Goat milk protein digestibility in relation to intestinal function

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ABSTRACT

Background: Milk is an important high-quality animal protein source in low- and middle-income countries (LMICs). Although the true ileal digestibility and absorption of milk has been shown to be high in French adults, this may be lower in individuals from LMICs who are at risk of environmental enteropathy.

Objective: To determine the true ileal indispensable amino acid (IAA) digestibility of intrinsically labeled goat milk protein in South Indian women of reproductive age (WRA), using the dual-isotope tracer technique, and to measure intestinal absorption of amino acid and inert sugar in the same participants using L-allo-isoleucine and a dual-sugar assay.

Methods: Milk with 2H-labeled protein collected from a lactating goat fed intrinsically 2H-labeled fodder (maize and cowpea) was spray dried. Labeled milk protein was administered in a plateau feeding protocol to WRA with normal BMI, in whom urinary lactulose and mannitol recovery and the lactulose/mannitol ratio (LMR) were measured, to determine its true ileal IAA digestibility by the dual-isotope tracer technique with a reference U-13C–amino acid mixture. A phenylalanine absorption index was calculated from the plasma to meal ratio of 13C9 phenylalanine within the digestibility protocol. On a separate day, the allo-isoleucine absorption index was estimated from the ratio of plasma allo-isoleucine enrichments after oral 13C6-15N-L- and intravenous 2H10-L-allo-isoleucine administration.

Results: The means ± SDs of true ileal IAA digestibility of goat milk protein, lactulose and mannitol recovery, LMR, allo-isoleucine and phenylalanine absorption index were 94.0 ± 2.9%, 0.09 ± 0.03%, 7.9 ± 2.3%, 0.012 ± 0.004, 88.4 ± 3.8% and 24.5 ± 1.6%, respectively. The LMR correlated with the allo-isoleucine absorption index (r_s = -0.93, P = 0.008).

Conclusion: The true ileal digestibility of goat milk protein in South Indian WRA with normal intestinal absorptive function and integrity was comparable to earlier estimates in healthy French adults. Am J Clin Nutr 2021;113:845–853.

Keywords: milk protein, intrinsic labeling, dual-isotope tracer technique, lactulose to mannitol ratio, amino acid absorption

Introduction

Animal source foods (ASFs) have been shown to be beneficial for growth, cognitive performance, physical activity, and health, particularly in children and pregnant women (1–3). Milk is a common ASF in many low- and middle-income countries (LMICs), and a recent analysis of a nationally representative Indian survey underlined the association of household milk consumption, represented by maternal consumption, with a moderate reduction (3%) in stunting of their preschool children (4). Milk protein consumption during pregnancy in South Indian women was also shown to be positively associated with birth weight (5). These observations emphasize the importance of ASF consumption, particularly in India, where the risk of quality-corrected protein inadequacy is high (6).

The quality of an ASF protein is dependent not only on its amino acid score but also on its true ileal indispensable amino acid (IAA) digestibility, which is the combination of

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Supplemental Tables 1–3 and Supplemental Figures 1 and 2 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn.

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Abbreviations used: ASF, animal source food; EE, environmental enteropathy; ELU, Experimental Livestock Unit; HIC, high-income country; IAA, indispensable amino acid; IV, intravenous; LMIC, low- and middle-income country; LMR, lactulose/mannitol ratio; NIANP, National Institute of Animal Nutrition and Physiology; ppm, parts per million excess; r_s, Spearman’s Rho; TCF, transamination correction factor; WRA, women of reproductive age.

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protein digestion and IAA absorption at the ileal level (7). Milk is shown to be a high-quality protein with optimum amino acid score and high digestibility in high-income countries (HICs) (8). Although food processing and the food matrix can affect protein digestibility (9), another factor that could limit digestibility is the impairment of intestinal function by environmental enteropathy (EE) mostly prevalent in LMICs (10). Earlier reports suggest lower brush-border enzyme activity and reduced sugar, amino acid, and dipeptide absorption in asymptomatic adults from LMICs compared with their Western counterparts (11, 12). In comparison to US and Jamaican women, healthy South Indian women showed lower intestinal absorptive capacity and functional enterocyte mass, as measured by mannitol absorption (37% and 41% compared with 15%) and citrulline flux (12 and 10 compared with 9 μmol/kg fat-free mass/h), along with a unique gut microbiota with functional correlates (13). Asymptomatic Indians residing in the United Kingdom also showed altered intestinal morphology and greater intestinal permeability compared with the native population (14). The question therefore arises whether milk protein is equally well digested and absorbed in LMICs compared with HICs.

The true ileal IAA digestibility of a protein can be indirectly measured by the minimally invasive dual stable isotope tracer technique (15) by comparing the plasma appearance of individual IAAAs from an intrinsically (1) labeled test protein with that of a differently (13C) labeled reference protein/free IAAAs of known digestibility (15). The technique, when referenced against free IAAAs, represents digestion, with an assumption of similar absorption of the test and reference amino acids. Small intestinal permeability and absorption can be assessed by the noninvasive dual-sugar assay with lactulose and mannitol (16). Intestinal amino acid absorption can be measured as an absorption index resulting from the administration of isotopically labeled nonprotein isotopeologues of L-amino acids, such as allo-isoleucine, via oral and intravenous routes (17, 18). This study aimed to measure the true ileal digestibility of goat milk protein in healthy South Indian women of reproductive age (WRA) by the dual-isotope tracer technique while also characterizing their intestinal absorption by the allo-isoleucine absorption index and intestinal permeability by the dual-sugar assay.

