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Compared to milk protein, a wheat and pea protein blend reduces high-fat high-sucrose induced metabolic dysregulations while similarly supporting tissue protein anabolism in rats

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List of abbreviations:

ASR: absolute synthesis rate; AA: amino acids; AP: animal protein; ACLY: ATP-citrate lyase; BCAA: branched-chain amino acids; CHO: carbohydrate; DNL: *de novo* lipogenesis; FAS: fatty acid synthase; FSR: fractional synthesis rate; GK: glucokinase; G6Pase: glucose-6-phosphatase; G6PDH: glucose-6-phosphate dehydrogenase; GOT: glutamic oxaloacetic transaminase; GPT: glutamine-pyruvate transaminase; HFS: high fat and sucrose; HOMA-IR: homeostasis model for the assessment of insulin resistance; IR: insulin resistance; ME: malic enzyme; NEFA: non-esterified fatty acids; NFS: normal fat and sucrose; PP: plant protein; PK: pyruvate kinase; PS: protein synthesis; TG: triglycerides; T2D: type 2 diabetes

Abstract (max 300 words):

Background: Plant proteins (PP) have been associated with better cardiovascular health than animal proteins (AP) in epidemiological studies. However, the underlying metabolic mechanisms remain mostly unknown.

Objective: Using a combination of cutting-edge isotopic methods, we aimed at better characterizing the differences in protein and energy metabolisms induced by dietary protein sources (PP *vs* AP), in a prudent or western dietary context.

*Methods***:** Male Wistar rats (n=44, 8 weeks old) were fed for 4.5 months with isoproteic diets differing in their protein isolate sources, either AP (100% milk) or PP (50%:50% pea: wheat), and being normal (NSF) or high (HFS) in sucrose (6 or 15%kcal, respectively) and saturated fat (7 or 20%kcal, respectively). We measured body weight and composition, hepatic enzyme activities and lipid content, and plasma metabolites. In the intestine, liver, adipose tissues and skeletal muscles, we concomitantly assessed the extent of amino acid (AA) trafficking using a ^{15}N natural abundance method, the rates of macronutrient routing to dispensable AA using a ^{13}C natural abundance method, and the metabolic fluxes of protein synthesis (PS) and *de novo* lipogenesis (DNL) using a ^{2}H labelling method. Data were analyzed using ANOVA and mixed models.

Results: At the whole-body level, PP limited HFS-induced insulin resistance (-27% in HOMA-IR between HFS groups, $P<0.05$). In the liver, PP induced lower lipid content (-17%, $P<0.01$) and DNL (-24%, $P<0.05$). In the different tissues studied, PP induced higher AA transamination accompanied by higher routings of dietary carbohydrate and lipid towards dispensable AA synthesis by glycolysis and β-oxidation, resulting in similar tissue PS and protein mass.

Conclusions: In growing rats, compared to AP, a balanced blend of PP similarly supports protein anabolism while better limiting whole-body and tissue metabolic dysregulations, through mechanisms related to their less optimal AA profile for direct channeling to PS.

Keywords (5 to 10): protein and amino acid metabolism, protein synthesis/turnover, natural isotopic abundance, metabolic tracer, *de novo* lipogenesis

Introduction

Diets in high-income countries are high in animal products, refined carbohydrate (CHO) and saturated fats. A shift toward more plant-based diets is actively promoted to achieve more sustainable diets, better for both planetary and human health. The partial replacement of animal protein (AP) by plant protein (PP) sources is a key element of this transition, as the relative proportions of AP and PP produced and consumed both have considerable environmental impacts and broad public health implications (1,2). Regarding human health, although PP are considered to be less efficient than AP to sustain overall protein anabolism and muscle function (3,4), there is growing evidence that PP sources are better for cardiometabolic health (5–8). Indeed, substituting AP with PP, while accounting for dietary cofounders, was associated with lower cardiovascular and all-cause mortality (9,10). Conversely, high AP intake has been associated with metabolic syndrome features, such as dyslipidemia and insulin resistance (IR) (11,12), and with increased risk of cardiovascular diseases (13) and type 2 diabetes (T2D) (14,15). The differential effects of AP or PP intake on cardiometabolic features could be mediated by a better overall lifestyle, including diet which differs greatly depending on the predominant dietary protein sources (16–18), and/or by other nutrients and bioactive compounds present in the food matrix (7).

Those health effects could also be mediated by the intrinsic amino acid (AA) composition of AP and PP sources (7,8). Thus, AP are richer in indispensable AA such as branched-chain AA (BCAA) and aromatic AA, while PP are richer in dispensable AA such as glycine, glutamine, cysteine and arginine, with most of these AA already reported to be implicated in cardiometabolic health (19). In addition to individual AA, the whole dietary AA pattern, accounting for potential interactions between AA, could also contribute to the health effects of protein sources. However, little is known regarding the health effects of specific AA intake patterns, and few studies have investigated associations with cardiovascular endpoints (20–22). Interestingly, AA intake patterns have been shown to be strongly associated with cardiovascular mortality: negatively for some of the dispensable AA (more abundant in PP) and positively for some of the indispensable AA (more abundant in AP) (20).

In rodents, the specific effects of some protein sources or dietary AA on cardiometabolic features have been studied and seem to be dependent on the dietary context (23–28). For instance, while fasting or postprandial glycemia after a glucose tolerance test were reported to be similar between AP and PP sources when fed within a prudent energy context (23–25), PP were reported to lower hepatic triglyceride accumulation and lipogenic gene expression in a high fat dietary context (26,27). We hypothesized that the differential effects of AP and PP on cardiometabolic health could relate to the fact that their AA profiles differentially modulate protein metabolism on one hand, and other pathways connected to intermediary metabolism on the other hand, with potentially higher differences in a western than a prudent dietary context. To date, no single study has explored the interrelated changes in protein, fat and CHO metabolisms between dietary protein sources in the different organs concomitantly. Thus, exploring the specific and concomitant tissue responses to protein sources on protein and energy metabolisms in different dietary energy contexts may contribute to explain the differential effects of AP and PP on cardiometabolic health.

In this study, we aimed at characterizing, at both the systemic and tissue levels, the various metabolic orientations specifically related to the dietary protein source (PP vs AP) within a prudent or western dietary pattern. This multi-pathway metabolic study was endeavored by using a combination of cutting-edge isotopic methods at both natural (^{15}N and ^{13}C) and enriched abundance levels (^{2}H), to respectively estimate the extent of AA metabolic trafficking, the rates

of macronutrients routing to dispensable AA and the fluxes of protein synthesis (PS) and *de novo* lipogenesis (DNL) in tissues.

Materials and methods

Animals, diets and experimental procedures

Male Wistar rats (n=44) of 8 weeks of age and weighing 272.8g (SEM ± 1.5g) (Janvier), were kept on their breeding diet and adapted to the individual housing conditions (12-h light-dark cycle, 20°C) for 3 weeks. Then, the animals were randomly divided into 4 groups (n=11) and fed *ad libitum* for 4.5 months with 4 experimental diets (**Table 1**) differing in their energy level, either normal (NFS) or made high (HFS) by enrichment in dietary fat and sucrose content, and in their protein source, either AP or PP. AP consisted of total milk protein isolate (PRODIET 90, Ingredia) and PP on a 50:50 blend of wheat: pea protein isolates (NUTRALYS® W and NUTRALYS® S85 PLUS for wheat and pea respectively, Roquette Frères). All diets contained the same protein proportion relative to dietary energy content (15%) and met the requirements for rodent growth regarding indispensable AA (29). The difference in AA composition between AP and PP sources was calculated based on protein isolate AA compositions, as provided by suppliers (Supplemental Figure S1). Mixing cereal and legume protein sources with a 50:50 ratio resulted in a PP blend with a well-balanced AA composition, since wheat and pea protein compensated each other for low contents in lysine and sulfur AA, respectively, while presenting very good digestibility of individual AA (30). In comparison to AP, the AA content of the PP blend was lower in indispensable AA, such as BCAA (- 20%), and higher in dispensable AA like glycine (+81%), arginine (+89%) and cysteine (+131%). All the experiments were performed in accordance with the guidelines formulated by the European Community for the use of experimental animals (L358-86/609/EEC, Council Directive, 1986) and authorized by the Regional Ethics Committee (agreement no.12050-2017103015435153V4).

