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# Phenylalanine stable isotope tracer labeling of cow milk and meat and human experimental applications to study dietary protein-derived amino acid availability

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**Abbreviations:** AA, amino acid; EAA, essential amino acid; GC-MS/MS, gas chromatography-triple-stage quadrupole-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MPE, mole percent excess; RMR, resting metabolic rate; TTR, tracer to tracee ratio

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## SUMMARY

*Background & aims:* Availability of dietary protein-derived amino acids (AA) is an important determinant for their utilization in metabolism and for protein synthesis. Intrinsic labeling of protein is the only method to directly trace availability and utilization. The purpose of the present study was to produce labeled milk and meat proteins and investigate how dietary protein-derived AA availability is affected by the protein-meal matrix.

*Methods:* Four lactating cows were infused with L-[ring-$d_5$]phenylalanine and one with L-[$^{15}$N]phenylalanine for 72 h. Milk was collected, and three of the [$d_5$]phenylalanine cows were subsequently slaughtered. Two human studies were performed to explore plasma AA availability properties utilizing the labeled proteins. One study compared the intake of whey protein either alone or together with carbohydrates-fat food-matrix. The other study compared the intake of meat hydrolysate with minced beef. Cow blood, milk, meat and human blood samples were collected and analyzed by mass spectrometry.

*Results:* Whey and caseinate acquired label to 15-20 mole percent excess (MPE), and the meat proteins reached 0.41-0.73 MPE. The [$d_5$]phenylalanine appeared fast in plasma and peaked 30 min after whey protein alone and meat hydrolysate intake, whereas whey protein with a food-matrix and the meat minced beef postponed the [$d_5$]phenylalanine peak until 2 and 1 h, respectively.

*Conclusions:* Phenylalanine stable isotope-labeled milk and meat were produced and proved a valuable tool to investigate AA absorption characteristics. Dietary protein in food-matrices showed delayed postprandial plasma AA availability as compared to whey protein alone and meat hydrolysate.

## 1. Introduction

One major stimulator of protein turnover rates and especially protein synthesis in healthy adults is circulatory hyperaminoacidemia [1–4]. The temporal pattern of hyperaminoacidemia from nutritive proteins is affected by processing of the protein [5,6], chewing efficiency [7], and concomitant intake of other macronutrients [8–10]. Recent decades of research endeavors have provided evidence for defining good protein: high content of essential amino acids (EAA) [11,12], especially leucine inheres a stimulatory effect that exceeds that of all other AA [13], and a quick availability of food protein-derived AA in the postprandial period is instantly more anabolic than a slow availability [1,4]. In accordance, protein quality is defined by the digestibility and content of EAA [14]. Importantly, it should be emphasized that it is the protein net balance that will decide the long term impact on body protein mass, such as gain or loss of muscle mass.

Protein ingredient innovation is evolving resulting in specialized protein ingredients targeting various nutritive purposes for groups with special needs. To study how these ingredients are utilized in the body golden-standard research methods must be applied to include the complexity of splanchnic circulation [15] and the influence on the utilization of absorbed dietary AA by gut epithelia and liver [16], to follow the fate of dietary protein-derived AA as well as their utilization for protein synthesis.

Adding an oral free AA tracer to a ‘mixed meal’ composed of crystalline AA allow the determination of the gastro-intestinal absorption kinetics and the first pass splanchnic extraction ratio [16]. However, when the aim is to study the uptake of AA from peptides or intact proteins the digestion process is added on top and a crystalline

AA tracer is no longer representative of the dietary amino acids. To validly investigate these questions, the intrinsically labeled proteins – although recently discussed [17] – provide the model, and their further application in nutritional research formulates the next level of state-of-the-art approach for investigating complex digestive and dietary protein utilization questions.

The concept of labeling proteins intrinsically by provision of AA tracers or intermediates that will transfer stable isotope atoms in *de novo* AA/protein synthesis is not new. Some studies report the production of intrinsically labeled milk proteins but as secondary findings, since the primary purposes were e.g. to improve the understanding of milk protein synthesis for optimization of milking outputs in lactating animals [18] or to study the nitrogen transport and metabolism [19]. In another case, with the purpose to investigate how AA appear as essential, non-essential or conditionally essential in hens, uniformly $^{13}C$-labeled feed-ingredients were produced by growing algal in an atmospheric pure $^{13}CO_2$ environment and fed to the animals [20]. More recently, hens have been fed with $^{15}N/^{13}C$-labeled AA mixtures [21] or [$d_3$]leucine [22] with the purpose to produce egg proteins and poultry meat proteins that were sufficiently labeled to make up a feed model for use in human metabolic studies. Also, ileal indispensable amino acid appearance has been measured by use of deuterium-labeled hen's egg and meat and some vegetable food sources with a minimally invasive dual-stable-isotope approach [23–25]. One of the earliest examples of production of intrinsically labeled milk proteins provided oral as well as intravenous stable isotope labeled AA to lactating women and established that both approaches can be used to label human breast milk, and that the labeled milk was suitable for investigation of protein digestion and AA utilization in human nutritional studies [26,27]. Similar approaches have been used in

cows to produce milk proteins [4,28,29] and meat proteins [30,31]. Also, soy proteins have been labeled and investigated in a human setting [32].

In this study we report a Danish setup for producing intrinsically labeled milk and meat proteins suitable for human consumption and with sufficient phenylalanine enrichment to trace its appearance into the circulation and to determine fate and utilization for *de novo* protein synthesis. The setup builds on our previous experience [4] and the work mentioned above. Further, the aim was to demonstrate how the intrinsic tracer can be used to study characteristics of protein digestion and AA absorption by measures of protein-derived AA availability when fed in different formats in human nutrition studies.

## 2. Materials and methods

### *2.1. Overall study design*

This project consists of three separate parts. The first part is the production of phenylalanine stable isotope-labeled milk and meat protein, the second and third part are human studies performed to explore the appearance of the labeled protein-derived phenylalanine when ingested in different forms of milk and meat products.

### *2.2. Cow infusion protocol*

The production of labeled milk was performed at Aarhus University Foulum (Department of Animal Science, Aarhus University, Tjele, Denmark) and complied with the guidelines of the Danish Ministry of Justice (Act No. 726, 1993) with respect to animal experimentation and care of animals under study (journal no. 2014-15-2934-01018). The protocol was a modification of our previous work [4].

Two days before experimental onset, five high-yielding Danish Holstein Friesian cows had a catheter (1.02-mm id, 1.78-mm od catheters (Tygon, S-54-HL; Buch & Holm, Herlev, Denmark)) inserted 15 cm into the right and left jugular veins by percutaneous venipuncture using a hypodermic needle (2.5 x 110 mm; Mediplast, Malmö, Sweden). Prior to insertion, the veins were visually blotted by shaving, skin was then disinfected by chlorhexidine wiping, and the incision site anaesthetized by subcutaneous injection of 5 mL of Xylocaine (20 mg/mL lidocaine; AstraZeneca, Albertslund, Denmark). The catheters were secured by skin sutures kept in place on the catheters by two cuffs (5- to 8-mm-long pieces of Tygon blue/yellow pump tubing;

Buch & Holm) slid over the catheters using a pair of hemostats after removal of the hypodermic needle.

