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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

1 **Abstract (max 300 words):**

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

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

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

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

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

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

## 28 Introduction

29 Diets in high-income countries are high in animal products, refined carbohydrate (CHO) and  
30 saturated fats. A shift toward more plant-based diets is actively promoted to achieve more  
31 sustainable diets, better for both planetary and human health. The partial replacement of animal  
32 protein (AP) by plant protein (PP) sources is a key element of this transition, as the relative  
33 proportions of AP and PP produced and consumed both have considerable environmental  
34 impacts and broad public health implications (1,2). Regarding human health, although PP are  
35 considered to be less efficient than AP to sustain overall protein anabolism and muscle function  
36 (3,4), there is growing evidence that PP sources are better for cardiometabolic health (5–8).  
37 Indeed, substituting AP with PP, while accounting for dietary cofounders, was associated with  
38 lower cardiovascular and all-cause mortality (9,10). Conversely, high AP intake has been  
39 associated with metabolic syndrome features, such as dyslipidemia and insulin resistance (IR)  
40 (11,12), and with increased risk of cardiovascular diseases (13) and type 2 diabetes (T2D)  
41 (14,15). The differential effects of AP or PP intake on cardiometabolic features could be  
42 mediated by a better overall lifestyle, including diet which differs greatly depending on the  
43 predominant dietary protein sources (16–18), and/or by other nutrients and bioactive  
44 compounds present in the food matrix (7).

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

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

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

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

## 77 **Materials and methods**

### 78 *Animals, diets and experimental procedures*

79 Male Wistar rats (n=44) of 8 weeks of age and weighing 272.8g (SEM  $\pm$  1.5g) (Janvier), were  
80 kept on their breeding diet and adapted to the individual housing conditions (12-h light-dark  
81 cycle, 20°C) for 3 weeks. Then, the animals were randomly divided into 4 groups (n=11) and  
82 fed *ad libitum* for 4.5 months with 4 experimental diets (**Table 1**) differing in their energy level,  
83 either normal (NFS) or made high (HFS) by enrichment in dietary fat and sucrose content, and  
84 in their protein source, either AP or PP. AP consisted of total milk protein isolate (PRODIET  
85 90, Ingredia) and PP on a 50:50 blend of wheat: pea protein isolates (NUTRALYS® W and  
86 NUTRALYS® S85 PLUS for wheat and pea respectively, Roquette Frères). All diets contained  
87 the same protein proportion relative to dietary energy content (15%) and met the requirements  
88 for rodent growth regarding indispensable AA (29). The difference in AA composition between  
89 AP and PP sources was calculated based on protein isolate AA compositions, as provided by  
90 suppliers (Supplemental Figure S1). Mixing cereal and legume protein sources with a 50:50  
91 ratio resulted in a PP blend with a well-balanced AA composition, since wheat and pea protein  
92 compensated each other for low contents in lysine and sulfur AA, respectively, while presenting  
93 very good digestibility of individual AA (30). In comparison to AP, the AA content of the PP  
94 blend was lower in indispensable AA, such as BCAA (- 20%), and higher in dispensable AA  
95 like glycine (+81%), arginine (+89%) and cysteine (+131%). All the experiments were  
96 performed in accordance with the guidelines formulated by the European Community for the  
97 use of experimental animals (L358-86/609/EEC, Council Directive, 1986) and authorized by  
98 the Regional Ethics Committee (agreement no.12050-2017103015435153V4).

99 Food intake was recorded weekly and body composition was assessed by MRI (EchoMRITM-  
100 700) at 0, 1, 2, 3, 4 and 4.5 months. Blood was collected from the tail vein with heparinized  
101 syringes at 0, 2 and 4 months after an overnight fast. After centrifugation (3000  $\times$  g, 10 min,  
102 4°C), plasma was rapidly collected, frozen in liquid nitrogen and stored at -80°C until further  
103 analyses. Two days before euthanasia (at 4.5 months), rats received a bolus of <sup>2</sup>H<sub>2</sub>O saline  
104 solution (20 mL/kg of body weight at 99.9%, Sigma) intraperitoneally under light anesthesia in  
105 order to measure PS and DNL (31–34) concomitantly. The dose was set to result in ~3%  
106 enrichment in <sup>2</sup>H of body water, which was then maintained until euthanasia by providing  
107 access to 3% <sup>2</sup>H drinking water. Then, after an overnight fast, rats were euthanized under  
108 isoflurane anesthesia. Blood was withdrawn from the abdominal aorta and handled as above.  
109 The liver, small intestine, a sample of epididymal adipose tissue and skeletal *gastrocnemius*  
110 and *tibialis anterior* muscles were rapidly collected, weighed, freeze-clamped in liquid  
111 nitrogen, and stored at -80°C for further analyses.