Methods

Intrinsic labeling of goat milk

The intrinsic labeling of goat milk protein (hereafter referred to as milk protein) was performed in 2 stages. In the first stage, a fodder crop for goat feed was intrinsically labeled with deuterium oxide (2H2O, 99.9%; Sercon Ltd), and in the second stage, the 2H-labeled fodder was administered to a lactating goat and her milk collected.

Fodder crops, maize (Zea mays; var. African black) and cowpea (Vigna unguiculata; var. KM 5), were grown in the rainy (kharif) season of 2018 at the University of Agricultural Sciences, Bengaluru, India. A primed pulse labeling as previously described was followed with modifications in the gravimetric irrigation protocol, where the field capacity of the soil was maintained at 100% and 70% for maize and cowpea, respectively, until harvest and the 2H2O dosing (19). Based on a pilot study to ensure sufficient enrichment of fodder for subsequent adequate labeling of milk protein for a human digestibility study, the 2H2O pulsed dosing was initiated after the 50% flowering stage with a bolus of 400 mL (35% 2H2O, w/w) on the first day, followed by 100-mL pulses of 7.5% 2H2O (w/w) on days 3, 5, 7, and 9 for both crops. An extra 100-mL pulse of 7.5% was administered on day 11 for the maize crop. The plants were harvested 10 d after the last pulse to ensure maximal uptake of residual 2H2O in the soil, shredded, air dried, and stored for feed.

The Institutional Animal Ethical Committee of St. John’s Medical College approved the goat milk intrinsic labeling experiment and handling procedures. A lactating doe (Capra aegagrus hircus; common name, Beetal; 4 y old, 44-kg weight) and its kid were housed in the Experimental Livestock Unit (ELU) of the National Institute of Animal Nutrition and Physiology (NIANP), Bengaluru, India. The doe was dewormed, vaccinated against enterotoxaemia, and acclimatized to the ELU and the feed (unlabeled dried maize and cowpea fodder, with concentrate mixture prepared by the NIANP) for 45 d before the actual labeling protocol began. For the intrinsic labeling protocol of milk, the dried 2H-labeled fodder was substituted into the feed administered to the doe for 17 d. The amount provided was 750 g/d of 2H-labeled maize and 160 g/d of 2H-labeled cowpea with 500 g/d of unlabeled protein concentrate mixture. The quantity was determined based on the feed consumed by the doe during the acclimatization period and adjusted to provide 60% protein from the labeled fodder and the remaining from the concentrate mixture. The feed administered met the daily protein and energy requirements of a lactating goat in accordance with the National Research Council (20). The amount of leftover feed was quantified each day, ranging from 117 to 493 g and 0 to 21 g of maize and cowpea, respectively. Water was provided ad libitum throughout the study duration.

During this period, the kid was weaned from doe to cow milk to ensure that all the 2H-labeled milk was available for the human protein digestibility study. The doe was milked twice daily (morning and evening), and the collected milk was pasteurized immediately at 72°C for 1 min, then cooled to room temperature and stored in a ~20°C freezer, until it was taken for spray drying. The milk was collected from 2 d before until the 21st day of the labeled fodder feeding protocol. Milk from days 4 to 20 was pooled and spray dried (Bowen Engineering; inlet temperature, 150°C; outlet temperature, 90°C) at the Central Food Technological Research Institute, Mysuru, India. The spray-dried milk powder was stored at 4°C for subsequent human experiments.

Human studies

All participants were recruited from the staff population of St. John’s Research Institute, Bengaluru, India. Healthy nonpregnant and nonlactating women (n = 7) with a normal BMI (between 18.5 and 25 kg/m2), aged between 20 to 35 y, were included in the study. Participants who were nonsmokers, without any food allergies, and reporting no medical or surgical illness within 3 mo of the study were selected. Medical history and clinical examination were performed to rule out history of gastrointestinal symptoms, antibiotic and iron supplement intake within 4 wk of the study, nonsteroidal anti-inflammatory drug use, and alcohol consumption in the past 1 wk. All experiments were conducted during the proliferative or secretory phase of their...
menstrual cycle. Informed written consent was obtained from the participants. The study protocol was approved by the Institutional Ethical Review Board of St. John’s Medical College Hospital. Participant screening and enrollment details are provided in Supplementary Figure 1.

Measurement of intestinal permeability, allo-isoleucine absorption, and milk protein digestibility

Along with the measurement of milk protein digestibility, intestinal function was characterized by the evaluation of intestinal permeability by a dual-sugar test and the measurement of allo-isoleucine absorption. While the noninvasive dual-sugar test was performed first, the order of the invasive (intravenous administration and blood sampling) allo-isoleucine absorption and milk protein digestibility was randomly allocated using a research randomizer tool. In total, there were 3 separate experiment days conducted within 2 wk.

Dual-sugar absorption test

After an overnight fast of 10 h, participants reported to the Division of Nutrition of St. John’s Research Institute at 08:00 on the study day. A baseline urine sample was collected from each participant, after which an oral solution of lactulose (5 g; Tokyo Chemical Industry) and mannitol (1 g; Merck) in 75 mL of water was administered to them (21). Urine samples were collected hourly after that, up to 5 h postdose (Figure 1). Participants were not allowed any food or beverages except water until the end of the experiment. Postdose hourly urine samples were proportionally pooled for 1–2 and 1–5 h, aliquoted, and stored at –20°C until analysis.