Four cows were allocated to infusion of L-[ring-$d_5$]phenylalanine and one cow was allocated to infusion of L-[$^{15}$N]phenylalanine. The cows were housed in tie stalls bedded with rubber mats and sawdust and had free access to ad libitum feed and water. Average body weight of the cows was 676 ± 92 kg and at experimental onset the cows were 78 ± 23 days after calving. Four cows were in their third lactation, and one cow was in her second lactation. During the experiment, the cows were milked three times daily in order to maximize yield. Prior to the experiment, milk and milk protein yields were 43.2 ± 2.0 kg/d and 1399 ± 97 g/d, respectively. The cows' feed were mixed similar to ratios feed for Danish dairy cows and the ratio was composed in correspondence to NorFor recommendations [33]. Cows were fed once a day (08:00) and feed residue was measured daily in order to determine daily intake. Average feed intake during the experiment was 58.5 ± 2.5 kg feed/d and 23.5 ± 1.0 kg dry matter/d.

Each of four cows received 180 g of L-[ring-$d_5$]phenylalanine (98 atom %; Cambridge Isotope Laboratories, Tewksbury, MA) and one cow received 180 g of L-[$^{15}$N]phenylalanine (98 atom %; Cambridge Isotope Laboratories). The solution for each cow was made into 3 x 5 L of 0.9% NaCl by sterile technique. The cows received the tracer infusion in one jugular vein catheter, and infusion started on day 1 (13:00) and continued until day 4 (13:00), in total 72 h equivalent to an infusion rate of 208 mL/h (corresponding to 14.7 mmol/h for [$d_5$]phenylalanine and 15.1 mmol/h for [$^{15}$N]phenylalanine). The other catheter was used for frequent blood sampling. A blood sample was obtained before initiation of the infusion period, at 30 min, 1, 2, 3, 4, 12, 24, 36, 48, 60 and 72 h during the infusion, and at 72.5, 73, 74, 75, 76, and 88 h after the

179 infusion was terminated (time point 0 h is infusion start and 72 h is infusion stop). Milk
180 was collected from 9 milkings during the tracer infusion period (each day at 05:00,
181 13:00, 21:00) and from 2 milkings (21:00 and 05:00) after the infusion had ended at
182 13:00. Hence, milk from a total of 11 milkings from each cow was collected. This
183 milking protocol was argued in the aim of balancing yield and tracer abundance.
184 Immediately after each milking, the collected milk was stored in 25 L buckets at 2-3 ºC.
185 After milking no. 5, 8, and 11, the collected milk from the four cows infused with L-
186 [ring-$d_5$]phenylalanine was pasteurized (71-72 ºC, 15 sec) on a small scale equipment at
187 Aarhus University Foulum. Milk from the cow infused with L-[$^{15}$N]phenylalanine was
188 pasteurized as one portion after the 11$^{th}$ milking to reduce loss. The total yield of
189 pasteurized milk was approximately 700 kg of [$d_5$]phenylalanine-labeled and 150 kg of
190 [$^{15}$N]phenylalanine-labeled milk that on day 6 were transported to the dairy company
191 (Arla Foods, Nr. Vium, Denmark) and further processed as described below. Also on
192 day 6, three cows infused with L-[ring-$d_5$]phenylalanine were transported to a slaughter
193 house and slaughtered as described below. A schematic overview of the experimental
194 cow infusion protocol is illustrated in Fig. 1.
195
196 *2.3. Milk processing and protein fractionation*
197 Upon receiving the milk in cooled tanks containing the [$d_5$]phenylalanine-labeled
198 and [$^{15}$N]phenylalanine-labeled milk separately, the dairy company (Arla Foods)
199 pasteurized (71-72 °C, 15 sec) and skimmed the milk. The cream fraction was
200 discarded. Thereafter, the casein was precipitated by addition of 10% HCl under strong
201 agitation at 52°C, until a pH of 4.6 was reached. The mixture was agitated for 10 min
202 after which the casein was allowed to settle. The whey was then drained, collected and

cooled to 4°C. The casein was washed three times with half the initial volume of water (pH 4.6, 50°C) to remove any remaining whey and lactose traces. All washing water was discarded. The final casein protein pellet was slowly dissolved in water (65°C), to a final volume corresponding to twice the volume of casein mass, under thorough agitation by repeated addition of 5% $Ca(OH)_2$ to obtain a pH of 8-9. Once all casein was solubilized, the reconstitution of $Ca(OH)_2$ was stopped at pH 7.5. The caseinate concentrate solution was then heated to 120°C for 6 seconds and then spray dried. The dried powder was collected and stored in plastic bags.

The acidic whey solution was concentrated at <10°C on a standard Ultrafiltration Membrane (5kDa, Kock Membrane Systems, Wilmington, MA) until a retentate brix of 20° was reached. Diafiltration was started and run until permeate brix <2° was reached using a diafiltration flow equal to permeate flow. The retentate (whey protein concentrate 80%, WPC80) was adjusted to pH 6.5 with a mix of NaOH/KOH, and then heat-treated at 67°C for 10 s and finally spray dried. The dried powder was collected and stored in plastic bags. All protein fractions were analyzed for chemical and bacteriological specifications by the dairy and showed to be suitable for human consumption.

*2.4. Meat protein processing*

Three of the [$d_5$]phenylalanine infused cows were slaughtered 48 h after the 72-h tracer infusion period. The slaughter was conducted at Danish Crown Beef (DC Beef, Aalborg, Denmark) according to Danish legislation for conventional slaughtering of cattle for human food consumption. After slaughter the meat servings were sliced into

226 standard cuts for bovine meat and stored at -40°C. Small cuts and leftovers were minced
227 or discarded such as heart, liver, kidney.

228 Upon preparation for research trial usage, the meat cuts were further cleaned for
229 connective tissue and fat. Hereafter, the cuts were minced using a 3 mm disc. The
230 portions for minced meat servings were packed in sous vide packs and formed as beefs
231 and cooked at 90°C for 20 min and stored at -40°C until usage. The minced meat used
232 for hydrolysate was mixed up in water and under constant stirring heated to 60°C.
233 Hereafter, enzymes (0.1% of meat weight of both the endoprotease Protamex® and the
234 exopeptidase Flavourzyme®, Novozymes, Bagsvaerd, Denmark) were added and the
235 solution was heated under constant stirring: 60°C for 1 h and subsequently 90°C for 15
236 min. The slurry was drained and the pellet (mainly connective tissue proteins) was
237 discarded. The watery hydrolysate was portioned and stored at -40°C until usage.
238

239 *2.5. Human study 1: milk protein*

240 Six young, healthy male participants were recruited by announcement on the
241 internet. Participants were recruited with the following criteria: age 20-30 y, body mass
242 index 20-30 kg/m$^2$, non-diabetic, no regular medication, lactose tolerant, and alcohol
243 consumption below 21 units/wk. Study design, purpose, and possible risks were
244 explained to each participant before informed written consent to participate was given.
245 The study 1 protocol adhered to the Declaration of Helsinki II and was approved by the
246 local Ethics Committee of the Capital Region of Denmark (H-15005598). Subject
247 characteristics are displayed in Table 1.

