Indispensable Amino Acid Digestibility of Moroccan Fava Bean Using the Dual Isotope Method in Healthy Adults

Meriam Khayour, Aziza Benmhimdane, Juliane Calvez, Naima Saeid, N. Khodorova, Hakim Belghiti, Asmaa El Hamdouchi, Khalid El Kari, Victor Owino, Daniel Tomé, et al.

Indispensable amino acid digestibility of Moroccan fava bean using the dual isotope method in healthy adults

Meriam Khayour[1], Aziza Benmhimdane[2], Juliane Calvez[3], Naima Saeid[1], Nadezda Khodorova[3], Hakim Belghiti[4], Asmaa El Hamdouchi[1], Khalid El Kari[1], Victor Owino[5], Daniel Tomé[3], Hassan Aguenaou[1], Rachid Mentag[2], Mohammed El Mzibri[1] and Claire Gaudichon[3]

1. National Center for Energy, Nuclear Sciences and Techniques - Ibn Tofail University Joint Research Unit in Nutrition and Food, RDC-Nutrition Associated to AFRA/IAEA, Morocco.
2. Biotechnology Research Unit, Regional Center of Agricultural Research of Rabat, National Institute of Agricultural Research (INRA), Rabat, Morocco
3. Université Paris-Saclay, AgroParisTech, INRAE, UMR PNCA, Paris, France
4. Nutrition Unit. Military hospital of Instruction Mohammed V. Rabat. Morocco.
5. Division of Human Health, International Atomic Energy Agency, Vienna, Austria.

Corresponding Author

M. EL Mzibri
Life Sciences Division
CNESTEN. BP 1382 RP. 10001 Rabat. Maroc
Tél: +212.537.71.27.51, Fax: +212.537.71.18.46
GSM: +212.661.47.91.24
Mail: mzibri@yahoo.com
elmzibri@cnesten.org.ma

Financial support

The project was partly funded by the International Atomic Energy Agency under the CRP E.4.30.31. All authors report no conflicts of interest.

Conflict of interesting

Pr. Daniel Tome is an Associate Editor of the Journal of Nutrition and played no role in the Journal's evaluation of the manuscript; he reports no conflicts of interest.

Short title

Amino acid digestibility of fava bean in humans

Abbreviations

AA: amino acid, AAA: aromatic amino acid, AP: atom percent, APE: atom percent excess, Asx: asparagine + aspartate, DIAAR: digestible indispensable amino acid ratio, DIAAS: digestible indispensable amino acid score, Dig: digestibility, EA: elementary analyzer, EDTA: ethylene-di-amine-tetra-acetic acid, GC: gas chromatography, GC-C-IRMS: gas

chromatography combustion isotope ratio mass spectrometry, Glx: glutamine + glutamate, Hb: haemoglobin, IAA: indispensable amino acid, IRMS: isotopic ratio mass spectrometry, NS: non-significant, SAA: sulphur amino acid, SD: standard deviation, STD: standard, TCF: transamination correction factor, U-HPLC: ultra-high performance liquid chromatography, WHO: World Health Organization.

Clinical Trial Registry number and website

NCT04866927

www.clinicaltrials.gov

Data described in the manuscript will be made available upon request, pending application and approval.

ABSTRACT

Background. Assessment of protein quality is necessary to satisfy the nutritional needs of populations across the world. In addition to Indispensable Amino Acid (IAAs) composition, protein digestibility is a major component of IAA bioavailability, playing a crucial role in human health and affecting the linear growth of children.

Objectives. This study aimed to evaluate IAA digestibility of fava beans, a legume widely consumed in Morocco, using the dual-tracer method.

Study design. ^{2}H- intrinsically labelled Fava beans supplemented with 12 mg/kg BW of ^{13}C spirulina was given to five healthy volunteers (3 males and 2 females), aged 25.8 ± 3.3 y old, with a mean BMI of 20.0 kg/m^2. The meal was spread in small portions and given hourly throughout 7 h. Blood was sampled at baseline and hourly from 5 to 8 h after meal ingestion. IAA digestibility was evaluated by gas chromatography - combustion - isotope ratio mass spectrometry using the $^{2}H/^{13}C$ ratio in plasma IAA. Digestible Indispensable Amino Acid Ratios (DIAAR) were calculated using the scoring pattern for people older than 3 y.

Results. Fava beans had an adequate level of lysine but were limiting in several IAAs, especially methionine. Under our experimental conditions, the average IAA digestibility of fava bean was 61.1 ± 5.2%. Valine had the highest digestibility (68.9 ± 4.3%) and threonine had the lowest (43.7 ± 8.2%). In consequence, the lowest DIAAR was 67% for threonine and only 47% for sulphur amino acids (SAA).

Conclusion. The present study is the first to determine the digestibility of fava bean amino acids in humans. The mean IAA digestibility was moderate, and consequently, we conclude that fava bean provides a limited amount of several IAAs, especially SAA, but adequately for lysine. Preparation and cooking methods of fava beans should be improved to increase digestibility.

This study was registered at ClinicalTrials.gov as NCT04866927.