Food intake was recorded weekly and body composition was assessed by MRI (EchoMRITM-700) at 0, 1, 2, 3, 4 and 4.5 months. Blood was collected from the tail vein with heparinized syringes at 0, 2 and 4 months after an overnight fast. After centrifugation (3000 × g, 10 min, 4°C), plasma was rapidly collected, frozen in liquid nitrogen and stored at −80°C until further analyses. Two days before euthanasia (at 4.5 months), rats received a bolus of $^{2}H_2O$ saline solution (20 mL/kg of body weight at 99.9%, Sigma) intraperitoneally under light anesthesia in order to measure PS and DNL (31–34) concomitantly. The dose was set to result in ~3% enrichment in ^{2}H of body water, which was then maintained until euthanasia by providing access to 3% ^{2}H drinking water. Then, after an overnight fast, rats were euthanized under isoflurane anesthesia. Blood was withdrawn from the abdominal aorta and handled as above. The liver, small intestine, a sample of epididymal adipose tissue and skeletal *gastrocnemius* and *tibialis anterior* muscles were rapidly collected, weighed, freeze-clamped in liquid nitrogen, and stored at -80°C for further analyses.

Analytical procedures

Plasma metabolites and insulin levels

At 4.5 months, plasma non-esterified fatty acids (NEFA), glucose, lactate, urea, uric acid, creatinine, triglycerides (TG), total cholesterol, HDL-cholesterol and LDL-cholesterol levels were measured using a commercial kit (NEFA-HR(2), Fujifilm Wako) and a microplate reader or different kits on an ABX Pentra C400 analyzer (Horiba Medical). At 0, 2 and 4 months, plasma glucose levels were as above and plasma insulin levels were assayed using a commercial ELISA kit (10-1250-01, Mercodia). The homeostasis model for the assessment of insulin

resistance (HOMA-IR) was calculated as the product of insulin (µU/ml) and glucose (mg/dl) concentrations.

Liver enzyme activities

Hepatic activities of enzymes involved in glucose, AA and lipid metabolisms were assessed on liver homogenates as previously described (35,36), including ATP-citrate lyase (ACLY), fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), glucokinase (GK), pyruvate kinase (PK), glucose-6-phosphatase (G6Pase), glutamic oxaloacetic transaminase (GOT) and glutamine-pyruvate transaminase (GPT). Measurements were performed at 37 °C using an Infinite M200Pro spectrophotometer (Tecan) by monitoring the oxidation rate of NAD(P)H. Activities were expressed in mU/mg protein.

Tissue protein masses and synthesis fluxes

Protein masses and synthesis fluxes were measured in the liver, the intestine and muscles as previously described (37). Briefly, for each tissue, the protein mass (mg N) was determined from the tissue weight, the protein content per mg fresh tissue, and the percentage nitrogen content in protein measured using elemental analysis (Vario Micro Cube; Elementar). PS was measured using the precursor-product ^2H labeling method, with ^2H enrichment measured in the free and protein-bound alanine of liver, intestine and muscles by GC-MS after ~2 days of ^2H_2O exposure before euthanasia. Enrichments were calculated on the basis of a standard curve of known enrichments corrected for variations in abundance, as previously described (37). As explained in Supplemental Text S1, in each sampled tissue (intestine, liver and muscles), the net fluxes of protein synthesized and stored *in situ* during the last ~2d before euthanasia were calculated as:

$$Tissue\ protein\ FSR\ (\%/d) = \frac{100}{t\ (d)} \times ln\left[\frac{E_{AA} - E_{P0}}{E_{AA} - E_P}\right] \quad [1]$$

$$Tissue\ protein\ ASR\ (mg\ N/d) = \frac{FSR\ (\%/d)}{100} \times tissue\ proteins\ (mg\ N) \quad [2]$$

where t (d) is the period of ^2H labeling from ^2H_2O injection to tissue sampling, E_{AA} (%) is the ^2H enrichment of tissue free alanine, E_P and E_{P0} (%) are the ^2H enrichments of protein-bound alanine at the end of the labeling period and before ^2H_2O administration, respectively (with E_{P0} measured in 4 rats from the same batch that were euthanized at baseline). FSR (%/d) and ASR (mg/d) are the fractional and absolute synthesis rates, respectively.

Tissue lipid content and de novo lipogenesis (DNL) fluxes

Lipid masses and DNL fluxes were measured in the liver and epididymal adipose tissue. Neutral lipids (mostly exclusively triglycerides) were extracted quantitatively by the Soxhlet procedure (38), for calculating tissue lipid content and mass. The lipid mass in whole-body adipose tissues was calculated as the product of the percentage of lipid in epididymal adipose tissue by the total fat mass measured by echo-MRI. DNL was estimated using the precursor-product ^2H labeling method, with ^2H enrichment measured in the precursor (with plasma used as a proxy) and product (tissue neutral lipids) by EA-IRMS and GC-MS, respectively. As explained in Supplemental Text S1, in the liver and total adipose tissues, the net fluxes of lipids synthesized by DNL and stored *in situ* during the last ~2d before euthanasia were calculated as:

$$Tissue\ DNL(\%/d) = \frac{100}{t\ (d)} \times ln\left[\frac{0.48 * E_{BW} - E_{Lip0}}{0.48 * E_{BW} - E_{Lip}}\right] \quad [3]$$

$$Tissue\ DNL\ (mg\ lipids/d) = \frac{FSR\ (\%/d)}{100} \times tissue\ lipids\ (mg) \quad [4]$$

where t (days) is the period of ^2H labeling from ^2H_2O injection to tissue sampling, E_{BW} (%) is the ^2H enrichment of body water (with 0.48*E_{BW} taken as a proxy of the DNL precursor enrichment (34)), E_{Lip} and E_{Lip0} (%) are the ^2H enrichments of the tissue neutral lipids at the end of the labeling period and before ^2H_2O administration, respectively (with E_{Lip0} measured in 4 rats from the same batch that were euthanized at baseline), and tissue lipids are the lipid masses in the liver or adipose tissues.

Finally, whole-body DNL was calculated as the sum of DNL in the liver and adipose tissues and expressed as the % of CHO intake for comparisons with findings in the literature (with glucose-to-fat conversion calculated as 2.8 g glucose/g fatty acids):

$$Whole\text{-}body\ DNL\ (\%\ CHO\ intake) = \frac{100 \times 2.8 \times [\ Liver + adipose\ tissues\ DNL\ (mg\ lipids/day)\ ]}{CHO\ intake\ (mg/day)} \quad [5]$$

Natural abundances of stable N and C isotopes in tissue proteins

Natural abundances of N and C stable isotopes were measured in the proteins of the different tissues sampled and in dietary proteins, CHO and lipids, and expressed relative to standards (atmospheric N_2 for $^{15}N/^{14}N$ and Vienna Pee Dee Belemnite for $^{13}C/^{12}C$, respectively) using the delta notation:

$$\delta(‰) = \left(RR_{sample} - R_{standard}\right) / \ R_{standard} \quad [6]$$

where δ is the parts-per-thousand difference in the ratio of heavy to light isotopes ($^{15}N/^{14}N$ and $^{13}C/^{12}C$) in the samples (R_{sample}) and standards ($R_{standard}$, 0.0112372 for C and 0.0036765 for N). Isotopic measurements were performed with an isotope-ratio mass spectrometer (Isoprime; VG Instruments) coupled to an elemental analyzer (EA Vario Micro Cube; Elementar). Tyrosine was used for calibration and drift correction. $\delta^{15}N$ values for tissue proteins were expressed relative to the δ-value of dietary proteins using the capital delta notation ($\Delta_{tissue\ proteins} = \delta_{tissue\ proteins} - \delta_{dietary\ proteins}$).