Allo-isoleucine absorption

A dual differentially labeled stable isotope nonprotein amino acid, allo-isoleucine, study was conducted to assess amino acid absorption (18). On the day of the experiment, participants were allocated to the metabolic ward at 06:30 after an overnight fast of 10 h. Two indwelling intravenous (IV) cannulas (Jelco 22 G; Medex Medical Ltd.) were secured in each arm, one for IV infusion of the tracer and the other for venous blood sampling. The participants remained in a reclining position with minimal physical activity throughout the experiment. The experimental protocol started at 07:00 and continued for a duration of 8 h. A continuous IV infusion of $^{2}$H$_{10}$-allo-isoleucine (0.3 μM/kg/h, >99%; Cambridge Isotope Laboratories) was started simultaneously with half-hourly oral boluses of $^{13}$C$_{6}$,$^{15}$N-allo-isoleucine (0.3 μM/kg/h, >99%; Cambridge Isotope Laboratories) until the end of the protocol. The participants were provided one-third of their daily sedentary energy and protein requirement (Table 1) during the protocol, composed of ultra-heat-treated milk (Goodlife; Karnataka Milk Federation) with beet sugar and protein-free wheat starch cookies (Supplementary Table 1). The total meal amount was divided into 16 equal portions, each portion representing a mini meal, which was fed half-hourly from the start of the protocol, to ensure a steady plateau pattern of feeding. A basal blood sample (4 mL) was collected prior to starting the infusion, followed by hourly samples from 5 h onward, until the end of experiment (Figure 1). Whole blood was transferred into EDTA-coated evacuated tubes (Becton Dickenson) and centrifuged at 1098 × g at 4°C for 10 min to separate plasma, which was aliquoted and stored at –80°C until analysis.

Milk protein digestibility

On a separate day, participants reported at 06:30 to the metabolic ward after an overnight fast of 10 h. An indwelling venous cannula (Jelco 22 G; Medex Medical Ltd.) was secured at the beginning of the experiment for blood sample collection. The participants remained in a reclining position with minimal physical activity throughout the experiment. The measurement protocol started at 07:00 and continued for a duration of 8 h. A continuous IV infusion of $^{2}$H$_{10}$-allo-isoleucine (0.3 μM/kg/h, >99%; Cambridge Isotope Laboratories) was started simultaneously with half-hourly oral boluses of $^{13}$C$_{6}$,$^{15}$N-allo-isoleucine (0.3 μM/kg/h, >99%; Cambridge Isotope Laboratories) until the end of the protocol. The participants were provided one-third of their daily sedentary energy and protein requirement (Table 1) during the protocol, composed of ultra-heat-treated milk (Goodlife; Karnataka Milk Federation) with beet sugar and protein-free wheat starch cookies (Supplementary Table 1). The total meal amount was divided into 16 equal portions, each portion representing a mini meal, which was fed half-hourly from the start of the protocol, to ensure a steady plateau pattern of feeding. A basal blood sample (4 mL) was collected prior to starting the infusion, followed by hourly samples from 5 h onward, until the end of experiment (Figure 1). Whole blood was transferred into EDTA-coated evacuated tubes (Becton Dickenson) and centrifuged at 1098 × g at 4°C for 10 min to separate plasma, which was aliquoted and stored at –80°C until analysis.

| Meal nutrient | Allo-isoleucine absorption (n = 6) | Milk protein digestibility (n = 7) |
|--------------|-----------------------------------|----------------------------------|
| Energy, kcal | $672.0 \pm 25.1$                   | $671.3 \pm 30.4$                 |
| Protein, g   | $19.7 \pm 1.3$                    | $26.5 \pm 1.7$                   |
| Carbohydrate, g | $80.6 \pm 2.3$              | $65.0 \pm 2.7$                   |
| Fat, g       | $30.4 \pm 1.3$                    | $34.0 \pm 1.8$                   |
| Protein to energy ratio, % | $11.7 \pm 0.4$ | $15.8 \pm 0.6$ |

1Values are mean ± SD
2The nutrient composition presented is representative of the whole test meal, which was divided into smaller portions for half-hourly administration in the plateau feeding protocol.
of true ileal digestibility of the milk protein was performed using
the dual-isotope method (15).

The test meal (which was fed in a plateau feeding protocol) consisted of the spray-dried intrinsically labeled milk powder reconstituted in water with beet sugar and protein-free wheat starch cookies (Supplementary Table 1). It provided one-third of the daily sedentary energy requirement (22), and protein was provided at 1.3 g/kg body weight. A higher protein intake in the meal compared with the sedentary protein requirement was given to ensure quantifiable individual plasma IAA enrichments, based on previous measurements of ileal protein digestibility by the same method (19, 23). The nutrient composition of the test meal is provided in Table 1. An 8 h plateau feeding protocol was adopted, in which the test meal was portioned into 21 portions, each portion representing a single mini-meal. Here, an initial priming portion (containing 6 mini-meals) was fed at the beginning of the protocol, followed by single mini-meals every half hour. Meals were warmed in a microwave oven before administration to the participants. One mini-meal aliquot was retained for IAA isotopic enrichment analysis.

The intrinsically $^2$H-labeled milk protein made up the total protein in the test meal, with a trace amount (~0.1%) contributed by a U-$^{13}$C-labeled algal amino acid mixture (1.25 mg/kg, 98%; Cambridge Isotope Laboratories). The latter served as the standard protein that is fully digested (15, 19) for comparison in the dual-isotope method. $^{13}$C$_{11}$-L-tryptophan (0.04 mg/kg, 99% purity; Cambridge Isotope Laboratories) was also added to the meal, as it was absent in the labeled algal amino acid mixture. $^{13}$C$_{11}$-L-tryptophan was added to allow for digestibility measurement at a later date on standardizing an appropriate method.

