential to overcome the drawbacks of the classical method in this model. Both ^{15}N- and ^{2}H-

labeling may be used in this approach since AA are supposed to be minimally affected by transamination in the digestive tract.

In this pilot study, we explored a dual isotope tracer approach in rats by using ^{2}H and ^{15}N doubly-labeled whey proteins and a tracer dose of ^{13}C-spirulina as a reference protein to evaluate AA bioavailability. We determined the true bioavailability of whey AA at the cecal level. We showed that the bioavailability was slightly underestimated with ^{2}H in comparison to ^{15}N. Digestive absorption indexes determined at the cecal level gave consistent values with true orocecal bioavailability, especially for IAA, for the highly digestible protein tested in our study. In conclusion, a dual isotope tracer approach and the enrichment ratio method seem to be a possible approach to determine AA bioavailability in rats. Additional studies with different sources of proteins (with variable bioavailability) are warranted to further develop this approach and applied it at the ileal level.

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Code availability: Not applicable.

Authorship: The authors' responsibilities were as follows - CG, DT: designed the research; AE: labeled the goat milk; SB: extracted and purified the goat milk proteins; CG, DT, JC, NK: conducted the research, analyzed data and wrote the paper; JC, CG: had primary responsibility for final content; and all authors: read and approved the final manuscript.

Ethics approval: This study was conducted in compliance with the EU directive 2010/63/EU for animal experiments and approved by the Ethics committee in animal experiments of INRAE (n° 17-20, Jouy-en-Josas, France) and the French Ministry of Higher Education and Research (APAFIS#11921-2017091818236657).

Consent to participants: This research involved animals.

Consent for publication: Not applicable in this study.

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Tables

Table 1. Meal composition of standard diet and goat milk.

	Standard diet		**Goat milk calibrated meal**	
	g/kg DM	% of energy	g/kg DM	% of energy
Protein	140.0	14.5	120.0	13.9
Soybean oil	40.0	10.4	42.5	10.8
Starch	622.4	63.1	630.0	62.5
Sucrose	100.3	10.9	110.0	11.8
Cellulose	50.0	0.0	50.0	0.0
Minerals	35.0	0.0	35.0	0.0
Vitamins	10.0	1.1	10.0	1.0
Others	2.3	0.0	2.5	0.0

Composition in mass (g/kg of dry matter (DM)) and energy supply (% of energy provided).

Table 2. Orocecal bioavailability (in % of amino acids ingested) of individual amino acids from tracer dose of ^{13}C-spirulina determined from cecal ^{13}C enrichment 6 h after meal ingestion.

	Orocecal bioavailability of spirulina (%)	
	Mean	SD
Indispensable AA		
Isoleucine	92.9	0.9
Leucine	91.5	1.1
Lysine	92.6	0.9
Methionine	*n.d.*	
Phenylalanine	90.8	1.3
Threonine	93.5	1.3
Valine	92.0	1.1
Dispensable AA		
Alanine	89.6	1.3
Glutamate + glutamine	96.4	0.4
Glycine	71.3	5.0
Serine	71.2	6.2
Tyrosine	94.0	1.1
Mean AA bioavailability	90.2	1.5

n = 6 rats/group. Mean AA bioavailability was calculated from AA bioavailability weighted by the proportion of each AA in spirulina. SD, standard deviation; AA, amino acid.

Table 3. Whey orocecal bioavailability (in % of amino acids ingested) of individual amino acids obtained from the standard method and digestive absorption index obtained from the dual isotope approach measured 6 h after meal ingestion for ^{15}N and ^{2}H.