248 All participants underwent two experiment days in a balanced and randomized
249 crossover design. The participants were blinded for the order of the test meals prior to

the test day, and the interval between experiment days was at least 14 d. The study protocol started at 08:00 with subjects arriving at the laboratory in an overnight-fasted state from 21:00 the evening before. Subjects were instructed to refrain from alcohol and strenuous activities the day before each experiment day. At arrival, the subjects were weighed and their height was measured. Afterwards they were placed comfortably in beds and instructed to stay in bed throughout the day, except from toilet visits. A catheter (18G Venflon, Vasofix safety, Braun, Melsungen, Germany) was inserted in the antecubital vein of one arm, and a baseline blood sample was obtained just before consumption of the test meal or test beverage. Thereafter, the experiment blood sampling protocol was conducted as shown in Fig. 2A, and the experiment day was finished approximately at 16:00 in the afternoon. The subjects had the catheter removed and received a small lunch.

The mixed meal, which consisted of whey protein, mashed potatoes, and butter, and the whey drink both contained the [$d_5$]phenylalanine-labeled whey protein mixed in the ratio 1/10 with unlabeled whey protein (Lacprodan® 80, Arla Foods Ingredients Group P/S, Viby J., Denmark). The aim was to provide the subjects with 20 g of whey protein in total in each of the two different test meals. 2 g of protein from the [$d_5$]phenylalanine-labeled whey, which contained 64% protein, and 18 g of protein from the unlabeled whey protein, which contained 80% protein. The protein content of 64% and 80% were taken into account when calculating the total weight of protein powder to be ingested to achieve the 20 g of protein.

The [$d_5$]phenylalanine-labeled whey drink were dissolved in 400 mL water. In the mixed meal with carbohydrates and fat, the dietary food items were selected to provide a low amount of food-derived protein. The provided food was analyzed in a nutritional

274 software program (Dankost 3000; Dansk Catering Center, Herlev, Denmark). The
275 mixed meal consisted of mashed potatoes with butter due to the low amount of proteins
276 and high content of carbohydrates and fat, respectively. However, the potatoes in the
277 given amount added approximately 10 g of protein to the 20 g of whey protein. The
278 amount of carbohydrates and fat in the meal was calculated to balance the nutritional
279 recommendations of a standard breakfast meal as 25% of the daily nutrient
280 requirements. Resting metabolic rate (RMR) was determined for each participant by the
281 Harris-Benedict equation using age, weight, and height multiplied by an activity factor
282 1.5 for sedentary individuals [34]. The content of energy, protein, carbohydrates, and fat
283 adhered to the general Nordic nutritional recommendations [35] as well as the
284 calculated RMR and are outlined in Table 2. The whey drink was served cold, and the
285 mixed meal was warmed in a microwave oven prior to ingestion. The test meals were
286 ingested in 5-10 min after which the blood samples were timed according to the
287 protocol.
288
289 *2.6. Human study 2: meat protein*
290 The six participants in study 2 were recruited in the same way and with the same
291 criteria as for human study 1. Study design, purpose, and possible risks were explained
292 to each participant before informed written consent to participate was given. The study
293 2 protocol adhered to the Declaration of Helsinki II and was approved by the Ethics
294 Committee of the Capital Region of Denmark (H-15012327). Subject characteristics are
295 displayed in Table 1. The experimental settings and the study protocol were identical
296 with study 1 except for the blood sampling, which was every 15 min in the first h, every
297 30 min from 1-3 h, and for one more h in the end. Furthermore, the tested meals were

298 based on the [$d_5$]phenylalanine-labeled meat. The experiment protocol for human study
299 2 is shown in Fig. 2B.

300 The meat test meals were given after a background blood sample. The meat
301 hydrolysate was given as a 140 mL drink, and the minced meat was given as single 70 g
302 beef. Both test meals were warmed in a microwave oven prior to ingestion and salt and
303 pepper could be added by the participant. The content of energy, protein, carbohydrates,
304 fat, and the AA composition is outlined in Table 3.

305

306 *2.7. Cow and human venous plasma analyses*

307 Cow venous plasma phenylalanine enrichment was measured by gas
308 chromatography-triple-stage quadropole-mass spectrometry (GC-MS/MS, TSQ
309 Quantum, Thermo Fischer Scientific, San Jose, CA) as described in detail previously
310 [36].

311 Human venous plasma phenylalanine enrichment and AA concentrations were
312 analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as
313 described in detail previously [58]. Briefly 100 μL of plasma was mixed with 100 μL
314 full AA profile internal standard solution (Cambridge Isotope Laboratories). The
315 combined AA were converted to their phenylthiocarbamyl derivatives and analyzed on
316 the LC-MS/MS equipment (TSQ Vantage, Thermo Fischer Scientific, San Jose, CA)
317 [37]. The total sum of EAA is comprised of histidine, threonine, valine, methionine,
318 isoleucine, leucine, tryptophan, phenylalanine, and lysine. Cysteine is not included in
319 the analyses.

Venous plasma insulin concentration was measured in human study 1 using a commercial ELISA kit (K6219; Dako Denmark; Agilent Technologies, Glostrup, Denmark).

*2.8. Milk and meat protein analyses*

The [$d_5$]- and [$^{15}$N]phenylalanine enrichment of the milk proteins was measured in four aliquots from each fraction. Eight mg protein powder was added 1 mL of 6 M HCl and left overnight (15 hours) at 110°C.

The [$d_5$]phenylalanine enrichment in various meat cuts and meat hydrolysates was determined. In whole meat proteins we cut out samples from the outside bottom round meat cut of the hind limb. From minced meat, which is a mix of various left over cuts, we randomly took eight samples and similarly we randomly took eight meat hydrolysate samples. From meat samples we isolated samples weighing ~10 mg wet weight (~2 mg protein) and from meat hydrolysates 20 uL (~2 mg protein) and added 1 mL of 6 M HCl and left it overnight (15 hours) at 110°C.

All hydrolyzed food protein samples were after hydrolysis run over acidified cation resin exchange (Dowex AG 50W-X8 resin 100-200 mesh, BioRad, Copenhagen, Denmark) columns (Medium HDPE Open tip column CC07, Intertech Medical Inc., Denver, CO) to purify constituent amino acids. From milk protein and the bottom round of hind limb samples the eluted aliquots of amino acids were derivatized using MTBSTFA + tBDMCS (Regis Technologies, Morton Grove, IL) and acetonitrile, 1:1 and the phenylalanine enrichment was finally measured by GC-MS/MS as described in detail previously [36]. The eluted aliquots of amino acids from the minced meat and the

meat hydrolysate were converted to their phenylthiocarbamyl derivatives and analyzed on the LC-MS/MS equipment as previously described [37].