### 112 *Analytical procedures*

#### 113 *Plasma metabolites and insulin levels*

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

120 resistance (HOMA-IR) was calculated as the product of insulin ( $\mu\text{U}/\text{ml}$ ) and glucose ( $\text{mg}/\text{dl}$ )  
 121 concentrations.

### 122 *Liver enzyme activities*

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

### 130 *Tissue protein masses and synthesis fluxes*

131 Protein masses and synthesis fluxes were measured in the liver, the intestine and muscles as  
 132 previously described (37). Briefly, for each tissue, the protein mass ( $\text{mg N}$ ) was determined  
 133 from the tissue weight, the protein content per  $\text{mg}$  fresh tissue, and the percentage nitrogen  
 134 content in protein measured using elemental analysis (Vario Micro Cube; Elementar). PS was  
 135 measured using the precursor-product  $^2\text{H}$  labeling method, with  $^2\text{H}$  enrichment measured in the  
 136 free and protein-bound alanine of liver, intestine and muscles by GC-MS after  $\sim 2$  days of  $^2\text{H}_2\text{O}$   
 137 exposure before euthanasia. Enrichments were calculated on the basis of a standard curve of  
 138 known enrichments corrected for variations in abundance, as previously described (37). As  
 139 explained in Supplemental Text S1, in each sampled tissue (intestine, liver and muscles), the  
 140 net fluxes of protein synthesized and stored *in situ* during the last  $\sim 2\text{d}$  before euthanasia were  
 141 calculated as:

$$142 \text{ Tissue protein FSR } (\%/d) = \frac{100}{t(d)} \times \ln \left[ \frac{E_{AA} - E_{P0}}{E_{AA} - E_P} \right] \quad [1]$$

$$143 \text{ Tissue protein ASR } (\text{mg N}/d) = \frac{\text{FSR } (\%/d)}{100} \times \text{tissue proteins } (\text{mg N}) \quad [2]$$

144 where  $t$  (d) is the period of  $^2\text{H}$  labeling from  $^2\text{H}_2\text{O}$  injection to tissue sampling,  $E_{AA}$  (%) is the  
 145  $^2\text{H}$  enrichment of tissue free alanine,  $E_P$  and  $E_{P0}$  (%) are the  $^2\text{H}$  enrichments of protein-bound  
 146 alanine at the end of the labeling period and before  $^2\text{H}_2\text{O}$  administration, respectively (with  $E_{P0}$   
 147 measured in 4 rats from the same batch that were euthanized at baseline). FSR ( $\%/d$ ) and ASR  
 148 ( $\text{mg}/d$ ) are the fractional and absolute synthesis rates, respectively.

### 149 *Tissue lipid content and de novo lipogenesis (DNL) fluxes*

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

$$159 \text{ Tissue DNL } (\%/d) = \frac{100}{t(d)} \times \ln \left[ \frac{0.48 * E_{BW} - E_{Lip0}}{0.48 * E_{BW} - E_{Lip}} \right] \quad [3]$$

$$160 \text{ Tissue DNL } (\text{mg lipids}/d) = \frac{\text{FSR } (\%/d)}{100} \times \text{tissue lipids } (\text{mg}) \quad [4]$$

161 where t (days) is the period of  $^2\text{H}$  labeling from  $^2\text{H}_2\text{O}$  injection to tissue sampling,  $E_{\text{BW}}$  (%) is  
 162 the  $^2\text{H}$  enrichment of body water (with  $0.48 \cdot E_{\text{BW}}$  taken as a proxy of the DNL precursor  
 163 enrichment (34)),  $E_{\text{Lip}}$  and  $E_{\text{Lip0}}$  (%) are the  $^2\text{H}$  enrichments of the tissue neutral lipids at the  
 164 end of the labeling period and before  $^2\text{H}_2\text{O}$  administration, respectively (with  $E_{\text{Lip0}}$  measured in  
 165 4 rats from the same batch that were euthanized at baseline), and tissue lipids are the lipid  
 166 masses in the liver or adipose tissues.

