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Metabolic Availability of Lysine in Milk and a Vegetarian Cereal–Legume Meal Determined by the Indicator Amino Acid Oxidation Method in Indian Men

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ABSTRACT

Background: Lysine rich foods such as milk and legumes serve as important food additions to the lysine deficient cereal-based diets of vegetarian populations in low- and middle-income countries (LMICs) to alleviate the risk of quality corrected dietary protein inadequacy. Dietary protein quality can be determined by estimating the metabolic availability (MA) of lysine.

Objectives: The study aimed to estimate the MA of lysine in spray-dried cow milk powder (SMP), heat-treated spray-dried cow milk powder (HSMP), and a habitually consumed cereal-legume based vegetarian meal (VM), using the indicator amino acid oxidation (IAAO) slope-ratio method.

Methods: The MA of lysine in SMP, HSMP, and VM was estimated in 7 healthy young men aged 19–24 y with BMI of 21.5 ± 0.5 kg/m^2 in a repeated measures design. The IAAO response slopes with 2 graded lysine intakes (10.5 and 15.0 mg·kg^{-1}·d^{-1}) from the SMP and VM were compared with the response slope generated with 3 graded crystalline lysine intakes (6.0, 10.5, and 15.0 mg·kg^{-1}·d^{-1}) at the subrequirement level. To produce HSMP, pasteurized cow milk was heat treated and spray dried. The MA of lysine in HSMP was tested at a single level of lysine intake (15 mg·kg^{-1}·d^{-1}). A total of 8 IAAO experiments were conducted on each participant in randomized order. The IAAO slopes were estimated using a linear mixed-effect regression model.

Results: The MA of lysine in SMP, HSMP, and VM was 91.9%, 69.9%, and 86.6% respectively.

Conclusions: Heat treatment reduced the MA of lysine by 22% in HSMP compared with SMP in healthy Indian adults. The lysine MA estimates can be used to optimize lysine limited cereal-based diets, with the addition of appropriately processed legumes and milk powder, to meet the protein requirement. This trial was registered at Clinical Trials Registry of India (http://ctri.nic.in) as CTRI/2019/08/020568. *J Nutr* 2020;00:1–7.

Keywords: Protein quality, metabolic availability, milk, heat-treatment, cereal-legume meal, indicator amino acid oxidation

Introduction

The risk of quality corrected protein inadequacy is high in low- and middle-income countries (LMICs) due to the cereal-dominated and lysine limited diets consumed by the population (1). This may be compounded by an increase in the daily lysine requirement due to subclinical infections and intestinal parasites, or an impaired intestinal absorptive capacity in poor environmental conditions (2). In addition, the ileal lysine digestibility of plant foods (including legumes) has been shown to be low in healthy South Indian adults and children (3, 4). This is because antinutritional factors such as phytates, polyphenols, tannins, and trypsin inhibitors in plant source foods can reduce the amino acid digestibility of an ingested food matrix (5). Food processing techniques can also modify amino acids, especially lysine, to further reduce their digestion and absorption, or rendering them unavailable for utilization (5). Therefore, a dietary approach to address the risk of quality corrected protein inadequacy in cereal-based diets is the inclusion of legumes in higher quantities or the modest addition of animal source foods such as milk, eggs and meat, which have highly digestible protein with a high lysine content (3, 6).

The quality of a dietary protein, in addition to its limiting indispensable amino acid (IAA) content, depends on the

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metabolic availability (MA) or utilization of that IAA (7). Milk serves as a high-quality protein food addition to lysine deficient diets of vegetarian populations (8) and has a high human digestibility (9), but milk and milk products are often subjected to varying degrees of high temperature treatments such as pasteurization, homogenization, concentration, spray drying, and heat sterilization leading to the production of modified lysine (such as Maillard reaction), which is not metabolically available (5, 10). The MA of lysine in heat-treated or spray-dried milk has not been estimated in humans. The MA of lysine in a cereal and legume food combination in a habitually consumed meal preparation has also not been estimated.

Although food protein quality has been evaluated in adults and children (<2 y) by the minimally invasive dual isotope tracer technique (3, 4) which measures the true ileal digestibility and metabolic utilization of all IAA in a single trial (7), the intrinsic labelling of a dietary protein is expensive, particularly for mixed meals, because it requires all the food protein sources in a mixed meal to be intrinsically labelled. In contrast, the noninvasive indicator amino acid oxidation (IAAO) slope-ratio method measures the MA of a single selected IAA by comparing the oxidation response slopes of a labelled indicator IAA to graded intakes of the selected IAA from an unlabeled test and reference proteins. This is a repeated measures study design, with a higher subject burden, but does not require labelled food protein (7). The IAAO slope-ratio method has been validated against the slope-ratio growth assay (standard assay) of measuring bioavailability of IAA in pigs (11) and has been used to measure MA of limiting IAAs (lysine and tryptophan) in rice and cornmeal in humans (12, 13). The present study aimed to determine the MA of lysine in spray-dried cow milk powder (SMP) and in a habitually consumed cereal-legume based vegetarian meal (VM) in Indian adult men, using the IAAO slope-ratio method. It also aimed to estimate the effect of heat treatment on the MA of lysine in heat-treated spray-dried cow milk powder (HSMP).

