Plant Protein Can Be as Efficient as Milk Protein to Maintain Fat Free Mass in Old Rats, Even When Fat and Sugar Intakes Are High

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Laurianne Dimina[1,2], Justine Landais[2], Véronique Mathe[1], Marianne Jarzaguet[2], Cindy Le Bourgot[3], Dominique Hermier[1], François Mariotti[1], Didier Rémond[2], Laurent Mosoni[2]

1 Université Paris-Saclay, AgroParisTech, INRAE, UMR PNCA, 75005, Paris, France

2 Université Clermont Auvergne, INRAE, UMR1019, Unité de Nutrition Humaine, 63122, Saint-Genès-Champanelle, France

3-Tereos, R§D department, 77230, Moussy-le-Vieux, France

Corresponding author: Laurent Mosoni; Tel. +33 4 73 62 48 27; email: laurent.mosoni@clermont.inrae.fr

Running title: Blended plant protein adequately replaces milk proteins

Abbreviations: AA: amino acids; AIN: American Institute of Nutrition; ANOVA; analysis of variance; ASR: absolute synthesis rates; FSR: fractional synthesis rates; IAA: indispensable amino acids; MHE: milk proteins, high energy; MSE: milk proteins, standard energy; PHE: plant proteins, high energy; PSE: plant proteins, standard energy; SE: standard error.

1 *Abstract*

2 Background: Alternative, sustainable and adequate sources of protein must be found

3 to meet global demand. Objective: The aim of this study was to assess the effect of a

4 plant-protein blend with a good balance of indispensable amino acids and high

5 contents of leucine, arginine and cysteine on the maintenance of muscle protein

6 mass and function during aging in comparison to milk proteins, and to determine if

7 this effect varied according to the quality of the background diet. Methods: Old male

8 Wistar rats (n = 96, 18 months old) were randomly allocated to four diet groups,

9 differing according to protein source (milk or plant blend) and energy content

10 (standard, with starch, or high, with saturated fat and sucrose), for 4 months. Plasma

11 biochemistry and body composition (echo MRI) were measured every two months.

12 Muscle functionality (Catwalk XT system) was assessed before and after the

13 experimental diet. After 4 months of diet, animals were injected with a flooding dose

14 of L-[1-13C] valine, and hind limb muscles, liver and heart were sampled and

15 weighed. Results: We found that the plant protein blend stimulated muscle protein

16 synthesis, maintained lean body mass, muscle mass, and muscle functionality during

17 aging as well as milk protein. Liver and heart weights were unchanged. The high fat

18 high sucrose diet increased body fat but, assessed by fasting plasma glucose and

19 insulin, had little impact on insulin sensitivity and related metabolisms. Therefore, we

20 could not test the hypothesis that in situations of higher insulin resistance, our plant

21 protein blend rich in leucine, arginine and cysteine may be better than milk protein.

22 Conclusion: Finally, the present findings offer significant proof of concept from the

23 nutritional standpoint that appropriately blended plant proteins can have high

24 nutritional value even in demanding situations such as ageing protein metabolism.

25

28

29   INTRODUCTION

30   By the year 2050, the combined increase in the world's population and living

31   standards in developing countries will lead to a very considerable rise in the demand

32   for dietary protein, especially from animal sources (1). This demand cannot be met

33   sustainably by increasing animal production, meaning that alternative sources of

34   protein must be developed. The simplest feasible option in the short term is to take

35   better advantage of the large amount and diversity of plant proteins already available.

36   The co-products of the oilseed and starch industry contain significant amounts of

37   proteins (15-50%). Their use in human nutrition is at present negligible and should be

38   developed (2).

39   Our objective falls in this context of diversifying protein resources for humans, with

40   the aim of tackling food insecurity while taking into account the sustainability of food

41   systems (exploitation of co-products). However, a key issue will be to use plant

42   proteins to develop protein-rich foods able to compete with animal products (meat,

43   eggs, milk), which are considered as providers of reference proteins in human

44   nutrition. Indeed, besides technical and organoleptic issues, one of the main

45   obstacles of using plant proteins in human nutrition is generally the low nutritive value

46   classically attributed to them, because of their unbalanced composition in

47   indispensable amino acids (IAA), and concerns regarding digestibility (3). Indeed,

48   amino acid (AA) bioavailability may be an issue for plant proteins, but when proteins

49   are extracted from their natural matrix and properly processed, their true ileal

50   digestibility is generally high (84-94%), and only slightly lower than that of animal

51   proteins (90-95%) (4). The unbalanced composition of individual plant proteins could

52   be readily solved by combining them. Finally, by taking advantage of a wider range of

53   available plant proteins and combining them, it should be possible to identify and

54   produce protein blends with tailored AA composition and high digestibility (5, 6).

55   This optimized AA composition can even be targeted for a specific population.

56   Undoubtedly, the most important problem to solve in the coming years is feeding the

57   elderly. Demographic trends in Western countries are characterized by the gradual

58   aging of the population and increased longevity (1/4 of the population in Europe and

59   North America will be over 65 in 2050 -

60   https://population.un.org/wpp/publications/files/wpp2019_highlights.pdf). Sarcopenia,

61   defined as the gradual decline in muscle mass and function, is a major feature of

62   aging. It is an inevitable physiological process, occurring even in healthy elderly

63   subjects, which increases the risk of loss of independence, falls and fractures, and

64   decreases resistance to nutritional, infectious or traumatic stresses (7). Muscle mass

65   is controlled by the balance between protein synthesis and degradation. It was

66   shown that the post-prandial stimulation of muscle protein synthesis and inhibition of

67   muscle protein degradation decreases during aging, with the development of

68   anabolic resistance (8). Thus, besides inactivity and protein undernutrition, other

69   factors like oxidative stress, inflammation (9), or insulin resistance (10) can

70   accelerate sarcopenia by worsening muscle anabolic resistance. Given these

71   phenomena, how can we optimize the AA composition of plant protein blends for

72   elderly subjects?

73   First, there is a risk of mismatch between requirements and intakes in the elderly,

74   because of an increased requirement and a decreased appetite (11). A significant

75   proportion of the elderly population may have marginal intake with regards to protein

76   requirements (12). In this specific context, the quality of protein intake in terms of IAA

77   supply is considered to be a key factor for optimal protein metabolism. In addition,

78   leucine is a potent stimulator of protein synthesis, and supplementation with this AA

79   was shown to restore muscle postprandial anabolism during aging (13, 14). Arginine,

80   as a precursor of NO, could play a major role in the provision of AA for muscles.