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

$$170 \text{ Whole-body DNL (\% CHO intake)} = \frac{100 \times 2.8 \times [\text{Liver} + \text{adipose tissues DNL (mg lipids/day)}]}{\text{CHO intake (mg/day)}} \quad [5]$$

### 171 *Natural abundances of stable N and C isotopes in tissue proteins*

172 Natural abundances of N and C stable isotopes were measured in the proteins of the different  
 173 tissues sampled and in dietary proteins, CHO and lipids, and expressed relative to standards  
 174 (atmospheric  $\text{N}_2$  for  $^{15}\text{N}/^{14}\text{N}$  and Vienna Pee Dee Belemnite for  $^{13}\text{C}/^{12}\text{C}$ , respectively) using the  
 175 delta notation:

$$176 \delta (\text{‰}) = (RR_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \quad [6]$$

177 where  $\delta$  is the parts-per-thousand difference in the ratio of heavy to light isotopes ( $^{15}\text{N}/^{14}\text{N}$  and  
 178  $^{13}\text{C}/^{12}\text{C}$ ) in the samples ( $R_{\text{sample}}$ ) and standards ( $R_{\text{standard}}$ , 0.0112372 for C and 0.0036765 for N).  
 179 Isotopic measurements were performed with an isotope-ratio mass spectrometer (Isoprime; VG  
 180 Instruments) coupled to an elemental analyzer (EA Vario Micro Cube; Elementar). Tyrosine  
 181 was used for calibration and drift correction.  $\delta^{15}\text{N}$  values for tissue proteins were expressed  
 182 relative to the  $\delta$ -value of dietary proteins using the capital delta notation ( $\Delta_{\text{tissue proteins}} = \delta_{\text{tissue}}$   
 183  $\text{proteins} - \delta_{\text{dietary proteins}}$ ).

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

### 196 *Statistical analyses*

197 Results are expressed as means and SEM.  $P$ -values  $< 0.05$  were considered to be significant.  
 198  $P$ -values close to the significance threshold ( $0.05 \leq P < 0.1$ ) were also reported. Analyses were  
 199 performed using SAS version 9.4 (SAS Institute, Cary, NC). For data obtained at a single time  
 200 point at the end of the experiment (natural  $\delta^{15}\text{N}$  abundances, PS and DNL measured by  $^2\text{H}$   
 201 labelling, hepatic lipid content, enzyme activities and some plasma biochemical values), the  
 202 effects of the protein source, the energy level and their interaction were assessed using ANOVA  
 203 (PROC GLM). No statistical analysis could be conducted on the nutrient routing results because  
 204 the equations used to estimate the routing coefficients from the  $\delta^{13}\text{C}$  data did not provide a



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

## 213 Results

### 214 Macronutrient intake, body weight and composition

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

### 221 Evolution of glucose homeostasis and insulin sensitivity

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

### 229 End point metabolite levels

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

### 238 Liver lipid content and liver and whole-body DNL fluxes

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

## 246 Protein synthesis and macronutrient contribution to AA synthesis and routing to 247 tissue proteins

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

## 260 Extent of AA metabolic trafficking in tissues

261 The tissue protein  $\Delta^{15}\text{N}$  values (i.e., the natural nitrogen isotopic distance between tissue  
262 proteins and dietary proteins, or trophic step) reflect the intensity of AA trafficking by  
263 transamination and deamination relative to their direct use for protein anabolism and turn-over,  
264 as these metabolic pathways are fractionating processes that amplify the  $\Delta^{15}\text{N}$  trophic step  
265 (37,39–42).  $\Delta^{15}\text{N}$  values measured in the protein of the intestine, liver and muscles are presented  
266 in **Figure 6**. Compared to AP, the PP groups had higher  $\Delta^{15}\text{N}$  values and thus higher rates of  
267 AA metabolic trafficking in all tissues. In addition, in all tissues except *gastrocnemius* muscle,  
268 there was a significant interaction (a trend in *tibialis anterior* muscle) between protein source  
269 and energy level, showing smaller differences between protein sources in the HFS context.

## 270 Hepatic enzyme activities

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

## 282 Discussion

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

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

#### 294 Protein source effects on protein and AA metabolism in tissues

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

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

#### 321 Protein source effects on insulin sensitivity

322 In our study, the long-term intake of PP compared to that of AP resulted in lower fasting plasma  
323 glucose and insulin levels, as well as reduced HOMA-IR (the latter two in the HFS context  
324 only). In the rodent literature, PP blends have often been compared to AP in a NFS dietary  
325 context, which may explain the lack of effects, or only small, effects on glucose homeostasis  
326 (23–25). Similarly, in short-term clinical interventions, no specific effects of the protein source  
327 on glucose homeostasis were observed (46–48). These contrasted findings could be related to  
328 the duration of the dietary exposure, shorter in both animal studies and human clinical trials  
329 than in our experiment (4 months), when accounting for the differences in life expectancy.  
330 Indeed, when long-term exposures were considered in human observational studies, AP intake  
331 was rather positively associated with IR (12) and T2D (14,15), in line with the current study.  
332 In this sense, because our study consisted in a long-term dietary exposure with highly controlled  
333 protein sources in a HFS dietary context, our findings add an important line of evidence in  
334 comparison to noisier human interventions, by highlighting the beneficial effects of PP-feeding  
335 on insulin sensitivity.