*2.9. Statistics*

Phenylalanine enrichment and AA concentration data in the human studies were compared by two-factor, repeated ANOVA. In case of main significant effects, Student-Newman-Keuls post hoc tests were performed. The area under the curve (AUC) was compared by two-sided and paired t-tests. All values are means ± SE except human subject characteristics and milk and meat protein enrichments, which are means ± SD. Statistical significance was considered at $P<0.05$, and all statistical analyses were carried out by using GraphPad Prism 7.00 (GraphPad Software, Inc., La Jolla, CA).

## 3. Results

### *3.1. Cow plasma phenylalanine enrichment*

Cow venous plasma phenylalanine enrichment is shown in Fig. 3 for the four [$d_5$]phenylalanine cows and the one [$^{15}$N]phenylalanine cow. Both enrichments rose quickly after the start of the infusion as measured in the first sample after start at time point 15 min. The gross mean enrichments from 12-72 h reached a level of 28±7 SD and 35±3 tracer to tracee ratio % (TTR%) for the four [$d_5$]phenylalanine cows and the one [$^{15}$N]phenylalanine cow, respectively. The enrichments quickly decreased and leveled off 4-16 h after the infusions were stopped at 4±1 and 3±0.2 TTR% for the [$d_5$]- and [$^{15}$N]phenylalanine cows, respectively.

### *3.2. Milk and meat yield and protein phenylalanine enrichment*

Milk and milk protein yields during the collection were 46.7 ± 3.6 kg/d and 1458 ± 50 g/d, respectively, equivalent to the gross delivery before experimentation. A total of 2.0 kg of [$d_5$]phenylalanine-whey and 1.0 kg of [$d_5$]phenylalanine-caseinate was obtained from the 700 kg of milk. The low yield of caseinate was due to unforeseen problems with the drying process. A total of 1.5 kg of [$^{15}$N]phenylalanine-whey, which could only be concentrated to a 35% protein content due to the relative low amount of milk (150 kg), and 2.0 kg of [$^{15}$N]phenylalanine-caseinate was obtained. The enrichments of the whey and caseinate are shown in Table 4. The [$d_5$]phenylalanine enrichment was higher than the [$^{15}$N]phenylalanine enrichment in both the whey and caseinate proteins.

The total yield of selected meat cuts was 9.7 kg tenderloin, 42.5 kg filet, 7.7 kg culotte, 13.0 kg cuvette, 25.8 kg inner thigh, and 175 kg minced meat. Meat mixed protein [$d_5$]phenylalanine enrichments at the 20 different sampled sites in the whole hind limb and in the meat hydrolysate and meat minced beef are shown in Table 4.

*3.3. Human study 1: milk protein*

All venous plasma results for human study 1 are displayed in Fig. 4. All data revealed a significant interaction (treatment x time, $P<0.001$). [$d_5$]phenylalanine enrichments (Fig. 4A) showed a faster response after the whey only intake as compared to the whey mixed meal intake, and the whey only response was significantly higher at 30 min and 1 h as compared to the whey mixed meal. However, at 3 h the whey mixed meal response was significantly higher than the whey only response. The AUC for [$d_5$]phenylalanine enrichments were $1.33 \pm 0.05$ and $1.50 \pm 0.04$ for whey only and whey mixed meal, respectively. The AUC was significantly highest after intake of the whey mixed meal ($P<0.01$). Concentrations of phenylalanine (Fig. 4B), leucine (Fig. 4C), total EAA (Fig. 4D), and the total AA (Fig. 4E) all showed similar responses, however, not with respect to the AUC. The AA concentration responses were faster and more pronounced after the whey only intake, and the concentrations after both types of test meal peaked in general at 1 h, except for phenylalanine that peaked at 30 min after the whey only intake. The peaks and also the 30 min time point was significantly higher after the whey only intake as compared to the whey mixed meal intake. The AUC were not significant different in any of the concentration measurements.

Venous plasma insulin concentrations showed a marked significant difference between the two test meals at 30 min to 1.5 h. The insulin response peaked at 30 min

after both meals, but whereas the peak was 315 pmol/L after the whey mixed meal intake, it was only 78 pmol/L after the whey only intake. The AUC was significantly higher after the whey mixed meal as compared to the whey only meal ($P<0.05$).

*3.4. Human study 2: meat protein*

All venous plasma results for human study 2 are displayed in Fig. 5. All data revealed a significant interaction (treatment x time, $P<0.001$). [$d_5$]phenylalanine enrichments (Fig. 5A) showed a faster response after meat hydrolysate intake as compared to the meat minced beef intake, and the meat hydrolysate response was significantly higher at 15 and 30 min as compared to the meat minced beef. Phenylalanine (Fig. 5B), leucine (Fig. 5C), total EAA (Fig. 5D), and the total AA concentration (Fig. 5E) all showed similar responses. The AA concentration responses were significantly faster after the meat hydrolysate intake as compared to the meat minced beef as 15 and 30 min concentrations were significantly highest after the meat hydrolysate intake. The concentrations peaked at 30 min after the meat hydrolysate intake and at 1 h after the meat minced beef intake. In the later phase after the test meal intakes, the AA concentrations were significantly higher after the meat minced beef intake as compared to the meat hydrolysate intake. This was at 1.5-2 h for the phenylalanine and the total AA concentrations, at 2 h only for the leucine concentration, and at 1.5 to 2.5 h for the EAA concentration. None of the AUC data were significantly different between the meat hydrolysate and the meat minced beef intake.

## 4. Discussion

The cow tracer infusion protocol, the milking schedule, and the meat collection all showed to be feasible and produced foods suitable for human consumption that were sufficiently intrinsically labeled to trace the phenylalanine label in *in vivo* human experimentation. The two human studies showed that the [$d_5$]phenylalanine label was a powerful tool to trace and determine the peripheral circulating availability of dietary protein-derived phenylalanine, which is not necessarily reflected in plain concentration measurements of phenylalanine or other AA. [$^{15}$N]phenylalanine labeled milk proteins are not used in the two human studies within this work, but having enrichments of around 20 MPE (Table 4) they are expected to show the same properties as the [$d_5$]phenylalanine labeled milk proteins.

### *4.1. Production of labeled dietary proteins*

Intrinsically labeled milk proteins [4,28,29] and also meat proteins [24, 30,31] have been produced before. The cost and industrial expertise of production of these intrinsically labeled proteins (from tracer purchase, dairy cow management and manufacturing of protein ingredient products) require an extensive cross-disciplinary collaboration with significant industry involvement. However, once produced the intrinsically labeled proteins are a very powerful methodological tool to assess the fate and utilization of nutrient-derived AA, although we would like to pay attention to some recent discussion with the use of the proteins in determining the exogenous rate of appearance [17].