81   Indeed, the role of NO in the peripheral extraction of nutrients in response to insulin

82   has been established (15, 16). Supplementation with arginine was shown to increase

83   muscle perfusion in the elderly (17), although the effect on anabolism depends on the

84   level of supply of the other AAs. Cysteine is involved in the determination of the redox

85   status, mainly because of its presence in glutathione, the main molecular antioxidant

86   of the body. This redox status is altered with aging (18). The requirement for cysteine

87   seems to increase with aging because of changes in metabolic demand in relation

88   with oxidative stress, low grade inflammation, and increased medication use (19). In

89   line with this, long-term supplementation with cysteine was shown to delay loss of

90   muscle mass in aging (20, 21). Thus, we reasoned that a blend of plant protein with

91   an excellent IAA balance and particularly rich in leucine, arginine, and cysteine may

92   be an adequate diet for maintaining muscle mass and function during aging.

93   Using linear programming (5), we designed a plant protein blend with an excellent

94   IAA balance and high levels of leucine, arginine and cysteine, and used it as the

95   unique protein source in the diet of aging rats (final age 22 months) in a 4-month

96   study. The objective was to test if this blend could maintain lean body mass, muscle

97   mass and function as well as muscle protein synthesis rates, in comparison to protein

98   obtained from milk, recognized as an excellent protein source. This experiment was

99   performed in the context of a standard diet (22), and a high fat high sucrose diet

100   which better reflects western diets, with the expectation of increased insulin

101   resistance and related metabolic alterations. We showed that this blend of plant

102   proteins was as efficient as milk proteins to prevent sarcopenia during aging.

103

METHODS

Animals and diet

This experiment was conducted in accordance with institutional guidelines on animal

experimentation in France and was approved by the Ethics Committee in the Matter

of Animal Experiments of Auvergne (registration number: CE 56-12). Male 15-month-

old Wistar rats (n=96) (Janvier, France) were housed under controlled environmental

conditions (21°C, hygrometry 55%, 12-h dark period starting at 07:00) and were

allowed free access to water and standard pellets (UAR 04, UAR, France). After

three months, animals were randomly divided into four groups (n=24 per group) fed

four different diets: 1) MSE (milk protein, standard energy) rats were fed the AIN 93

diet (22) with milk proteins replacing casein; 2) PSE (plant proteins, standard energy)

rats were fed the AIN 93 diet with a mix of plant proteins replacing casein; 3) MHE

(milk proteins, high energy) rats were fed a diet with milk proteins and high fat and

sucrose content; 4) PHE (plant proteins, high energy) rats were fed a diet with the

same mix of plant protein as PSE rats and a high fat and sucrose content (see

detailed diet composition in Table 1). The plant protein mix contained 22.1% lupine

protein isolate (10600 Prolupin GmbH, Grimmen, Germany), 24.1% potato protein

(Protimex®, Tereos, France), 9.5% zein (f4400c-phg flozein®, Flo Chemical

Corporation, MA, USA), 37% rice protein hydrolysate (Meripro® Rice H, Tereos,

France), 7.3% rapeseed albumin (Napine, Supertein®, Burcon, Canada). These diets

were given *ad libitum* for 4 months, between the ages of 18 and 22 months, either as

pellets (MSE, PSE diets) or as a paste (MHE, PHE diets). Food intake and body

weight were measured once a week. Muscle functionality was assessed with the

127 CatWalk XT system (Noldus, Netherlands) before and at the end of the experimental

128 period. After 0, 2 and 4 months of experimental diets: 1) a blood sample was taken

129 from a lateral tail vein in the post-absorptive state; 2) body composition was

130 measured using magnetic resonance imaging (Echo MRI international, TX, USA). On

131 the 4th month of the experiment, *in vivo* muscle protein synthesis rates were

132 measured either in the post-absorptive state (PA - after overnight food deprivation,

133 with room lights on) for half of the rats or in the post-prandial state (PP - 2h after

134 feeding, with lights off) for the other half. Due to normal age-related mortality, the final

135 number of rats was 75, distributed as follows: MSE PA: n=10; MSE PP: n=9; PSE

136 PA: n=9; PSE PP: n=9; MHE PA: n=10; MHE PP: n=10; PHE PA: n=9; PHE PP: n=9.

137 *Measurements of in vivo protein synthesis, euthanasia, sampling*

138 Protein synthesis rates were measured using the flooding dose method as previously

139 reported (23). Briefly, twenty minutes before euthanasia, each rat was injected

140 intravenously with L-[1-^{13}C] valine (Euriso-Top, France) (150 μmol/100 g body

141 weight). Rats were then killed under 4% isoflurane anaesthesia by exsanguination

142 from the abdominal aorta. Posterior leg skeletal muscles (gastrocnemius, tibialis

143 anterior, extensor digitorum longus and soleus) were quickly excised, weighed, and

144 frozen in liquid nitrogen until further analysis. Liver and heart were weighed. Protein

145 synthesis rates were measured in the gastrocnemius muscle by measuring muscle

146 free and protein bound valine enrichments after grinding in liquid nitrogen in a ball

147 mill (Dangoumeau, Prolabo, France), and protein extraction with trichloroacetic acid.

148 *Calculations*

149 *In vivo* muscle fractional synthesis rates (FSR, %.d) were calculated as described

150 previously (24): FSR = 100 × (EP-EN)/(EA × t) where t is the incorporation time

151 expressed in days, EP and EA (atom %) are the ^{13}C enrichments of protein-bound

152 valine and of muscle free valine, respectively. EN (atom %) is the natural ^{13}C

153 enrichment of protein-bound valine which was estimated in rats that were not injected

154 with the flooding dose (2 per group). Absolute synthesis rates (ASR) were calculated

155 from the product of FSR with protein content and expressed in milligrams per day.

156 *Plasma measurements*

157 Plasma glucose was determined by an enzymatic test using an automated system

158 (Olympus AU 400 analyzer, CRI biochemistry platform, France).

159 Plasma insulin concentrations were measured using a commercially available rat

160 insulin ELISA kit (80-INSRT-E01, Alpco Immunoassays; USA) at each time point,

161 optical densities were determined at 450 nm using a Tecan Infinite M200 Pro plate

162 reader (Tecan US, Inc., NC, USA).