Methods

Study participants

Apparently healthy adult men aged between 19 and 24 y, with a normal body mass index (BMI; 18.5–25.0 kg/m^2) participated in the study. Participants who did not smoke or consume alcohol and had no food allergies were recruited. Those reporting serious medical or surgical ailments 3 months prior to the study, on dietary or nutrient supplements, or who had taken antibiotics 4 w before the initiation of the study were excluded. All study participants were recruited from the student population of St. John's Medical College. Details of screening and enrollment of participants are provided in **Supplemental**

TABLE 1 Lysine contribution from reference amino acid mixture and test proteins in the experimental meals to determine the MA of lysine in SMP, HSMP, and VM in Indian men[1]

Lysine intake mg·kg^{-1}·d^{-1} (% requirement)[2]	Source of lysine, mg·kg^{-1}·d^{-1}	
	Crystalline lysine[3]	Test protein
For defining reference slope[4]		
6.0 (20%)	6.0	—
10.5 (35%)	10.5	—
15.0 (50%)	15.0	—
For defining SMP slope[5]		
10.5 (35%)	6.0	4.5
15.0 (50%)	6.0	9.0
For defining HSMP slope[6]		
15.0 (50%)	6.0	9.0
For defining VM slope[5]		
10.5 (35%)	6.0	4.5
15.0 (50%)	6.0	9.0

[1] HSMP, heat-treated and spray-dried cow milk powder; MA, metabolic availability; SMP, spray-dried cow milk powder; VM, vegetarian meal.
[2] Lysine requirement of adults (15).
[3] A *base* amount of 6 mg·kg^{-1}·d^{-1} as crystalline lysine was provided at each level of lysine intake to ensure that both reference and test protein slopes had a common origin.
[4] The reference slope was estimated with 3 graded intakes of lysine as crystalline lysine.
[5] The VM and SMP slopes were estimated with 2 graded intakes of lysine from test proteins.
[6] The HSMP slope was estimated with a single level of lysine intake from HSMP.

Figure 1. The Institutional Ethical Review Board of St. John's Medical College and Hospital approved the study. A written informed consent was obtained from all the participants at enrollment. The study was registered at Clinical Trials Registry, India, with a registration number of CTRI/2019/08/020568.

IAAO slope-ratio method to evaluate MA of lysine in test proteins

The MA of lysine in SMP, HSMP, and VM was assessed by use of the IAAO slope-ratio method (14). Briefly, at subrequirement levels of intake, the IAAO response (using [1–^{13}C] phenylalanine oxidation) shows a linear decline with the increasing intake of a test IAA (lysine) from the protein being tested. When this slope is compared to the IAAO slope estimated with the crystalline test IAA provided in a reference crystalline amino acid mixture (assumed to have an MA of 100%, see below), the resulting slope-ratio is an estimate of the MA of lysine in the test protein (14). Because a key condition of the slope-ratio method is obtaining a common origin for the IAAO slopes that are being compared, a fixed *base* intake of the test IAA from the reference crystalline amino acid mixture is included in the design (13, 14).

First, the reference crystalline amino acid mixture IAAO response slope was calculated from 3 graded intakes of lysine (6.0, 10.5, and 15.0 mg·kg^{-1}·d^{-1} lysine, respectively; **Table 1**), representing 20%, 35%, and 50% of the daily lysine requirement (15). Second, the test protein IAAO response slopes with similar graded intakes of lysine from the test proteins (SMP, HSMP, and VM) were calculated. The slope-ratio was then calculated to estimate the lysine MA in the test proteins. Two levels of lysine intake (10.5 and 15.0 mg·kg^{-1}·d^{-1}) were used for SMP and VM, but the *base* lysine intake (6 mg·kg^{-1}·d^{-1}) was provided as crystalline lysine. Thus, the remaining 4.5 and 9 mg·kg^{-1}·d^{-1} lysine came from the test proteins (Table 1). To determine the effect of heat-treatment, the HSMP was tested at a single level of lysine intake, i.e., 15 mg·kg^{-1}·d^{-1}. Here too, the *base* lysine intake was 6 mg·kg^{-1}·d^{-1} (Table 1). Therefore, a total of 8 IAAO experiments with 3 graded intakes of lysine were conducted on each participant in a repeated measures design with randomized order. Each IAAO experiment followed a 3-d experimental protocol with 2-d prior