*4.2. Human study application*

The applicability of intrinsically labeled proteins in human studies investigating effects of protein intake spans numerous topics and research questions. By having the milk and meat proteins labeled it is possible to detect and quantify the appearance of nutrient-derived AA into the circulation (covering digestion rate and splanchnic extraction), which has been demonstrated previously [1,3,38-43]. Depending on the availability of sampling sites and modeling the splanchnic outflow (arterial and hepatic vein blood), the peripheral whole-body (arterialized venous blood) and/or limb utilization (arterial and region of interest venous blood), the utilization in energy metabolism/oxidation (metabolite tracing in and blood or breath $CO_2$ collection), as well as the incorporation into newly synthesized proteins (protein sampling) can be assessed [44-47]. In general, access to sampling sites is a limiting factor both in the present and other human settings. Plasma labeled AA availability in the postprandial period can therefore be applied as an indirect indication of protein digestion and AA absorption rates, and dependent on the study setup different kinds of modeling can also be applied [23,24,44-48].

The use of meat proteins in this context is less applied [24,30,31]. Due to the slow turnover rate of meat proteins only little label is incorporated and tracing the label after ingestion and absorption is technically challenging. However, the enrichment in the meat products in the present study (Table 4) is sufficient to detect appearance in the circulation in the postprandial phase (Fig. 5A) with the analytical sensitivity on the LC-MS/MS equipment setup [37]. We used the model to investigate the characteristics of a newly developed quick-hydrolysate from meat protein. We found that the meat hydrolysate, just like e.g. casein hydrolysate, is digested and AA appearing fast in the

476 blood as compared to a minced meat beef, which previously has been shown to be faster
477 than steak [39]. Minced meat intake has been compared with mixed milk protein (20/80
478 mixture of whey and casein) and found to induce a similar muscle protein synthetic
479 response [48]. The present data reveal that the protein-derived phenylalanine
480 enrichment as well as AA concentrations peak at 30 min and 1 h after the intake of the
481 meat hydrolysate and the minced meat beef, respectively. These fast characteristics of
482 the meat hydrolysate could in part be facilitated by a faster gastric emptying. Future
483 studies should be conducted to reveal the anabolic potentials of meats and meat-derived
484 ingredients alone and when supplied as protein source in foods.
485 Another application that we tested in the present study was the impact of co-
486 ingestion of carbohydrate and fat on the appearance of protein-derived label in the
487 circulation. We used the [$d_5$]phenylalanine labeled whey and demonstrated a delayed
488 appearance of the intrinsic AA label in the circulation (Fig. 4A). The labeled
489 phenylalanine enrichment plateaued at 30 min and 1 h after the whey only intake at a
490 higher level than the plateau after the mixed meal from 30 min to 2 h (Fig. 4A). The
491 gross average of hyperaminoacidemia in the postprandial period turned out to be lower
492 after the mixed meal than after the whey protein alone, despite that a net of 50% more
493 protein was provided with the mixed meal (from mashed potatoes). While a postponed
494 uptake would be expected due to the content of carbohydrate and fat delaying gastric
495 emptying and absorption of AA [8-10], this would not be expected to affect the gross
496 average of AA concentrations. The explanation for the differences in concentrations is
497 most likely a change in the balance of the peripheral flux rates of AA possibly
498 accomplished by the insulin response [49], which was markedly higher after the mixed
499 meal (Fig. 4F). A combination of a stimulated influx into tissues and a dampened efflux

out of the tissues lowers the concentrations. Infusion of another phenylalanine tracer would have allowed us to determine these rates. However, this was not the purpose of the present study.

In summary, the strong methodological benefit of applying intrinsically labeled proteins either alone or in combination with other nutrients is the most valid and precise measure of how the protein-derived AA are handled by the splanchnic bed and appearing in the circulation. This advantage can be applied in wide ranging clinical research questions and only sampling sites limit the interpretation.

*4.3. Perspectives for tracer applications*

The advantages of use of labeled proteins in metabolic research are multiple and necessary to consider in order to gain valid data on many nutritional questions (e.g. a clinical setup after Roux-en-Y gastric by-pass surgery [50,51]). Extensive protocols involve infusion of one or more other tracers and/or blood sampling from various sites (arterial and venous) and tissue sampling. Further, it is the only approach that can directly assess digestion and/or splanchnic utilization of dietary protein-derived AA, which though require sampling access at specific sites. Another application is the use of the labeled protein as a mean of providing the tracer for the assessment of the protein fractional synthesis rate by the direct incorporation technique [52]. Yet another application is to combine the dietary labeled protein with a continuous infusion of another stable isotope AA tracer. The intake of intrinsically labeled whole proteins stimulates protein turnover differently when compared to crystalline AA intake or combinations of protein and a single AA tracer intake [53,54].

A pertinent question concerns dietary protein source. In this study, we obtained bovine milk and meat proteins. It is possible to label milk [4,28,29], meat [24,30,31], egg [21,22,24], and soy [32] proteins, in principle most proteins. An understudied source of proteins in relation to exercise, digestion, and whole-body and muscle protein metabolism is the plant-based proteins. Few studies have compared plant to milk protein, both acute [55] and long-term [56]. Wheat proteins are also demonstrated to induce a lower anabolic response as compared to milk proteins, but this lower response can be overcome by ingesting a greater total amount of protein [57]. However, ingestion of protein blends consisting of both animal- and plant-based proteins may be a promising strategy to stimulate whole-body and muscle protein synthesis [58-60]. Recently, the pros and cons on physiological response parameters of plant proteins and their individual AA compositions and digestive properties have been discussed [61], and it has been suggested that plant-based proteins can be fortified with respects to the AA composition by enhancing the EAA part to achieve a greater anabolic potential [62,63]. Therefore, future research could be directed to investigate the metabolic and health effects of this wide range of protein sources (animal- and plant-based) in the context of natural eating behavior containing mixed macronutrients and mixed protein sources.

## 5. Conclusions

The process of producing intrinsic labeled feed proteins is both challenging, expensive, and demanding in terms of facilities, legislation, and collaboration between academia and industry. However, once in house, the intrinsically labeled proteins allow unique possibilities of nutritional investigations, which would not be possible with the same accuracy and validity by other means. We here report two examples of human trials demonstrating the applicability and exemplifies results that warrants more investigations. The present findings clearly show that the dietary matrix has profound effects on the postprandial aminoacidemia. Furthermore, the perspectives for use of labeled dietary proteins are wide ranging and cover nutrition research topics within the clinic, sports, and age-related scientific fields.

## Statement of authorships

SR, BT, JA, KD, GH, MEM, ACS, KRP, ETH, GvH, PL, and LH planned and conducted the experimental work. SR, BT, JA, KD, GH, MEM, GvH and LH analyzed and interpreted the data. SR and LH designed the study and drafted the manuscript. All authors contributed and edited the manuscript, and all authors approved the final content and this version of the manuscript.