A basal blood sample (4 mL) was collected prior to the administration of mini-meals, followed by half-hourly samples from 5 h onward, until the end of the experiment (Figure 1). Whole blood was transferred into EDTA-coated anticoagulant evacuated tubes (Becton Dickinson), processed, and stored as described above.

Analyses of intrinsically labeled fodder and goat milk protein

The $^2$H-IAA enrichment in the fodder, as well as a precipitated aliquot of each day’s milk protein and pooled spray-dried milk powder, was measured as described in detail elsewhere (15, 24). Briefly, milk protein was precipitated using trichloroacetic acid (25%, v/v; Sigma-Aldrich), followed by acetone wash (75%, v/v; Merck) and vacuum drying (24). The milk protein precipitate and pooled spray-dried milk powder underwent a gas-phase acid hydrolysis followed by cation exchange purification of the IAA s and derivatization to N-ethoxycarbonyl ethyl esters. The $^2$H-IAA enrichments were measured using GC–pyrolysis–isotope ratio mass spectrometry (Delta V Advantage; Thermo Fisher Scientific) and expressed as parts per million excess (ppme) (15).

Baseline $^2$H-IAA abundance was measured in the milk protein collected before the start of the labeled fodder feeding protocol.

Urinary analysis for lactulose and mannitol

Urinary concentrations of lactulose and mannitol were measured as described earlier (25). Briefly, 100 μL of urine sample was deionized with 5 mg of AG 501-X8(D) resin (Bio-Rad) after spiking with $^{13}$C$_6$-mannitol and $^{13}$C$_{12}$-lactulose as internal standards (50 μL each from of 100 μM/L stock solution; Cambridge Isotope Laboratories) by vortex mixing for an hour and centrifuged at 1098 × g at 28 C. The supernatant was dried, and the sugars were derivatized as their silylated esters and quantified by GC-MS (SQ, 5975; Agilent Technologies) (25). Lactulose (an index of intestinal epithelial barrier/integrity) and mannitol (an index of intestinal absorption) recovery (as percent) pooled samples (2 h and 5 h) were calculated by multiplying the concentration of the sugar with the total volume of urine void and dividing by the dose of the respective sugars. The ratio of lactulose to mannitol recovery provided the lactulose/mannitol ratio (LMR). Eventually, the LMR at 2 h postdose was used since LMR at 2 h and 5 h correlated well ($r_s = 0.99, P < 0.0001$) and 2 h offers a closer representation of small intestinal absorption (21, 26, 27).

Analyses of plasma and meal IAA enrichment

Isotopic enrichments in plasma and lyophilized test meal samples were measured as explained elsewhere (15). The masses monitored for isotopes of allo-isoleucine accounted for the transamination of the isotopes administered (Supplementary Table 2). The allo-isoleucine absorption index was defined as a ratio of isotopic enrichment of oral ($^{13}$C$_6$, $^{15}$N-L-allo-isoleucine) to IV ($^2$H$_{10}$-L-allo-isoleucine) trace in the plasma. In addition to this, the phenylalanine absorption index, as previously estimated (15) using free $^{13}$C$_6$-L-phenylalanine, was computed with free $^{13}$C$_{12}$-L-phenylalanine, which was part of the free $^{13}$C IAA mix administered with the present protocol, and calculated as

\[
\frac{\text{Plasma}^{13}C_9-\text{phenylalanine (ppme)}}{\text{Meal}^{13}C_9-\text{phenylalanine (ppme) × 100}} \tag{1}
\]

The same equation can be applied to arrive at absorption indices using other free $^{13}$C IAA plasma to meal ratios. The true ileal digestibility percentage for each IAA of milk was calculated using the following equation:

\[
\frac{\text{Plasma}^2H - \text{IAA (ppme) / Meal}^2H - \text{IAA (ppme)}}{\text{Plasma}^{13}C - \text{IAA (ppme) / Meal}^{13}C - \text{IAA (ppme)}} \times 100. \tag{2}
\]

Statistical analysis

Five participants were required to obtain a standard deviation of 1.8 (8) with 95% confidence and 2% precision. All data are reported as arithmetic mean and SD unless specified. Spearman correlation was used to evaluate the associations. For all the comparisons, $P < 0.05$ was considered significant. All calculations were performed with SPSS, version 25 (SPSS, Inc.).

Results

Goat milk yield and enrichment

The total yield of intrinsically labeled maize and cowpea fodder was 15 kg and 2.5 kg, respectively. The average $^2$H-IAA enrichment of the fodder was 1607 and 1357 ppme for maize and
Goat milk protein digestibility

The average IAA $^2$H enrichment of the pooled milk was 257 ppme.

Cowpea, respectively (Supplementary Figure 2). The volume of $^2$H-intrinsically labeled milk collected during the study was 14.5 L. The milk collected on the first 3 d and the last day (day 21) was not used for the human digestibility study. The daily and pooled $^2$H-IAA enrichment of milk is represented in Figure 2. The average IAA enrichment of pooled goat milk (between 4 and 20 days) was 257 ppme.