	^{15}N					**^{2}H**					***T-test: ^{15}N vs ^{2}H***	
	Orocecal bioavailability (%)		**Digestive absorption index (%)**		***T-test*** [a]	**Orocecal bioavailability (%)**		**Digestive absorption index (%)**		***T-test*** [a]	***Orocecal bioavailability*** [b]	***Digestive index*** [c]
Indispensable AA	Mean	SD	Mean	SD		Mean	SD	Mean	SD			
Isoleucine	97.6	0.4	98.0	0.5	*0.01*	96.0	0.3	96.7	0.4	*0.01*	*<0.001*	*<0.001*
Leucine	98.6	0.3	98.7	0.3	*n.s.*	97.8	0.2	97.9	0.2	*n.s.*	*0.0011*	*<0.001*
Lysine	99.0	0.3	98.9	0.3	*n.s.*	97.3	0.5	97.2	0.3	*n.s.*	*<0.001*	*<0.001*
Methionine	98.8	0.3	*n.d.*		-	98.2	0.2	*n.d.*		-	*<0.001*	-
Phenylalanine	98.9	0.3	99.0	0.3	*0.04*	94.6	0.9	95.3	0.8	*0.03*	*<0.001*	*< 0.001*
Threonine	97.8	0.4	98.7	0.4	*<0.001*	91.3	1.0	94.9	0.6	*<0.001*	*< 0.001*	*< 0.001*
Valine	98.1	0.4	98.3	0.4	*n.s.*	95.6	0.6	96.1	0.6	*n.s.*	*< 0.001*	*< 0.001*
Dispensable AA												
Alanine	98.1	0.5	98.1	0.6	*n.s.*	97.9	0.3	98.0	0.3	*n.s.*	*n.s.*	*n.s.*
Glutamate + glutamine	98.5	0.4	99.2	0.2	*0.003*	97.8	0.7	98.9	0.2	*0.005*	*0.003*	*< 0.001*
Glycine	95.4	2.1	91.1	2.5	*0.008*	94.0	3.2	88.7	1.2	*0.003*	*n.s.*	*0.05*
Proline	99.3	0.1	99.2	0.2	*0.02*	99.0	0.1	98.8	0.2	*0.02*	*< 0.001*	*< 0.001*
Serine	95.8	0.7	93.9	1.3	*0.003*	92.1	1.1	88.7	1.5	*0.001*	*< 0.001*	*< 0.001*
Tyrosine	98.9	0.3	99.3	0.2	*<0.001*	92.2	1.6	95.0	0.7	*0.002*	*< 0.001*	*< 0.001*
Mean AA bioavailability	98.3	0.3	98.5	0.4	*n.s.*	95.7	0.5	97.0	0.7	*0.009*	*< 0.001*	*< 0.001*

Data are presented as mean ± SD. n = 6 rats/group. Mean AA bioavailability is calculated from AA bioavailability values weighted by the proportion of each AA in whey protein. AA, amino acid; SD, standard deviation; n.s., non significant; n.d., not determined. [a] The paired t-test compared the orocecal bioavailability and the digestive absorption index within each isotope. [b] The paired t-test compared the orocecal bioavailability obtained with ^{15}N and ^{2}H. [c] The paired t-test compared the digestive absorption index obtained with ^{15}N and ^{2}H.