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Address correspondence to AVK (e-mail: a.kurpad@sjri.res.in)
Supplementary Tables 1–3 and Supplementary Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
Abbreviations used: APE, atom % excess; BMI, body mass index; BMR, basal metabolic rate; CL, crystalline lysine; HSMP, heat-treated spray dried cow milk powder; IAA, indispensable amino acids; IAAO, indicator amino acid oxidation; IRMS, isotope ratio mass spectrometer; LMIC, low- and middle-income countries; MA, metabolic availability; SMP, spray dried cow milk powder; VM, vegetarian meal.

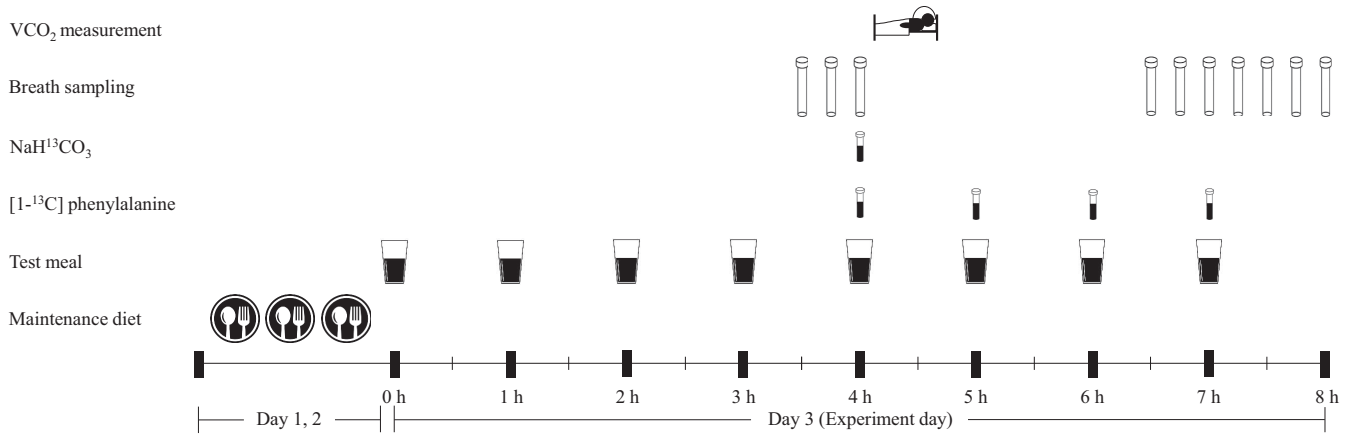

FIGURE 1 Experimental protocol of an IAAO study to determine MA of lysine in SMP, HSMP, and VM in Indian men. Each IAAO study followed a 3-d experimental protocol. During the initial 2-d maintenance period, participants were given a standardized diet, which was consumed as breakfast, lunch, and dinner. On the day 3, the tracer study was conducted for a period of 8 h, in which participants were administered 8 isonitrogenous and isocaloric hourly test meals. The priming doses of tracers, i.e., [^{13}C] sodium bicarbonate and [1–^{13}C] phenylalanine were provided with the 5th meal with subsequent hourly doses of [1–^{13}C] phenylalanine until the 8th h. Three fed-state baseline breath samples (before initiating the tracer protocol) and 7 post-dose samples (2.5 h after first tracer administration) were collected at 15-min intervals. The VCO_2 was measured using indirect calorimetry immediately after the 5th meal. HSMP, heat-treated and spray-dried cow milk powder; IAAO, indicator amino acid oxidation; MA, metabolic availability; $NaH^{13}CO_3$, [^{13}C] sodium bicarbonate; SMP, spray-dried cow milk powder; VM, vegetarian meal; VCO_2, volume of CO_2 production.

maintenance on a standardized diet to provide adequate energy and protein to the participants (**Figure 1**). This was followed by a 1-d IAAO tracer protocol. Each IAAO experiment was performed with an interval of ≥1 w to ensure complete washout of the tracer.