Keywords. Fava bean, digestibility, stable isotopes, dual-tracer method, humans

INTRODUCTION

Protein quality is critical in the World Health Organization's (WHO) 2025 targets, one of which aims to reduce the prevalence of stunting in children under five by 40%. Scientific evidence has shown that some amino acids are of absolute dietary necessity to maintain normal growth. These indispensable amino acids (IAAs) are provided through animal and plant foods (1). From a nutritional perspective, protein quality refers to the amino acid composition as well as digestibility and related amino acid absorption, in adequacy with the human requirement pattern (2). Protein quality plays a crucial role in meeting the nutritional needs of populations across the developing world throughout the course of life, and during pregnancy and early childhood, in particular.

The Moroccan diet is a Mediterranean diet based on a high consumption of cereals, fruits and vegetables (3). Diet diversification is clearly in progress, especially in urban households and wealthier classes, increasingly including more foods rich in micronutrients. However, the consumption of animal products is still limited. Cereals, legumes and fodder crops constitute the main pillars of Moroccan agriculture. There is growing interest in the cultivation of legumes, especially fava beans, which are by far the most cultivated legume in Morocco, representing 56% of the country's legume production.

Conventional methods of evaluating amino acid digestibility are based on ileal amino acid balance, which is possible with ileostomates or intubation in healthy volunteers. Although it provides reliable and accurate values, the ileal amino acid method is very invasive and can only be applied in healthy adult volunteers. Recently, a minimally invasive dual-tracer technique has been proposed to overcome these constraints (4), and has been applied to measure the digestibility of IAAs from numerous intrinsically labelled proteins (5–7). This technique is based on the simultaneous ingestion of an intrinsically ^{2}H- or ^{15}N-labelled test protein and a "standard" protein of known digestibility, labelled with ^{13}C. The relative

isotopic pattern of these labelled amino acids in the blood compared to the meal allows estimation of the test protein's amino acid digestibility (4,8).

The present study aimed to evaluate dietary IAA digestibility from fava beans in healthy, Moroccan volunteers with the dual-tracer method. Furthermore, it contributes to global efforts to validate this approach for the evaluation of food protein quality.

MATERIAL AND METHODS

Study population

The interventional study was performed in November 2019. The number of volunteers was determined to be 6, in accordance with previous studies on amino acid digestibility using the dual isotope tracer method (11,12) and to allow for external (inter-studies) comparison. Finally, due to insufficient amount of labelled fava bean, only five participants (three males and two females), aged between 25 and 35, were recruited with respect to inclusion/exclusion criteria. A flow chart participants' enrolment in the study is given in Supplemental Figure 1. A face-to-face interview was conducted with each participant to collect information on health problems (exclusion criteria) and consumption of medications and/or supplements. To be included in the study, participants had to meet the following criteria: (a) body mass index (BMI) < 25 kg/m^2, (b) non-pregnancy, (c) no anaemia symptoms, (d) not taking any medication, including NSAIDs and (5) no antibiotics within four weeks before the study.

Anthropometric measurements, including weight and height, were taken before the intervention study according to WHO recommendations (9). Body Mass Index (BMI) was calculated as the ratio between weight and height squared (kg/m^2). The haemoglobin (Hb) measurement in whole blood was performed in situ using the HemoCue portable spectrophotometer (HemoCue AB, Angelholm, Sweden) according to manufacturer's recommendations (10).

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (CERB/22/17), and written, informed consent was obtained from each participant. This intervention study was registered at ClinicalTrials.gov as NCT04866927.

Legume protein labelling

First, a pilot study was conducted to evaluate the labelling efficiency. Plants were grown in 5 L pots and labelled with deuterium oxide (D_2O, 99.8%, Sercon Ltd). Labelling started 15 days after flowering and four treatments were used. These treatments consisted of applying a pulse dose of 25% D_2O, followed by daily irrigation with 2.5% D_2O for 0 (T1), 5 (T2), 10 (T3) or 20 (T4) days. Control plants were irrigated with tap water. After plant maturation, pods were harvested and seeds were collected and dried. Seeds were then milled to fine flour in a grinder. The proteins were directly hydrolysed. Obtained amino acids were derived in N-ethoxycarbonyl-ethyl-esters and analysed using Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), coupled to an IRMS (Isoprime, GV Instrument, Manchester, UK) via the GC5 Isoprime interface to determine their ^2H enrichments, as detailed below.

Based on the results of the pilot study, plants for the intervention study were grown in 5 L pots and labelled with a pulse dose of 25% D_2O followed by daily irrigation with 2.5% D_2O for 15 days. After plant maturation, pods were harvested and seeds were collected, dried and stored.