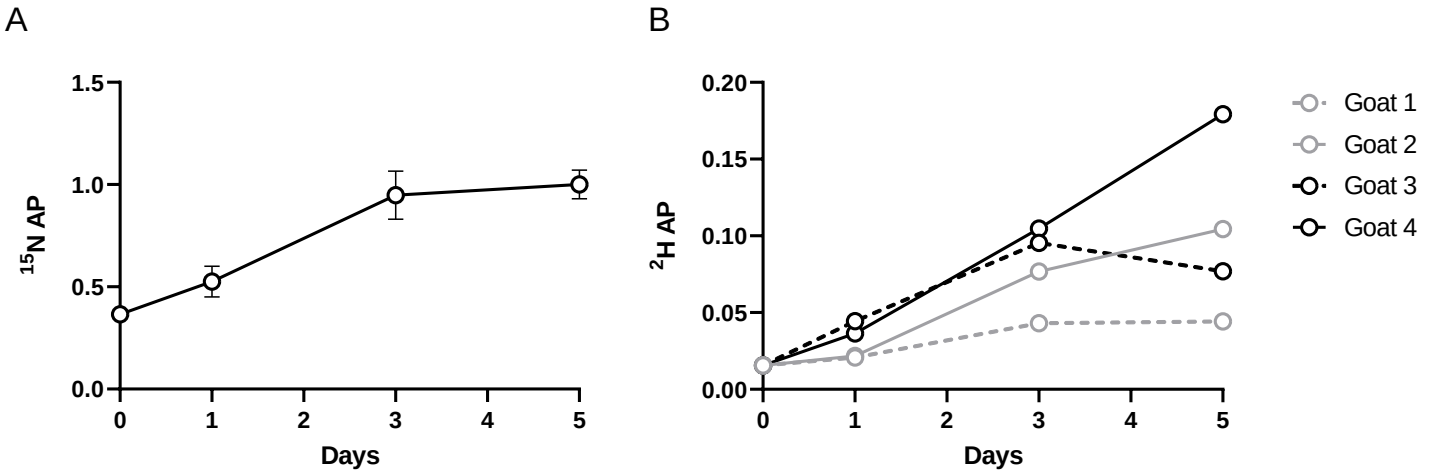
Legends of figures

Fig.1 Kinetics of ^{15}N (A) and ^{2}H (B) goat milk enrichment during the five-day labeling protocol. For ^{15}N labeling, the four goats received 5 g of ^{15}N ammonium salt for four consecutive days (from day 1 to day 4). For ^{2}H labeling, goats 1 and 3 received 80 and 160 mL of deuterated water, respectively, on one day (day 1) and goats 2 and 4 received 80 and 160 mL of deuterated water, respectively, for three consecutive days (from day 1 to day 3). Data are presented as mean of the 4 goats ± SD for ^{15}N. AP, atom percent.

Fig.2 ^{15}N (A) and ^{2}H (B) enrichment in individual amino acids of goat milk used to extract doubly labelled whey protein for the animal study. The dotted lines represent the natural abundance of ^{15}N (0.367) and ^{2}H (0.016). Data are presented as mean + SD for ^{15}N. AP, atom percent; IAA, indispensable amino acid; DAA, dispensable amino acid.

Fig.3 ^{2}H goat milk enrichment in individual amino acids on day 5 of the five-day labeling protocol in each goat (A) and kinetics of ^{2}H goat milk enrichment in individual amino acids during the five-day labeling protocol for goat 4 (B). Goats 1 and 3 received 80 and 160 mL of deuterated water, respectively, on one day (day 1) and goats 2 and 4 received 80 and 160 mL of deuterated water, respectively, for three consecutive days (from day 1 to day 3). AP, atom percent.

Fig.4 Recovery of dietary nitrogen (A) and hydrogen (B) in the different segments of the gastrointestinal tract 6 h after meal ingestion. Data are presented as mean + SD. n = 6 rats/group. * Significantly ($P < 0.05$) different from the dietary nitrogen within the same gastrointestinal segment and time**.**