163 *Statistical analysis*

164 Data were analysed using SAS® Studio (SAS Institute Inc) by variance analysis

165 (ANOVA) followed by the Tukey-Kramer test for post hoc comparison of means. We

166 used two class variables, "Protein" with two levels (milk and plant proteins), and

167 "energy content" with two levels (standard and high). When measurements were

168 repeated over time (body weight, food intake, plasma biochemistry, etc.), repeated

169 time ANOVA was performed. For muscle protein metabolism, we added "nutritional

170 state" as class variable with two levels (post absorptive, postprandial). For several

171 analyses, we also added covariates as factors in the variance analysis. To test

172 differences in death rates between groups, nonparametric estimates of the survivor

173 functions were computed to compare survival curves and perform a Log-Rank test.

174 Data are expressed as means ± SE. The level of significance was set at $P < 0.05$.

175

RESULTS

Survival, food intake, body weight, and body composition

From randomization to euthanasia, the total number of deaths over 4 months was 2 in the MSE group, 3 in the PSE group, 2 in the MHE group, and 4 in the PHE group. There was no significant difference in survival between groups (P = 0.998).

Spontaneous food intake (Figure 1) decreased over time, and this decline was more marked in high energy fed rats. As expected, rats fed with these high energy diets had a lower food intake (g) than rats fed with standard energy diets. There was no difference in food intake between MHE rats and PHE rats, whereas in the first months PSE rats ate significantly less than MSE rats. Energy intakes and protein intakes followed the same pattern. Over the whole period, the energy intake was still significantly higher in MHE rats (92.1 ± 1.6 kcal / day) and PHE rats (93.9 ± 1.7 kcal / day) than in MSE rats (87.5 ± 1.6 kcal / day) and PSE rats (81.7 ± 1.7 kcal / day)(P = 0.001). There was no difference between MHE and PHE rats, whereas the overall energy intake was significantly lower in PSE rats than in MSE rats (P = 0.01). Thus, the effect of protein type was different in rats fed the standard energy diet (lower energy intake with plant proteins) and in rats fed the high-energy diet (no difference), reflected by a significant protein x energy interaction (P = 0.02). Regarding protein intake, as we anticipated a lower spontaneous food intake in high energy fed rats, we chose to increase the protein content of these diets. Consequently, over the whole period, protein intake was similar in all groups (MSE rats: 2.7 ± 0.1 g / day; PSE rats: 2.6 ± 0.1 g / day; MHE rats: 2.5 ± 0.1 g / day; PHE rats: 2.7 ± 0.1 g / day). However, variance analysis detected one significant effect, protein x energy interaction

199 (P=0.02) due to the lower food intake with plant proteins specifically in standard

200 energy fed rats.

201 Weight slowly increased in all groups during the course of the experiment (P =

202 0.001), although it plateaued in the last month (Figure 2). Weight increased more in

203 high energy fed animals (P = 0.0001). Once again, due to the lower food intake with

204 plant proteins specifically in standard energy fed rats, there was also a significant

205 time x protein x energy interaction (P = 0.002). However, for a given energy level in

206 the diet, there was never a significant difference between the weight of milk protein

207 fed rats and plant protein fed rats.

208 *Body composition and tissue weight*

209 Before the start of the experiment, body composition was homogenous between rats,

210 and there was no significant difference between groups. The overall mean initial fat

211 mass was 109.3 ± 3.1 g, and the overall mean initial lean mass was 457.4 ± 3.7 g. As

212 shown in figure 3, during the first two months, the fat mass of all rats increased

213 significantly. Animals fed the high-energy diet almost doubled their fat mass. The

214 gain in fat mass was much lower for the standard-energy diet fed rats, but there was

215 a significant difference between MSE rats and PSE rats, with a lower gain for the

216 latter. Thus, the significant effects of variance analysis were energy (P = 0.0001) and

217 protein x energy (P = 0.04). During the same period, lean mass was preserved in

218 high-energy diet fed rats whereas the loss of lean mass of the rats fed the standard-

219 energy diet was significant. Thus, the high-energy diet induced increased lean mass

220 expressed in percent of initial value (P = 0.0003). There was no significant effect of

221 protein type on the evolution of lean mass.

222 During the last two months, the evolution of body composition was similar in all

223 groups of rats. All groups except PSE rats gained fat mass (+8-10%). Indeed, fat

224 mass, expressed in percent of value at 2 months, was significantly lower in PSE rats

225 than in MSE rats and PHE rats, as reflected by protein x energy interaction (P =

226 0.05). Regarding lean mass loss, it was significant for all the groups of rats, ~4%,

227 with no differences between groups.

228 At the end of the experiment, after 4 months of experimental diet, there was no

229 significant difference between the groups regarding muscle mass (Table 2) or muscle

230 total protein mass (data not shown). There was no negative impact of the high-

231 energy diet. In addition, plant proteins allowed the maintenance of muscle mass. In

232 particular, despite a slightly lower food intake in animals fed the standard energy diet,

233 muscle mass was well maintained in PSE rats compared with all the other groups.

234 Heart weight (Table 2) increased significantly in response to the high-energy diet, as

235 shown by variance analysis. There was no difference in liver weight between groups

236 (Table 2).

237 *Muscle functionality*

238 Among the >300 parameters of footfalls and locomotion characteristics assessed by

239 the CatWalk XT system, we selected simple integrative parameters, which are

240 related to muscle functionality and easy to interpret (Table 3). Among these

241 parameters, there was a significant effect of time only for the number of steps to

242 perform the run, cadence (steps / s) and swing speed (cm / sec). Cadence

243 significantly decreased with time. It could reflect a deleterious effect of aging.

244 However, the number of steps and swing speed improved with time. The high-energy

245 diet significantly reduced this gain with time in the number of steps and swing speed

246 (significant interaction time and energy). There were no other significant effects of the

247 high-energy diet. Regarding the protein effect, there was a significant time x protein

248 interaction for run duration, average speed, and step cycle. Compared to plant

249 protein, milk protein improved the performances of rats regarding run duration and

250 step cycle, but reduced their performances regarding average speed. There was also

251 a significant triple interaction (time x protein x energy) for the number of steps and

252 stride length. It was related to lower values in the PHE group.

