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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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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.

Abstract

Background: Alternative, sustainable and adequate sources of protein must be found to meet global demand. Objective: The aim of this study was to assess the effect of a plant-protein blend with a good balance of indispensable amino acids and high contents of leucine, arginine and cysteine on the maintenance of muscle protein mass and function during aging in comparison to milk proteins, and to determine if this effect varied according to the quality of the background diet. Methods: Old male Wistar rats (n = 96, 18 months old) were randomly allocated to four diet groups, differing according to protein source (milk or plant blend) and energy content (standard, with starch, or high, with saturated fat and sucrose), for 4 months. Plasma biochemistry and body composition (echo MRI) were measured every two months. Muscle functionality (Catwalk XT system) was assessed before and after the experimental diet. After 4 months of diet, animals were injected with a flooding dose of L-[1-13C] valine, and hind limb muscles, liver and heart were sampled and weighed. Results: We found that the plant protein blend stimulated muscle protein synthesis, maintained lean body mass, muscle mass, and muscle functionality during aging as well as milk protein. Liver and heart weights were unchanged. The high fat high sucrose diet increased body fat but, assessed by fasting plasma glucose and insulin, had little impact on insulin sensitivity and related metabolisms. Therefore, we could not test the hypothesis that in situations of higher insulin resistance, our plant protein blend rich in leucine, arginine and cysteine may be better than milk protein. Conclusion: Finally, the present findings offer significant proof of concept from the nutritional standpoint that appropriately blended plant proteins can have high nutritional value even in demanding situations such as ageing protein metabolism.

Keywords: plant proteins; aging; sarcopenia; obesity; muscle protein synthesis; muscle functionality.

INTRODUCTION

By the year 2050, the combined increase in the world's population and living standards in developing countries will lead to a very considerable rise in the demand for dietary protein, especially from animal sources (1). This demand cannot be met sustainably by increasing animal production, meaning that alternative sources of protein must be developed. The simplest feasible option in the short term is to take better advantage of the large amount and diversity of plant proteins already available. The co-products of the oilseed and starch industry contain significant amounts of proteins (15-50%). Their use in human nutrition is at present negligible and should be developed (2).

Our objective falls in this context of diversifying protein resources for humans, with the aim of tackling food insecurity while taking into account the sustainability of food systems (exploitation of co-products). However, a key issue will be to use plant proteins to develop protein-rich foods able to compete with animal products (meat, eggs, milk), which are considered as providers of reference proteins in human nutrition. Indeed, besides technical and organoleptic issues, one of the main obstacles of using plant proteins in human nutrition is generally the low nutritive value classically attributed to them, because of their unbalanced composition in indispensable amino acids (IAA), and concerns regarding digestibility (3). Indeed, amino acid (AA) bioavailability may be an issue for plant proteins, but when proteins are extracted from their natural matrix and properly processed, their true ileal digestibility is generally high (84-94%), and only slightly lower than that of animal proteins (90-95%) (4). The unbalanced composition of individual plant proteins could be readily solved by combining them. Finally, by taking advantage of a wider range of

available plant proteins and combining them, it should be possible to identify and produce protein blends with tailored AA composition and high digestibility (5, 6).

This optimized AA composition can even be targeted for a specific population. Undoubtedly, the most important problem to solve in the coming years is feeding the elderly. Demographic trends in Western countries are characterized by the gradual aging of the population and increased longevity (1/4 of the population in Europe and North America will be over 65 in 2050 - https://population.un.org/wpp/publications/files/wpp2019_highlights.pdf). Sarcopenia, defined as the gradual decline in muscle mass and function, is a major feature of aging. It is an inevitable physiological process, occurring even in healthy elderly subjects, which increases the risk of loss of independence, falls and fractures, and decreases resistance to nutritional, infectious or traumatic stresses (7). Muscle mass is controlled by the balance between protein synthesis and degradation. It was shown that the post-prandial stimulation of muscle protein synthesis and inhibition of muscle protein degradation decreases during aging, with the development of anabolic resistance (8). Thus, besides inactivity and protein undernutrition, other factors like oxidative stress, inflammation (9), or insulin resistance (10) can accelerate sarcopenia by worsening muscle anabolic resistance. Given these phenomena, how can we optimize the AA composition of plant protein blends for elderly subjects?