A
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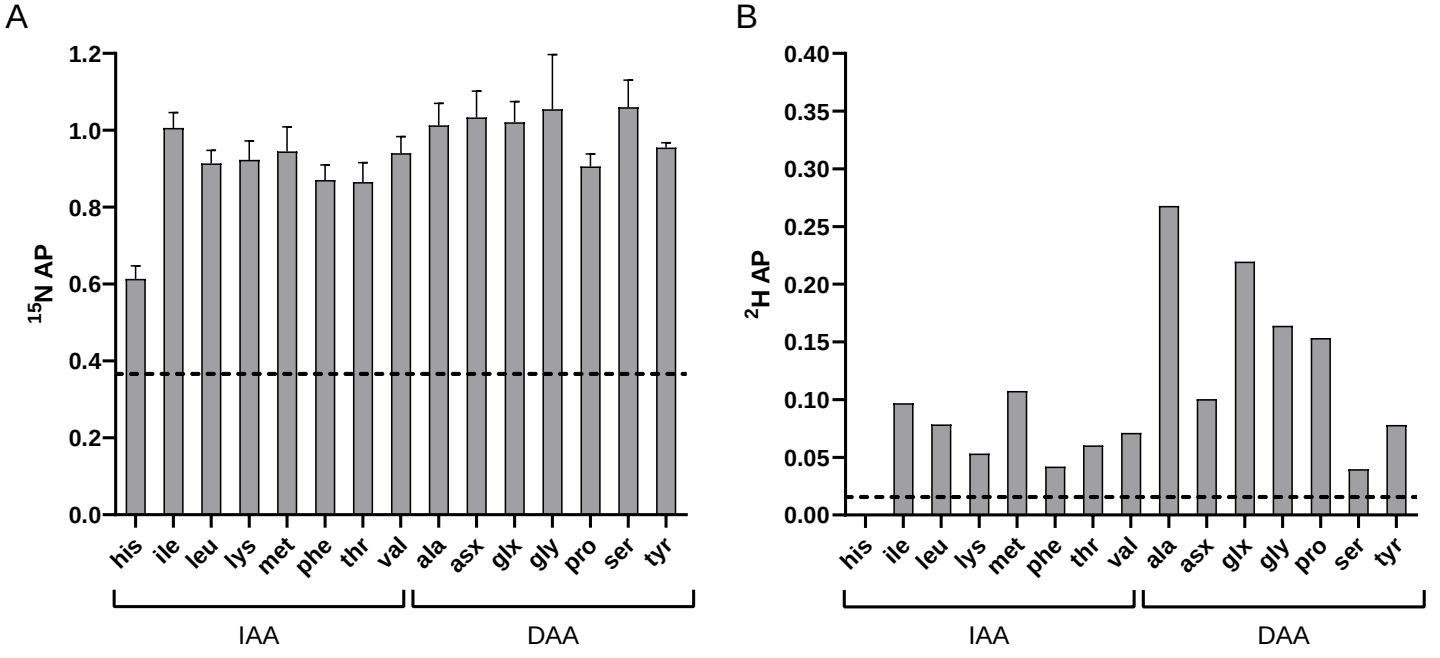
Goat 1
Goat 2
Goat 3
Goat 4