As previously explained (37), the natural ^{15}N and ^{13}C fingerprints in tissue proteins were respectively used to assess the extent of tissue AA metabolic trafficking and catabolism (relative to PS), and the relative use of the different macronutrients for tissue AA synthesis. Indeed, while tissue AA mainly originate from dietary proteins, dietary lipids and CHO are also used for the synthesis of dispensable AA via ß-oxidation and glycolysis that produce different AA precursors. Details on reasoning and equations are given in Supplemental Text S2. Briefly, during between-group comparisons, higher $\Delta^{15}N$ values in tissue proteins were interpreted as indicative of increased AA metabolic trafficking and catabolism relative to PS in the tissue considered, in the light of our previous results (39–41). In addition, using the tissue protein $\delta^{13}C$ values and assuming that dietary protein routing to tissue AA was affected only by the protein source (and not the energy level), we assessed the impact of the protein source on the contribution of each nutrient to tissue AA synthesis (37,42).

Statistical analyses

Results are expressed as means and SEM. *P*-values < 0.05 were considered to be significant. *P*-values close to the significance threshold ($0.05 \leq P < 0.1$) were also reported. Analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). For data obtained at a single time point at the end of the experiment (natural $\delta^{15}N$ abundances, PS and DNL measured by ^2H labelling, hepatic lipid content, enzyme activities and some plasma biochemical values), the effects of the protein source, the energy level and their interaction were assessed using ANOVA (PROC GLM). No statistical analysis could be conducted on the nutrient routing results because the equations used to estimate the routing coefficients from the δ13C data did not provide a

separate estimate for each rat but a single estimate for each group or protein source (see Supplementary Text S2). TG and G6PDH values were log transformed before analysis to meet normality. The 4-month kinetics of food intake, body weight, body composition, glucose and insulin levels were analyzed using a mixed model (PROC MIXED) with time as the repeated factor, and protein source and energy level as between-group factors. Interactions between protein source, energy level and time were also tested on the whole kinetics and at the final time. Finally, the differences between PP and AP were tested as preplanned comparisons at each level of energy, using ad hoc contrast in the ANOVA and MIXED models.

Results

Macronutrient intake, body weight and composition

Protein intake did not differ between groups while, as expected, lipids and CHO intake differed between HFS and NFS groups (125% higher lipid intake and ~50% lower CHO intake in HFS, $P < 0.0001$), but not between the PA and PP groups. For all groups, body weight, fat and lean masses increased with time (**Figure 1**). The increase observed in body weight was higher in the HFS groups than in the NFS groups, due to the higher fat mass gain and similar lean mass gain. The protein source had no effect on body weight or composition.

Evolution of glucose homeostasis and insulin sensitivity

The post-absorptive levels of plasma glucose, insulin and HOMA-IR increased with time (**Figure 2**). At 4 months, the energy level (i.e., the fat and sucrose dietary contents) impacted glucose and insulin levels and HOMA-IR, which were systematically greater in the HFS groups than in the NFS groups. In contrast, the protein source affected only glucose levels, which were lower in the PP groups than in the AP groups. However, as shown by the significant interactions between the protein source and the energy level, the differences between protein sources in all these parameters were greater within the HFS context.

End point metabolite levels

To further characterize the metabolic status of the animals by the end of the study, we measured the plasma concentration of various metabolites related to lipid, nitrogen and glucose metabolisms (**Figure 3**). Urea and lactate concentrations were lower in the PP groups than in the AP groups while NEFA concentrations were higher. Regarding lactate, while the protein source effect was significant ($P < 0.05$) on all energy levels, it was only a trend ($P < 0.1$) at each energy level. TG levels were also lower in the PP groups than in the AP groups, but only in the NFS context (P-interaction = 0.012). No differences were found for total, HDL- and LDL-cholesterol, uric acid and creatinine concentrations.

Liver lipid content and liver and whole-body DNL fluxes

Liver lipid content and DNL were affected by both the energy level and the protein source, with values higher by 41% and 49% respectively ($P < 0.001$) in the HFS groups than in the NFS groups and values lower by 17% and 24% ($P < 0.05$) in the PP groups than in the AP groups (**Figure 4**). Regarding liver DNL, while the protein source effect was significant ($P < 0.05$) on all energy levels, it was only a trend ($P < 0.1$) at each energy level. Adipose tissue DNL (data not shown) and whole-body DNL were affected only by the energy level, with higher levels in the HFS groups than in the NFS groups ($P < 0.001$).

Protein synthesis and macronutrient contribution to AA synthesis and routing to tissue proteins

Protein synthesis was affected only in two organs (see **Table 2).** Compared to NFS, HFS groups had higher protein mass and ASR in the liver but lower protein FSR and ASR in the *gastrocnemius* muscle. The results obtained for macronutrient routing to tissue proteins are presented in **Figure 5**. Only the effect of the protein source was explored, as the energy level effect on the routing of dietary protein to tissue protein was assumed to be comparatively negligible (Supplemental Text 2). Compared to AP, PP groups had a lower direct contribution of dietary protein-bound AA to tissue protein-bound AA in all the sampled tissues (up to a ~3% absolute decrease in liver, where protein routing dropped from 79% in AP to 76% in PP groups). In contrast, in PP *vs* AP groups, dietary CHO and lipids contributed slightly more to tissue deposition of dispensable AA via glycolysis and ß-oxidation (up to 1.5% and 1% absolute increases in liver for CHO and lipid routings, respectively, on average for both energy levels but with the same trend for each).

Extent of AA metabolic trafficking in tissues

The tissue protein $\Delta^{15}N$ values (i.e., the natural nitrogen isotopic distance between tissue proteins and dietary proteins, or trophic step) reflect the intensity of AA trafficking by transamination and deamination relative to their direct use for protein anabolism and turn-over, as these metabolic pathways are fractionating processes that amplify the $\Delta^{15}N$ trophic step (37,39–42). $\Delta^{15}N$ values measured in the protein of the intestine, liver and muscles are presented in **Figure 6**. Compared to AP, the PP groups had higher $\Delta^{15}N$ values and thus higher rates of AA metabolic trafficking in all tissues. In addition, in all tissues except *gastrocnemius* muscle, there was a significant interaction (a trend in *tibialis anterior* muscle) between protein source and energy level, showing smaller differences between protein sources in the HFS context.

Hepatic enzyme activities

The activity levels of limiting hepatic enzymes related to DNL, glucose and AA metabolism are presented in **Figure 7**. Regarding DNL, FAS and G6PDH (which contribute to the NADPH production) activities were lower in the HFS groups than in the NFS groups. Regarding the protein effect, the activity levels were lower in the PP groups than the AP groups for ACL and G6PDH irrespectively of the energy context, while for FAS this reduction was visible in the NFS context only (P-interaction = 0.040). ME, another NADPH producer, had similar activities across groups. GK and PK, both key enzymes of glycolysis, were down-regulated in HFS *vs* NFS groups, and GK activity was also lower in the PP groups than in the AP groups. The activity of G6Pase, involved in the glucose export into the blood stream, was similar across diets. Regarding AA metabolism, the GPT activity was higher in the PP and HFS groups compared to the AP and NFS groups. GOT activity was not modified by experimental diets.