Amino acid and protein content

For amino acid quantification (other than tryptophan), 10 mg of ground fava beans were hydrolyzed with HCl 6N at 110°C for 24 h. Norvaline was added before hydrolysis and used

as an internal standard. Performic acid oxidation was carried out before hydrolysis for analysis of sulphur amino acids (SAA), in order to convert methionine and cysteine to the acid-stable derivatives methionine sulfone and cysteic acid, respectively. For tryptophan analysis, a base hydrolysis was carried out and 15 mg of ground fava beans were hydrolyzed with barium hydroxide 2N at 110°C for 20 h. 5-methyl-tryptophan was added before hydrolysis and used as an internal standard. Calibration standards were composed of an amino acid mixture (Waters), to which specific amino acids were added (norvaline, methionine sulfone, cysteic acid, tryptophan, 5-methyl-tryptophan). Fava bean hydrolysates and standards were then derivatised using the AccQTag Ultra Derivatisation Kit (Waters) according to the manufacturer's protocol. The amino acid analysis was performed with an Acquity HClass ultra-HPLC (UHPLC) system with a photodiode array detector (PDA detector; Waters). Amino acids were separated using an AccQ-Tag AA C18 column (2.1 × 100 mm; 1.7 μm bead size; Waters) and quantified as mmol/g of dry matter. Amino acid concentrations were converted to g/kg dry matter using "in chain" amino acid molecular weights. Total nitrogen of ground fava beans was determined by the Dumas method using an elemental analyser (Vario Micro Cube; Elementar), with atropine as the elemental standard. Protein content of fava beans was calculated using the nitrogen-to-protein conversion factor of 5.4 (total N content × 5.4). Each analysis was done on five replicates.

Fava bean protein digestibility

In this study, ^{2}H-labelled Fava beans soup was prepared as it is typically done in Moroccan households. To facilitate cooking, beans were firstly dehulled and then soaked overnight. Beans were then cooked in a casserole or a pressure cooker for 1 hour. After that, cooked fava beans were poured in a food blender until we got a soup. The soup is then seasoned with salt and some spices.

For the study, the test meal given to each participant was composed of a fava bean soup (equivalent to 100 g of fava beans), much olive oil, bread and two boiled eggs, supplemented with 12 mg/kg body weight of ^{13}C-labelled spirulina (Sercon Ltd), used as the standard protein (6). The total energy of the meal was estimated as 600 kcal.

For each participant, the test meal was divided into 11 portions. After an overnight fast, three portions were given at T0 and seven portions were then given hourly for 7 h, the remaining portion was kept for the evaluation of deuterium enrichment. Blood samples were collected before feeding the first mini-doses (T0) and at 5, 6, 7 and 8 h after the beginning of the protocol.

Blood samples were collected into EDTA-coated anticoagulant tubes (Becton Dickenson), centrifuged immediately at 2500 g for 15 min at 4°C. The plasma was then recovered and stored at -80°C until analysis.

^{2}H and ^{13}C enrichment assessment

The meal samples were hydrolyzed (6N HCl at 110°C for 24 h), filtered (0.22 µm) and purified using the cation-exchange Dowex AG50X8 resin (H^+-form). Serum samples were acidified by 1N HCl and directly mixed with cation-exchange resin. The amino acids were eluted with 6N NH_4OH and dried using the vacuum dryer. The dried samples were dissolved in 4 mL of 0.1N HCl and mixed with 3.2 mL of an ethanol/pyridine solution (60:40, v/v). Then 630 µL of ethyl chloroformate (ECF) was added, and the solution was gently shaken until no bubbles were formed. To separate ECF derivatives from the reactive mixture, 2 mL of dichloromethane:hexane (50:50, v:v) was added, the solution was vigorously vortexed, and the upper organic phase was transferred into a glass tube and dried under nitrogen stream. The obtained residue was dissolved in 50 µL of ethylacetate, transferred to a GC vial and stored at -20°C until analysis.

The ^{13}C and ^{2}H enrichments of derived amino acids were determined using Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an IRMS (Isoprime, GV Instrument, Manchester, UK) *via* the GC5 Isoprime interface. For ^{13}C analysis, the temperature of the combustion oven was regulated at 850°C. For ^{2}H analysis, the high temperature conversion (HTC) reactor was maintained at 1050°C.

A 30-m Rxi-17 capillary column (Restek, Evry, France; 0.25 mm i.d. and 0.25 µm film thickness) was used to separate amino acids. The inlet temperature was set at 270°C. Samples were injected in split mode (10:1 for ^{13}C and 3:1 for ^{2}H analysis). The initial oven temperature was 150°C, thereafter the temperature was raised to 200°C by 4°C/min and then to 270°C by 25°C/min. The final temperature was maintained for 15 min at 270°C. The stable isotopic compositions of carbon and hydrogen were reported using the conventional delta per mill notation: the $\delta^{13}C$ and $\delta^{2}H$ values are expressed relative to the international standards (PDB and VSMOW, respectively).