Reference amino acid mixture and test proteins

The reference crystalline amino acid mixture (Ajinomoto) was based on egg protein (USDA, NDB: 1123) (16), and contained all amino acids (~120% of the requirement) except lysine, phenylalanine, and tyrosine (**Supplemental Table 1**), which were added separately to this mixture after adjusting for their contribution from the test proteins. The milk used for SMP and HSMP production were sourced from the same dairy (Karnataka Milk Federation). The SMP was procured as a single batch of whole-milk powder that had been spray dried, without prior heat treatment, at the Karnataka Milk Federation. To produce SMP, the inlet and outlet temperature of the spray dryer was maintained at 180°C and 80°C, respectively. To produce HSMP, pasteurized cow milk was heat-treated at 120°C for 40 min and spray dried (LU-222 Advanced, Labultima), in a similar method to that used to produce SMP, at the National Dairy Research Institute. The inlet and outlet temperature of the spray dryer maintained to produce HSMP was 200°C and 75°C, respectively.

The VM was prepared in the form of a habitually consumed rice–lentil Indian meal (*khichdi*) (17). It consisted of milled rice, dehulled and split pigeon pea, potato, and green beans in a 3.4:1:1.4:4.3 ratio (17), which was pressure cooked (~120°C for 60 min) with water (10 times the rice weight), oil (0.28 times the rice weight), and garlic and salt for flavor. After standardization and acceptability testing, the quantity of VM required for the study was prepared in a single batch, aliquoted in smaller portions, and stored in a food-grade freezer at −20°C. Required portions were thawed overnight in a refrigerator at 4°C before portioning and administration to the participants on the day of the IAAO experiment.

The SMP, HSMP, and raw ingredients of VM (i.e., rice, pigeon pea, potato, and green bean) were analyzed for their protein and amino acid content (Eurofins Analytical Services), as previously described (18–20). The precision (CV) for protein estimation was ±5% and for amino acid analysis was ±10%. The amino acid contribution from the individual ingredients of VM was summed to obtain the amino acid composition of the meal. Other proximate nutrients (fat, ash, and moisture) and dietary fiber were analyzed at the National Institute of Nutrition using AOAC methods (21) with a precision of ±5% for all assays. The reactive (unmodified) lysine, furosine, and lysinoalanine (the most commonly measured markers of early and advanced Maillard reactions, respectively) were measured (Ansynth service BV) using standard methods (22, 23) with a precision of ±5%. The concentration of Amadori products (lactulosyllysine/fructosyllysine) in the test proteins was calculated using measured values of furosine (24). The total lysine in SMP and HSMP was calculated as a sum of reactive and modified lysine content in the test proteins.

Experimental protocol

A week prior to the initiation of the first IAAO study, participants were housed overnight in the metabolic ward of the Division of Nutrition, St John's Research Institute, for the measurement of their basal metabolic rate (BMR) and anthropometry next morning. BMR was measured for 20 min using open-circuit indirect calorimetry with a ventilated hood system (25), calibrated by ethanol combustion, with an error of <2%. Participants were weighed in minimal clothing using a calibrated digital scale (Goldtech, AE038) with a precision of 0.01 kg. Body weight was measured at least once per week for the entire study duration. The height of the participants was recorded without footwear on a vertically mobile scale to the nearest of 0.1 cm (Seca 213).

During the 2-d maintenance period, participants were given a standardized diet, providing energy at BMR × 1.4 (their recorded habitual physical activity level), protein at 1.0 $g \cdot kg^{-1} \cdot d^{-1}$, and lysine at 56–58 $mg \cdot kg^{-1} \cdot d^{-1}$. Eggs were the only animal-source protein in the standardized diet. Other lysine-rich foods such as milk and milk products and flesh foods were not given. The diet plan was based on their habitual dietary pattern using a 3-d dietary recall (2 weekdays and 1 weekend) and was consumed as 3 meals (breakfast, lunch, and dinner), providing ~10%, 35%, and 55% of energy from protein, fat, and carbohydrate, respectively. Participants consumed all meals under the supervision of the study staff and their daily food records were maintained. They were encouraged to maintain their customary physical activity level and refrain from any nonhabitual or excessive physical activity.

On the 3rd d, after a 10–12 h overnight fast, participants arrived at the metabolic ward to take part in 1 of the 8 randomly assigned IAAO experiments, each of which lasted for 8 h (Figure 1). The test meals were portioned into 8 isonitrogenous and isocaloric mini-meals that were fed every h, providing 2/3 of the daily energy (BMR × 1.3) and protein (1 $g \cdot kg^{-1} \cdot d^{-1}$) requirement. The protein was supplied either as the reference amino acid mixture or as a combination of the reference

TABLE 2 Furosine, Amadori product, lysinoalanine, modified lysine and total lysine concentration in the test proteins consumed by Indian men to determine the MA of lysine in SMP, HSMP and VM[1]