Discussion

We have shown that compared to the standard AP used in reference rodent diets (milk protein), a balanced blend of PP (50:50 wheat:pea) similarly supports the lean mass gain and tissue protein turnover in growing rats, while protecting against metabolic dysregulations induced by excessive intakes of saturated fat and sucrose. Thus, in such a western dietary context, dietary PP limit whole-body IR and fat deposition in the liver, with lower hepatic lipid storage and DNL and higher rates of nutrient oxidation in the intestine, liver and muscles. We evidenced a higher routing of dietary lipids and CHO oxidation products towards PS after PP feeding than AP feeding, accompanying and possibly compensating for the higher AA trafficking by

transamination and deamination. Since, in the present study, differences between the AP and PP diets specifically lie in their protein isolate fraction, in the next sections we discuss these metabolic differences in the light of the distinct AA profiles of AP and PP.

Protein source effects on protein and AA metabolism in tissues

Dietary AP and PP (when provided as a well-balanced 50:50 wheat:pea blend) had an equivalent ability to support lean mass growth and tissue PS in growing rats fed *ad libitum*. These results, in line with other findings in the rodent literature (43), support the idea that the balanced AA profile of wheat and legume protein blends are equivalent to the reference AP for sustaining protein anabolism.

In contrast with the tissue protein accretion and turnover, the metabolic trafficking of AA in tissues differed between the dietary protein sources studied. Compared to AP, PP induced higher AA transamination and/or deamination in tissues, as shown by the higher natural nitrogen isotopic distances between tissue and dietary proteins ($\Delta^{15}N$). This evidences an enhancement of such fractionating processes (37,39), in line with previous $\Delta^{15}N$ findings in rats fed with other PP and AP (44). Here, higher $\Delta^{15}N$ values with PP more likely reflect increased transamination than increased deamination, as supported by the higher activity of hepatic GPT and lower plasma urea levels. Of note, the lower plasma urea levels in the PP-fed rats may not only result from lower urea production, but also from higher enterohepatic urea hydrolysis and nitrogen salvage, in line with our previous findings showing the increase in both urea production and urea recycling with PP (45). Furthermore, we found that dispensable AA supply for tissue PS relied less on dietary AA with PP than AP, while dietary CHO and lipids contributed more through enhanced glycolysis and ß-oxidation, as evidenced by the natural carbon isotopic distances between tissue and dietary proteins ($\Delta^{13}C$). Overall, the observed metabolic differences between PP and AP reflected specific responses to their distinct AA profiles, given that AA intake from AP better matches metabolic tissue demands and requires less metabolic trafficking prior to incorporation into tissue proteins (4). The AA profile of PP not only enhances AA transamination but also glycolysis and lipolysis, which sustains the routing of AA toward PS in tissues. In contrast, the AA profile of AP could increase urea production to dispose AA in excess to the metabolic demand and/or decrease urea recycling when nitrogen salvage is useless (45).

Protein source effects on insulin sensitivity

In our study, the long-term intake of PP compared to that of AP resulted in lower fasting plasma glucose and insulin levels, as well as reduced HOMA-IR (the latter two in the HFS context only). In the rodent literature, PP blends have often been compared to AP in a NFS dietary context, which may explain the lack of effects, or only small, effects on glucose homeostasis (23–25). Similarly, in short-term clinical interventions, no specific effects of the protein source on glucose homeostasis were observed (46–48). These contrasted findings could be related to the duration of the dietary exposure, shorter in both animal studies and human clinical trials than in our experiment (4 months), when accounting for the differences in life expectancy. Indeed, when long-term exposures were considered in human observational studies, AP intake was rather positively associated with IR (12) and T2D (14,15), in line with the current study. In this sense, because our study consisted in a long-term dietary exposure with highly controlled protein sources in a HFS dietary context, our findings add an important line of evidence in comparison to noisier human interventions, by highlighting the beneficial effects of PP-feeding on insulin sensitivity.

Interestingly, we also reported lower plasma lactate in PP, compared to AP. To the best of our knowledge, this is the first time that this metabolite, whose elevated levels are often associated

with obesity and TD2 (49), has been related to protein sources. Therefore, the role of this particular pathway deserves further exploration in future studies.

Protein source effects on lipid metabolism

Compared to AP, long-term PP intake favored liver homeostasis with a lower lipid accumulation and DNL, whatever the dietary energy level. In particular, we found that the metabolic machinery of the liver better responded to HFS exposure when the protein source was PP, with coordinated changes in intermediary metabolism resulting in limiting DNL and lipid accumulation. These changes consisted in direct changes on DNL, like lower ACL activity, but also indirect adaptations like reduced CHO influx *via* glycolysis (lower GK activity) ultimately feeding DNL, or restricted pentose phosphate cycle limiting the availability of NADPH donors (lower G6PD activity) to DNL. These results are in line with other studies in rodents involving PP like soy (50–52) or beans (26,27), and are also supported by the positive association found in humans between AP intake and non-alcoholic fatty liver disease in humans (53).

In conjunction with lower hepatic lipid storage and DNL, we observed better insulin sensitivity following PP feeding than AP. There are two possible intertwined explanations for these concomitant findings. On the one hand, hepatic lipid accumulation is known to suppress signaling through the insulin pathway, thereby promoting IR. On the other hand, IR favors DNL and lipid storage, which in turn promotes further IR in a vicious cycle (54). In addition, the glycolytic potential was lower after PP than AP intake, while oxidation products of dietary CHO and lipids from both glycolysis and lipolysis were routed more to PS. These metabolic shifts could also contribute to a lower substrate availability for DNL and thus restrain lipid accumulation.

Causal role of distinct protein source AA profiles

The differences in AA composition between protein sources provide mechanistic hypotheses for their metabolic effects evidenced here, as AP are richer in indispensable (BCAA) and PP in dispensable AA (cysteine, arginine and glycine), which have been positively and negatively linked to cardiometabolic risk, respectively (55).

Regarding BCAA, dietary restriction in rodents was reported to improve glucose homeostasis (56–58), while BCAA or leucine supplementation in a high fat context either promoted IR (59) or preserved insulin sensitivity (60,61). These contrasted findings could be related to differences in background diets, as suggested by a recent meta-analysis of the rodent literature (28). In humans, BCAA intake has generally been associated with impaired glucose homeostasis (62,63). On the other hand, glycine, cysteine and arginine intake have been associated with improved glucose homeostasis (64–66). In rodents, supplementation studies with these AA have reported reduced oxidative stress, a potential explanatory mechanism for preserved insulin sensitivity (67–70). Beneficial effects of glycine and arginine supplementation on insulin sensitivity have been also reported in humans (71,72), their respective circulating concentrations being rather low in T2D (73,74), often in conjunction with elevated BCAA levels regarding glycine (75).

This study has several strengths. With the use of purified isoproteic and isocaloric diets, the observed metabolic effects could be directly attributed to the protein sources, and with the use of protein isolates, even more specifically to their AA profiles. The relatively long experimental duration in rats also represents long-term dietary exposure. With the combined use of cutting-edge isotopic methods and traditional approaches, we obtained an integrative view of the metabolic shifts between PP and AP at both whole-body and tissue levels. Our study has also

some limits, like the exclusive nature of the protein source in the diet (100% AP or 100% PP), favoring a straight-forward mechanistical interpretation of the effects, but rather difficult to translate into the classical range of AP:PP consumption ratio in human diets (about 70:30 in western countries (76)).

Conclusion

PP intake adequately supported tissue PS and body weight gain in growing rats while limiting whole body and liver metabolic dysregulations induced by HFS diets. These effects could be related to the fact that the AA profile of PP, which is less optimal for direct channelling to protein synthesis than that of AP, stimulated other metabolic pathways like AA transamination, glycolysis and lipolysis while down-regulating others, such as DNL. These combined effects involving highly intertwined metabolic pathways could explain, at least in part, the beneficial effect on cardiometabolic health of PP *per se*, which could therefore account for some of the beneficial effects of plant-based diets. These metabolic adaptations induced by specific protein sources deserve further exploration at the molecular level using omics approaches and within more realistic dietary settings in human dietary interventions.