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

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

### 340 Protein source effects on lipid metabolism

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

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

### 361 Causal role of distinct protein source AA profiles

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

366 Regarding BCAA, dietary restriction in rodents was reported to improve glucose homeostasis  
367 (56–58), while BCAA or leucine supplementation in a high fat context either promoted IR (59)  
368 or preserved insulin sensitivity (60,61). These contrasted findings could be related to  
369 differences in background diets, as suggested by a recent meta-analysis of the rodent literature  
370 (28). In humans, BCAA intake has generally been associated with impaired glucose  
371 homeostasis (62,63). On the other hand, glycine, cysteine and arginine intake have been  
372 associated with improved glucose homeostasis (64–66). In rodents, supplementation studies  
373 with these AA have reported reduced oxidative stress, a potential explanatory mechanism for  
374 preserved insulin sensitivity (67–70). Beneficial effects of glycine and arginine  
375 supplementation on insulin sensitivity have been also reported in humans (71,72), their  
376 respective circulating concentrations being rather low in T2D (73,74), often in conjunction with  
377 elevated BCAA levels regarding glycine (75).

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

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

## 388 Conclusion

389 PP intake adequately supported tissue PS and body weight gain in growing rats while limiting  
390 whole body and liver metabolic dysregulations induced by HFS diets. These effects could be  
391 related to the fact that the AA profile of PP, which is less optimal for direct channelling to  
392 protein synthesis than that of AP, stimulated other metabolic pathways like AA transamination,  
393 glycolysis and lipolysis while down-regulating others, such as DNL. These combined effects  
394 involving highly intertwined metabolic pathways could explain, at least in part, the beneficial  
395 effect on cardiometabolic health of PP *per se*, which could therefore account for some of the  
396 beneficial effects of plant-based diets. These metabolic adaptations induced by specific protein  
397 sources deserve further exploration at the molecular level using omics approaches and within  
398 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
<i>Ingredients (g/kg diet):</i>				
Total milk protein isolate (90% protein on DM <sup>1</sup> )	174	209	0	0
Pea protein isolate (85% protein on DM <sup>1</sup> )	0	0	95	106
Wheat protein isolate (89% protein on DM <sup>1</sup> )	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
<i>Energy and macronutrient:</i>				
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) <sup>2</sup>	7%	20%	6%	19%

<sup>1</sup> DM: dry matter

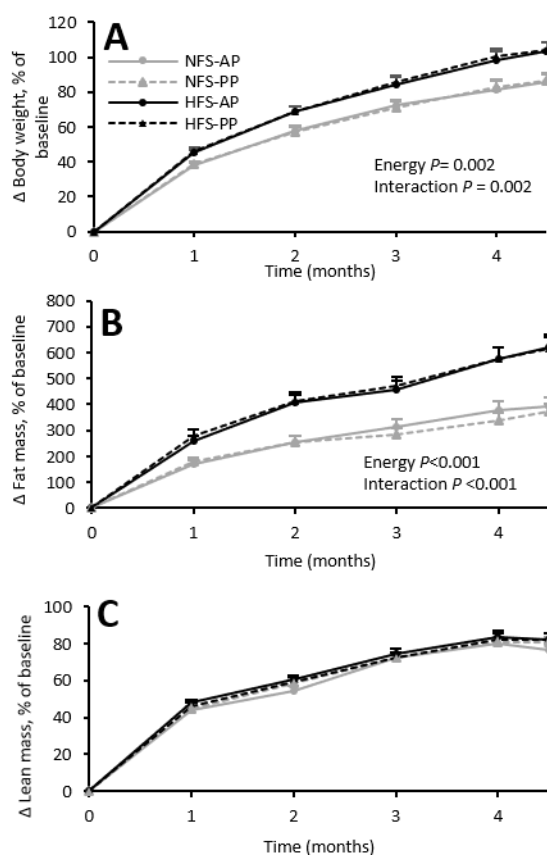
<sup>2</sup>Saturated fatty acid content was estimated based on lard and soy oil USDA food database composition