Test proteins	Furosine, mg/100 g food	Amadori product,[2] mg/100 g food	Lysinoalanine, mg/100 g food	Modified lysine,[3] %	Total lysine,[4] g/100 g food
SMP	40.4	234	<3.0	3.41	2.1
HSMP	90.8	525	27.4	9.67	1.8
Pasteurized milk[5]	25.2	146	<3.0	2.22	2.0
VM[6]	1.61	6.10	<3.0	0.61	0.15

[1]HSMP, heat-treated spray-dried cow milk powder; MA, metabolic availability; SMP, spray-dried cow milk powder; VM, vegetarian meal.
[2]Lactulosyllysine (SMP and HSMP) and fructosyllysine (VM) were calculated using the measured furosine concentration in the test protein.
[3]Modified lysine was calculated based on the furosine and lysinoalanine values.
[4]Total lysine was calculated as a sum of reactive and modified lysine.
[5]Analyzed lyophilized pasteurized milk, which was heat-treated and spray dried to produce HSMP.
[6]VM was analyzed in cooked form.

amino acid mixture and one of the test proteins. The amount of each test protein provided at each level of lysine intake (to provide 35% and 50% of the lysine requirement) was calculated based on the total lysine concentration (reactive + modified per g of protein) and the protein content in the test proteins except for the HSMP experiments (**Supplemental Tables** 1 and 2). Because heat-treatment substantially increased amino acid cross links (such as lysinoalanine) in HSMP (**Table 2**) and there could be possible formation of other advanced Maillard reaction products that were not measured in this study, the total lysine content of HSMP (1.8 mg/100 g) was underestimated by 10% to that of pasteurized milk (2.0 mg/100 g) before it was heat treated and spray dried to produce HSMP (Table 2). Therefore, the total lysine content of the pasteurized milk was used to plan the test meal for the HSMP experiments (Table 2). The SMP and HSMP were reconstituted to liquid milk by addition of water in a ratio of 1:10 before administration. The nonprotein energy in the test diets was provided in the form of a flavored (Mango Tang, Mondelez India Foods Pvt Ltd) sugar-oil drink, maltodextrin (Polycal, Nutricia), and protein-free wheat starch (Kroner K Starke) cookies. The reference amino acid mixture, nonlabelled crystalline phenylalanine, tyrosine, lysine, and maltodextrin, were added and mixed with the sugar-oil drink before administering the hourly mini-meals. The test proteins were separately administered to the participants with each mini-meal. Participants stayed in a reclining position and did not sleep or consume any food or beverage other than the test meal and water provided throughout the study duration.

During the IAAO protocol, 4 hourly mini-meals were administered to achieve a stable fed state in breath $^{13}CO_2$ background abundance before introducing the oral tracers (Figure 1). The tracers [1–^{13}C] phenylalanine and [^{13}C] sodium bicarbonate (99%; Cambridge Isotope Laboratories), prepared with 0.9% saline (Infutec Healthcare, Ltd), were provided in priming doses of 11.98 μmol/kg and 2.07 μmol/kg, respectively, with the 5th meal (12, 13). Subsequently, an hourly oral dose of [1–^{13}C] phenylalanine (7.99 μmol·kg^{-1}·h^{-1}) was provided with remaining meals until 8th h (12, 13). The amount of phenylalanine provided as tracer was subtracted from the total dietary provision of phenylalanine to maintain a constant phenylalanine intake (36 mg·kg^{-1}·d^{-1}) across the experiments. Tyrosine intake in the study diet was set at 40 mg·kg^{-1}·d^{-1} (1.6 times of the total aromatic amino acid requirement), to ensure that no labelled phenylalanine was hydroxylated to meet the demand for tyrosine (12, 13). The total phenylalanine and tyrosine levels were kept constant across the study days by adjusting the contribution of nonlabelled crystalline phenylalanine and tyrosine in the test meals.

Breath sample collection and analyses

Breath samples were collected for the measurement of isotopic enrichment by the participant breathing out into a specially designed bag with a 1-way valve, avoiding breath from the respiratory dead space. At each time point (Figure 1), samples were collected and transferred into 2 10 mL nonsilicon-coated tubes (Vacutainer; Becton Dickinson). Three fed-state baseline breath samples were collected at 30, 15, and 2 min before the tracer protocol started and 7 samples were collected at 15-min intervals, from 2.5 h after the tracer administration began and continued until the end of the study (Figure 1). When breath collection coincided with an hourly meal, the breath sample was collected first, followed by the meal. The breath-sample tubes were stored at room temperature until analysis. The $^{13}CO_2$ abundance (atom %) in the breath samples was analyzed using an isotope ratio mass spectrometer (IRMS, Delta V Advantage, Thermo Fisher Scientific Inc.). The $^{13}CO_2$ enrichment in the breath samples due to isotopic administration during the fed state was expressed as atom % excess (APE) over baseline. The rate of CO_2 production was measured for a period of 15 min using indirect calorimetry immediately after the 5th meal.