Human intestinal permeability and allo-isoleucine absorption

The participant characteristics for the study are provided in Table 2. The mean ± SD hourly lactulose and mannitol excretion for 2-h pooled urine samples was 6.8 ± 2.1 and 216.2 ± 66.5 μM/h, respectively. The mean lactulose and mannitol recovery (percent) and LMR for 2-h pooled samples are provided in Table 3, and the LMR ranged from 0.008 to 0.020. The mean ± SD lactulose and mannitol recovery (percent) and LMR for 5-h pooled urine samples were 0.24 ± 0.06%, 16.9 ± 4.4%, and 0.015 ± 0.004, respectively. The allo-isoleucine absorption index at plateau (5–8 h) is provided in Figure 3, and the mean allo-isoleucine absorption index is provided in Table 3. The mean interindividual CV of $^{13}$C$_6$-$^{15}$N-L-allo-isoleucine and $^2$H$_{10}$-L-allo-isoleucine enrichment at plateau was 15% and 12%, respectively. The mean $^{13}$C$_9$ phenylalanine absorption index was 24.5 ± 1.6%.

Goat milk protein digestibility

The mean $^2$H and $^{13}$C IAA enrichments (ppme) of the protein in the test meals are provided in Supplementary Table 3. The $^2$H and $^{13}$C enrichments of each plasma IAA at plateau (5–8 h) are represented in Figure 4. The mean interindividual CV of $^2$H and $^{13}$C plasma enrichment at plateau was 15% and 19%, respectively. The CVs for both $^2$H and $^{13}$C plasma enrichments were lowest for phenylalanine at 10% and 14%, respectively, and highest for methionine at 23% and 25%, respectively. The mean ± SD true ileal digestibility of spray-dried milk powder

### Table 2: Characteristics of study participants

| Variable     | Value     |
|--------------|-----------|
| Age, y       | 27.4 ± 4.8 |
| Weight, kg   | 58.1 ± 3.4 |
| Height, m    | 1.6 ± 0.02 |
| BMI, kg/m$^2$| 23.0 ± 1.2 |

1 Values are mean ± SD, $n = 7$.

### Table 3: Lactulose and mannitol recovery (%), lactulose/mannitol ratio and allo-isoleucine absorption index in apparently healthy South Indian women

| Intestinal function parameter$^2$ | Value     |
|-----------------------------------|-----------|
| Lactulose recovery, %             | 0.09 ± 0.03 |
| Mannitol recovery, %              | 7.90 ± 2.34 |
| Lactulose/mannitol ratio          | 0.012 ± 0.004 |
| Allo-isoleucine absorption index, %| 88.4 ± 3.8 |

1 Values are mean ± SD.

2 $n = 7$ for lactulose and mannitol recovery, as well as lactulose/mannitol ratio; $n = 6$ for allo-isoleucine absorption index.
was 94.0 ± 2.9% and ranged from 89.9% for threonine to 97.9% for methionine (Table 4). There was a significant inverse correlation ($r_s = -0.93$, $P = 0.008$) between the LMR and allo-isoleucine absorption index ($r^2 = 0.49$, slope = -6.9323 and intercept = +0.9714) but not between either of these variables and phenylalanine absorption index or IAA digestibility.

**Discussion**

This study determined the true ileal IAA digestibility of intrinsically labeled, spray-dried goat milk powder, using the dual stable isotope tracer technique, in healthy South Indian WRA whose intestinal integrity was assessed by a dual-sugar test and amino acid absorption estimated using a nonprotein
ILEAL PROTEIN/IAA DIGESTIBILITY MEASUREMENTS, THROUGH KILLING TO THE LACTATING ANIMAL, FOR USE IN DIRECT OROFECAL OR TRUE DIGESTIBILITY. PREVIOUSLY, GOAT OR COW MILK HAS BEEN LABELED WITH THE 2H AND 15N ISOTOPE TRACER TECHNIQUE (15, 19, 30). THE HIGH DIGESTIBILITY OF GOAT MILK PROTEIN MAKES IT AN IMPORTANT ASF IN POPULATIONS THAT ARE PREDOMINANTLY VEGETARIAN AND IN COUNTRIES LIKE INDIA, WHERE THE PROPORTION OF VEGETARIANS IS HIGH. THE INTRINSIC LABELING OF DIETARY PROTEIN WITH STABLE ISOTOPIES IS ASSUMED TO BE TRANSPORTED BY THE SAME SECONDARY ACTIVE TRANSPORTER MECHANISM AS NEUTRAL AMINO ACIDS (7 OF 19). THE MEAN TRUE ILEAL IAA DIGESTIBILITY OF GOAT MILK PROTEIN WAS 94.0% IN THESE WOMEN, WITH NORMAL INTESTINAL FUNCTION AS ASSESSED BY LMR AND ALLO-ISOLEUCINE ABSORPTION INDEX.

**Goat milk protein digestibility**

The mean true ileal IAA digestibility of goat milk protein (94.0%) was similar to that of cow milk protein isolate and skimmed milk protein (95.1% and 95.5%) in healthy French adults (8, 28). It also compared reasonably well (94.0% compared with 97.2%) with the true ileal digestibility of goat milk protein concentrate in rats (29). The individual IAA digestibility was within the range (93–100%) of previously reported values (8). The mean true ileal IAA digestibility of goat milk protein was similar to that of other ASF and greater than plant source protein determined in healthy Indian adults of both sexes by the dual-isotope tracer technique (15, 19, 30). The high digestibility of milk protein makes it an important ASF in populations that are predominantly vegetarian and in countries like India, where frequency of meat consumption is low, to alleviate the risk of quality protein inadequacy (6, 31).