## Conflicts of interest statement

Søren Reitelseder and Lars Holm have received funding from The Danish Dairy Research Foundation, Arla Foods Ingredients Group P/S and DC Ingredients. Peter Lund has received funding from The Danish Dairy Research Foundation. Kristian Raaby Poulsen is employee at Arla Foods Ingredients Group P/S, and Erik T. Hansen is employee at DC Ingredients. Otherwise, the authors declare no conflicts of interest.

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812 **Figure legends**

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817 **Fig. 1.** The cow infusion protocol. Five Holstein cows were infused for 72 h (started at
818 13:00), venous blood samples were collected before, during and after infusion, and milk
819 was collected three times daily during and at 8 and 16 h after the infusion was stopped.
820 Four cows received L-[ring-$d_5$]phenylalanine, and one cow received L-
821 [$^{15}$N]phenylalanine. Three of the L-[ring-$d_5$]phenylalanine infused cows were
822 slaughtered after the infusion and milk collection, and the meat was parted, vacuum
823 packed, frozen, and stored at -40° C.

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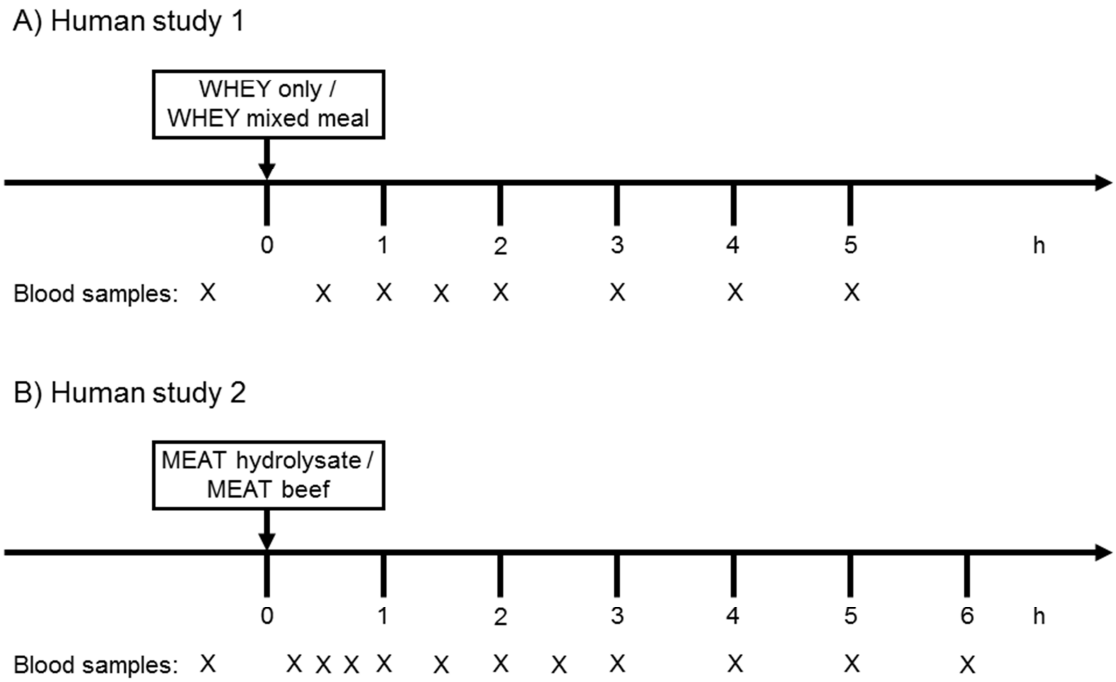


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828 **Fig. 2.** The human study 1 and 2 protocols. A) Human study 1: six young participants
829 ingested whey protein alone or as part of a mixed meal after an overnight fast. B)
830 Human study 2: six young participants ingested meat protein in the form of hydrolysate
831 or minced beef after an overnight fast. Both study 1 and 2 were cross-over trials with a
832 minimum of 14 d between each trial. Venous blood samples were collected in the fasted
833 state and for 5-6 h after protein ingestion as shown.

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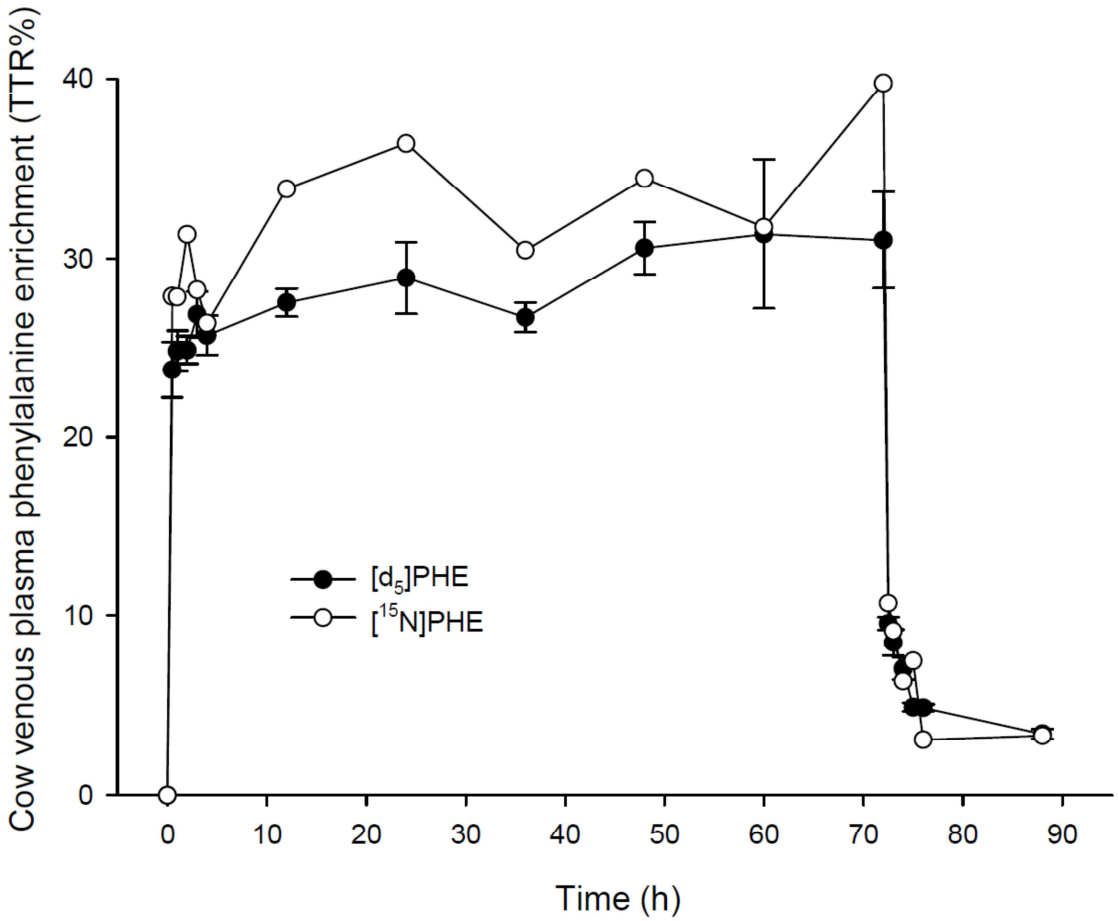


836

837 **Fig. 3.** Cow venous plasma phenylalanine enrichment. Four cows were infused with L-

838 [ring-$d_5$]phenylalanine, and one cow was infused with L-[$^{15}$N]phenylalanine. Values are

839 means ± SE for the [$d_5$]phenylalanine curve.