A
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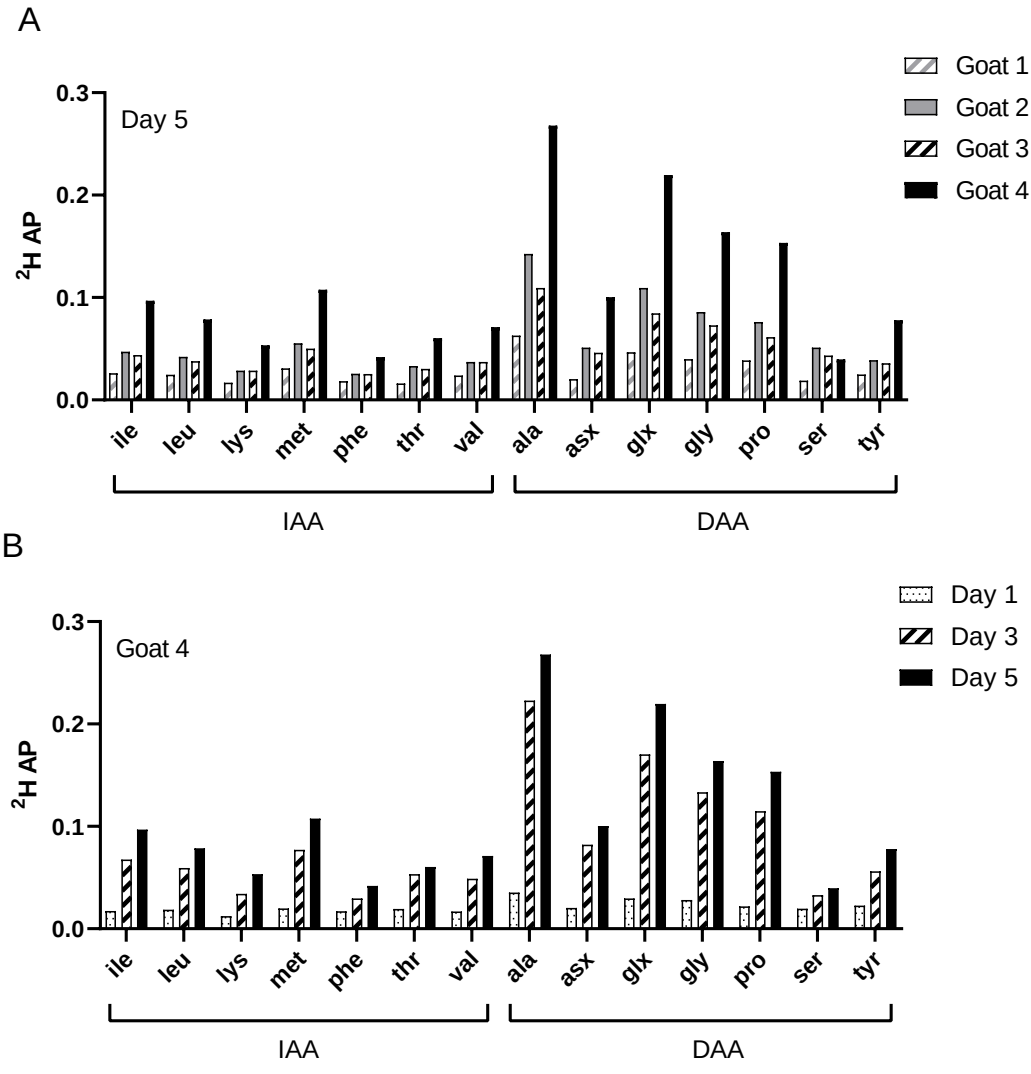
A
Goat 1
Goat 2
Goat 3
Goat 4
Day 5
^{2}H AP
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B
Day 1
Day 3
Day 5
Goat 4
^{2}H AP
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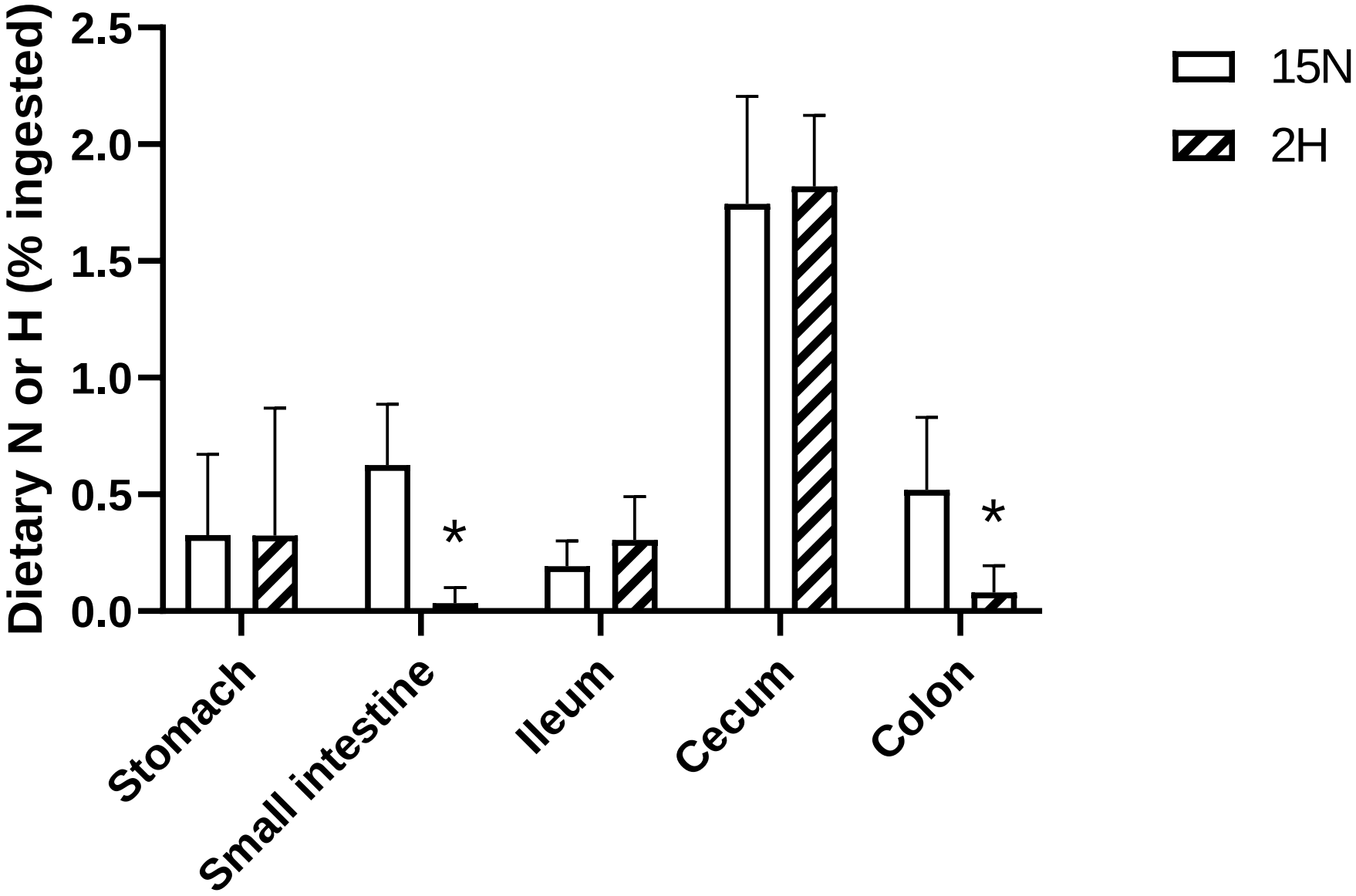
Dietary N or H (% ingested)
2.5
2.0
1.5
1.0
0.5
0.0
15N
2H
*
*
Stomach
Small intestine
Ileum
Cecum
Colon

