Antibiotic usage during or around the time of a probiotic intervention would certainly influence any potential effects, and this was therefore considered in our study. Women were asked before and after completion of the capsule intervention if they had taken any antibiotics, and a total of 6 women (2 from the probiotic group, 4 from the placebo group) confirmed that they had. Although details of antibiotic strain or duration of usage were not recorded, a secondary analysis of results that excluded antibiotic users and poor compliers to the capsule intervention showed that no significant differences in any of the metabolic variables or pregnancy outcomes remained (1).

As highlighted by Griffin, the ingestion of fermented milk products, live probiotics, or prebiotics by our study participants could also have influenced our results. All participants were asked to avoid fermented milk and probiotic and prebiotic products from recruitment until the end of their pregnancy, allowing a sufficient ''washout'' period before capsule commencement at 24 wk of gestation. To aid in this, an information sheet outlining the sources of fermented, probiotic, and prebiotic products on the market was developed and explained by the research dietitian to all study participants on recruitment at 12–19 wk of gestation, along with a list of appropriate nonprobiotic yogurts available in local shops. However, we did not directly investigate the consumption of fermented or probiotic products because we gathered information on dietary intakes during the intervention period by using a 3-d food diary rather than a food-frequency questionnaire.

We hope we have sufficiently answered the queries raised by Griffin and have instilled confidence in the robustness of our trial's methodology. As we conclude in our article, further research is warranted into the effects of probiotics in pregnancy on metabolic outcomes, particularly among obese women who may be at higher risk of adverse outcomes. Furthermore, there is a need to establish the optimal species, timing, and dosage of probiotics that may benefit this important patient group.

The authors declared no conflicts of interest.

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doi: 10.3945/ajcn.114.094425.

Protein requirements and aging

Dear Sir:

The recent article involving the use of the indicator amino acid oxidation (IAAO) method to assess protein requirements of octogenarian women (1) represents yet another attempt to show that there is an increase in protein requirements with age, a debate that has existed for decades. One reason for lack of resolution of this debate is that protein and amino acid metabolism is by far the most elaborate of any nutrient. Assuming that the protein requirement is an intake that allows maintenance of an acceptable body composition phenotype and associated normal function, we know that this can occur in population groups exposed to a wide range of habitual protein intakes, through metabolically complex adaptations. Evaluating exactly how adaptations to variation in protein intakes occur, at what cost, if any, and the lower and upper limits of protein intakes at which successful adaptation can occur is extremely challenging. In the absence of functional indicators of protein status of the adult population, all methods to date have been based on some measure of protein

balance, either nitrogen balance or amino acid balance, through measures of amino acid oxidation by using stable isotopes. Concern for inadequacies of the nitrogen balance approach has resulted in investigators adopting acute postprandial studies, such as the IAAO method (1), to evaluate the response to protein or amino acid intakes as a proxy for the "requirement." Assuming here that such studies can show useful information, it is certainly necessary that investigators adopting postprandial experimental protocols fully understand the metabolic complexity of the response of protein metabolism to protein intake and show that the model assumptions inherent in their studies are correct and that any metabolic response or endpoint does indeed directly relate to the "requirement." The principal advocates of the IAAO method, Pencharz and Ball (2), have always argued that the change in the oxidation rate, the breakpoint, of a nonlimiting indicator amino acid ([1-^{13}C]phenylalanine) in response to graded intakes of a test amino acid or of protein shows the intake that maximizes protein synthesis and minimizes indicator oxidation: ie, their definition of the "requirement." In response to a study of the protein requirement of healthy school-aged children determined by the IAAO method (3), Millward and Jackson (4) argued that the use of the IAAO method to assess protein requirements, as opposed to requirements for amino acids, was invalid. This is because, in this specific case, the [^{13}C]phenylalanine indicator does become limiting and this limitation determines the breakpoint. The experimental design of this approach measures [^{13}C]phenylalanine oxidation in response to meals containing increasing amounts of protein (as an amino acid mixture based on egg protein) containing a fixed amount of phenylalanine. This is shown in **Figure 1**, which plots phenylalanine content of protein meals at each amount of "protein" intake expressed as the content relative to the amount that would have been present if the amino acid mixture was balanced. Thus, intakes of phenylalanine are in excess at the 2 lowest intakes and are deficient