The fraction of $^{13}CO_2$ ($F^{13}CO_2$) from tracer phenylalanine oxidation in relation to the amount administered was calculated as follows (26):

$$F^{13}CO_2\,(\%) = [(ECO_2 \times VCO_2 \times 44.6 \times 60)/(W \times R \times 100 \times D)] \times 100$$

where ECO_2 was the $^{13}CO_2$ enrichment (in APE) in the expired breath, VCO_2 was the CO_2 production rate (expressed as mL/min), W was the weight (kg) of the participants, R was the recovery factor for $^{13}CO_2$, which was assumed to be 0.82 (27), and D was the dose of [1–^{13}C] phenylalanine administered (μmol·kg^{-1}·h^{-1}). The constants 44.6 (μmol/mL) and 60 (min) were used to convert VCO_2 to μmol/h, and the factor 100 (within parenthesis) expressed APE in a fraction.

The reference and test IAAO response slopes were calculated using $F^{13}CO_2$ measurements and the respective lysine intakes. The MA of lysine in the test proteins was determined by the slope-ratio method (14).

Statistical analysis

The sample size of the study was calculated based on the reported slope difference of 0.0028 and 0.0024 for IAAO oxidation in earlier estimations of the MA of lysine in cooked cornmeal against a reference amino acid mixture (13) and in oven-browned cooked rice against cooked white rice (12). Considering similar slope differences of 0.0028 in VM and SMP against the reference amino acid mixture and 0.0024 in HSMP against SMP, and assuming a SD of 60% for the reported slope differences, to detect a difference at 5% level of significance and 80% power, the minimum sample size required to test the MA of lysine in the test proteins was 6.

A sensitivity analysis was performed with the $^{13}CO_2$ enrichment (APE) of post-dose breath samples across the experiments to identify a plateau with least variability to calculate $F^{13}CO_2$. The slopes of $F^{13}CO_2$ against increasing test lysine intakes were calculated using a linear mixed effect regression model. Here, an additional independent and identical normal random component for each participant was included in the linear regression equation of the phenylalanine oxidation against the lysine intakes to estimate the reference and test protein slopes. The slopes were determined by a common origin of $F^{13}CO_2$ response at

base lysine intake (6 mg·kg^{-1}·d^{-1}). Data are presented as mean ± SD, median, and IQR. The slope estimates of $F^{13}CO_2$ for the reference amino acid mixture and test proteins are provided with standard error (SE). The MA of lysine in the test proteins were estimated by the slope–ratio method (14). Statistical significance was considered at $P < 0.05$. All statistical analyses were conducted using R version 3.6.1 (28).

Results

The amino acid profile and proximate composition of the test proteins are provided in Supplemental Tables 1 and 2. The furosine, Amadori compounds (lactulosyllysine/fructosyllysine), lysinoalanine, modified lysine and total lysine content in the test proteins are presented in Table 2. The modified lysine content ranged from 0.6% in cooked VM to 9.7% in HSMP. Although spray drying resulted in a small increase (1.2%) in the modified lysine content of SMP, heat-treatment and spray drying increased the modified lysine content by 7.5% in HSMP compared with pasteurized milk. The lysinoalanine was formed only in HSMP (27.4 mg/100 g). The total lysine in SMP was similar to HSMP (2.1 and 2.0 g/100 g of food, respectively) before it was heat-treated and spray dried (Table 2).

Seven participants were recruited in the study. Their anthropometric characteristics and BMR are summarized in **Supplemental Table 3**. There were no significant changes (within 2% of initial weight) in their body weight during the 8 w of experimentation. A total of 56 oxidation studies (7 participants × 8 experiments each) were conducted with no dropouts. All participants completed their 8 experiments within 3 months and on the study days were in good health and reported no discomfort.

The sensitivity analysis of the $^{13}CO_2$ enrichment (APE) of post-dose breath samples across the experiments showed a plateau with the least variability (19.6%) for the final 45 min breath collection, and these values were used to calculate the $F^{13}CO_2$. The overall $F^{13}CO_2$ variability (16.7%) across the experiments was consistent with that reported in previous studies (11, 29). The mean ± SD and median (IQR) of F $^{13}CO_2$ with graded intakes of lysine from reference amino acid mixture and test proteins are provided in **Table 3** and **Supplemental Figure 2**, respectively. The $F^{13}CO_2$ response against increasing test lysine intakes determined negative slopes ($P < 0.001$) for both reference and test proteins (Supplemental Figure 2). The slope estimates (SE) for reference amino acid mixture, SMP, HSMP, and VM were −0.186 (0.026), −0.171 (0.026), −0.130 (0.031), −0.161 (0.026), respectively. The slope equation and MA estimate of each test food are presented in **Table 4**. The MA of lysine in SMP and VM was 91.9 and 86.6%, respectively. Heat-treatment significantly reduced the MA of lysine by 22% in HSMP (69.9%) when compared with SMP (Table 4).

