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

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Running title: Dual isotope method in rats

Key words: Protein digestibility, amino acid bioavailability, dual isotope method, rat model.

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.

1 **Abstract**

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

17 **Introduction**

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

31 The FAO has proposed a minimally invasive but indirect method to measure AA bioavailability
32 in humans (FAO 2014). This dual isotope tracer method is based on measurement of plasma isotopic
33 enrichment of individual AA in subjects having received a test protein intrinsically labeled with ^2H or
34 ^{15}N and a reference protein intrinsically labeled with ^{13}C . ^{15}N -labeling has often been used to
35 determine digestibility and metabolic utilization of animal or plant proteins in humans, pigs or rodents
36 (Deglaire et al. 2009; Gaudichon et al. 1999; Mariotti et al. 1999; Oberli et al. 2016). For the
37 digestibility dual isotope method, ^2H -labeled proteins have been recommended because in contrast to
38 ^{15}N , there are few exchanges of deuterium through transamination processes.

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

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

51

52 **Methods**

53 **^{15}N and ^2H intrinsic labeling of goat milk protein**

54 Goat milk was ^{15}N and ^2H -labeled using a five-day labeling protocol at the INRAE (French
55 National Institute for Agricultural Research and Environment) experimental unit of Thiverval-
56 Grignon. Four goats received orally 5 g of ^{15}N ammonium sulfate (99%, Eurisotop, Saint-Aubin,
57 France) for four consecutive days to reach a target enrichment of about 1 atom percent (AP) at the
58 isotopic plateau, based on our previous milk labeling assays (Lacroix et al. 2006a; Mahe et al. 1994).
59 In the absence of any previous experiments on ^2H -labeling of milk, the goats received either 80 or 160
60 mL of deuterated water ($^2\text{H}_2\text{O}$, 98%, Eurisotop, Saint-Aubin, France) for one or three consecutive days
61 in drinking water (**Supplemental Table 1**). Milk was collected daily throughout the protocol to
62 determine the ^{15}N and ^2H enrichment patterns. The milk with the highest ^2H enrichment was then
63 processed at the LRGP (UMR CNRS 7274, Université de Lorraine, Nancy, France) to extract proteins.
64 Milk was defatted and whey proteins were purified by diafiltration.

65

66 **Rats and experimental design**

67 This animal study was conducted in compliance with the EU directive 2010/63/EU for animal
68 experiments and approved by the Ethics committee in animal experiments of INRAE (n° 17-20, Jouy-
69 en-Josas, France) and the French Ministry of Higher Education and Research (APAFIS#11921-
70 2017091818236657). Twelve adult, male Wistar Rcc:Han rats initially weighing 300 g were purchased
71 from Harlan Laboratories and used in the study. Rats were housed individually under controlled
72 conditions (12-h light/dark cycle, lights off at 10:00, constant room temperature of 22°C) in cages with