**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<sup>1</sup>.

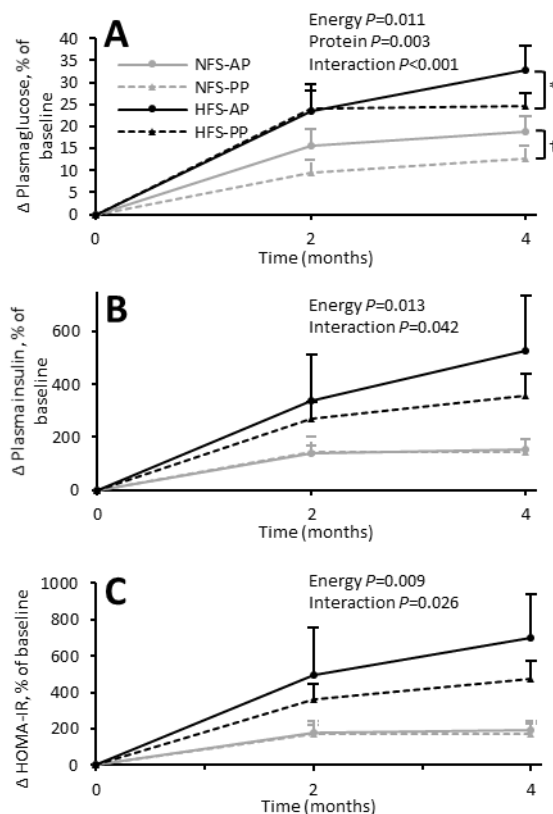
		NFS-AP	NFS-PP	HFS-AP	HFS-PP	Effects
Intestine	FSR (%/d)	59.4 ± 2.4	50.5 ± 3.4	52.7 ± 3.0	54.9 ± 9.9	
	ASR (mg N/d)	93.3 ± 6.9	84.6 ± 9.4	94.9 ± 9.0	94.8 ± 6.4	
	Mass (mg N)	157.0 ± 10.1	164.9 ± 9.2	179.5 ± 11.6	175.6 ± 9.9	
Liver	FSR (%/d)	36.9 ± 1.4	35.3 ± 1.3	35.3 ± 1.2	35.2 ± 1.4	
	ASR (mg N/d)	164.3 ± 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)
<i>Tibialis anterior</i> 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	
<i>Gastrocnemius</i> 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)
	ASR (mg N/d)	3.4 ± 0.2	3.7 ± 0.2	3.2 ± 0.2	2.8 ± 0.1	Interaction $P=0.071$ 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	

<sup>1</sup> 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).