The derivatisation process adds exogenous carbon and hydrogen atoms to the carbon and hydrogen atoms of amino acids. The $\delta^{13}C$ and $\delta^{2}H$ values of the amino acids in samples were calculated using the following equations:

$$\delta^{13}C_{AAsample} = (n\,\delta^{13}C_{\text{der AA sample}} - (n-m)\,\delta^{13}C_{ECF}))/m$$

$$\delta^{2}H_{AAsample} = (n\,\delta^{2}H_{\text{der AA sample}} - (n-m)\,\delta^{2}H_{ECF}))/m$$

Where $\delta^{13}C_{\text{AA sample}}$ and $\delta^{2}H_{\text{AA sample}}$ are the δ values of the given amino acid in the sample, $\delta^{13}C_{\text{der AA sample}}$ and $\delta^{2}H_{\text{der AA sample}}$ are the δ values measured for the derived amino acid in the sample. $\delta^{13}C_{ECF}$ and $\delta^{2}H_{ECF}$ are the δ values of the derivatisation agent ECF (measured off-line by EA-IRMS), n is the number of C (H) atoms in the derived amino acid and m the initial number of C (H) atoms in the non-derived molecule of amino acid.

$$\text{Relative IAA digestibility} = \frac{\text{plasma } {}^{2}H - \text{IAA (APE) /meal } {}^{2}H - \text{IAA (APE)}}{\text{plasma } {}^{13}C - \text{IAA (APE) /meal } {}^{13}C - \text{IAA (APE)}}$$

$$\text{IAA digestibility (\%)} = \text{Relative digestibility x 100 x } Dig_{Std} \times \text{TCF}$$

Dig_{Std} is the digestibility of each IAA from the ^{13}C-labelled spirulina protein, and TCF is an IAA-specific term used to correct for loss of a ^{2}H atom during transamination, as previously determined by Devi et al. (6). For tryptophan, methionine and histidine, all three of which the digestibility could not be determined, the mean digestibility of other IAAs was used as a proxy.

The digestible indispensable amino acid ratio (DIAAR) in relation to the human requirement pattern was also calculated. The DIAAR represented the content of each IAA, corrected by the individual digestibility of each IAA.

$$\text{DIAAR (\%)} = 100 \text{ x } \frac{\text{mg of digestible dietary IAA in 1 g of the dietary protein}}{\text{mg of the same dietary IAA in 1g of the reference protein}}$$

The Digestible Indispensable Amino Acid Score (DIAAS) was determined as the lowest DIAAR for fava bean seeds. Data are presented as mean ± standard deviation (SD).

RESULTS

Subject characterization

The subjects were 25.8 ± 3.3 y old. Anthropometric characteristics of these volunteers were a mean weight of 57.6 ± 7.7 kg, a mean height of 169.6 ± 6.5 cm, and consequently a mean BMI of 20.0 ± 1.8 kg/m^2. The mean Hb level was 14.2 ± 1.6 g/dl, ranging from 12.6 to 16.9 g/dl.

Total amino acid content evaluation in fava bean

Overall, the total protein content of fava bean seeds was 19.1%. Amino acid composition of fava bean seeds (Aguadulce cultivar) is reported in **Table 1**. Among non-IAAs, arginine, aspartate and glutamate prevailed and represented 8.62 ± 0.23, 9.09 ± 0.25 and 13.82 ± 0.25 g/100 g of protein, respectively. Cysteine was the least abundant amino acid, with only 0.86 ± 0.07 g/100 g of protein. Regarding IAAs, the lowest concentration was found for methionine (0.89 ± 0.04 g/100 g of protein), whereas lysine and leucine exhibited the highest concentrations with 7.42 ± 0.10 and 7.35 ± 0.13 g/100 g of protein, respectively.

Fava bean labelling

The pilot study was conducted to optimize the protocol for fava bean deuterium labelling. Accordingly, four treatments were performed, and results are reported in Figure 1. Natural abundances were obtained with control plants irrigated with tap water.

For all amino acids, lower levels of enrichments were obtained by labelling with only one pulse dose of 25% D_2O, which was not enough to significantly enrich the plant IAAs. Daily irrigation, with 2.5% of D_2O after a pulse labelling with 25% D_2O, increased plant protein labelling for almost all IAAs in respect to the labelling duration. Increased IAA labelling was obtained with a pulse labelling with 25% D_2O followed by a daily irrigation with 2.5% D_2O for 20 days.

Furthermore, there was a difference between amino acid labelling. In fact, for all treatments, methionine was the most labelled amino acid, followed by proline, with an enrichment of 0.70 APE and 0.36 APE, respectively. These levels were obtained with a 25% D_2O pulse followed by a daily labelling with 2.5% D_2O for 20 days. Other amino acids, including valine, leucine, isoleucine, phenylalanine and lysine were less labelled, with an enrichment of about 0.2 APE, obtained with the best treatment protocol. Finally, for the other amino acids such as alanine,

glycine, threonine, serine, aspartate and glutamate, labelling was the lowest with an enrichment that did not exceed 0.15 APE.

Fava bean IAA digestibility

The dual-isotopic method, using ^{13}C spirulina as the standard protein, was performed to evaluate the digestibility of intrinsically labelled fava bean IAA. Enrichments of both ^{13}C and ^{2}H in test meal IAAs are reported in **Table 2**. Enrichments of both ^{13}C and ^{2}H were evaluated in IAA plasma before the study intervention, and 5, 6, 7 and 8 h after the first test meal consumption. Results are reported in Figure 2. They clearly show that for the studied IAAs, plateaus of ^{13}C and ^{2}H enrichments were obtained between 5 h and 8 h after the first meal. Mean enrichments of ^{13}C and ^{2}H in plasma IAAs at plateaus are reported in **Table 2**.