73 wire bottoms to prevent coprophagia. Environmental enrichment included plastic tubes in the cage and
74 the rats were manipulated every week day for body weight evaluation and health check. They were
75 adapted over one week to the animal facility conditions. Rats were fed an AIN modified standard milk
76 protein diet that provided 14.5% of energy from proteins (**Table 1**). It was available *ad libitum* from
77 12:00 to 18:00. At 10:00, they received a small 4 g calibrated meal of this diet, which was consumed
78 in less than 15 minutes. This specific dietary intake pattern made it possible to include the labeled test
79 protein in the calibrated meal, as previously described (Lacroix et al. 2006a). Criteria for exclusion of
80 rats of the study were body weight decrease of $\geq 20\%$ and refusal to eat the calibrated meal. No rats
81 were excluded from the study. After 10 days of adaptation, 0.48 g of ^{15}N and ^2H -labeled goat milk
82 proteins were included in the calibrated meal (Table 1). A tracer dose of 5 mg of ^{13}C -spirulina (97%,
83 Eurisotop, Saint-Aubin, France) was incorporated in the meal. Approximately 1 to 1.5 mL of blood
84 was also collected with a heparinized syringe from the tail vein of rats in the fasting state, and 1 h and
85 3 h after meal intake. Six rats were euthanized 4 h after the test meal, and six rats were euthanized 6 h
86 after test meal ingestion, all with pentobarbital sodium anesthesia (100 mg/kg intraperitoneally).
87 Allocation of the rats in the 2 groups was done alternatively and this was a not blinded experiment.
88 The timing was chosen to collect the digesta 6 h after meal ingestion, allowing a compromise between
89 complete digestion and minimal duration of cecal fermentation. In addition, this provided enough
90 plasma samples at 4 h after meal ingestion for analyses of isotopic enrichment in individual plasma
91 AA in order to test the dual isotope tracer method for the determination of a proxy of AA
92 bioavailability. Isotopic enrichment is at the highest level in rat plasma 4 h after meal ingestion
93 (Morens et al. 2000). At euthanasia, heparin was injected into the abdominal cavity, and
94 approximately 10 mL of blood was withdrawn from the abdominal cavity following rupture of both
95 the caudal vena cava and the aorta. Blood was ice-stored in individual glass tubes and centrifuged (20
96 min, 3000 g, 4°C). The plasma was stored at -80°C for subsequent analyses. Gastrointestinal segments
97 were identified as stomach, small intestine, ileum (defined as the last 10 cm before the cecum), cecum
98 and colon. Feces were also collected. Luminal contents of these segments were rinsed with NaCl
99 solution (9‰), collected entirely and stored at -20°C.

101 **Analytical procedures**

102 *Total ¹⁵N and ²H enrichments*

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

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

110

111 *¹⁵N, ¹³C and ²H individual amino acid enrichments*

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

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

122

123 *Amino acid concentrations*

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

128

129 **Determination of protein digestibility and amino acid bioavailability**

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

142

143 *Protein digestibility*

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

$$147 \quad N_{\text{diet } x} = N_{\text{tot}} \times \frac{APE_s}{APE_m} \quad \text{or} \quad H_{\text{diet } x} = H_{\text{tot}} \times \frac{APE_s}{APE_m} \quad [Formula 1]$$

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

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

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

$$158 \quad \text{Orocecal digestibility (\%)}_N = 100 \times \frac{N_{\text{ing}} - (N_{\text{diet ileum}} + N_{\text{diet cecum}})}{N_{\text{ing}}}$$

159 or

$$160 \quad \text{Orocecal digestibility (\%)}_H = 100 \times \frac{H_{\text{ing}} - (H_{\text{diet ileum}} + H_{\text{diet cecum}})}{H_{\text{ing}}} \quad [Formula 2]$$

161
 162 where N_{ing} or H_{ing} is the total amount of ingested nitrogen or hydrogen (in mmol), N or $H_{\text{diet ileum}}$ and N
 163 or $H_{\text{diet cecum}}$ are the amounts of dietary nitrogen or hydrogen (in mmol) recovered in the ileum and the
 164 cecum, respectively, 6 h after the test meal. The true whey protein orofecal digestibility was calculated
 165 according to the same formula as true whey protein orocecal digestibility, except that dietary nitrogen
 166 or hydrogen recovered in the colon and feces was taken into account.

167

168 *Individual amino acid bioavailability*

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

$$173 \quad \text{AA}_{\text{diet } x} = \text{AA}_{\text{tot}} \times \frac{\text{APE}_s}{\text{APE}_m} \quad [Formula 3]$$

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

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

$$181 \quad \text{AA orocecal bioavailability (\%)} = 100 \times \frac{\text{AA}_{\text{ing}} - \text{AA}_{\text{diet cecum}}}{\text{AA}_{\text{ing}}} \quad [Formula 4]$$

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

184 The principle of the dual isotope tracer method was to estimate the cecal dietary AA from whey
 185 not absorbed 6 h after meal ingestion, relative to not absorbed cecal dietary AA from ^{13}C -spirulina,
 186 and to calculate digestive AA absorption index as a proxy of AA orocecal bioavailability, using either
 187 ^{15}N or 2H . The percentage of dietary AA from spirulina not absorbed and found in the cecum
 188 corresponds to 100 - orocecal AA bioavailability of spirulina calculated by formula 4. This value was
 189 corrected by the ratio of ^{15}N or $^2H/^{13}C$ AA enrichment in cecum on ^{15}N or $^2H/^{13}C$ AA enrichment in
 190 meal. Then, the digestive AA absorption index was calculated according to formula 5:

191

$$192 \quad \text{Digestive } ^{15}N \text{ AA absorption index} = 100 - \left[(100 - \text{AA bioav. spi}) \times \frac{\frac{^{15}N}{^{13}C} \text{ cecum}}{\frac{^{15}N}{^{13}C} \text{ meal}} \right]$$

193 And

$$194 \quad \text{Digestive } ^2H \text{ AA absorption index} = 100 - \left[(100 - \text{AA bioav. spi}) \times \frac{\frac{^2H}{^{13}C} \text{ cecum}}{\frac{^2H}{^{13}C} \text{ meal}} \right]$$

195

[Formula 5]

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

199

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

$$203 \quad \text{Plasma AA absorption index} = \text{AA bioav. spi} \times \frac{\frac{^{15}N}{^{13}C} \text{ plasma}}{\frac{^{15}N}{^{13}C} \text{ meal}} \quad [Formula 6]$$

204 where $^{15}N/^{13}C$ plasma and meal are the enrichment ratios of the AA in plasma and meal, respectively,
 205 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 ^2H 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 ^2H 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 $^2\text{H}_2\text{O}$ dose, three-day
228 administration (goats 2 and 4) enabled a greater ^2H 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 ^2H 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**).

234 The ^2H enrichment was determined in 14 AA (**Fig. 2B**). The highest dose of deuterated water
235 combined with three-day administration enabled greater ^2H enrichment in AA on the last day of the
236 protocol (goat 4, day 5, **Fig. 3A**). In this goat, the ^2H enrichment in individual AA gradually increased
237 during and after $^2\text{H}_2\text{O}$ administration (day 1 to day 5, **Fig. 3B**). However, labeling covered a wider
238 range across AA, varying from 0.04 AP for phenylalanine to 0.27 AP for alanine (Fig. 2B). The highest
239 enrichments were observed in dispensable AA (alanine, proline, glutamate, glycine; Fig. 2B). The milk
240 from the last day of collection from goat 4 was selected and processed for extraction and purification
241 of whey proteins used in the *in vivo* study.

242

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

244 We determined the amount of dietary nitrogen and hydrogen, expressed as the percentage of
245 nitrogen or hydrogen from whey proteins ingested (originating from labeled goat milk) and that
246 remained in the gastrointestinal tract 6 h after the meal (**Fig. 4**). Most of the dietary nitrogen or
247 hydrogen non-absorbed was found in the cecum. The comparison of dietary nitrogen and hydrogen
248 remaining in the stomach, ileum or cecum revealed no differences, whereas in the small intestine and
249 colon, recovery of dietary hydrogen was significantly reduced compared to nitrogen ($P < 0.001$ and P
250 $= 0.02$, respectively).

251 The true oro-faecal digestibility of whey proteins was comparable between both isotopes, with
252 values of $97.7 \pm 0.4\%$ and $97.8 \pm 0.2\%$, respectively ($P > 0.05$). Similarly, the true oro-cecal
253 digestibility of whey proteins was comparable ($98.2 \pm 0.4\%$ for dietary nitrogen and $97.9 \pm 0.1\%$ for
254 hydrogen, $P > 0.05$).

255

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

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

262 99.3 ± 0.1% for proline, with an average AA bioavailability of 98.3 ± 0.3%. When assessed with ²H,
263 true orocecal bioavailability of nearly all whey individual AA (except alanine and glycine) were
264 significantly lower than when assessed with ¹⁵N (paired *t*-test, P < 0.001 to P = 0.002 depending on the
265 AA). Hence, true orocecal bioavailability of whey AA determined from ²H enrichment varied from
266 91.3 ± 1.0% for threonine to 99.0 ± 0.1% for proline, with an average AA bioavailability of 95.7 ±
267 0.5%.