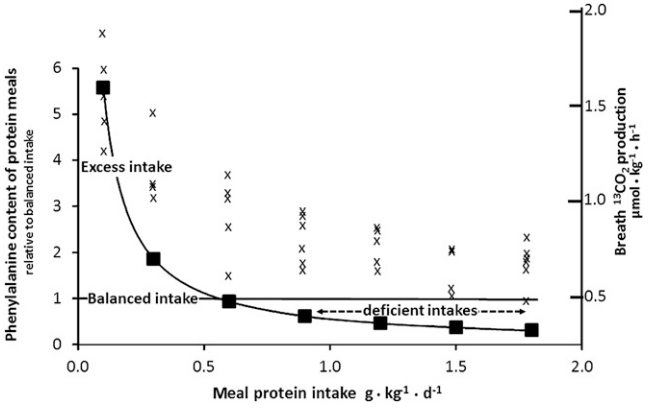

FIGURE 1. Concentrations of the "indicator" amino acid (phenylalanine) in meal protein intakes relative to those of a balanced intake at each protein amount. Values shown are calculated from studies in 6 octogenarian women (1) and are the amounts of phenylalanine in each amount of the test meals of protein relative to its content that would have occurred if the test amino acid mixture pattern was that of the reference (egg) amino acid pattern (■). Also shown are the reported rates of expired $^{13}CO_2$ measured at the end of each test amount of intake, which reflect phenylalanine oxidation rates (X). In the studies, L-[1-^{13}C]phenylalanine oxidation was measured during the feeding of frequent small meals of the test intake. These test intakes contained the varying amounts of an amino acid mixture patterned on egg protein that was equivalent to the daily protein intakes as shown but with a fixed amount of phenylalanine (30.5 mg · kg^{-1} · d^{-1}) and tyrosine (40.7 mg · kg^{-1} · d^{-1}). This results in a relative excess of phenylalanine at low intakes, limiting amounts at high intakes, and a balanced intake at protein intakes equivalent to 0.56 g · kg^{-1} · d^{-1}. It is argued that this variation in the relative concentration of the phenylalanine "indicator" with protein intake will be the primary influence on the reported shape and breakpoint of the indicator oxidation curve.

in the 4 highest intakes. Because of this, the indicator oxidation rate, shown in Figure 1, reflects the excess or deficiency of the indicator, not the amount of protein intake. The authors refer to the criticism by Millward and Jackson (4) of their approach in their article (1) and argue that "regardless of the protein intake, the total aromatic amino acid concentration is always 70 mg · kg^{-1} · d^{-1}, which is higher than the aromatic amino acid requirement and thus could not be 'balanced' by increasing protein intake. With sufficient tyrosine, the concept of the IAAO approach is to keep the phenylalanine content constant and sufficient at any protein (amino acid mixture) level to reflect the protein oxidation rate." This statement shows a lack of understanding of the postprandial response to varying protein intakes. In fact, with this study design, the protein oxidation rate, which is not measured, will be the opposite of the observed phenylalanine oxidation rate. As pointed out previously (4), on the basis of many published tracer studies of the feeding response, it can be confidently predicted that at low amounts of intake the meal protein will be fully used with low levels of overall amino acid oxidation but with high levels of [^{13}C]phenylalanine oxidation because of its excess. However, as the intake of the amino acid mixture exceeds 0.6 g · kg^{-1} · d^{-1}, overall utilization of the amino acid mixture for net protein synthesis will decrease as it becomes limited by the relative availability of phenylalanine. In consequence, overall amino acid oxidation will increase and [^{13}C]phenylalanine oxidation will decrease. The validity of this argument is easily tested by measuring the response of blood concentrations of phenylalanine (predicted to be high in the excess intake range and low in the deficient range), changes that will be the opposite of other amino acids (eg, leucine). The argument that the total aromatic amino acid concentration always exceeds the aromatic amino acid requirement is irrelevant because in such acute feeding studies the "requirement" for phenylalanine is that which allows efficient utilization of the meal amino acid mixture for net protein deposition. As shown in Figure 1, the meals are phenylalanine deficient at intakes >0.6 g · kg^{-1} · d^{-1}. Thus, these studies tell us nothing about the protein requirement of octogenarian women.