The intrinsic labeling of dietary protein with stable isotopes allows for the precise measurement of true ileal protein digestibility. Previously, goat or cow milk has been labeled with 2H and 15N by oral administration of 2H2O or (15NH4)2SO4 to the lactating animal, for use in direct orofecal or true ileal protein/IAA digestibility measurements, through killing (rats) or nasoileal intubation (humans), respectively (8, 28, 32). However, milk obtained in this manner is mostly likely to be labeled on either the α-H or α-N moiety of the constituent IAs (33, 34) through transamination, and these are prone to losses in the body by the same process. The isotopic label lost through transamination cannot be traced to its parent molecule, thereby limiting its use for the dual-isotope tracer approach. Hence, for this study, it was necessary to label the fodder fed to the goat with 2H2O, which leads to the stable incorporation of 2H at random positions on the amino acid carbon backbone efficiently labeling all amino acids, with minimal losses of α-2H that could be corrected using a transamination correction factor (TCF) when labeled milk is administered in humans (15). However, the TCF was not applied for the human digestibility calculation in this study, since transamination losses of α-2H have already occurred in the lactating goat (35).

**Amino acid absorption: Allo-isoleucine and phenylalanine absorption index**

The mean allo-isoleucine absorption index obtained was similar (88% compared with 80%) to the estimated value in healthy US adults (18). Absorption of individual test and reference amino acids is assumed to be equivalent (after protein digestion) in the dual-tracer approach for protein digestibility. Because this equivalent absorption cancels out, the dual-isotope method gives a readout of true ileal digestibility but not absorption. The absorption is also assumed to be maximum; if a value lower than this is used, the net transfer of amino acids into the body could be lower. This is important, as earlier reports have shown mean glycine (amino acid) and glycylglycine (peptide) absorption to be lower by 31% in healthy asymptomatic Indian men compared with age-matched English men (36). Here, the absorption of a nonprotein amino acid (allo-isoleucine), which is assumed to be transported by the same secondary active transporters (Na+ dependent, B0AT1) as neutral amino acids (7 of 19) across the intestinal epithelium, was close to 100% (37), which is the normal absorptive capacity of the proximal small intestine epithelial amino acid transporters in the participants. Furthermore, the small bowel reserve capacity for absorption is unlikely to be affected unless extensive gut damage occurs or surgical resections are performed (38, 39). Therefore, even if absorptive losses are observed, their clinical relevance needs to be established.

Traditionally, amino acid absorption has been studied directly using intestinal infusion studies (40) that are invasive or by the “glycine tolerance test” (41), which has multiple metabolic fates precluding accurate measurement. Stable amino acid isotopes can be exploited to estimate an absorption index. For example, the use of the plasma to meal ratio of enrichments of orally administered 13C6 phenylalanine and 13C9 phenylalanine in the present study (15) is an approximation for intestinal absorption of this essential amino acid but includes first-pass metabolism by splanchnic tissues, which is assumed to be less variable in a fed steady state (42). The method used in the present study is a better reflection of intestinal absorption, as amino acid isomers such as D-phenylalanine and L-allo-isoleucine are thought to be metabolically inert, with no metabolic fate except for oxidation in

**Table 4: True ileal digestibility of spray-dried goat milk powder in apparently healthy South Indian women**

| Amino acid     | True ileal digestibility, % (n = 7) |
|----------------|-------------------------------------|
| Methionine     | 97.9 ± 1.8                          |
| Phenylalanine  | 93.0 ± 2.4                          |
| Threonine      | 89.9 ± 1.2                          |
| Lysine         | 93.0 ± 2.1                          |
| Leucine        | 95.2 ± 2.5                          |
| Isoleucine     | 97.3 ± 1.4                          |
| Valine         | 92.0 ± 2.7                          |
| Mean           | 94.0 ± 2.9                          |

1Values are mean ± SD.
humans (18, 43). Although L-allo-isoleucine, a by-product of L-isoleucine transamination, could revert to L-isoleucine, this might not be quantitatively significant considering that it is a poor substrate for branched-chain amino transferases (44, 45). Nevertheless, the robustness and sensitivity of these methods to assess impairment in intestinal amino acid absorption need to be tested in clinical populations with a variable degree of amino acid malabsorption.

Associations between intestinal permeability, amino acid absorption, and protein digestibility

The LMR in the present study showed a significant inverse relation with the allo-isoleucine absorption index. This finding implies that disrupted intestinal barrier function may impair amino acid absorption, which needs further evaluation involving participants with a wider distribution of LMR or abnormal values. LMR and allo-isoleucine absorption did not correlate with either the mean digestibility or individual IAA digestibility. Experimental evidence indicates that irradiated minipigs with intestinal mucosal barrier disruption (similar to EE) impairing absorption and citrulline production did not show a significant decrease in protein digestion, thereby pointing to the reserve capacity of the pancreas whose cumulative postprandial enzyme production (10- to 15-fold) far exceeds the quantity required for digestion under normal physiologic conditions (17, 46). Otherwise, the digestibility of milk protein is mainly dependent on the quantity of antinutritional components produced during processing (9) or the antinutrients contributed by other ingredients in a mixed meal.