268

269 **Digestive and plasma absorption indexes of whey amino acids determined with a dual isotope** 270 **tracer method**

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

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

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

296

297

298 **Discussion**

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

307 Goat milk protein was intrinsically and uniformly labeled. Classically, ^{15}N or ^{13}C intrinsic
308 labeling of protein has been used to assess the digestive and metabolic fate of dietary proteins (Boirie
309 et al. 1997; Daenzer et al. 2001; Gaudichon et al. 1999; Mahe et al. 1994). Intrinsically ^{15}N -labeled
310 milk can be easily obtained by adding ^{15}N ammonium sulfate to the diet of lactating ruminants
311 (Gaudichon et al. 1999), as in the present study where proteins were uniformly labeled at a level of
312 approximately 1 AP. Recently, ^2H -labeled proteins were used to determine true ileal digestibility of
313 IAA using a minimally invasive dual isotope tracer method (Devi et al. 2020; Shivakumar et al. 2019;
314 Kashyap et al. 2019a; Kashyap et al. 2018; Devi et al. 2018). Regular watering with deuterated water
315 during plant growth was used to obtain rice, finger millet, mung bean and chick pea ^2H -labeled
316 proteins (Shivakumar et al. 2019; Devi et al. 2018). Feeding animals with a mixture of [^2H]-labeled
317 crystalline AA mix enabled production of ^2H -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 ^2H -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 ($^1\text{H}_2\text{O}$) during hydrolysis or exchange between ^2H and ^1H
326 during reactions of intermediary metabolism. By doubly-labeling milk protein, we were able to
327 compare the performance of both tracers. Lower ^2H enrichment was found in the colon and small
328 intestine; this can be ascribed to the dilution of ^2H 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 ^2H . 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 ^2H 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 ^2H -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 ^2H 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 ^2H 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 $^2\text{H}/^{13}\text{C}$ 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

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

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

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

404
405 In this pilot study, we explored a dual isotope tracer approach in rats by using ^2H and ^{15}N
406 doubly-labeled whey proteins and a tracer dose of ^{13}C -spirulina as a reference protein to evaluate AA
407 bioavailability. We determined the true bioavailability of whey AA at the cecal level. We showed that
408 the bioavailability was slightly underestimated with ^2H in comparison to ^{15}N . Digestive absorption
409 indexes determined at the cecal level gave consistent values with true orocecal bioavailability,
410 especially for IAA, for the highly digestible protein tested in our study. In conclusion, a dual isotope
411 tracer approach and the enrichment ratio method seem to be a possible approach to determine AA
412 bioavailability in rats. Additional studies with different sources of proteins (with variable
413 bioavailability) are warranted to further develop this approach and applied it at the ileal level.

414

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421

422 **Conflict of Interest:** The authors declare no conflict of interest.

423

424 **Availability of data and material:** Data described in the manuscript will be made available upon
425 reasonable request, pending application and approval.

426

427 **Code availability:** Not applicable.

428

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

433

434 **Ethics approval:** This study was conducted in compliance with the EU directive 2010/63/EU for
435 animal experiments and approved by the Ethics committee in animal experiments of INRAE (n° 17-
436 20, Jouy-en-Josas, France) and the French Ministry of Higher Education and Research
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438

439 **Consent to participants:** This research involved animals.

440

441 **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	<i>n.d.</i>	
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 ^2H .

	^{15}N					^2H					<i>T-test: ^{15}N vs ^2H</i>	
	Orocecal bioavailability (%)		Digestive absorption index (%)		<i>T-test</i> ^a	Orocecal bioavailability (%)		Digestive absorption index (%)		<i>T-test</i> ^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 \pm 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 ^2H . ^c The

paired t-test compared the digestive absorption index obtained with ^{15}N and ^2H .