It is the case that previous studies by the lead author of this article showed no difference with age in the protein requirements of adults as measured by both nitrogen balance (5) or by [1-^{13}C]leucine balance (6). These 2 reports are separate publications from the same study that, together, comprise the most comprehensive study in the literature on the protein requirements of healthy adults. The study shows quite clearly no effect of age and sex, similar to our own findings (7), with the authors concluding that "there are no compelling data that the dietary protein needs of old people are different from those of young people when expressed per kg body weight." It is a puzzle, therefore, that in this most recent study (1), the [1-^{13}C]leucine balance article (6) is not quoted at all and the nitrogen balance arm of the study (5) is only briefly mentioned together with a list of reports arguing for an increased protein requirement, none of which include any unequivocal evidence. One would expect experienced investigators to have a consistent message in their published work or at the least explain why they have changed their view.

My understanding of the literature in terms of well-conducted nitrogen balance or ^{13}C oxidation studies is that the experimental evidence to date shows that requirement values do not change significantly with advancing age. As indicated in an editorial about this recent article (8), what is really needed are studies that show that incremental increases in protein intake make a difference—ie, that they do affect clinically important outcomes. Sarcopenia has been widely discussed as a potential consequence of inadequate protein intake, although there is very little, if any, unequivocal evidence that the loss of muscle mass and function with age can be influenced by protein intake (9). In the absence of

clinical outcomes from well-conducted randomized controlled trials, the identification of a suitable experimental approach that could be adopted by different investigators could allow the requisite much larger numbers of volunteers to be studied and might settle the debate if agreement could be reached on a suitable method. It is quite clear to me that the IAAO method could not serve such a purpose.

The author had no conflicts of interest.

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doi: 10.3945/ajcn.114.089540.

Reply to DJ Millward

Dear Sir:

With Millward's letter, he continues to criticize the merits of the indicator amino acid oxidation (IAAO) method to assess human protein requirements. The issues raised are old and repeatedly described and discussed in the literature, as well as carefully considered during a rigorous review of the article before acceptance for publication, and noted in Fukagawa's editorial published with the article (1).

Apparently, Millward's chief criticism of the IAAO method is that the intake of the indicator amino acid phenylalanine was inadequate at the higher intakes of protein and thus the breakpoint in the response was due to a deficiency of phenylalanine. He used a circular argument with regard to whether phenylalanine intake was adequate or deficient. We have previously measured the phenylalanine requirement in the presence of an excess of all other amino acids (ie, protein), including tyrosine, and found it to be 13.6 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} (population upper 95% CI) (2). During the present experiment, we provided phenylalanine at an intake of 30.5 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}, which is well in excess of the phenylalanine requirement in the presence of excess tyrosine (40 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}) and other amino acids. Therefore, very clearly, phenylalanine would not be limiting at any intake of protein in the current experiment. In which case, as we discussed in the article, the breakpoint was due to the plateau in protein synthesis that occurred when the intake of protein was adequate. To argue that phenylalanine was deficient because it plateaued in oxidation is therefore incorrect, and ignores the extensive work done to show how the IAAO method works.

Two main principles of the IAAO method are *1*) that the excess intake of phenylalanine is proportioned between protein synthesis and oxidation and *2*) phenylalanine oxidation progressively declines with increasing intake of the limiting amino acid (or of total protein) from deficient to adequate and reaches a steady nadir (breakpoint) when a sufficient amount is consumed. Millward has suggested that the intake of phenylalanine, as the indicator, be allowed to change in proportion to the protein intake. If there was a change in the intake of phenylalanine along with protein intake, then the percentage of dose oxidized would not change with each increment of protein because the degree of excess of phenylalanine would be the same for every protein intake. This means that the percentage of dose oxidized would not vary or vary only very little with intakes between deficient and adequate, and it would increase thereafter because phenylalanine would be in excess. This is the same as using the direct oxidation approach, which has other well-recognized issues (3). This is also the key reason why the indicator amino acid must be controlled to the same intake in all treatments; otherwise, the slope of the response line may be due to changes in intake of the indicator amino acid rather than intake of the test protein or amino acid (3).

Millward repeatedly argues that the requirement of every indispensable amino acid varies directly with protein intake, the "adaptive metabolic demand." However, this theory is untested by direct experimentation. In contrast, there is no evidence currently available that the requirement for any amino acid, other than the single most limiting amino acid in the diet, varies with protein intake. This principle of the limiting amino acid defining the protein requirement is the principle whereby the amino acid score of a protein is derived. If one accepts that amino acid score is a valid concept, as Millward does (4), then "adaptive metabolic demand" cannot also be correct.

The authors had no conflicts of interest to declare.

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