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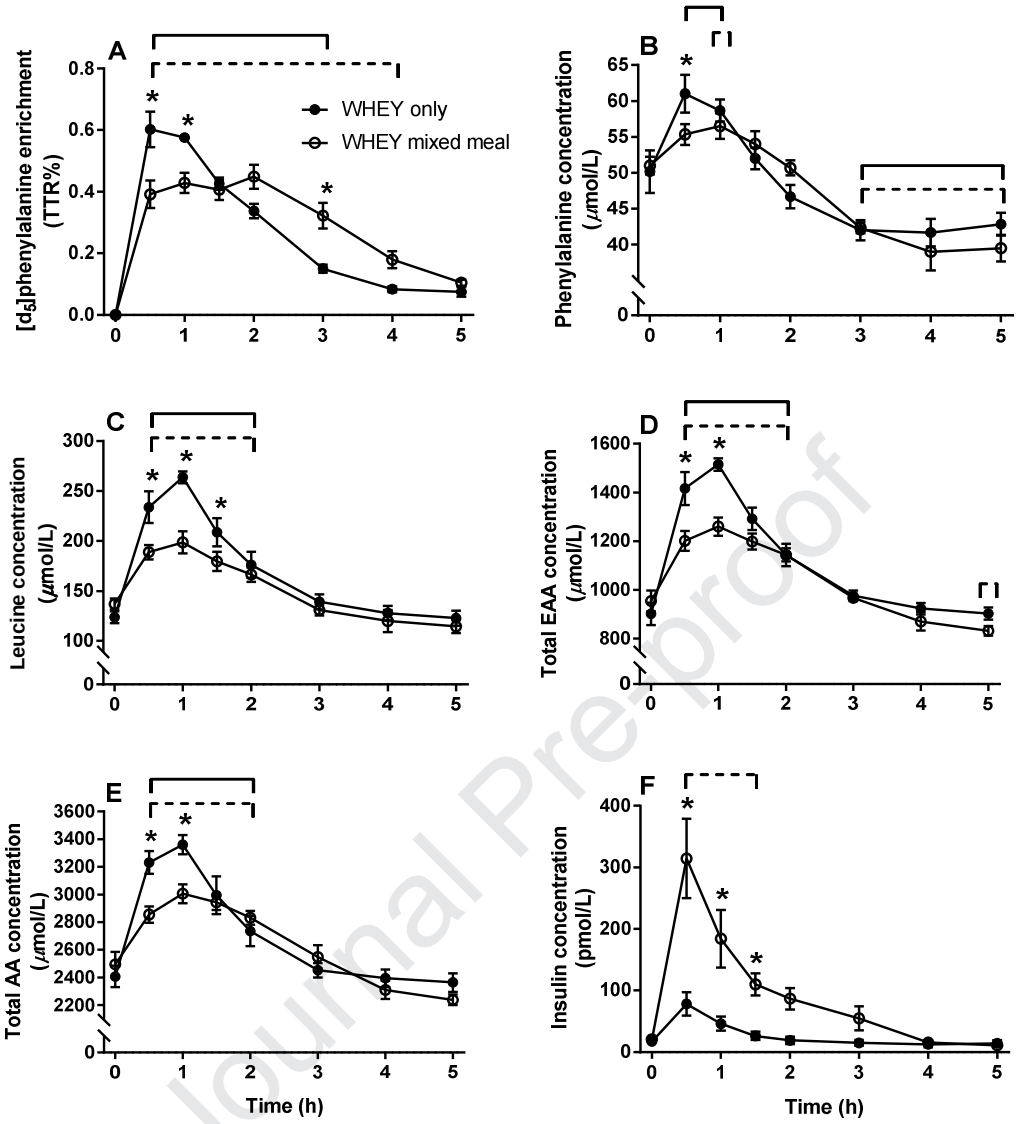


842

843 **Fig. 4.** Human study 1 milk proteins, venous plasma results. Means ± SE are shown for
844 [$d_5$]phenylalanine enrichment (A), and concentrations of phenylalanine (B), leucine (C),
845 total EAA (D), total AA (E), and insulin (F) at baseline in the fasted state (0 h) and
846 following intake of whey only and whey mixed meal. Data were analyzed with 2-factor
847 repeated measures ANOVA, and all measures had significant interaction (treatment x
848 time, $P<0.001$). Student-Newman-Keuls post-tests showed: *) treatment difference at
849 time point ($P<0.05$); solid line) time point different from baseline within WHEY only

850 (P<0.05); dashed line) time point different from baseline within WHEY mixed meal

851 (P<0.05).

852

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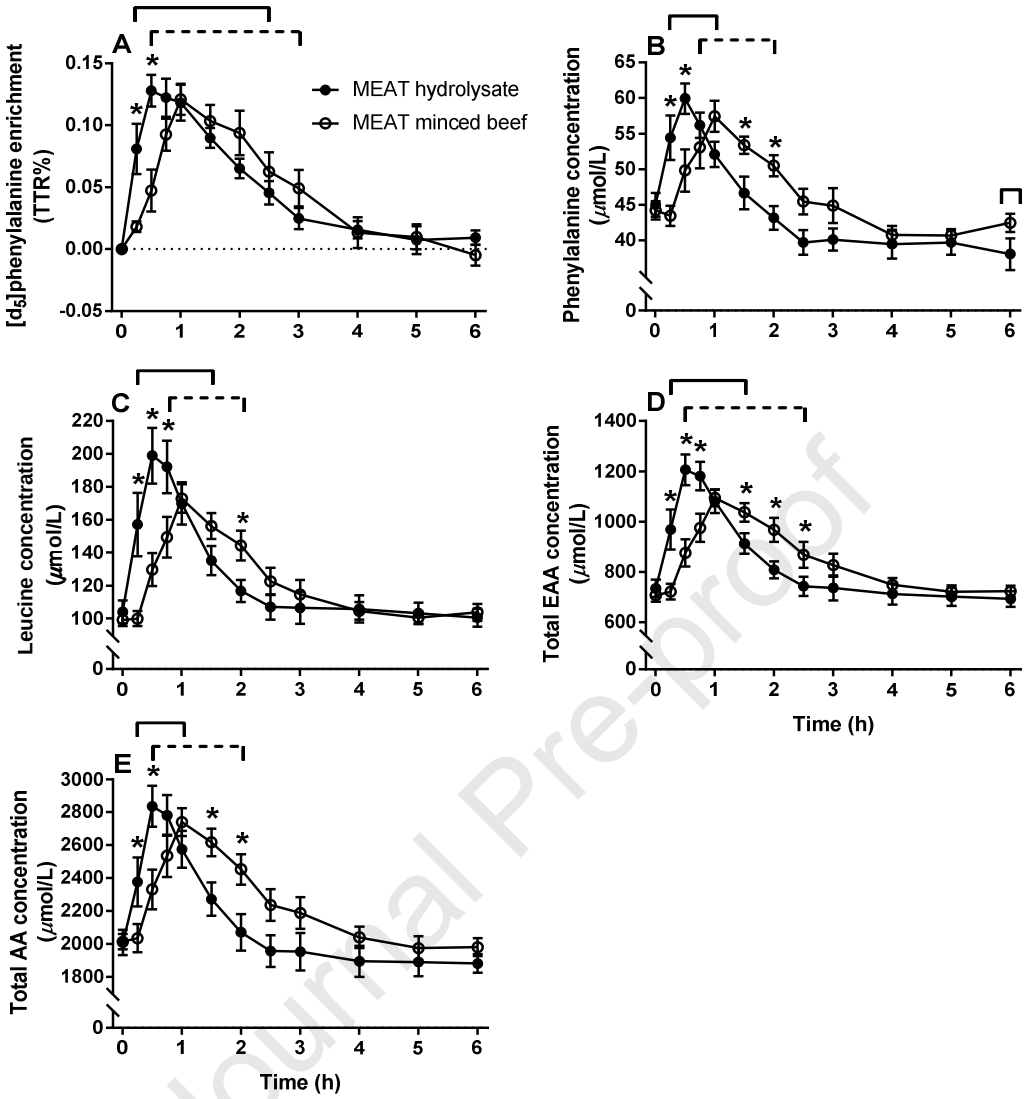