Legends of figures

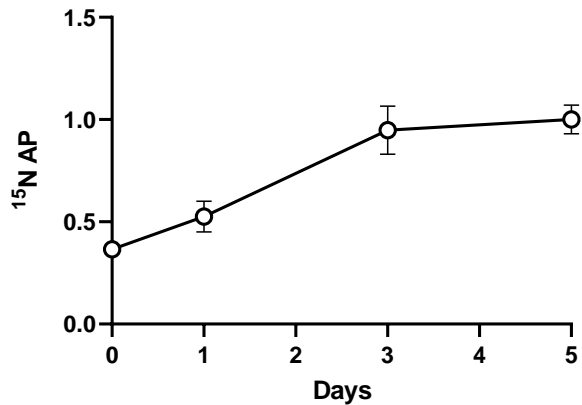
Fig.1 Kinetics of ^{15}N (A) and ^2H (B) goat milk enrichment during the five-day labeling protocol. For ^{15}N labeling, the four goats received 5 g of ^{15}N ammonium salt for four consecutive days (from day 1 to day 4). For ^2H labeling, goats 1 and 3 received 80 and 160 mL of deuterated water, respectively,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

Fig.2 ^{15}N (A) and ^2H (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 ^2H (0.016). Data are presented as mean + SD for ^{15}N . AP, atom percent; IAA, indispensable amino acid; DAA, dispensable amino acid.

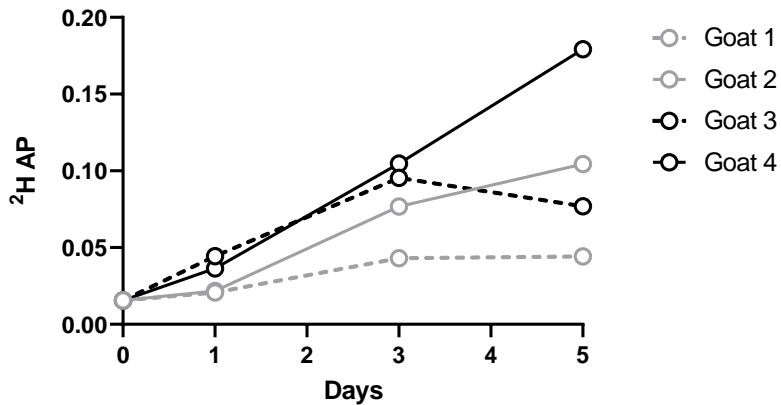
Fig.3 ^2H goat milk enrichment in individual amino acids on day 5 of the five-day labeling protocol in each goat (A) and kinetics of ^2H goat milk enrichment in individual amino acids during the five-day labeling protocol for goat 4 (B). Goats 1 and 3 received 80 and 160 mL of deuterated water, respectively, 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.