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Tables

Table 1. Composition of experimental diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose

	NFS-AP	**HFS-AP**	**NFS-PP**	**HFS-PP**
*Ingredients (g/kg diet)**:***				
Total milk protein isolate (90% protein on DM[1])	174	209	0	0
Pea protein isolate (85% protein on DM[1])	0	0	95	106
Wheat protein isolate (89% protein on DM[1])	0	0	88	114
Corn starch	531	188	537	195
Sucrose	60	189	60	190
Lard	60	260	53	260
Soy oil	60	40	53	20
AIN 93G Mineral mix	40	40	40	40
AIN 93VX Vitamin mix	13	13	13	13
Alpha-cellulose	60	60	60	60
Choline (chloride)	1.4	1.4	1.4	1.4
*Energy and macronutrient**:***				
Energy (kcal/kg)	4044	4928	4068	4924
Protein (%kcal)	15%	15%	15%	15%
Carbohydrates (%kcal)	58%	31%	59%	31%
Lipid (%kcal)	27%	55%	27%	54%
Saturated fatty acids (%kcal)[2]	7%	20%	6%	19%

[1] DM: dry matter

[2]Saturated fatty acid content was estimated based on lard and soy oil USDA food database composition

Table 2: Fractional synthesis rates (FSR, %/d), absolute synthesis rates (ASR, mg N/d) and masses (mg N) of tissue proteins in intestine, liver and skeletal muscles at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose[1].

		NFS-AP	NFS-PP	HFS-AP	HFS-PP	Effects
Intestine	FSR (%/d)	59.4 ± 2.4	50.5 ± 3.4	52.7 ± 3.0	54.9 ± 9.9	
	ASR (mg N/d)	93.3 ± 6.9	84.6 ± 9.4	94.9 ± 9.0	94.8 ± 6.4	
	Mass (mg N)	157.0 ± 10.1	164.9 ± 9.2	179.5 ± 11.6	175.6 ± 9.9	
Liver	FSR (%/d)	36.9 ± 1.4	35.3 ± 1.3	35.3 ± 1.2	35.2 ± 1.4	
	ASR (mg N/d)	164.3 ± 8.7	145.2 ± 10.1	183.0 ± 13.0	177.7 ± 13.0	Energy *P*=0.03 (HFS>NFS)
	Mass (mg N)	446.3 ± 21.8	409.2 ±20.1	518.7 ± 34.9	500.6 ± 22.8	Energy *P*=0.003 (HFS>NFS)
Tibialis anterior muscle	FSR (%/d)	4.5 ± 0.3	4.6 ± 0.3	4.3 ± 0.2	4.3 ± 0.3	
	ASR (mg N/d)	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	
	Mass (mg N)	26.9 ± 0.9	27.0 ± 1.3	26.3 ± 0.8	26.4 ± 0.7	
Gastrocnemius muscle	FSR (%/d)	3.6 ± 0.2	3.9 ± 0.2	3.5 ± 0.2	3.1 ± 0.2	Energy *P*=0.019 (HFS<NFS) Interaction *P*=0.071
	ASR (mg N/d)	3.4 ± 0.2	3.7 ± 0.2	3.2 ± 0.2	2.8 ± 0.1	Energy *P*=0.018 (HFS<NFS)
	Mass (mg N)	94.0 ± 2.5	93.2 ± 3.9	91.8 ± 1.9	91.4 ± 2.5	

[1] Data are mean ± SEM values, n=44. * *P*<0.05; ** *P*<0.01: effects of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA. No significant difference was observed for all variables in preplanned comparisons (NFS-AP *vs* NFS-PP and HFS-AP *vs* HFS-PP).

Figures

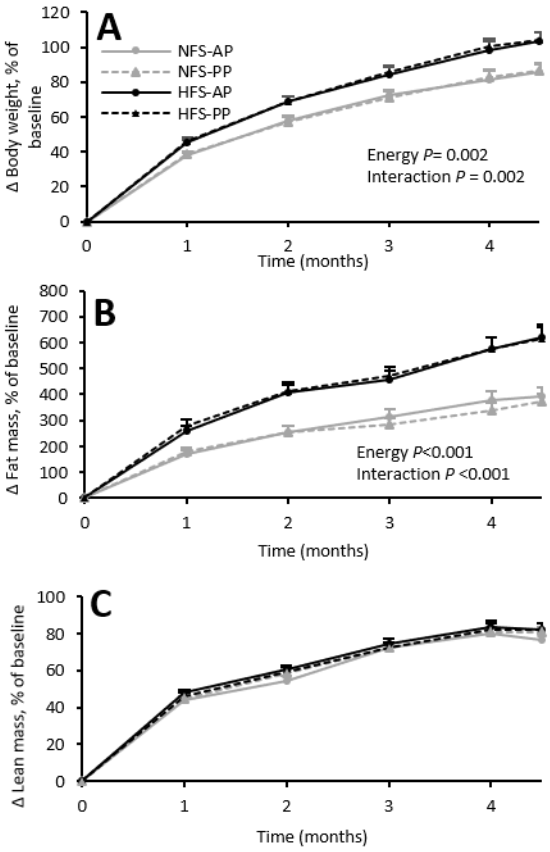


Figure 1: Changes in body weight (A), fat mass (B) and lean mass (C) in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM (n=44) expressed as increase in percentage of their baseline values. Effects at the final time of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with mixed models.

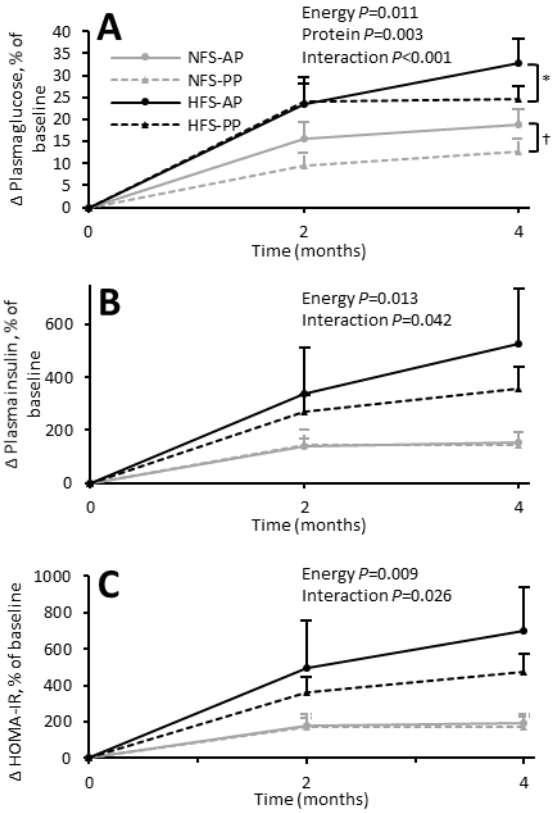


Figure 2: Changes in plasma glucose (A), plasma insulin (B) and HOMA-IR (C) in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values (n=44) expressed as increase in percentage of their baseline values. † 0.05 <*P*<0.1; * *P*<0.05. Effects at the final time of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA.