253 In addition, we tested the impact of body weight using multivariate models (data not

254 shown). Body weight significantly and independently influenced swing speed and run

255 duration, without affecting the other significant effects. Swing speed and run duration

256 increased when body weight increased. Body weight was also significantly

257 associated with the number of steps, with a size effect similar to that of the effect of

258 the high-energy diet. When body weight was included as a covariate in the statistical

259 model, the interaction time x energy level was no longer significant. Thus, it is likely

260 that the significant effect of a higher energy level on the number of steps was mostly

261 mediated by increases in body weight.

262 *Gastrocnemius muscle protein synthesis*

263 Absolute protein synthesis rates (ASR) were measured in the gastrocnemius muscle

264 in each group, either in the post absorptive or in the postprandial state. The only

265 significant effect was nutritional state (Figure 4). Overall, feeding increased by 13%

266 ASR in the gastrocnemius muscle. ASR could be maintained at the same level

267 whatever the protein type or energy level. Regarding fractional synthesis rates (FSR),

268 there was no significant effect. However, when using a covariate such as

269 gastrocnemius muscle mass or lean body mass, both significantly correlated with

270 FSR, a significant increase in FSR, independent of feeding, was detected in rats fed

271 plant proteins (P < 0.001). Adjusted means ± SE for FSR were 6.5 ± 0.2 % / h for

272 milk proteins and 7.6 ± 0.2 % / h for plant proteins.

273 *Plasma biochemistry*

274 Overall, plasma glucose (Figure 5A) declined over time irrespective of energy level or

275 protein type, even if after two months the energy level tended to increase fasting

276 glycaemia.

277 There was no clear trend regarding plasma insulin (Figure 5B), probably in relation

278 with heterogeneous values in animals, even before the start of the experiment.

279 Overall, we found a significant time x protein x energy interaction (P = 0.02) when

280 analyzing global values, and at 4 months we found lower plasma insulin after feeding

281 with plant proteins compared to milk protein (P = 0.04).

282

283 DISCUSSION

284 The aim of this study was to assess the capacity of a mix of purified plant proteins

285 rich in all the indispensable amino acids (IAA) and with relatively high leucine,

286 arginine, and cysteine contents to limit sarcopenia, compared to milk protein. Using

287 aging rats fed a normal diet or a diet rich in fat and sugar for 4 months as a model,

288 we focused our analyses on postprandial protein metabolism and lean muscle mass

289 and function.

290 We showed that despite a slightly lower food intake in rats fed with the standard

291 energy plant protein diet, lean mass, muscle mass and functionality were maintained

292   in all the groups of rats fed plant proteins to the same extent as in rats fed milk

293   proteins.

294   Likewise, the response of muscle protein synthesis to feeding was also similar

295   between plant protein and milk protein. This is a key point because we showed that

296   during healthy aging, a defect in the stimulation of muscle protein synthesis during

297   feeding is thought to drive the slow erosion of muscle mass (25). To overcome this

298   resistance of muscle anabolism, one strategy is to improve peripheral amino acid

299   (AA) or leucine availability after feeding (25): 1) Leucine is a key AA for optimizing the

300   stimulation of muscle protein synthesis after feeding in old age (26); 2) Arginine, as a

301   precursor of NO, can increase muscle perfusion and thus the peripheral extraction of

302   nutrients in response to insulin (15, 17). Another strategy would be to mitigate

303   oxidative stress, inflammation, and insulin resistance that increase during aging (9,

304   27). Because of its presence in glutathione, cysteine can help to prevent the

305   alteration of the redox status observed during aging (18, 19), while long-term

306   supplementation with cysteine was shown to delay loss of muscle mass in aging (20).

307   This was the rationale for designing a protein blend with relatively high amounts of

308   leucine, arginine and cysteine contents and testing its effect in both standard and

309   high fat high sucrose diets.

310   The possibility that our plant protein blend could stimulate muscle protein synthesis

311   independently of feeding, as suggested by our results on adjusted FSR, was a

312   surprise. The fact that muscle mass was not increased implies a corresponding

313   increase in muscle proteolysis, and thus a higher turnover. To the best of our

314   knowledge, such a result has not been described previously.

315   Currently, numerous studies are performed to assess the suitability of plant proteins

316   to partially replace animal proteins. Single proteins and mixtures of proteins are

317   tested, as are crude food products (lentils for instance) and purified fractions. Plant

318   crude products with a high protein content are more likely to have low digestibility

319   (presence of anti-nutritional factors), and to be marginally deficient in certain IAA

320   (lysine in cereals, sulfur AA in legumes).

321   Our approach was to mix numerous highly purified plant proteins, using industrial co-

322   products when possible, thus lowering the risk of poor digestibility, mainly through the

323   inactivation of trypsin inhibitors.

324   Blending sources also offer the opportunity to tailor AA composition. It was easy to

325   find a blend with an excellent AA balance (5). A deficiency in one AA, especially in

326   the long term, can affect tissue protein anabolism. However, another additional effect

327   could occur. Indeed, studies using soy and wheat as protein sources observed an

328   increase in urea synthesis (28). This was attributed to poor utilization of dietary AA in

329   the intestine, leading in turn to a marked increase in free AA content in the portal

330   vein, which could constitute a signal for the liver to increase urea synthesis, thus

331   further limiting the availability of dietary AA for peripheral protein synthesis (28). The

332   reason for the poor utilization of dietary AA after the ingestion of wheat or soy is not

333   completely clear. It could result from a suboptimal IAA balance (for instance lysine in

334   wheat) and / or in the lower proportion of total IAA among total AA (38% for soy *vs*

335   43% for milk proteins). It was also proposed that this phenomenon could result from

336   modifications in digestion kinetics (29). Thus, it was shown that soy proteins could

337   not stimulate muscle proteins in old rats to the same extent as whey (30) .

338   In our plant blend, the content of all the IAA was similar to or even slightly higher than

339   that of milk proteins, except for lysine. Lysine content was 56% of milk protein

340   content but 163% of wheat protein content and allowed a lysine intake much higher

341   than the requirement of mature rats (31). However, the proportion of IAA was 38% in

342   our plant protein blend, likewise for soy. Given the excellent results we obtained with

343   our plant blend regarding the long-term effect on muscle mass and the short-term

344   effect on muscle protein synthesis, this suggests that the stimulation of urea

345   synthesis observed with soy (29) is not related to the slightly lower IAA proportion but

346   to possible specific digestion kinetics issues.