Based on these data, the digestibility of intrinsically labelled fava bean IAAs was evaluated, and results are reported in **Table 3**. The digestibility of IAA varied between 68.9 ± 4.3% for valine and 43.7 ± 8.2% for threonine. Accordingly, the mean IAA digestibility was 61.1 ± 5.2%.

In this study, DIAARs were calculated according to recommended amino acid scoring patterns for older children (> 3 years), adolescents and adults (2). Results are reported in **Table 4**. The lowest DIAARs observed in our study were for threonine (0.67) and SAA (0.47). In consequence, the fava bean has a low DIAAS of < 50%.

DISCUSSION

This study aimed to evaluate the protein quality of Moroccan fava beans in healthy adults using the dual isotope method to measure digestibility. For this purpose, fava beans were intrinsically labelled with ^{2}H and cooked in a soup. It was fed to volunteers in a repeated meal

protocol according to that proposed by the group from Bangalore to enable external comparisons of IAA digestibility in legumes (6).

Scientific evidence has shown that animal proteins are the best quality sources owing to their IAA composition, their high digestibility and bioavailability. They are consumed in large amounts in high-income and developed countries. However, in developing countries, despite a gradual increase in their consumption, animal protein intake is still insufficient to meet the IAA requirements of children and adults alike (13). Thus, it has been recommended to evaluate new alternative sources, including plant-sourced proteins (14). In this field, there is great interest in legumes for their nutritional properties and beneficial effects on human health, including mitigation of conditions such as heart disease, high blood pressure, stroke and type 2 diabetes (15). In some developing countries, fava bean is already a significant source of protein. It is most commonly found in Middle Eastern and Mediterranean diets (16). Fava bean is rich in some amino acids such as aspartic acid, glutamic acid and arginine (17,18), but does not meet the requirement for several IAAs, including SAAs (18). However, as with other legumes, fava bean showed adequate amounts of lysine compared to IAA requirements.

The use of DIAAS has become popular because it accounts for both the IAA composition and their individual ileal digestibility. It thus simply reflects the nutritional value of dietary proteins in respect to their capacity to satisfy IAA body requirements (2). However, its calculation requires the determination of individual IAA digestibility. In this study, the dual-tracer approach was implemented to measure IAA digestibility of ^{2}H-labelled fava bean using a tracer dose of ^{13}C-labelled spirulina in the test meal (6,11). Fava bean amino acids were heterogeneously labelled. Some amino acids, including methionine and proline, were well labelled, whereas labelling of other amino acids was reduced, in particular, glycine, serine and aspartate. This differential labelling could be due to the number of H atoms in their chemical

276 structures as well as in their precursors. Uniformly labelled ^{13}C-spirulina protein was used as
277 the standard protein, in accordance with the protocol proposed by Devi et al. (6). The values
278 of IAA digestibility from spirulina were previously estimated relative to a mixture of ^{2}H-
279 labelled crystalline IAAs, and the mean digestibility of the IAAs was 85.2%, ranging from 77
280 to 95% (6). These values, as well as the transamination correction factors previously reported,
281 were used for our digestibility calculation.
282 In our study, fava bean seeds were first soaked and then dehulled before test meal preparation.
283 Previous studies have shown that dehulling of beans before preparation may increase its
284 average IAA digestibility by 5.7% (6). Furthermore, it was reported that soaking and
285 dehulling reduce the amounts of phytate and trypsin inhibitor contents, widely reported as
286 anti-nutritional factors (19,20). Fava beans were then prepared as a soup, one of the most
287 popular fava bean-based recipes in Morocco.
288 The dual-isotope tracer method is gaining of interest and great efforts are widely made to
289 promote the use of this minimally invasive technique to assess amino acid true digestibility.
290 In particular, this method can overcome the constraints related to the invasiveness of the
291 intubation protocol for ileal balance method in humans.
292 The repeated meals procedure over 7 h enabled achieving a plateau for ^{2}H and ^{13}C IAAs in
293 plasma of all subjects. Under these experimental conditions, the average IAA digestibility of
294 dehulled fava beans was 61.1 ± 5.2%. Valine had the highest digestibility (68.9 ± 4.3%) and
295 threonine had the lowest digestibility (43.7 ± 8.2%). Devi *et al.* have evaluated the
296 digestibility of other legumes using the same approach, and reported mean IAA digestibility
297 of chick peas at 56.6%, of mung beans at 57.7%, and of dehulled mung beans at 63.4% (6).
298 The mean digestibility we report for fava beans is thus close to that observed for dehulled
299 mung beans using the same method, but lower than that reported for pinto beans (77%)(12).