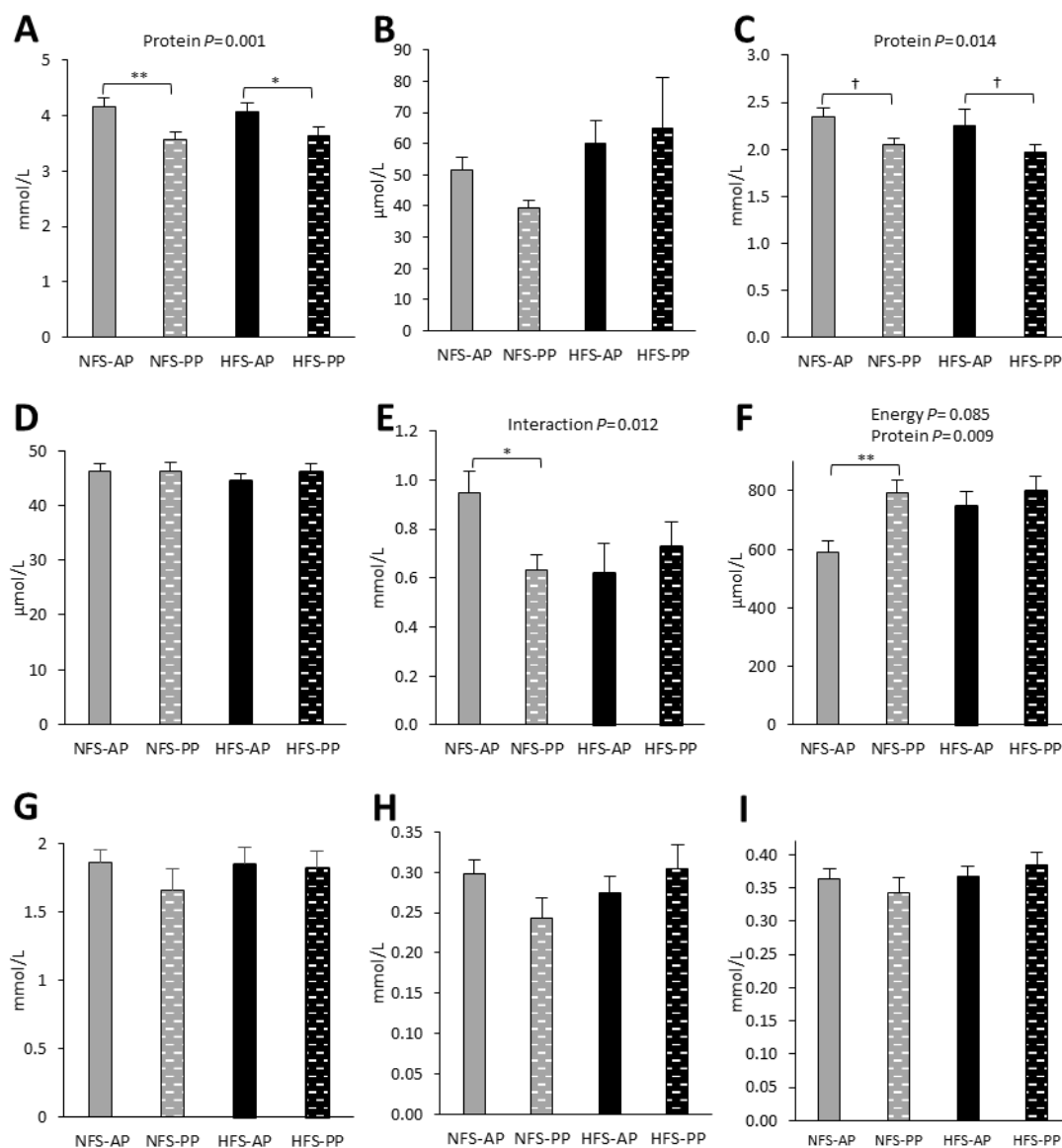
## Figures



**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.

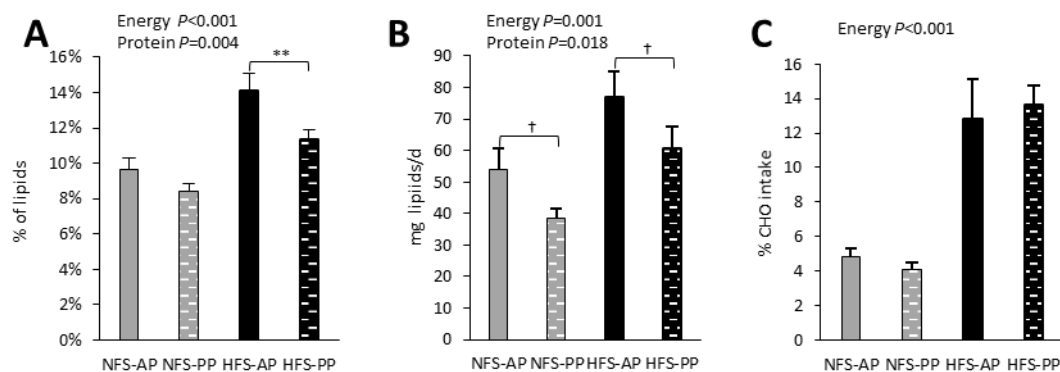


**Figure 2:** Changes in plasma glucose (A), plasma insulin (B) and HOMA-IR (C) in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values ( $n=44$ ) expressed as increase in percentage of their 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.

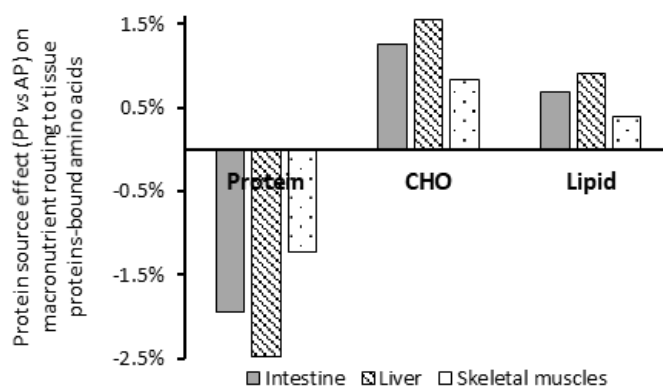


**Figure 3:** Plasma metabolite concentrations at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values for urea (A,  $n=43$ ), uric acid (B,  $n=42$ ), lactate (C,  $n=42$ ), creatinine (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.