TABLE 3 IAAO response ($F^{13}CO_2$) with graded intakes of lysine from reference amino acid mixture and test proteins for determining the MA of lysine in SMP, HSMP, and VM in Indian men[1]

Lysine intake mg·kg^{-1}·d^{-1},% requirement[2]	$F^{13}CO_2$, %[3]
Reference amino acid mixture	
6.0 (20%)	18.7 ± 2.66
10.5 (35%)	15.6 ± 3.41
15.0 (50%)	13.3 ± 1.96
SMP	
10.5 (35%)	15.4 ± 0.93
15.0 (50%)	13.9 ± 3.15
HSMP	
15.0 (50%)	15.6 ± 2.03
VM	
10.5 (35%)	17.2 ± 4.26
15.0 (50%)	13.4 ± 2.19

[1]Values are means ± SDs; $n = 7$, number of experiments conducted on each participant = 8. $F^{13}CO_2$, fraction of $^{13}CO_2$; HSMP, heat-treated and spray-dried cow milk powder; IAAO, indicator amino acid oxidation; MA, metabolic availability; SMP, spray-dried cow milk powder; VM, vegetarian meal.
[2]Lysine requirement of adults (15).
[3]$F^{13}CO_2$ (%) was calculated from [1–^{13}C] phenylalanine oxidation in relation to the dose administered.

lyophilized cow's milk powder (mean ± SD: 94.9% ± 2.7%), measured in humans (9).

The MA of lysine in HSMP was 22% lower than that of SMP (70% compared with 92%), which could be due to the greater modified lysine content in HSMP (9.7% compared with 3.4%) formed by the additional high-temperature heat treatment for a prolonged duration. When heat treated, the reactive lysine in milk is involved in chemical reactions leading to the formation of early (lactulosyllysine) and advanced Maillard reaction products (cross-linked protein aggregates) and D-enantiomers (through racemization), which cannot be metabolically utilized (5, 31, 32). This is because lactulosyllysine cannot undergo enzymatic hydrolysis and the less soluble cross-linked aggregated proteins are inaccessible to digestive enzymes (31, 33). The D-amino acids cannot be utilized for protein synthesis due to the absence of racemases in humans, which convert D-amino acids to their L forms (32). In addition, the Maillard products competitively inhibit the activity of pancreatic and intestinal proteolytic enzymes, particularly trypsin, carboxypeptidase A and B, and aminopeptidase N (33), and cause a further reduction in the digestion of proteins. This

TABLE 4 MA of lysine in SMP, HSMP, and VM based on IAAO studies in Indian men[1]

Source of lysine	Slope equation[2]	MA (%)[3]	*P* value[4]
Reference amino acid mixture	$-0.186x + 18.7$	100.0[5]	<0.001
SMP	$-0.171x + 18.7$	91.9	<0.001
HSMP	$-0.130x + 18.7$	69.9	<0.001
VM	$-0.161x + 18.7$	86.6	<0.001

[1]Number of experiments conducted on each participant = 8. HSMP, heat-treated and spray-dried cow milk powder; IAAO, indicator amino acid oxidation; MA, metabolic availability; SMP, spray-dried cow milk powder; VM, vegetarian meal.
[2]Slopes were determined by a common origin (18.7) at *base* lysine intake (6.0 mg·kg^{-1}·d^{-1}).
[3]MA estimated by the slope–ratio method (14).
[4]All slopes were significantly different from zero ($P < 0.001$).
[5]MA of lysine in the reference amino acid mixture was assumed to be 100%.

Discussion

In this study, the MA of lysine in SMP, HSMP, and a habitually consumed cereal-legume based VM was estimated by the IAAO slope-ratio method in healthy adult men. The mean MA of lysine in SMP, HSMP, and VM was 92%, 70%, and 87%, respectively. For SMP, the MA of lysine was within the previously reported range of lysine MA in skim milk powder (from 78% to 98%) as estimated by traditional slope–ratio growth assay method in growing pigs (30). The MA of lysine in SMP was similar to the true ileal lysine digestibility of

has been shown in a porcine cannulation model, in which the MA of lysine at the level of liver was 60% lower in heat-treated milk powder (with 50% modified lysine as lactulosyllysine) than in lyophilized milk powder (with no detectable modified lysine) (24). In the same study, the appearance of fructoselysine (an early Maillard reaction product) in the portal blood was ~8% and this was entirely excreted in the urine, providing evidence for the metabolic inertness of modified lysine (24).