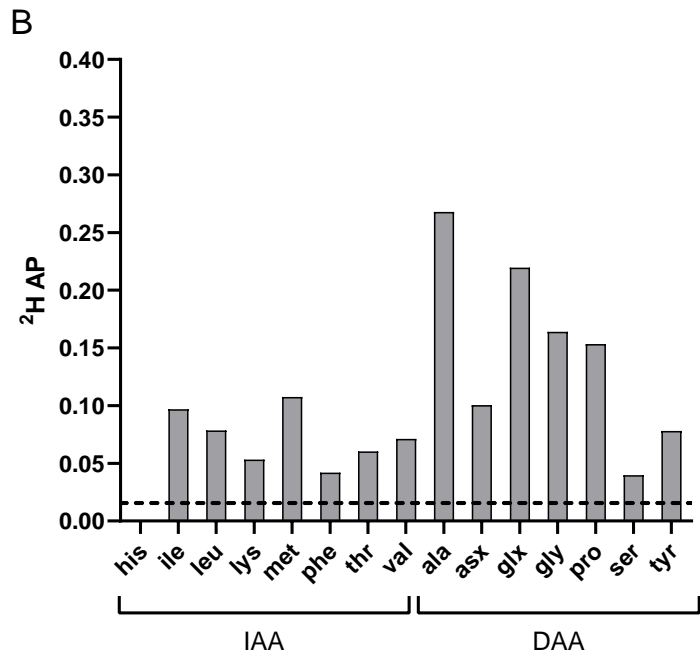
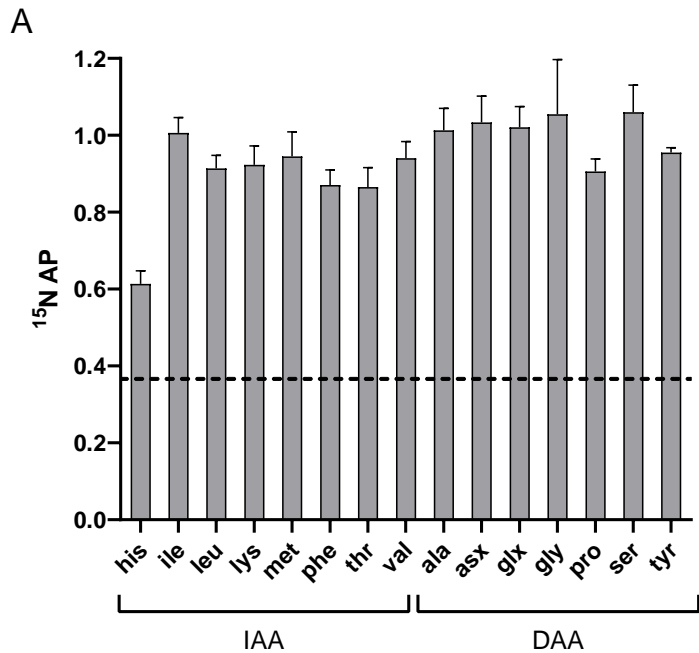
Fig.4 Recovery of dietary nitrogen (A) and hydrogen (B) in the different segments of the gastrointestinal tract 6 h after meal ingestion. Data are presented as mean + SD. $n = 6$ rats/group. * Significantly ($P < 0.05$) different from the dietary nitrogen within the same gastrointestinal segment and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