300 Hence, in comparison to these studies using the dual-tracer method, it seems that fava beans
301 presented a relatively lower IAA digestibility in our study.
302 To our knowledge, there is no data in the literature on ileal AA digestibility of fava beans in
303 humans. A few studies have been conducted in animals with the standard ileal balance and
304 revealed higher values than ours. In broilers, standard ileal digestibility ranged from 67% for
305 lysine to 87% for valine (21). In growing pigs, values ranged from 61% for tryptophan to 87%
306 for arginine, and lysine digestibility being 82% (22). The differences compared to our values
307 can be partly explained by the concentration of anti-nutritional factors, especially for tannins.
308 These concentrations partly depend on the cultivar. Among the six cultivars analysed by
309 Jezierny et al., digestibility varied by 13% for threonine and 8% for lysine (22). Nevertheless,
310 we cannot exclude the possibility that values obtained with the dual isotope method may have
311 underestimated IAA digestibility, as suggested in a recent study (8). More investigations in
312 humans would be useful to draw further conclusions.
313 The DIAAS has been recommended for protein quality evaluation. In our study, and due to
314 moderate digestibility of IAAs, the lowest DIAAR was only 47% for SAA. This result
315 corresponds with those reported previously for legumes (24, 25). The second lowest DIAAR
316 was 67% for threonine, which exhibited a particularly low digestibility of 43%. Such a low
317 threonine digestibility identified with the dual isotope method has previously been reported
318 for mung bean, but not chickpea (6, 11). Scientific evidence has shown that plant proteins
319 have lower DIAAS as compared to animal proteins, and several of them lie in the no quality
320 claim category (DIAAS <75) (23). This suggests the need to combine these plant proteins
321 with adequate, complementary sources to enhance their nutritional efficiency (18). Owing to
322 the adequate amount of lysine in fava bean, and despite its moderate digestibility, the DIAAR
323 we measured for lysine was close to 1 (0.98). It is thus an important nutritional attribute,
324 making for a suitable complementary source to cereal-based diets known to be deficient in

lysine. In Morocco, for example, fava bean is usually eaten with bread. This is an example of a typical meal where cereal-based proteins, scoring low in lysine but well-balanced in SAAs, can complement to some extent leguminous proteins scoring high in lysine but lower in SAAs.

This study presents some limitations. First, due to a limited quantity of labelled seeds, the test was performed on only five volunteers. Nevertheless, inter-individual variability was comparable to other studies using the same methodology (5,6). Second, the enrichment of some IAAs was not detected in the test meal and/or blood samples, and the digestibility of three IAAs, including the most limiting one, could not be determined. However, the digestibility of other IAAs serve as a good indicator of their digestibility, even for moderate digestibility values (26). Therefore, we can consider the mean digestibility as a good proxy. Also, the use of spirulina IAA digestibility and transamination correction factors, measured independently in a group of Indians, may be not accurate for another population. However, the protocol used for the dual isotope method is the same as that developed by the Kurpad group, allowing external comparisons for IAA digestibility values in various legumes.

The present study is the first to evaluate the digestibility of fava bean in humans. It showed both a moderate digestibility but an adequate content of some IAAs, particularly lysine that was not limiting. In contrast, several AAs were limiting, especially threonine and SSA. However, we cannot exclude that an underestimation of the digestibility using the dual isotope method contributed to the low scoring. Further experiments are necessary to validate this result, especially those using the classical ileal balance method.

CONCLUSION

Although fava bean is limiting in several IAAs, the adequate lysine content makes fava bean an appropriate food to complement the Moroccan diet, which is largely based on cereals, for a well-balanced IAAs intake to better satisfy human body requirements.

Acknowledgments

We would to kindly thank all participants in the present study for their patience, time and consent.

Authors' contributions

The authors' responsibilities were as follows: HA, DT, MEM and CG: conceived and designed the study; AB, MEM, KEK and RM were in charge of plant growth and grain labelling; MK, NS, AEH and MEM were in charge of recruitment and the intervention study; MK, MEM, JC, NK and CG were in charge of experimental analysis, data collection and interpretation; HB, was in charge of the medical visit and blood sampling; MK and MEM have drafted the paper; HA, JC and CG read, revised and approved the final manuscript. All authors have read and approved the final manuscript.

REFERENCES

1. World Health Organization. Food and Agriculture Organization, United Nations University. Protein and Amino Acid Requirements in Human Nutrition: Report of a Joint WHO/FAO/UNU Expert Consultation. Geneva, Switzerland: World Health Organization; WHO technical report series 935. 2007.
2. FAO. Dietary protein quality evaluation in human nutrition. Report of an FAO Expert Consultation. Rome, Food and Nutrition. 2013.
3. FAO. Profil Nutritionnel du Maroc - Division de la nutrition et de la protection des consommateurs. 2011.

4. FAO. Research Approaches and Methods for Evaluating the Protein Quality of Human Foods: Report of an FAO Expert Working Group 2 – 5 March 2014 Bangalore, India. Food and Agriculture Organization of the United Nations, Rome. 2014.
5. Kashyap S, Shivakumar N, Varkey A, Duraisamy R, Thomas T, Preston T, Devi S, Kurpad A. 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(5):980–7.
6. 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(6):984–91.
7. van der Wielen N, Khodorova NV, Gerrits WJJ, Gaudichon C, Calvez J, Tomé D,Mensink M. Blood 15N:13C Enrichment Ratios Are Proportional to the Ingested Quantity of Protein with the Dual-Tracer Approach for Determining Amino Acid Bioavailability in Humans. J Nutr. 2020;150(9):2346–52.
8. Tessier R, Calvez J, Airinei G, Khodorova N, Kapel R, Quinsac A, Galet O, Piedcoq J, Benamouzig R, Tomé D et al. The true amino acid digestibility of 15N-labelled sunflower biscuits determined with ileal balance and dual isotope methods in healthy humans. The Journal of Nutrition. 2021;nxab423.
9. World Health Organization (WHO). Physical status: the use and interpretation of anthropometry. Report of a WHO expert committee technical. Report series no 854. WHO, Geneve. 1995.
10. Burger S, Pierre-louis J. Micronutrient Global Leadership Project: A procedure to estimate the accuracy and reliability of HemoCueTM measurements of survey workers. Washington, D.C.ILSI. 2003.
11. Kashyap S, Varkey A, Shivakumar N, Devi S, Reddy B H R, Thomas T, Preston T, Sreeman S, Kurpad A. True ileal digestibility of legumes determined by dual-isotope