347   To the best of our knowledge, no similar plant protein mix has been studied in the

348   long term in the context of muscle aging. In previous studies, combinations of cereals

349   and legumes were studied, showing most of the time that protein retention was better

350   with the mix than with cereals or legumes alone, but still lower than with animal

351   proteins (32). Recently, Salles et al. (33) concluded that nitrogen balance, lean body

352   mass, muscle mass and post-absorptive protein synthesis could be maintained

353   during aging at the same level after two months of feeding with purified pea proteins

354   compared with casein or whey. The same team compared casein, whey and legume

355   enriched pasta (lentil, split pea, and faba bean), and showed that after 6 weeks in 22

356   month-old rats, despite lower digestibility and leucine content, legume enriched pasta

357   allowed maintaining lean body mass, nitrogen balance and post-absorptive muscle

358   protein synthesis (34). The authors argued that higher post-prandial insulin secretion

359   and high glycine and arginine content could have been contributive factors. Our plant

360   protein mix had similar glycine and arginine levels to that of legume enriched pasta

361   (34), and higher levels of leucine and cysteine than pea proteins (33). If the results

362   on muscle mass and function during aging were indeed mediated by these AA, our

363   findings would confirm those of Salles et al. (33).

364   Another unexpected result of our study was that the high-energy diet (rich in fat and

365   sucrose) seemed to have protective effects regarding lean body mass, independently

366   of the type of ingested protein. Indeed, the high-energy diet prevented a decrease in

367 lean body mass during the first two months of the experiment. Our initial objective

368 was to challenge the metabolism of aging rats by inducing insulin resistance to test

369 the capacity of our plant protein mix with high arginine and cysteine contents to

370 counteract the potential deleterious effects of the high-energy diet. It turned out that

371 insulin and glucose levels were not affected by energy, although there was a marked

372 gain in body weight and fat mass. Previously, we showed that replacing all starch by

373 sucrose induced insulin resistance in old rats (27). However, such changes are not

374 always obtained after high fat high sucrose diets in rats (23). In the present study,

375 food intake remained moderate and decreased during aging, in particular in rats fed

376 the high-energy diet. This may have attenuated the negative impacts of the high-

377 energy diet, in particular on the regulation of lean body mass.

378 In situations where higher energy intake does not lower insulin action, it is considered

379 favorable to protein metabolism, through classical sparing effects on intermediary

380 metabolism (35). In addition, the high-energy diet increased the weight of animals,

381 which then had to use more force to move in the cage, perhaps also explaining the

382 better maintenance of lean body mass during the first two months of the experiment.

383 A similar or slightly higher lean mass was also observed in other animal studies using

384 high fat diets during aging (36-38). In humans, obesity is often associated with a

385 higher lean body mass, and it was suggested that "carrying greater weight might

386 have physical training-like effects" (39).

387 However, these "training effects" on lean body mass did not translate into

388 improvements in muscle functionality. Indeed, two measurements obtained with the

389 CatWalk XT system, the number of steps to perform the run and the swing speed,

390 improved with time, but the high-energy diet significantly reduced this improvement.

391 It was surprising to observe an improvement with time in muscle functionality.

392 Recently, comparing 6 and 24-month-old Wistar rats, Le Bacquer et al. (40) obtained

393 a higher average speed, lower step number, and higher cadence in adult rats

394 compared to older rats. This is what could be expected in response to the deleterious

395 effect of aging. For the same parameters, we saw no direct effect of time on average

396 speed, a contrary result for step number, and a similar result for cadence. Our

397 studies are different because there was a large age difference in the study by Le

398 Bacquer et al. (40), while in our study the measurements were repeated for the same

399 animals. That is why we postulate that the improvements that we saw with time may

400 have been due to habituation. Animals were more familiar with the task they were

401 required to perform the second time. It makes sense that, despite habituation, high-

402 energy fed animals would have greater difficulty in performing the task due to the fact

403 that they were heavier the second time.

404 Besides energy, we found other significant effects of the type of protein with the

405 CatWalk XT system. Compared to milk protein, plant proteins reduced the

406 performances of rats regarding run duration and step cycle but improved their

407 performances regarding average speed. These results are difficult to interpret,

408 because 1) there was no effect of time alone; 2) they cannot be clearly linked to

409 differences in body weight, lean body mass or muscle mass; 3) the same type of

410 protein had positive and negative effects. Analyzing footfalls and locomotion

411 characteristics by the CatWalk XT system is a complex process, probably involving

412 not only neuro-muscular function but also brain function (memory, learning capacity,

413 confidence). It is possible that these differences observed between animal and plant

414 proteins could result from effects on brain function. Indeed, the type of protein

415 ingested can affect brain function by changing the blood concentration of large

416   neutral AA and hence serotonine and catecholamine synthesis. Specific peptides

417   generated during protein digestion could also influence brain function and some are

418   found in dairy products (41). However, the tryptophan, tyrosine, and branched chain

419   AA content was similar between milk proteins and our mix of plant proteins, and the

420   amount and nature of bioactive peptides generated after the ingestion of milk

421   proteins or our blend of plant protein remains poorly understood.

422   In conclusion, in this aging rat model, we showed that a tailored blend of plant protein

423   can adequately replace milk proteins in the long term, as it resulted in no difference in

424   the response of muscle protein synthesis to feeding, or in the evolution of lean body

425   mass and muscle mass during aging. We could not test the capacity of these plant

426   proteins to protect muscle in a situation of altered insulin sensitivity because the high

427   fat high sucrose diet only induced a marked increase in body fat without obvious

428   deleterious effects on insulin sensitivity. Many other aspects of substituting animal

429   proteins with plant protein need to be examined in other studies, but the present

430   findings offer an important proof of concept from the nutritional standpoint that

431   appropriately blended plant proteins can have a high nutritional value even in

432   demanding situations such as protein metabolism and sarcopenia in the elderly.

433

441   manuscript. The data described in the manuscript will be made available upon

442   request.

443

Figure Legends

445   **Figure 1** Evolution of food intake in MSE, PSE, MHE and PHE rats.