A

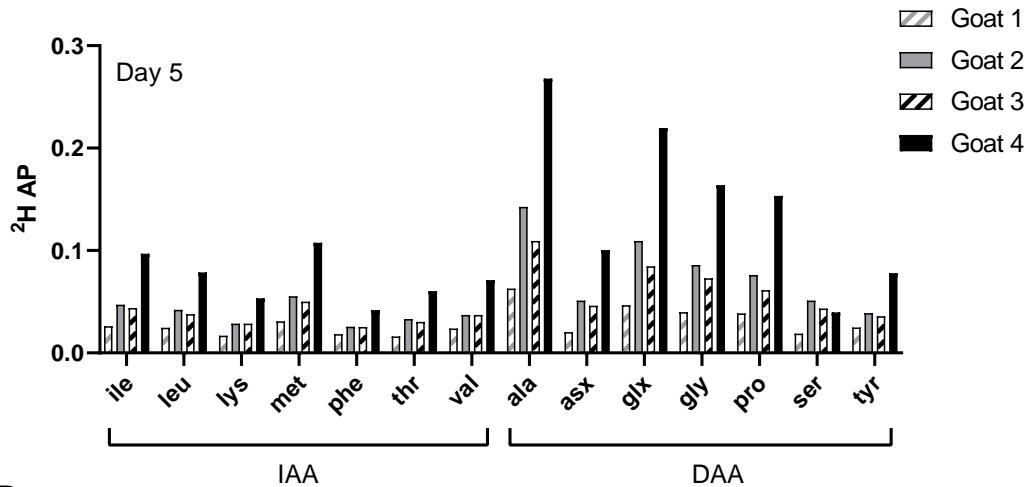


B

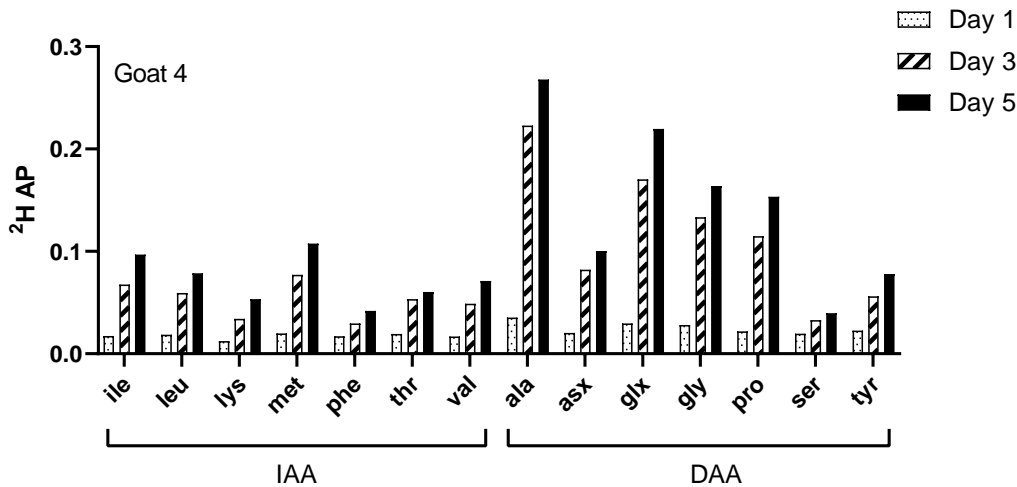


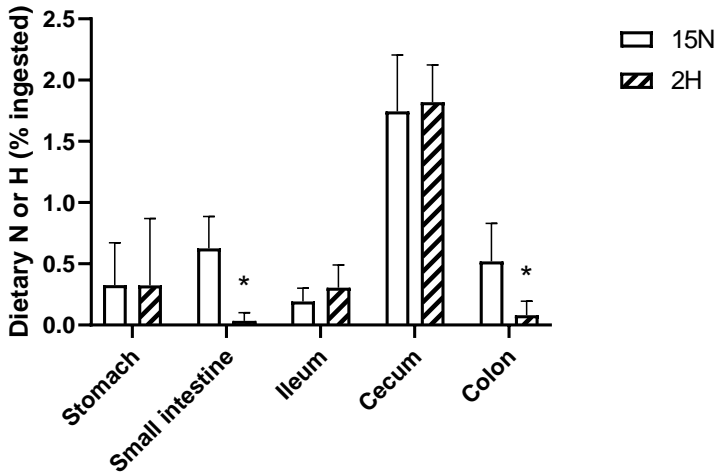


A



B





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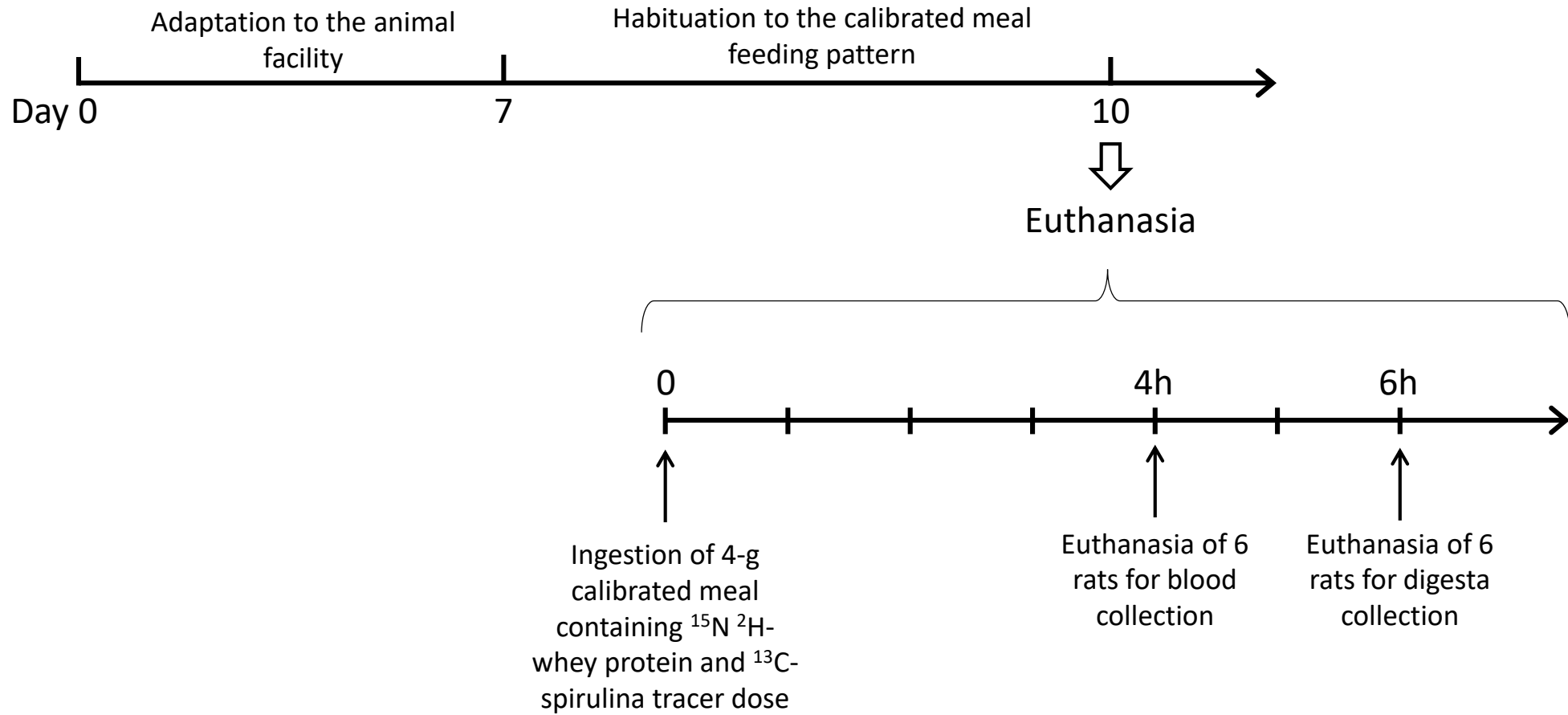
Supplemental Table 1. Five-day labeling protocol of administration of ^{15}N ammonium salt and deuterated water to goats.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Goat 1		^{15}N 5 g $^2\text{H}_2\text{O}$ 80 mL	^{15}N 5 g	^{15}N 5 g	^{15}N 5 g	
		Milk sampling				
Goat 2		^{15}N 5 g $^2\text{H}_2\text{O}$ 80 mL	^{15}N 5 g $^2\text{H}_2\text{O}$ 80 mL	^{15}N 5 g $^2\text{H}_2\text{O}$ 80 mL	^{15}N 5 g	
		Milk sampling				
Goat 3		^{15}N 5 g $^2\text{H}_2\text{O}$ 160 mL	^{15}N 5 g	^{15}N 5 g	^{15}N 5 g	
		Milk sampling				