tracer method in Indian adults. The American Journal of Clinical Nutrition. 2019;110(4):873–82.

12. Calderón de la Barca AM, Martínez-Díaz G, Ibarra-Pastrana ÉN, Devi S, Kurpad AV, Valencia ME. Pinto Bean Amino Acid Digestibility and Score in a Mexican Dish with Corn Tortilla and Guacamole, Evaluated in Adults Using a Dual-Tracer Isotopic Method. The Journal of Nutrition. 2021;151(10):3151–7.
13. Godfray HCJ, Aveyard P, Garnett T, Hall JW, Key TJ, Lorimer J, Pierrehumbert R T, Scarborough P, Springmann M, Jebb S A. Meat consumption, health, and the environment. Science. 2018;361(6399):eaam5324.
14. Pyett S, de Vet EWML, Trindade LM, van Zanten HHE, Fresco LO. Chickpeas, crickets and chlorella: our future proteins. Wageningen. 2019.
15. Polak R, Phillips EM, Campbell A. Legumes: Health Benefits and Culinary Approaches to Increase Intake. Clin Diabetes. 2015;33(4):198–205.
16. Marrou H, Ghanem ME, Amri M, Maalouf F, Ben Sadoun S, Kibbou F, Sinclair T R. Restrictive irrigation improves yield and reduces risk for faba bean across the Middle East and North Africa: A modeling study. Agricultural Systems. 2021;189:103068.
17. Alghamd S. Chemical Composition of Faba Bean (Vicia faba L.) Genotypes under Various Water Regimes. Pakistan J of Nutrition. 2009;8(4):477–82.
18. Herreman L, Nommensen P, Pennings B, Laus MC. Comprehensive overview of the quality of plant- And animal-sourced proteins based on the digestible indispensable amino acid score. Food Sci Nutr. 2020;8(10):5379–91.
19. Nalle CL, Ravindran G, Ravindran V. Influence of dehulling on the apparent metabolisable energy and ileal amino acid digestibility of grain legumes for broilers. J Sci Food Agric. 2010;90(7):1227–31.

20. Luo Y-W, Xie W-H. Effect of different processing methods on certain antinutritional factors and protein digestibility in green and white faba bean (*Vicia faba* L.). CyTA - Journal of Food. 2013;11(1):43–9.
21. Olukosi OA, Walker RL, Houdijk JGM. Evaluation of the nutritive value of legume alternatives to soybean meal for broiler chickens. Poultry Science. 2019;98(11):5778–88.
22. Jezierny D, Mosenthin R, Sauer N, Roth S, Piepho H-P, Rademacher M, Eklund M. Chemical composition and standardised ileal digestibilities of crude protein and amino acids in grain legumes for growing pigs. Livestock Science. 2011;138(1–3):229–43.
23. Marinangeli CPF, House JD. Potential impact of the digestible indispensable amino acid score as a measure of protein quality on dietary regulations and health. Nutrition Reviews. 2017;75(8):658–67.
24. Mathai JK, Liu Y, Stein HH. Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS). Br J Nutr. 2017;117(4):490–9.
25. Reynaud Y, Buffière C, Cohade B, Vauris M, Liebermann K, Hafnaoui N, Lopez M, Souchon I, Dupont D, Rémond D. True ileal amino acid digestibility and digestible indispensable amino acid scores (DIAASs) of plant-based protein foods. Food Chem. 2021;338:128020.
26. Calvez J, Benoit S, Piedcoq J, Khodorova N, Azzout-Marniche D, Tomé D, Benamouzig R, Airinei G, Gaudichon C. Very low ileal nitrogen and amino acid digestibility of zein compared to whey protein isolate in healthy volunteers. The American Journal of Clinical Nutrition. 2021;113(1):70–82.