446   18-month-old rats were fed for 4 months with a diet containing as protein source

447   either milk proteins or a plant protein mix combined with either standard energy

448   content or high energy content with a high level of fat and sucrose: MSE = milk

449   protein standard energy, PSE = plant protein standard energy, MHE = milk protein

450   high energy, PHE = plant protein high energy. Rats ate *ad libitum*. Significant effects

451   (SE) of repeated time variance analysis are given and the results showed that the

452   time effect was significant, as were all the interactions (time x protein, time x energy,

453   time x protein x energy). *: at this time point, there was a significant difference

454   between MSE rats and PSE rats, $P < 0.05$. #: at this time point there was a significant

455   difference between rats fed standard energy diets and rats fed high energy diets, $P <$

456   0.05. Values are means ± SE (n=18-20 per group).

457   **Figure 2** Evolution of body weight in MSE, PSE, MHE and PHE rats.

458   See figure 1 for group description. Significant effects of repeated time variance

459   analysis are given and the results showed that the time effect was significant, as

460   were time x energy and time x protein x energy interactions. # : at this time point,

461   there was a significant difference between rats fed standard energy diets and rats fed

462   high energy diets, $P < 0.05$. Values are means ± SE (n=18-20 per group)

463   **Figure 3** Evolution of body composition in MSE, PSE, MHE and PHE rats.

464 See figure 1 for group description. Body composition was measured before and after

465 2 and 4 months of the experimental diet. Fat mass and lean mass measurements are

466 expressed in percent of the initial measurement for the first 2 months, and in percent

467 of the value at 2 months for the last 2 months. Significant effects (SE) of variance

468 analysis are given. a, b: means assigned with the same letter were not significantly

469 different. *: significantly different from 0 (P < 0.05). Values are means ± SE (n=18-20)

470 **Figure 4** Absolute synthesis rates (ASR) in the gastrocnemius muscle after 4 months

471 of experimental diets.

472 See Figure 1 for group description. Absolute protein synthesis rates were measured

473 in the gastrocnemius muscle either in the post absorptive state (PA) or in the post-

474 prandial state (PP) in MSE, PSE, MHE and PHE rats, and are expressed in mg

475 protein synthetized per day. Bigger red dots represent mean values, and smaller blue

476 dots individual values (n= 5-9 per group). SE: variance analysis significant effects.

477 Only nutritional state had a significant effect (P = 0.02).

478 **Figure 5** Evolution of glycemia and insulinemia in MSE, PSE, MHE and PHE rats.

479 See figure 1 for group description. Plasma glucose (A) and insulin (B) levels were

480 measured in the post- absorptive state before and after 2 and 4 months of

481 experimental diets. SE: significant effect of repeated time variance analysis. *:

482 Variance analysis performed at this time point detected a significant effect of energy

483 (P = 0.01); $: Variance analysis performed at this time point detected a significant

484 effect of protein (P = 0.04).

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Table 1: Diet composition

Ingredients and dietary composition Per kg of diet	Standard energy Milk protein	Standard energy Plant mix	High energy Milk protein	High energy Plant mix
Milk proteins (g)	140	0	168	186.69
Plant protein mix (g)	0	155.8	0	0
L-cystine (g)	0.69	0.69	0.83	0.83
Wheatstarch (g)	495	479	144	125
Sucrose (g)	127	127	230	230
Lactose (g)	100	100	60	60
Canola oil (g)	20	20	20	20
Sunflower oil (g)	2	2	2	2
Peanut oil (g)	18	18	18	18
Lard (g)	0	0	260	260
Cellulose (g)	50	50	50	50
Mineral mix (ain-93g-mx) (g)	35	35	35	35
Vitamin mix (ain-93-vx) (g)	10	10	10	10
Choline bitartrate (41.1% choline) (g)	2.5	2.5	2.5	2.5
Energy (kcal)	**3624.07**	**3657.62**	**4889.76**	**4929.68**
% as protein	13.54	14.55	12.66	13.55
% as CHO	76.40	71.41	35.15	33.71
% as fat	10.30	10.39	50.14	50.50
Amino acids (g)	**121.97**	**128.13**	**146.36**	**153.53**
Alanine	3.72	7.40	4.47	8.86
Arginine	4.14	9.39	4.97	11.26
Asparagine/aspartate	8.62	11.94	10.35	14.30
Cystine	1.82	2.49	2.18	2.98
Glutamine/glutamate	24.78	22.68	29.74	27.17
Glycine	2.20	5.22	2.64	6.26
Histidine	3.14	3.02	3.76	3.62
Isoleucine	5.74	5.70	6.89	6.83
Leucine	11.21	12.30	13.46	14.74
Lysine	10.47	5.86	12.57	7.02
Methionine	3.19	2.49	3.83	2.98
Phenylalanine	5.60	6.53	6.72	7.82
Proline	10.99	7.04	13.19	8.44
Serine	6.36	6.69	7.63	8.01
Threonine	5.03	5.20	6.03	6.23
Tryptophan	1.68	1.60	2.02	1.92
Tyrosine	6.58	6.11	7.90	7.32
Valine	6.69	6.49	8.03	7.78

Table 2 : Tissue weight after 4 months of experimental diet in MSE, PSE, MHE and PHE rats

Groups		MSE	PSE	MHE	PHE	ANOVA SE
Muscles						
Gastrocnemius	g	2.12 ± 0.11	2.22 ± 0.09	1.95 ± 0.09	2.24 ± 0.14	ns
Tib anterior	g	0.65 ± 0.03	0.65 ± 0.03	0.57 ± 0.03	0.65 ± 0.05	ns
Soleus	mg	215 ± 11	226 ± 8	219 ± 14	243 ± 13	ns
EDL	mg	198 ± 9	215 ± 8	190 ± 7	202 ± 12	ns
Heart	g	1.65[a] ± 0.03	1.75[ab] ± 0.06	1.81[ab] ± 0.05	1.85[b] ± 0.06	E (P=0.01)
Liver	g	14.6 ± 0.5	13.8 ± 0.6	14.3 ± 0.5	15.1 ± 0.7	ns
Body weight	g	657[ab] ± 17	629[a] ± 21	710[ab] ± 26	737[b] ± 28	E (P=0.001)
Lean mass	g	424 ± 9	433 ± 11	440 ± 10	447 ± 13	ns

See figure 1 for group description. Body weight and lean mass are given again here for readers that prefer calculating tissue weight in proportion of body weight or lean mass. MSE = milk protein standard energy; PSE = plant protein standard energy; MHE = milk protein high energy; PHE = plant protein high energy; ANOVA SE: significant effects of variance analysis; Tib = tibialis; EDL = Extensor Digitorum Longus muscle; E = energy; ns = not significant. a, b = means affected by the same letter were not significantly different. Values are means ± SE (n=18-20).