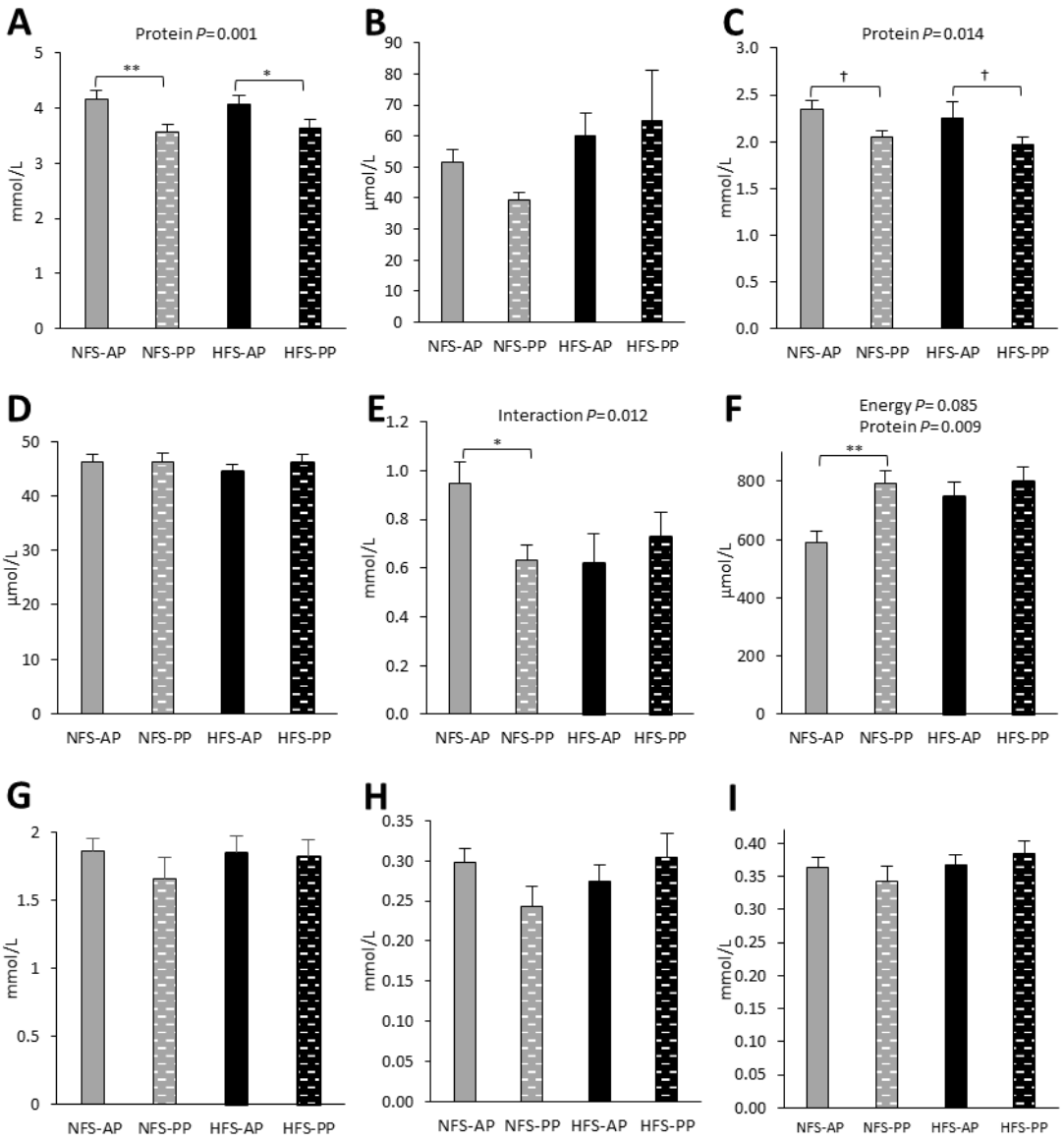


Figure 3: Plasma metabolite concentrations at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values for urea (A, n=43), uric acid (B, n=42), lactate (C, n=42), creatinine (D, n=44), triglycerides (E, n=43), non-esterified fatty acids (F, n=44), total cholesterol (G, n=43), LDL-cholesterol (H, n=43) and HDL-cholesterol (I, n=43) levels. † 0.05 <*P*<0.1; * *P*<0.05; ** *P*<0.01: effects of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA.

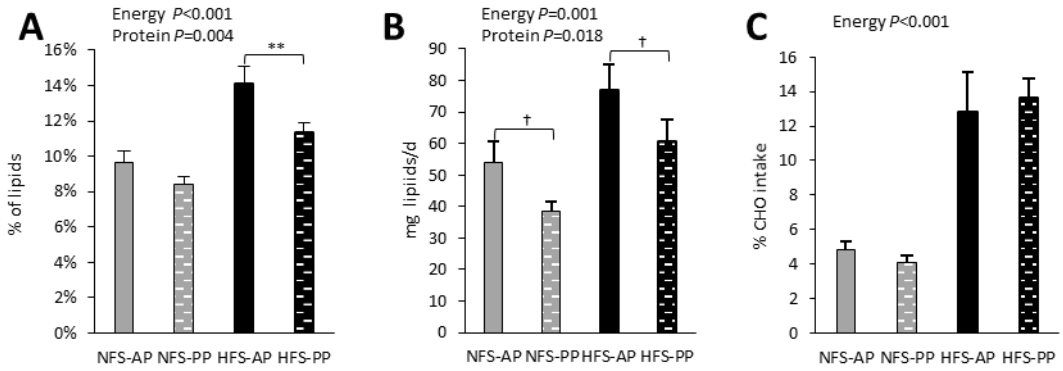


Figure 4: Liver lipid content (A), liver de novo lipogenesis (DNL) (B) and whole-body DNL (C) levels at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values (n=44). † 0.05 <*P*<0.1; ** *P*<0.01: effects of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA.

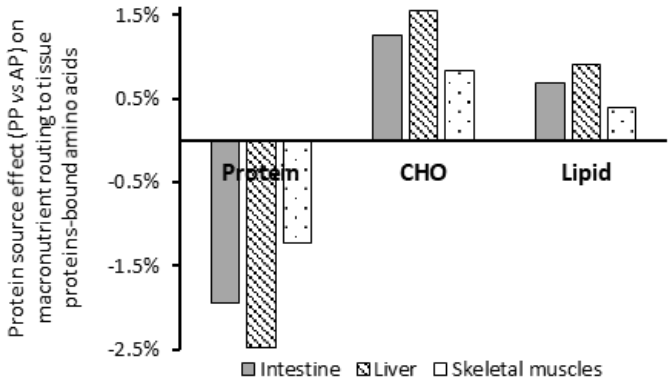


Figure 5: Dietary protein source effect on the relative contribution of macronutrients to tissue protein-bound amino acids. Values represent the absolute differences between plant (PP) and animal (AP) protein diets in their dietary protein, CHO and lipid routings to the proteins of intestine, liver and muscles (see Supplemental text 2 for explanations). Data from of *tibialis anterior* and *gastrocnemius* muscles were similar and thus merged. n=44

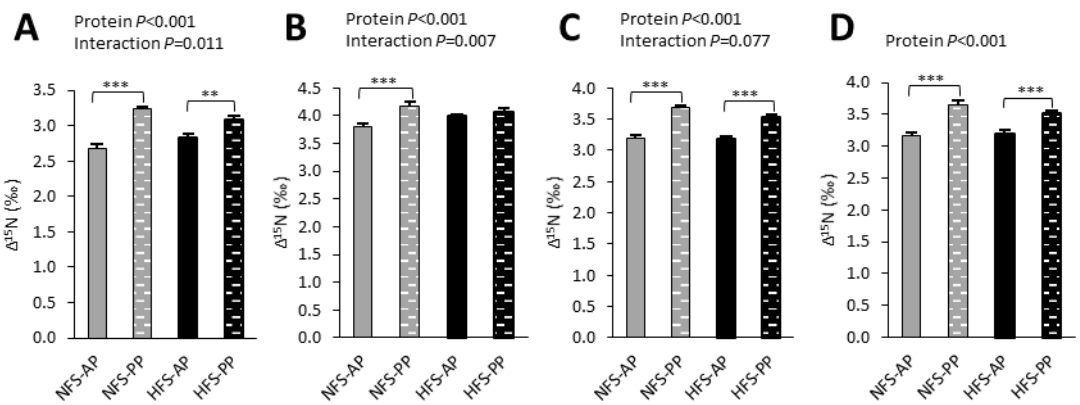


Figure 6: Natural nitrogen isotopic distances between tissue and dietary proteins ($\Delta^{15}N$) representing the extent of amino acid metabolic trafficking in tissues at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values for $\Delta^{15}N$ (‰) in intestine (A), liver (B), *tibialis anterior* muscle (C) and *gastrocnemius* muscle (D, n=44). * $P<0.05$; ** $P<0.01$; *** $P<0.001$: effects of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA.