Strengths and limitations

The strength of this study lies in the near-simultaneous estimation of intestinal function, especially amino acid absorption, along with the determination of protein digestibility, in an LMIC group of participants. A limitation is that the absorption of allo-isoleucine is assumed to represent neutral amino acid transporters, particularly the absorption of branched-chain amino acids, which is reasonable, as it is an isomer of isoleucine (37); however, the function of peptide transporters that demonstrate a kinetic advantage over amino acid transporters was not tested (47). Measurement of the allo-isoleucine absorption index is invasive and therefore has limited application in vulnerable populations, particularly children. The dual-isotope tracer technique is an indirect method and still needs direct validation against amino acid balances measured by nasojejunal intubation or ileostomates, but the present study values compared well with those measured by nasojejunal intubation (8, 28). In addition, the assessment of other biomarkers of EE could complement the intestinal function measurements in this study.

Conclusion

In conclusion, this study provides true ileal IAA digestibility of spray-dried goat milk powder in healthy South Indian women with normal intestinal function, which is comparable to estimates from HICs and adds to the expanding literature for protein quality assessment using the Digestible Indispensable Amino Acid Score (DIAAS).

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Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request to the corresponding author.

References

1. Cuco G, Aria V, Iranzo R, Vila J, Prieto MT, Fernández-Ballart J. Association of maternal protein intake before conception and throughout pregnancy with birth weight. Acta Obstet Gynecol Scand 2006;85:413–21.
2. Murphy SP, Allen LH. Nutritional importance of animal source foods. J Nutr 2003;133:3932S–3S.
3. Adesogan AT, Havelaar AH, McKune SL, Eilittä M, Dahl GE. Animal source foods: sustainability problem or malnutrition and sustainability solution? Perspective matters. Glob Food Secur 2019;25:100325.
4. Headey DD, Palloni G. Stunting and wasting among Indian preschoolers have moderate but significant associations with the vegetarian status of their mothers. J Nutr 2020;150:1579–89.
5. Mukhopadhyay A, Dwarkanath P, Bhanji S, Devi S, Thomas A, Kurpad AV, Thomas T. Maternal intake of milk and milk proteins is positively associated with birth weight: a prospective observational cohort study. Clin Nutr 2018;25:103–9.
6. Swanminathan S, Vaz M, Kurpad AV. Protein intakes in India. Br J Nutr 2012;108:S50–8.
7. FAO. Research approaches and methods for evaluating the protein quality of human foods. Report of an FAO Expert Working Group. Rome (Italy): FAO; 2014.
8. Gaudichon C, Bos C, Mores C, Petzke KJ, Mariotti F, Everwand J, Benamouzig R, Daré S, Tomé D, Metges CC. Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. Gastroenterology 2002;123:50–9.
9. Gilani GS, Cockell KA, Sepehr E. Effects of antinutritional factors on protein digestibility and amino acid availability in foods. J AOAC Int 2005;88:967–87.
10. Korpe PS, Petri WA Jr. Environmental enteropathy: critical implications of a poorly understood condition. Trends Mol Med 2012;18:328–36.
11. Swaminathan N, Mathan VI, Baker SJ, Radhakrishnan AN. Disaccharidase levels in jejunal biopsy specimens from American and South Indian control subjects and patients with tropical sprue. Clin Chim Acta 1970;30:707–12.
12. Baker SJ. Subclinical intestinal malabsorption in developing countries. B World Health Organ 1976;54:885.
13. Kao CC, Cope JL, Hsu JW, Dwarkanath P, Barnes JM, Luna RA, Hollister EB, Thame MM, Kurpad AV, Jhoo F. The microbiome, intestinal function, and arginine metabolism of healthy Indian women are different from those of American and Jamaican women. J Nutr 2015;145:706–13.
14. Iqbal TH, Lewis KO, Gearty JC, Cooper BT. Small intestinal permeability to manitol and lactulose in the three ethnic groups resident in west Birmingham. Gut 1996;39:199–203.
15. Devi S, Varkey A, Sheshshayee MS, Preston T, Kurpad AV. Measurement of protein digestibility in humans by a dual-tracer method. Am J Clin Nutr 2018;107:984–91.

16. Denno DM, VanBuskirk K, Nelson ZC, Musser CA, Hay Burgess DC, Tarr PI. Use of the lactulose to mannitol ratio to evaluate childhood environmental enteric dysfunction: a systematic review. Clin Infect Dis 2014;59:S213–19.

17. Kaur A, ten Have GA, Hritz B, Deutz NE, Olsen C, Moroni M. Morphological and functional impairment in the gut in a partial body irradiation minigip model of GI-ARS. Int J Radiat Biol 2020;96:112–28.

18. Deutz NE, Lightart-melis GC, Engelen M, ten Have G. Methods for diagnosing impaired absorption of amino acids, monosaccharides and fatty acids [Internet]. Available from: https://www.freepatentsonline.com/20170097335.pdf.

19. Kashyap S, Varkey A, Shivakumar N, Devi S, Reddy BHR, Thomas T, Preston T, Sreeman S, Kurpad AV. True ileal digestibility of legumes determined by dual-isotope tracer method in Indian adults. Am J Clin Nutr 2019;110:873–82.

20. National Research Council. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelsid. 6th ed. Washington (DC): National Academies Press; 2007.

21. Camilleri M, Nadeau A, Lamsam J, Linker Nord S, Ryks M, Burton D, Sweeter S, Zinsmeister AR, Singh R. Understanding measurements of intestinal permeability in healthy humans with urine lactulose and mannitol excretion. Neurogastroenter Motil 2010;22:e15–26.

22. ICMR NR. Recommended Dietary Allowances for Indians. Hyderabad (India): National Institutes of Nutrition, Indian Council of Medical Research; 2010.