Table 3: Evolution of muscle functionality before and after nutritional treatments

Parameter	Time	MSE	PSE	MHE	PHE	RSE	ANOVA SE
Run duration (s)	Before	4,00	4,12	4,38	3,98	0,85	T x P (P=0.02)
	After	4,02	4,32	3,98	4,49		
Average speed (cm / s)	Before	30,9	30,5	28,5	31,3	6,00	T x P (P=0.04)
	After	30,3	28,7	30,0	27,6		
Number of steps	Before	62,4	64,4	66,5	62,4	6,03	T (P=0.01), T x E (P=0.04), T x P x E (P=0.01)
	After	59,6	58,1	61,8	65,7		
Cadence (steps / s)	Before	8,05	8,13	7,91	8,08	1,07	T (P=0.01)
	After	7,70	7,17	7,86	7,65		
Stride length (cm)	Before	14,6	14,4	13,9	14,9	1,37	T x P x E (P=0.01)
	After	14,2	14,7	14,3	13,8		
Step cycle (s)	Before	0,47	0,46	0,48	0,48	0,06	T x P (P=0.02)
	After	0,45	0,49	0,46	0,48		
Swing speed (cm / s)	Before	104,7	99,3	97,4	99,8	13,70	T (P=0.001), T x E (P=0.001)
	After	78,8	75,2	88,7	86,0		

See figure 1 for group description. We give means and residual standard errors (RSE) before and after nutritional treatments in each group (n=17-18). We performed repeated time variance covariance analysis. MSE: milk protein standard energy; PSE: plant protein standard energy; MHE: milk protein high energy; PHE: plant protein high energy; RSE: residual standard error; ANOVA SE: significant effects of variance analysis. T = time; E = energy.
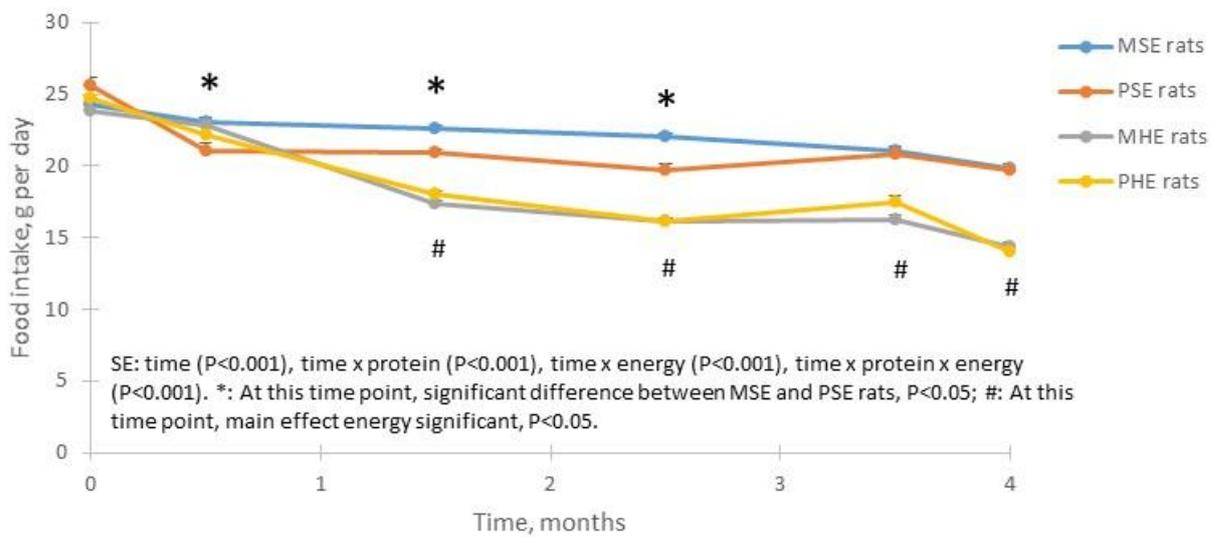
Figure 1:

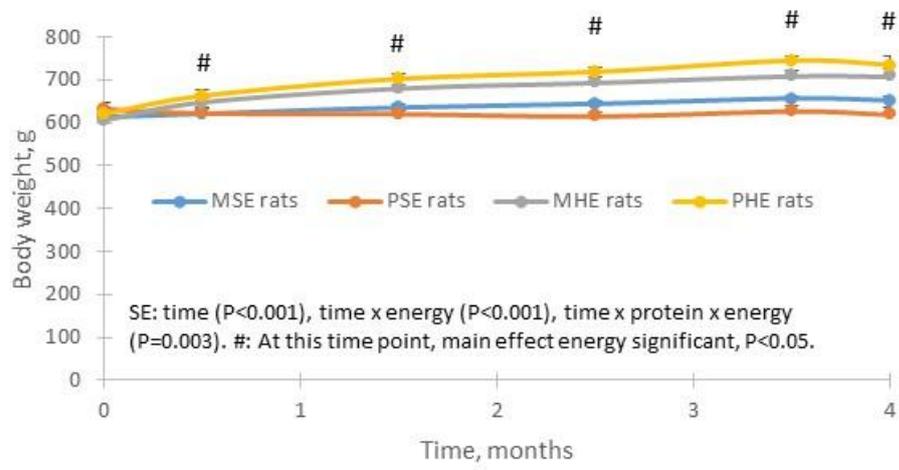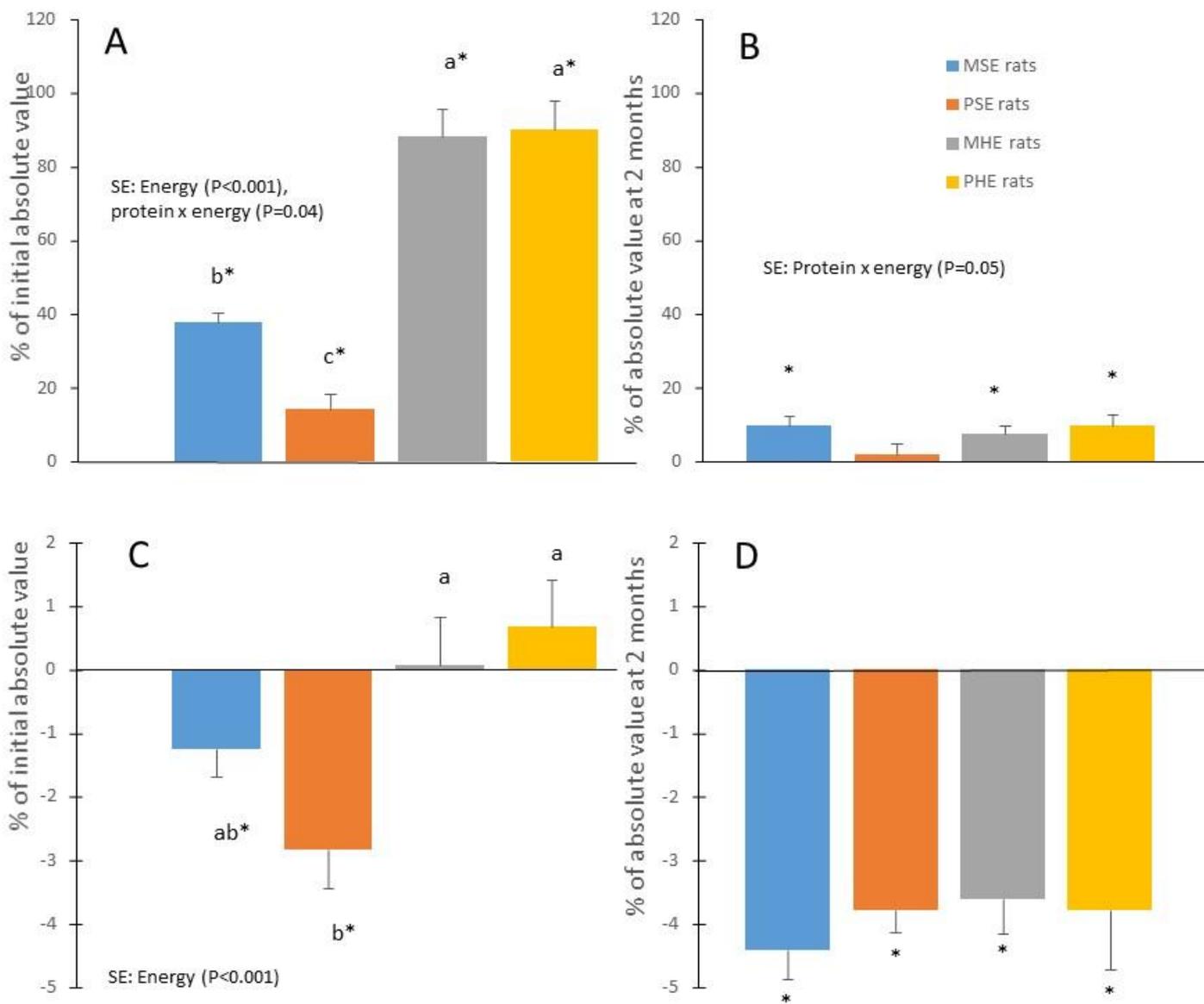SE: time (P<0.001), time x protein (P<0.001), time x energy (P<0.001), time x protein x energy (P<0.001). *: At this time point, significant difference between MSE and PSE rats, P<0.05; #: At this time point, main effect energy significant, P<0.05.

Figure 2 :

SE: time (P<0.001), time x energy (P<0.001), time x protein x energy (P=0.003). #: At this time point, main effect energy significant, P<0.05.

Figure 3:

a,b,..: labeled means without a common letter differ, P<0.05; *: significantly different from 0, P<0.05.

Figure 4:

Figure 5 :

A

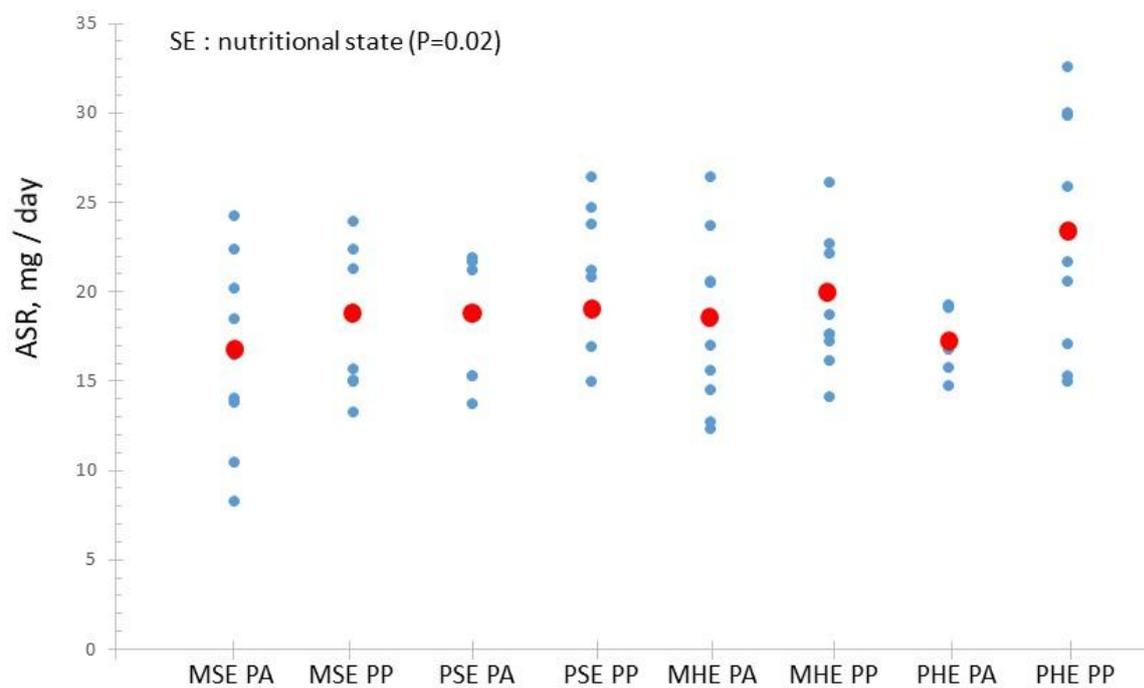SE : time (P<0.001). * At this time point, main effect energy significant, P<0.05.

MSE rats
PSE rats
MHE rats
PHE rats

B

SE : time x protein x energy (P=0.02). $: At this time point, main effect protein significant, P<0.05.

MSE rats
PSE rats
MHE rats
PHE rats