Tables

Table 1. Amino acid concentration of fava bean [1]

Dispensable amino acids			Indispensable amino acids		
Amino acid	**concentration g/kg DM**	**concentration g/100 g of proteins**	**Amino acid**	**concentration g/kg DM**	**concentration g/100 g of proteins**
ala	7.9 ± 0.1	4.16 ± 0.1	lys	14.2 ± 0.2	7.4 ± 0.1
arg	16.4 ± 0.5	8.62 ± 0.2	met	1.7 ± 0.1	0.9 ± 0.1
asx	17.3 ± 0.5	9.09 ± 0.3	phe	8.2 ± 0.2	4.3 ± 0.1
cys	1.6 ± 0.1	0.86 ± 0.1	his	4.4 ± 0.1	2.3± 0.1
glx	26.4 ± 0.5	13.82 ± 0.3	val	9.0 ± 0.2	4.7 ± 0.1
gly	7.3 ± 0.2	3.83± 0.1	thr	7.3 ± 0.1	3.9 ± 0.1
pro	8.1 ± 0.2	4.23 ± 0.01	trp	1.9 ± 0.1	1.0 ± 0.1
tyr	6.6 ± 0.3	3.46 ± 0.2	leu	14.0 ± 0.2	7.4 ± 0.1
ser	9.3 ± 0.2	4.88 ± 0.1	ile	8.6 ± 0.2	4.5 ± 0.1

[1]Values are means ± SDs; n = 5 replicates; DM: dry matter.

Table 2. Test meal and plateau plasma amino acid enrichment of ^{13}C and ^{2}H (APE)[1]

		Valine	Leucine	Isoleucine	Threonine	Phenylalanine	Lysine
Test meal	^{2}H enrichment	0.262 ± 0.003	0.268 ± 0.003	0.231 ± 0.003	0.289 ± 0.037	0.366 ± 0.007	0.240 ± 0.003
	^{13}C enrichment	2.644 ± 0.379	2.306 ± 0.324	2.803 ± 0.403	3.229 ± 0.459	1.989 ± 0.284	1.613 ± 0.228
Plasma	^{2}H enrichment	0.019 ± 0.005	0.019 ± 0.005	0.018 ± 0.004	0.013 ± 0.003	0.021 ± 0.006	0.019 ± 0.006
	^{13}C enrichment	0.255 ± 0.036	0.222 ± 0.044	0.310 ± 0.053	0.280 ± 0.030	0.187 ± 0.045	0.151 ± 0.031

[1]Values are presented as means ± SDs (N=5). APE: Atom percent excess.

Table 3. Digestibility of amino acids in fava bean proteins[1]

IAA	TCF[2]	Dig_{Std} (%)[3]	Relative digestibility (%)					IAA digestibility (%)
			V1	V2	V3	V4	V5	
Valine	1.048	87.1	83.1	71.0	73.9	72.7	76.8	68.9 ± 4.3
Leucine	1.081	86.0	75.3	66.7	72.4	68.0	74.2	66.3 ± 3.5
Isoleucine	1.070	84.2	73.6	64.9	74.2	62.2	76.6	63.4 ± 5.7
Threonine	1.016	82.5	68.9	47.3	44.1	52.1	48.3	43.7 ± 8.2
Phenylalanine	1.053	95.3	58.0	55.2	57.1	67.1	66.7	61.0 ± 5.7
Lysine	1.002	77.5	89.2	79.2	80.7	75.9	82.8	63.5 ± 3.8

[1]Values are presented as means ± SDs.

[2] Transamination correction factors (TCF) were obtained from Devi et al. ~~(2018)~~(6)

[3] Digestibility values of each IAA from spirulina used as a standard protein were obtained from Devi et al. ~~(2018)~~(6).

Table 4. DIAAR of fava beans

IAA	concentration mg/g	IAA digestibility	Digestible IAA mg/g	Recommended IAA scoring patterns[1]	**DIAAR**
Valine	47	68.9%	32.4	40 mg	0.81
Leucine	73	66.3%	48.4	61 mg	0.79
Isoleucine	45	63.4%	28.5	30 mg	0.95
Threonine	38	43.7%	16.6	25 mg	0.67
Lysine	74	63.4%	46.9	48 mg	0.98
AAA	77.6	61.0%[2]	47.3	41 mg	1.15
Histidine	23	61.1%[3]	14.1	16 mg	0.88
Tryptophan	10	61.1%[3]	6.1	6.6 mg	0.92
SAA	17.6	61.1%[3]	10.7	23 mg	0.47

[1] Recommended amino acid scoring patterns for older children (> 3 years), adolescents and adults (2).
[2] Digestibility of Phe was applied to evaluate the DIAAR of AAA (N=5).
[3] Mean IAA digestibility was applied to evaluate the DIAAR of histidine, tryptophan, and SAA (N=5), as ^{2}H enrichment could not be determined in the plasma.

AAA, aromatic amino acid; DIAAR, digestible indispensable amino acid ratio; IAA, indispensable amino acid; SAA, sulfur amino acid.

Legends for figures

Figure 1. Evaluation of deuterium enrichment in the cultivated fava bean in APE, according to the treatment.

Treatments consisted of applying a pulse dose of 25% D_2O to the growing plant, followed by daily irrigation with 2.5% D_2O for 0 (T1), 5 (T2), 10 (T3) or 20 (T4) days (n=4).

D_2O: deutered water; APE: atom percent excess; T: treatment

Figure 2. Plasma enrichment of IAA after consumption of test meal.

Plasma appearance of ^{13}C (A) and ^{2}H (B) isotopic enrichments of IAA (APE) after consumption of intrinsically labeled Fava beans (n = 5). Data are presented as means, SDs are omitted for clarity
IAA: indispensable amino acid; APE: atom percent excess.