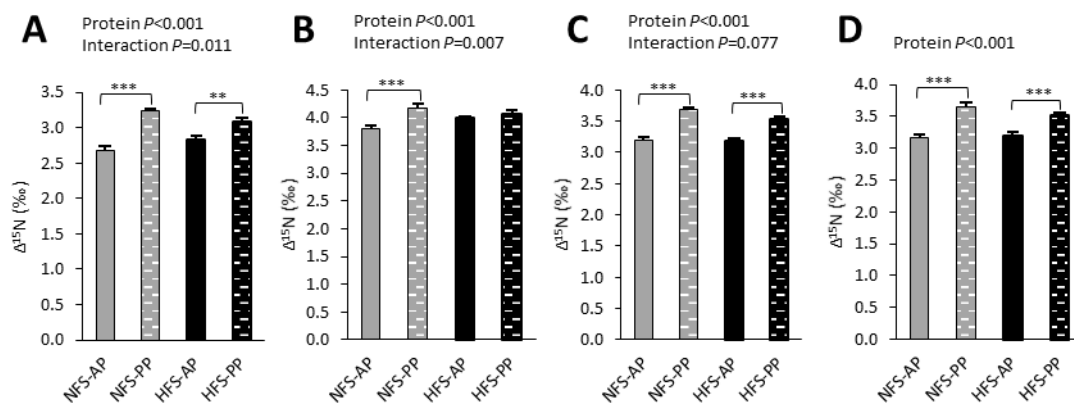




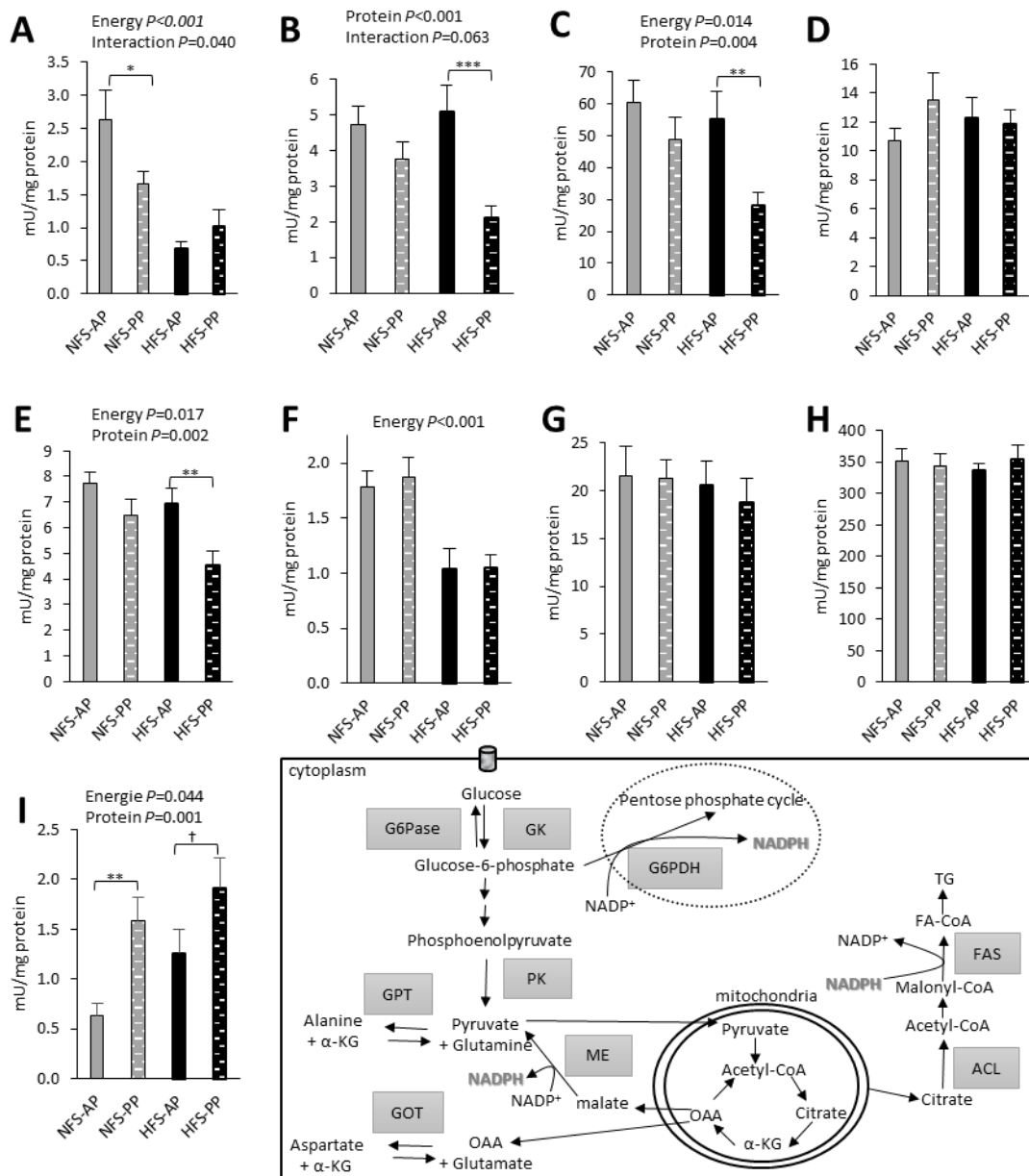
**Figure 4:** Liver lipid content (A), liver de novo lipogenesis (DNL) (B) and whole-body DNL (C) levels at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values ( $n=44$ ). †  $0.05 < P < 0.1$ ; \*\*  $P < 0.01$ : 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.  $n=44$