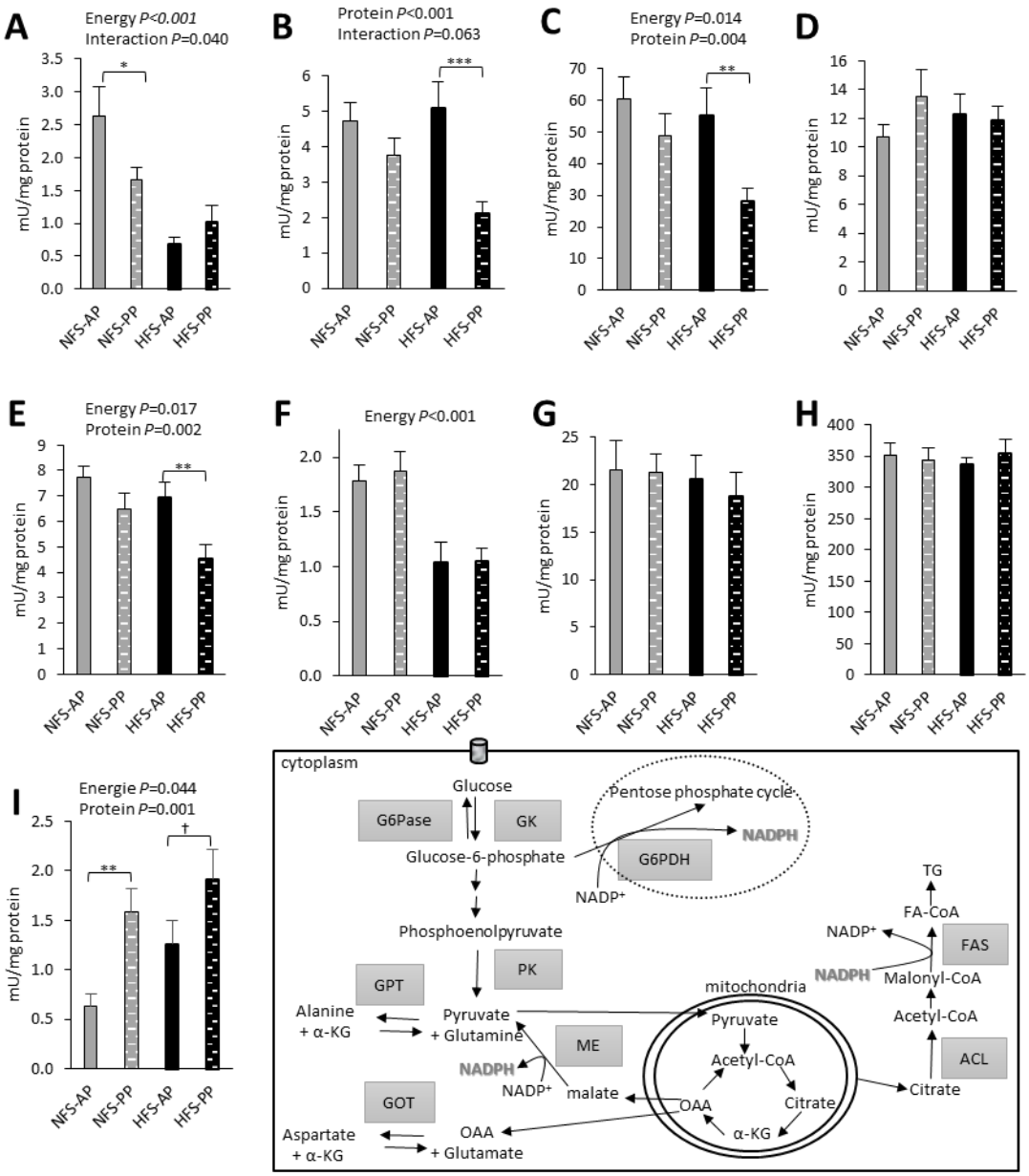
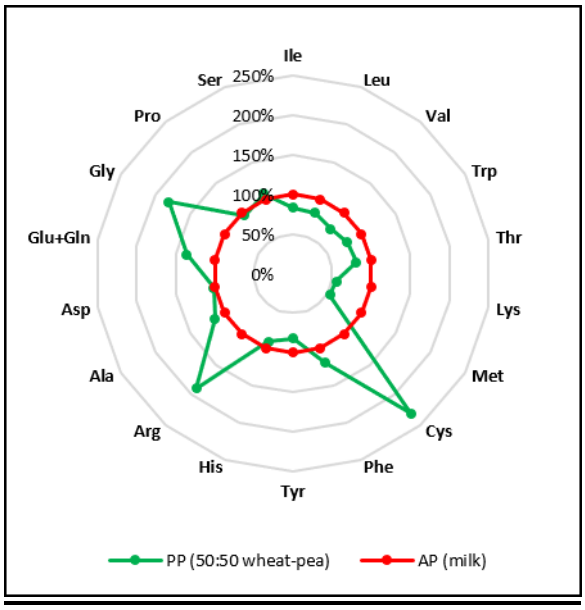


Figure 7: Hepatic enzyme activities at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values in mU/mg of protein of fatty acid synthase (FAS) (A, n=37), ATP-citrate lyase (ACL) (B, n=44), glucose-6-phosphate dehydrogenase (G6PDH) (C, n=44), malic enzyme (ME) (D, n=44), glucokinase (GK) (E, n=42), pyruvate kinase (PK) (F, n=44), glucose-6-phosphatase (G6pase) (G, n=44), glutamic oxaloacetic transaminase (GOT) (H, n=44) and glutamine-pyruvate transaminase (GPT) (I, n=44) enzymatic activities. ** $P<0.01$; * $P<0.05$: effects of the dietary energy level (Energy), protein source (Protein) and their interaction (Energy x Protein), as tested with ANOVA.

Supplementary data



<u>Supplemental Figure S1:</u> Relative amino acid composition of animal protein (milk) and plant protein (50% pea : 50% wheat) isolates, expressed as percentage of the animal protein isolate composition and calculated with information provided by protein isolate suppliers

<u>Supplemental Text S1</u>: Protein synthesis and de novo lipogenesis assessments by ^{2}H precursor-product labeling

In this work, we used the deuterated method to concomitantly estimate protein synthesis and *de novo* lipogenesis by ^{2}H labeling of their respective precursors and products (1–4). Whatever the precursor-product couple considered, the enrichment in the product is known to increase exponentially with time towards its final equilibrium value, i.e., the precursor's enrichment being stable over time, according to the following general equation (5):

$$E_{product}(t) = E_{precursor} + \left(E_{product_baseline} - E_{precursor}\right) * exp^{-FSR*t} \quad [1]$$

where t (day) is the labeling period (i.e., the period of ^{2}H exposure from ^2H_2O injection to tissue sampling), $E_{precursor}$ (%) is the ^{2}H enrichment in precursor being stable over the labeling period, $E_{product}$ and $E_{product_baseline}$ (%) are the ^{2}H enrichments in product at time t and before labeling (i.e., before ^2H_2O injection), respectively, and FSR (%/day) is the fractional rate of product synthesis from precursor. The fractional (FSR, %/day) and absolute (ASR, mg/day) rates of product synthesis from precursor can thus be calculated as:

$$FSR\ (\%/day) = \frac{100}{t\ (day)} \times ln\left[\frac{E_{precursor} - E_{product_baseline}}{E_{precursor} - E_{product}}\right] \quad [2]$$

$$ASR\ (mg/day) = \frac{FSR\ (\%/day)}{100} \times product\ pool\ size\ (mg) \quad [3]$$

In each sampled tissue, this product synthesis estimate corresponds to its mean value over both the postprandial and postabsorptive states over the labeling period, i.e., over the last ~2d of ^{2}H labeling before euthanasia (the labeling duration having been chosen to reach reliably detectable ^{2}H enrichment levels). As during this period, some newly synthesized product may have left the tissue by product degradation or export, this estimate also only represents the *net* flux of product that was synthesized and stored in the considered tissue over the period.