Supplementary data

Using the dual isotope method to assess cecal amino acid absorption of goat whey protein in rats, a pilot study

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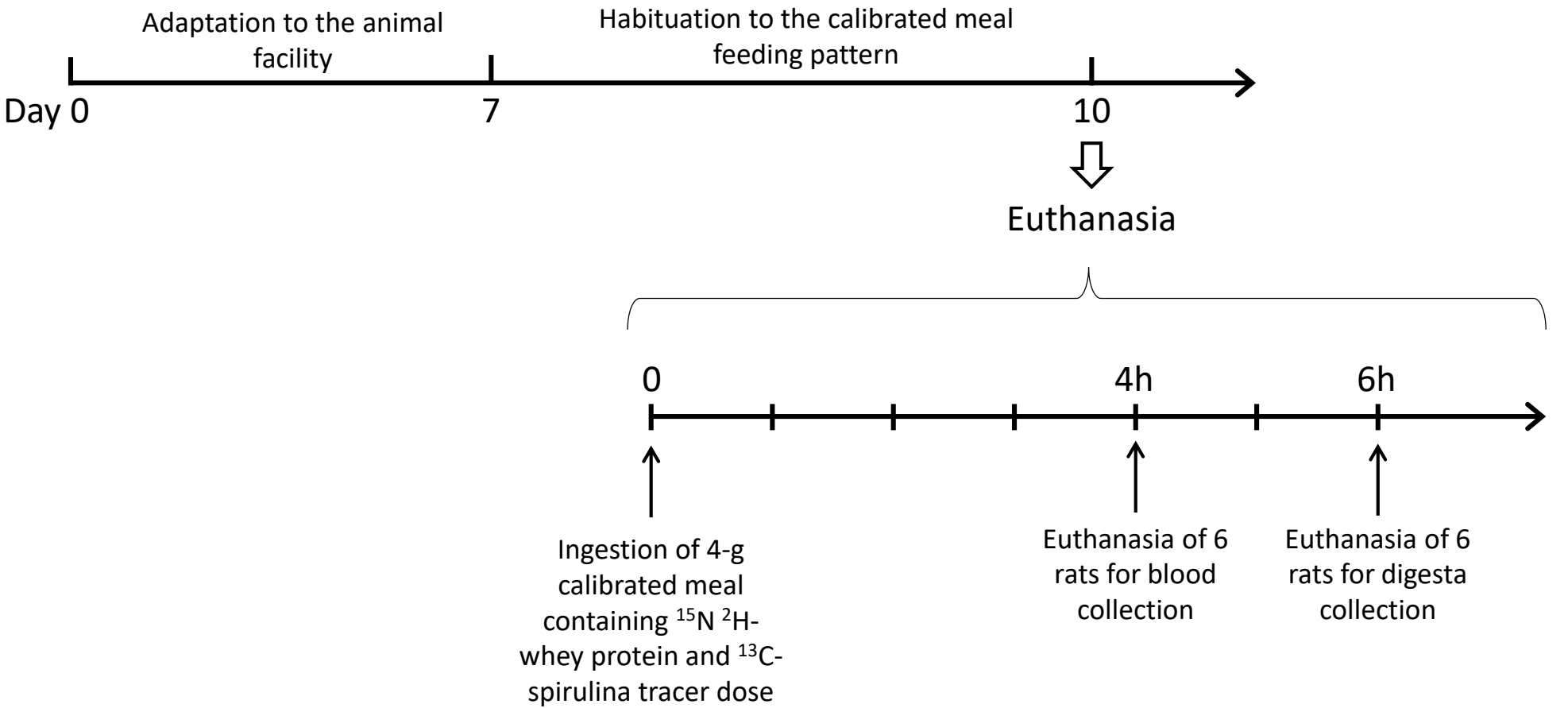
Supplemental Table 1. Five-day labeling protocol of administration of ^{15}N ammonium salt and deuterated water to goats.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Goat 1		^{15}N 5 g $^{2}H_2O$ 80 mL	^{15}N 5 g	^{15}N 5 g	^{15}N 5 g	
	Milk sampling					
Goat 2		^{15}N 5 g $^{2}H_2O$ 80 mL	^{15}N 5 g $^{2}H_2O$ 80 mL	^{15}N 5 g $^{2}H_2O$ 80 mL	^{15}N 5 g	
	Milk sampling					
Goat 3		^{15}N 5 g $^{2}H_2O$ 160 mL	^{15}N 5 g	^{15}N 5 g	^{15}N 5 g	
	Milk sampling					
Goat 4		^{15}N 5 g $^{2}H_2O$ 160 mL	^{15}N 5 g $^{2}H_2O$ 160 mL	^{15}N 5 g $^{2}H_2O$ 160 mL	^{15}N 5 g	
	Milk sampling					