The present study also provides the first reported estimate of the MA of lysine in a habitually consumed cereal and legume–based VM (87%) in humans. In the absence of the MA and true ileal digestibility data, the MA of lysine in VM was compared with the true orofecal crude protein (nitrogen) digestibility in human and rat models, where available. The MA of lysine in VM compared well with the true orofecal protein digestibility of North American vegetarian diets (88–94%) but was 9% higher than the reported crude protein digestibility (78%) of an Indian rice–bean vegetarian diet in humans (34). The lower crude protein digestibility in the Indian vegetarian diet could be attributable to the use of unrefined cereals and beans with high antinutrient contents (such as phytic acids, polyphenols, tannins, hemagglutinins, trypsin, and protease inhibitors) compared with North American vegetarian diets that were prepared using refined plant-based foods (34). In the present study, the higher MA of lysine in VM was possibly due to the use of polished and dehulled grains (i.e., milled rice, split and dehulled pigeon pea), differences in the food matrix, particle size of the meal, and preparation method (5, 34). In support of this, the true protein digestibility of pigeon pea after dehulling significantly improved by 12% for raw and by 11% for pressure-cooked pigeon pea cultivars in rats (35). This finding could be attributable to the reduction in phytic acids (by 89–93%) and tannins (by 52–60%) by the dehulling process (36). In addition, when tested individually, the lysine MA in cooked polished rice was found to be as high as 97% in humans (12), pointing to the possible impact of the milling process. Studies in South Indian adults and children reported the true ileal lysine digestibility and metabolic utilization of lysine in legumes and rice to be 60–69% and 78%, respectively, using the dual isotope tracer method (3, 4). The lower lysine digestibility and utilization values could be attributable to the use of unpolished rice and legumes (whole) and differences in the varieties of legumes and rice, food matrices, and methods of preparation (3, 4). Equally important is the formation of Maillard reaction products during cooking, which renders lysine unavailable for utilization (5). However, in the present study, formation of Maillard products in the VM was negligible (0.6%) and would have had a minimal impact on the MA of lysine.

To meet the protein requirements of LMIC populations, the MA of lysine in SMP and VM from the present study can be used to optimize the lysine-limited predominantly cereal-based diets that are usually consumed by these populations. According to an Indian national dietary survey (37) based on monthly household purchases, cereals and legumes are habitually consumed in a mean ratio of 13:1 and the risk of quality corrected protein inadequacy in such a diet was found to be 24% in adults (8). Using the MA estimates from this study, an addition of 15 g of SMP and 50 g of legumes to improve the cereal-legume ratio to 3.4:1 (as tested in the present study) would reduce the risk of quality protein inadequacy to <10%. Of course, this cereal–legume ratio also requires a reduction in the dietary cereal quantity, otherwise the energy content of the diet would be too high. The MA estimates from the present study can also be extrapolated to optimize the protein quality of Indian supplementary nutrition programs such as the Integrated Child Development Scheme and the Mid-day Meal Scheme. These supplementary programs deliver predominantly cereals with varying amounts of legumes, but without consideration of MA of the limiting IAA (lysine). The protein quality of these meals can be enhanced using animal source foods such as SMP and eggs, which are presently provided in an irregular and fragmented manner. In addition, the MA of lysine in HSMP obtained from the study indicates that commercial food products (such as sterilized, condensed, and evaporated milk and milk products; infant formulas; confectionaries; ready-to-eat cereals; and snacks) with similar or higher furosine and lysinoalanine content (10, 38) compared with HSMP could have a low-lysine MA. There is need for future research on evaluating the protein quality of commercial food products.

A limitation of this study is that the MA estimate derived for VM was specific to the cereal–legume combination (~3:1) that was tested in the study, and this might vary with other cereal–legume combinations. Another limitation was the measurement of lysinoalanine as the sole advanced heat-induced marker in the test proteins; there are several markers (such as deoxyosones and furfurals) that are difficult to measure because of their highly reactive nature (39).

In conclusion, this study provides estimates of the MA of lysine in SMP, HSMP, and VM, which could inform the protein quality of the foods that are habitually consumed or used in dietary interventions and supplementary nutrition programs to meet protein requirements in LMICs. Further studies will be required to determine the MA of limiting IAAs in relevant food groups and combinations to build into the expanding database on protein quality and to evaluate the effects of environmental stresses (intestinal parasites and infections) on MA of limiting IAAs in different age groups, particularly in growing children, women of reproductive age, and elderly adults, with the potential to adversely impact growth and functionality.

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