23. Shivakumar N, Kashyap S, Kishore S, Thomas T, Varkey A, Devi S, Preston T, Jhaor F, Sheshshayee MS, Kurpad AV. Protein-quality evaluation of complementary foods in Indian children. Am J Clin Nutr 2019;109:1319–27.

24. Link AJ, LaBauer J. Trichloroacetic acid (TCA) precipitation of proteins. Cold Spring Harb Protoc 2011:8:993–4.

25. Rodriguez H, Suchodolski JS, Berghoff N, Steiner JM. Development and analytic validation of a gas chromatography–mass spectrometry method for the measurement of sugar probes in canine serum. Am J Vet Res 2009;70:320–9.

26. Akram S, Moursani S, Ou CN, Rognerud C, Sadiq R, Goodgame RW. Assessment of intestinal permeability with a two-hour urine collection. J Pediatr Gastroenterol Nutr 2016;62:568–72.

27. Musa MA, Kabir M, Hossain MI, Ahmed E, Siddique A, Rashid H, Mahfuz M, Mondal D, Ahmed T, Petri WA, Haque R. Measurement of intestinal permeability using lactulose and mannitol with conventional five hours and shortened two hours urine collection by two different methods: HPAE-PAD and LC-MSMS. PLoS One 2019;14:e202397.

28. Bos C, Mahf S, Gaudichon C, Benamouzig R, Gaussnerès N, Luengo C, Ferrière F, Rautureau J, Tomé D. Assessment of milk protein nutritional quality by net postprandial utilization of [15N]-labeled milk nitrogen in humans. Br J Nutr 1999;81:221–6.

29. Tessier R, Khodorova N, Calvez J, Tomé D, Gaudichon C. Challenging in rats the use of 13C spirulina as reference protein for the dual isotope method to determine amino acid bioavailability (P08-061-19). Curr Dev Nutr 2019;3:nzz044–P08-061-19.

30. Kashyap S, Shivakumar N, Varkey A, Duraisamy R, Thomas T, Preston T, Devi S, Kurpad AV. Ileal digestibility of intrinsically labeled hen's egg and meat protein determined with the dual stable isotope tracer method in Indian adults. Am J Clin Nutr 2018;108:980–7.

31. Nutritional intake in India (2011–12), National Service Scheme (68th round). National Sample Survey Office, New Delhi, Government of India. Report No: 560. 2014.

32. Tessier R, Khodorova N, Calvez J, Kapel R, Quinsac A, Piedcog J, Tomé D, Gaudichon C. 15N and 2H intrinsic labeling demonstrate that real digestibility in rats of proteins and amino acids from sunflower protein isolate is almost as high as that of goat whey. J Nutr 2020;150:450–7.

33. Commerford SL, Carsten AL, Cronkite EP. The distribution of tritium among the amino acids of proteins obtained from mice exposed to tritiated water. Radiat Res 1983;94:151–5.

34. Schadhareti R, Krawielitzki K, Herrmann U. 15N transamination in the administration of various tracer substances. 1. Whole body studies in rats. Arch Tierernahr 1986;36:783–92.

35. Tibbo M, Jibril Y, Woldemeskel M, Dawo F, Aragau K, Rege JE. Serum enzymes levels and influencing factors in three indigenous Ethiopian goat breeds. Trop Anim Health Prod 2008;40:657–66.

36. Hellier MD, Radhakrishnan AN, Ganapathy V, Gammon A, Baker SJ. Intestinal absorption in normal Indian and English people. BMJ 1976;1:186–8.

37. Böhmer C, Bröer A, Munzinger M, Kowalczik S, Rasko JE, Lang F, Bröer S. Characterization of mouse amino acid transporter BOAT1 (slc6a19). Biochem J 2005;389:745–51.

38. Milne MD. Disorders of intestinal amino-acid transport. J Clin Pathol 1971;3:5–41.

39. Craft IL, Geddes D, Hyde CW, Wise IJ, Matthews DM. Absorption and malabsorption of glycine and glycine peptides in man. Gut 1968;9:425.

40. Adibi SA, Gray SJ. Intestinal absorption of essential amino acids in man. Gastroenterology 1967;52:837–45.

41. Butterworth CE, Santini R, Perez-Santiago E. The absorption of glycine and its conversion to serine in patients with sprue. J Clin Invest 1958;37:20–7.

42. Biolo GI, Tessari PA, Inchiostro SA, Bruttomesso DA, Fongher CR, Sabadin LU, Fratton MG, Valerio AN, Tiengo AN. Leucine and phenylalanine kinetics during mixed meal ingestion: a multiple tracer approach. Am J Physiol-Endoc M 1992;262:E455–63.

43. Murtas G, Sacchi S, Valentino M, Pollegioni L. Biochemical properties of human D-amino acid oxidase. Front Mol Biosci 2017;4:88.

44. Schadewald P, Dalle-Feste C, Langenbeck U, Wendel U. Oral L-alloisoleucine loading studies in healthy subjects and in patients with maple syrup urine disease. Pediatr Res 1991;30:430–4.

45. Schadewald P, Wendel U. Comparison of the catabolism of branched-chain l-amino acids in cultured human skin fibroblasts. Pediatr Res 1987;22:591–4.

46. Keller J. Gastrointestinal digestion and absorption. In: Lennarz WJ, Lane MD, editors. Encyclopedia of biological chemistry. 2nd ed. Waltham (MA): Academic Press; 2013. p 354–9.

47. Adibi SA, Soleimanepour MR. Functional characterization of dipeptide transport system in human jejenum. J Clin Invest 1974;53:1368–74.