Supplemental Table 2. ^{15}N amino acid plasma absorption indexes determined 4 h after meal ingestion and ^{15}N orocecal amino acid digestibility measured in cecal digesta collected 6 h after meal ingestion.

	^{15}N plasma absorption index (%)		**^{15}N orocecal digestibility (%)**		***T-test***
	Mean	SD	Mean	SD	
Indispensable AA					
Isoleucine	58.0	15.2	97.6	0.4	*0.004*
Leucine	77.7	6.7	98.6	0.3	*0.002*
Lysine	74.6	4.1	99.0	0.3	*< 0.001*
Methionine	63.4	12.3	98.8	0.3	*0.003*
Phenylalanine	137.0	25.5	98.9	0.3	*0.03*
Threonine	93.0	13.1	97.8	0.4	*n.s.*
Valine	67.8	6.8	98.1	0.4	*< 0.001*
Dispensable AA					
Alanine	337.3	85.0	98.1	0.5	*0.003*
Glutamate + glutamine	158.4	37.1	98.5	0.4	*0.02*
Glycine	175.1	51.2	95.4	2.1	*0.03*
Proline	*n.d.*		99.3	0.1	*n.d.*
Serine	*n.d.*		95.8	0.7	*n.d.*
Tyrosine	78.0	38.1	98.9	0.3	*n.s.*

n = 6 rats/group. AA, amino acid; n.d., not determined; n.s., non significant.


Adaptation to the animal facility
Habituation to the calibrated meal feeding pattern
Day 0
7
10
Euthanasia
0
4h
6h
Ingestion of 4-g calibrated meal containing ^{15}N ^{2}H-whey protein and ^{13}C-spirulina tracer dose
Euthanasia of 6 rats for blood collection
Euthanasia of 6 rats for digesta collection

Supp. Fig1