<u>Supplemental Text S2</u>: Assessment of tissue amino acid (AA) trafficking and macronutrients contribution to tissue AA synthesis by natural nitrogen and carbon isotopic distances between tissue proteins and nutrients ($\Delta^{15}N$ and $\Delta^{13}C$), respectively

1. Extent of tissue AA metabolic trafficking and catabolism relative to protein synthesis

Although dietary protein N is the sole source of body protein N, body proteins are naturally enriched over dietary protein in the heavier stable N isotope (^{15}N). This ^{15}N bioaccumulation ($\Delta^{15}N$) is globally due to discrimination against this heavy isotope during N waste formation by urea production, with the existence of several isotope effects associated with amino acid (AA) transaminations and deamination (6–8). Indeed, as variations in the relative orientation of tissue AA towards catabolic or anabolic processes can result in $\Delta^{15}N$ changes, $\Delta^{15}N$ values differ between tissues and subjects because of their particular AA metabolism (7,9). Through mechanistic modeling, we have shown that $\Delta^{15}N$ measurements in tissue proteins represent integrated biomarkers for tissue AA partitioning between oxidation and protein synthesis, and vary as a function of the extent of AA metabolic trafficking and catabolism (7). In the present study, during between-group comparisons, higher $\Delta^{15}N$ values were interpreted as indicative of increased AA metabolic trafficking and catabolism, in the light of these previous modelling outputs (7) and other consistent experimental results (8,10).

2. Assessment of macronutrients contribution to AA synthesis and routing to tissue proteins

By contrast, the heavier stable C isotope (^{13}C) bioaccumulates only modestly and its natural abundance in tissues mainly reflects that of the diet and provides information on the dietary origin of tissue AA (11–13). Even though dietary proteins are preferentially routed to tissue proteins as AA, dietary lipids and CHO are used for the biosynthesis of non-indispensable AA, via ß-oxidation and glycolysis that produce different AA precursors (11), and the extent of each nutrient routing to protein synthesis can vary with the dietary protein source and energy level (5,11,12,14). As the three macronutrients differed in terms of their $\delta^{13}C$ value, the $\delta^{13}C$ of tissue proteins represents the sum of macronutrient $\delta^{13}C$ values weighted by their respective contributions to C in tissue proteins (routing coefficients), plus the difference in $\delta^{13}C$ between diet and tissue proteins due to isotopic fractionation along metabolic pathways (the trophic step). As previously explained (5,14), we thus derived a system of equations explaining the $\delta^{13}C$ of tissue proteins by the $\delta^{13}C$ of macronutrients, where the unknowns were the routing coefficients (p, c and l, representing the proportions of protein, CHO and lipids routed to C in tissue proteins, respectively) and the trophic step (TS). This equation system has to be solved theoretically for each diet, as each parameter (p, c, l and TS) can depend on the dietary protein source and energy level. However for identifiability reasons and as in our previous study (14), p (but not c and l) was assumed to depend only of the dietary protein source (P = AP or PP) and not of the energy level (E = NFS or HFS), which allowed solving the system as:

$$p_P = (\delta^{13}C_{E_{NFS}} - \delta^{13}C_{E_{HFS}} + \delta^{13}C_{T_{HFS-P}} - \delta^{13}C_{T_{NFS-P}}) / (\delta^{13}C_{E_{NFS}} - \delta^{13}C_{E_{HFS}}) \quad [1]$$

$$TS_P = (\delta^{13}C_{T_{NFS-P}} - \delta^{13}C_{E_{NFS}}) - (\delta^{13}C_{E_{NFS}} - \delta^{13}C_{E_{HFS}} + \delta^{13}C_{T_{HFS-P}} - \delta^{13}C_{T_{NFS-P}})*(\delta^{13}C_{P_P} - \delta^{13}C_{E_{NFS}})/(\delta^{13}C_{E_{NFS}} - \delta^{13}C_{E_{HFS}}) \quad [2]$$

$$c_{E-P} = (\delta^{13}C_{T_{E-P}} - \delta^{13}C_{L_E} - TS_P + p_P*(\delta^{13}C_{L_E} - \delta^{13}C_{P_P})) / (\delta^{13}C_{C_E} - \delta^{13}C_{L_E}) \quad [3]$$

$$l_{E-P} = 1 - p_P - c_{E-P} \quad [4]$$

where $\delta^{13}C_{T_{E-P}}$, $\delta^{13}C_{P_P}$, $\delta^{13}C_{C_E}$, $\delta^{13}C_{L_E}$ and $\delta^{13}C_{E_E}$, are respectively the $\delta^{13}C$ values of proteins in a tissue, of dietary proteins, of dietary CHO, of dietary lipids and of dietary non-protein energy sources (both CHO and lipids) in the diet with the dietary protein source P (P = AP or PP) and the energy level E (E = NFS or HFS); p_P, c_{E-P} and l_{E-P} are the proportions of protein, CHO and lipids routed to C in tissue proteins (i.e., the routing coefficients) in the diet with the dietary protein source P and the energy level E, and TS_P the trophic step in the diet with the dietary protein source P.

Because of their different CHO and lipid contents, the NFS and HFS diets differed in terms of their $\delta^{13}C_E$ which was calculated as the average $\delta^{13}C$ of lipids and CHO weighted by their C content and dietary occurrence as follows (11,12):

$$\delta^{13}C_{E_E} = [(\%_{C_E}*0.444)/(\%_{L_E}*0.768 + \%_{C_E}*0.444)]*\delta^{13}C_{C_E} + [(\%_{L_E}*0.768)/(\%_{L_E}*0.768 + \%_{C_E}*0.444)]*\delta^{13}C_{L_E} \quad [5]$$

where $\%_{C_E}$ and $\%_{L_E}$ are dietary mass fractions of carbohydrates and lipids in the diet with the dietary energy level E and 0.444 and 0.768 are the reference C mass fractions of CHO and lipids, respectively.

Finally, using the mean isotopic data for each diet, we derived one estimate for each protein source (PP and AP) for p, and one estimate for each diet for c and l. In the results (**Fig. 5**), we presented the protein source effect (PP *vs* AP) on the nutrient routing coefficients p, c and l calculated as:

$$\Delta p_{PP-AP} = p_{PP} - p_{AP} \quad [6]$$

$$\Delta c_{PP-AP} = [(c_{NFS-PP} - c_{NFS-AP}) + (c_{HFS-PP} - c_{HFS-AP})] / 2 \quad [7]$$

$$\Delta l_{PP-AP} = [(l_{NFS-PP} - l_{NFS-AP}) + (l_{HFS-PP} - l_{HFS-AP})] / 2 \quad [8]$$

Of note, we here assumed that p depended only of the protein source and not of the energy level of the diet, this simplifying hypothesis being necessary to solve the equation system in our particular context. Nevertheless, in order to *a posteriori* test the validity of this simplifying hypothesis, we have verified that the individual estimates here obtained for each diet were consistent with those we previously obtained in another rat experiment where no simplifying hypothesis was required because of an isotopic experimental design fully adapted to the estimation of the nutrient routing coefficients (5). The individual estimates obtained here, ranging across diets from 76% to 79% for p values, from 8% to 18% for c values and from 5% to 14% for l values, were indeed fully in line with our previous estimates. Also in line with our hypothesis and results, other findings in the literature have shown that p varies only little under a broad range of dietary conditions, except for diets markedly deficient or excessive in protein (11).

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