Goat 4		^{15}N 5 g $^2\text{H}_2\text{O}$ 160 mL	^{15}N 5 g $^2\text{H}_2\text{O}$ 160 mL	^{15}N 5 g $^2\text{H}_2\text{O}$ 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 (%)		<i>T-test</i>
	Mean	SD	Mean	SD	
Indispensable AA					
Isoleucine	58.0	15.2	97.6	0.4	<i>0.004</i>
Leucine	77.7	6.7	98.6	0.3	<i>0.002</i>
Lysine	74.6	4.1	99.0	0.3	<i>< 0.001</i>
Methionine	63.4	12.3	98.8	0.3	<i>0.003</i>
Phenylalanine	137.0	25.5	98.9	0.3	<i>0.03</i>
Threonine	93.0	13.1	97.8	0.4	<i>n.s.</i>
Valine	67.8	6.8	98.1	0.4	<i>< 0.001</i>
Dispensable AA					
Alanine	337.3	85.0	98.1	0.5	<i>0.003</i>
Glutamate + glutamine	158.4	37.1	98.5	0.4	<i>0.02</i>
Glycine	175.1	51.2	95.4	2.1	<i>0.03</i>
Proline		<i>n.d.</i>	99.3	0.1	<i>n.d.</i>
Serine		<i>n.d.</i>	95.8	0.7	<i>n.d.</i>
Tyrosine	78.0	38.1	98.9	0.3	<i>n.s.</i>

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



Supp. Fig1