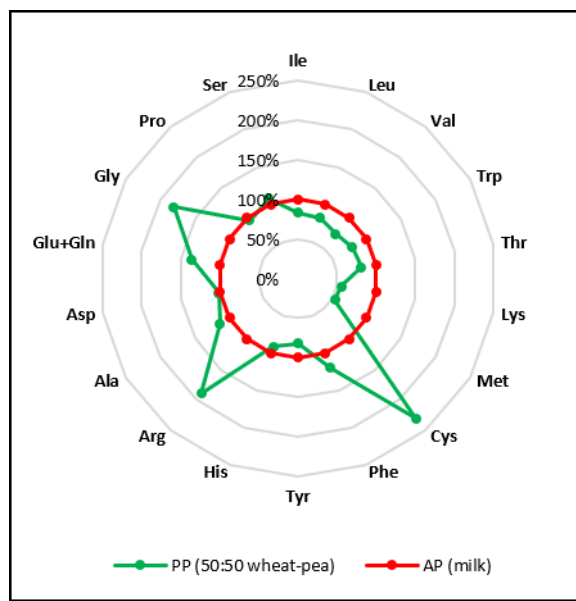


**Figure 6:** Natural nitrogen isotopic distances between tissue and dietary proteins ( $\Delta^{15}\text{N}$ ) representing the extent of amino acid metabolic trafficking in tissues at 4.5 months in rats fed with diets containing either animal (AP) or plant (PP) proteins and being either normal (NFS) or high (HFS) in fat and sucrose. Data are mean + SEM values for  $\Delta^{15}\text{N}$  (‰) 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



**Supplemental Figure S1: 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**

**Supplemental Text S1: Protein synthesis and de novo lipogenesis assessments by  $^2\text{H}$  precursor-product labeling**

In this work, we used the deuterated method to concomitantly estimate protein synthesis and *de novo* lipogenesis by  $^2\text{H}$  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):

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

where  $t$  (day) is the labeling period (i.e., the period of  $^2\text{H}$  exposure from  $^2\text{H}_2\text{O}$  injection to tissue sampling),  $E_{precursor}$  (%) is the  $^2\text{H}$  enrichment in precursor being stable over the labeling period,  $E_{product}$  and  $E_{product\_baseline}$  (%) are the  $^2\text{H}$  enrichments in product at time  $t$  and before labeling (i.e., before  $^2\text{H}_2\text{O}$  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  $^2\text{H}$  labeling before euthanasia (the labeling duration having been chosen to reach reliably detectable  $^2\text{H}$  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.

**Supplemental Text S2: 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 ( $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$ ), 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 ( $^{15}\text{N}$ ). This  $^{15}\text{N}$  bioaccumulation ( $\Delta^{15}\text{N}$ ) 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  $\Delta^{15}\text{N}$  changes,  $\Delta^{15}\text{N}$  values differ between tissues and subjects because of their particular AA metabolism (7,9). Through mechanistic modeling, we have shown that  $\Delta^{15}\text{N}$  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  $\Delta^{15}\text{N}$  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 ( $^{13}\text{C}$ ) 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  $\beta$ -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  $\delta^{13}\text{C}$  value, the  $\delta^{13}\text{C}$  of tissue proteins represents the sum of macronutrient  $\delta^{13}\text{C}$  values weighted by their respective contributions to C in tissue proteins (routing coefficients), plus the difference in  $\delta^{13}\text{C}$  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  $\delta^{13}\text{C}$  of tissue proteins by the  $\delta^{13}\text{C}$  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 ( $P = \text{AP}$  or  $\text{PP}$ ) and not of the energy level ( $E = \text{NFS}$  or  $\text{HFS}$ ), 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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