854
855

856 **Fig. 5.** Human study 2 meat protein, venous plasma results. Means ± SE are shown for
857 [$d_5$]phenylalanine enrichment (A), and concentrations of phenylalanine (B), leucine (C),
858 total EAA (D), and total AA (E) at baseline in the fasted state (0 h) and following intake
859 of MEAT hydrolysate and MEAT minced beef. Data were analyzed with 2-factor
860 repeated measures ANOVA, and all measures had significant interaction (treatment x
861 time, $P<0.001$). Student-Newman-Keuls post-tests showed: *) treatment difference at
862 time point ($P<0.05$); solid line) time point different from baseline within MEAT

863 hydrolysate only ($P<0.05$); dashed line) time point different from baseline within

864 MEAT minced beef only ($P<0.05$).

865

866

867 **Tables**

868

| | Human study 1 (n = 6) | | | Human study 2 (n = 6) | | |
|---|---|---|---|---|---|---|
| Age (y) | 25 | ± | 2 | 25 | ± | 3 |
| Weight (kg) | 90.6 | ± | 6.4 | 76.0 | ± | 10.1 |
| Height (m) | 1.89 | ± | 0.02 | 1.78 | ± | 0.06 |
| BMI (kg/m$^2$) | 25.52 | ± | 2.09 | 23.87 | ± | 2.81 |

869 **Table 1** Subject characteristics in human study 1 and 2. Values are means ± SD.

870

871

| Meal composition per serving | WHEY only | WHEY mixed meal* |
|---|---|---|
| Energy (kcal) | 101.8 | 759.4 ± 26.3 |
| Protein (g) | 20.0 | 30.4 ± 0.4 |
| Protein (kcal) | 80.0 | 121.5 ± 1.7 |
| Protein (E%) | 78.6 | 16.0 ± 0.3 |
| Carbohydrate (g) | 1.4 | 94.8 ± 3.8 |
| Carbohydrate (kcal) | 5.6 | 379.0 ± 15.0 |
| Carbohydrate (E%) | 5.5 | 49.9 ± 0.3 |
| Fat (g) | 1.8 | 28.8 ± 1.1 |
| Fat (kcal) | 16.2 | 258.9 ± 9.8 |
| Fat (E%) | 15.9 | 34.1 ± 0.2 |

872

873 **Table 2** Macronutrient of whey only and whey mixed meal (human study 1). All values
874 are in g, kcal, and energy% (E%), *) values are means ± SD due to the fact that the
875 amount of food ingredients (except the 20 g of whey) was based on 25% of the daily
876 nutrient requirements as determined by the individual resting metabolic rate with an
877 activity factor of 1.5.

878

879

| Meat composition per serving | MEAT hydrolysate drink | MEAT minced beef |
|---|---|---|
| Total served weight (g) | 140 | 70 |
| Energy (kcal) | 77 | 92 |
| Protein (g) | 17.2 | 17.3 |
| Protein (E%) | 89.5 | 75.1 |
| Carbohydrate (g) | 0.1 | 0.1 |
| Carbohydrate (E%) | 0.7 | 0.3 |
| Fat (g) | 0.8 | 2.5 |
| Fat (E%) | 9.8 | 24.6 |
| Water (g) | 121 | 49 |
| Amino acids per serving (g) | | |
| Alanine | 0.95 | 1.06 |
| Arginine | 0.96 | 1.08 |
| Asparagine | 1.53 | 1.69 |
| Cysteine | 0.13 | 0.11 |
| Glutamine | 2.55 | 2.61 |
| Glycine | 0.68 | 0.77 |
| Histidine | 0.64 | 0.63 |
| Isoleucine | 0.75 | 0.82 |
| Leucine | 1.34 | 1.47 |
| Lysine | 1.50 | 1.58 |
| Methionine | 0.37 | 0.33 |
| Phenylalanine | 0.72 | 0.81 |
| Proline | 0.59 | 0.61 |
| Serine | 0.64 | 0.74 |
| Threonine | 0.75 | 0.85 |
| Tryptophan | 0.20 | 0.21 |
| Tyrosine | 0.55 | 0.61 |
| Valine | 0.81 | 0.88 |
| Total essential amino acids | 8.04 | 8.65 |
| Total amino acids | 15.66 | 16.85 |

**Table 3** Macronutrient and amino acid composition of meat hydrolysate and meat minced beef (human study 2). All values are per serving in g, kcal, and energy% (E%).

| | [$d_5$]phenylalanine | [$^{15}$N]phenylalanine |
|---|---|---|
| Whey | 15.44 ± 0.24 | 19.18 ± 0.13 |
| Caseinate | 17.06 ± 0.07 | 20.81 ± 0.02 |
| Meat (hind limb) | 0.41 ± 0.04 | - |
| Meat hydrolysate | 0.73 ± 0.01 | - |
| Meat minced beef | 0.63 ± 0.04 | - |

884

885 **Table 4** Milk and meat protein phenylalanine enrichment. Milk protein enrichment was
886 analyzed in four aliquots from each protein. Meat protein enrichment was measured in
887 20 samples from the outside bottom round muscle of the hind limb, eight meat
888 hydrolysate samples, and eight meat minced beef samples. Values are means in mole
889 percent excess (MPE) ± SD.

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