First, there is a risk of mismatch between requirements and intakes in the elderly, because of an increased requirement and a decreased appetite (11). A significant proportion of the elderly population may have marginal intake with regards to protein requirements (12). In this specific context, the quality of protein intake in terms of IAA supply is considered to be a key factor for optimal protein metabolism. In addition,

leucine is a potent stimulator of protein synthesis, and supplementation with this AA was shown to restore muscle postprandial anabolism during aging (13, 14). Arginine, as a precursor of NO, could play a major role in the provision of AA for muscles. Indeed, the role of NO in the peripheral extraction of nutrients in response to insulin has been established (15, 16). Supplementation with arginine was shown to increase muscle perfusion in the elderly (17), although the effect on anabolism depends on the level of supply of the other AAs. Cysteine is involved in the determination of the redox status, mainly because of its presence in glutathione, the main molecular antioxidant of the body. This redox status is altered with aging (18). The requirement for cysteine seems to increase with aging because of changes in metabolic demand in relation with oxidative stress, low grade inflammation, and increased medication use (19). In line with this, long-term supplementation with cysteine was shown to delay loss of muscle mass in aging (20, 21). Thus, we reasoned that a blend of plant protein with an excellent IAA balance and particularly rich in leucine, arginine, and cysteine may be an adequate diet for maintaining muscle mass and function during aging.

Using linear programming (5), we designed a plant protein blend with an excellent IAA balance and high levels of leucine, arginine and cysteine, and used it as the unique protein source in the diet of aging rats (final age 22 months) in a 4-month study. The objective was to test if this blend could maintain lean body mass, muscle mass and function as well as muscle protein synthesis rates, in comparison to protein obtained from milk, recognized as an excellent protein source. This experiment was performed in the context of a standard diet (22), and a high fat high sucrose diet which better reflects western diets, with the expectation of increased insulin resistance and related metabolic alterations. We showed that this blend of plant proteins was as efficient as milk proteins to prevent sarcopenia during aging.

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

CatWalk XT system (Noldus, Netherlands) before and at the end of the experimental period. After 0, 2 and 4 months of experimental diets: 1) a blood sample was taken from a lateral tail vein in the post-absorptive state; 2) body composition was measured using magnetic resonance imaging (Echo MRI international, TX, USA). On the 4th month of the experiment, *in vivo* muscle protein synthesis rates were measured either in the post-absorptive state (PA - after overnight food deprivation, with room lights on) for half of the rats or in the post-prandial state (PP - 2h after feeding, with lights off) for the other half. Due to normal age-related mortality, the final number of rats was 75, distributed as follows: MSE PA: n=10; MSE PP: n=9; PSE PA: n=9; PSE PP: n=9; MHE PA: n=10; MHE PP: n=10; PHE PA: n=9; PHE PP: n=9.

Measurements of in vivo protein synthesis, euthanasia, sampling

Protein synthesis rates were measured using the flooding dose method as previously reported (23). Briefly, twenty minutes before euthanasia, each rat was injected intravenously with L-[1-^{13}C] valine (Euriso-Top, France) (150 *µ*mol/100 g body weight). Rats were then killed under 4% isoflurane anaesthesia by exsanguination from the abdominal aorta. Posterior leg skeletal muscles (gastrocnemius, tibialis anterior, extensor digitorum longus and soleus) were quickly excised, weighed, and frozen in liquid nitrogen until further analysis. Liver and heart were weighed. Protein synthesis rates were measured in the gastrocnemius muscle by measuring muscle free and protein bound valine enrichments after grinding in liquid nitrogen in a ball mill (Dangoumeau, Prolabo, France), and protein extraction with trichloroacetic acid.

Calculations

In vivo muscle fractional synthesis rates (FSR, %.d) were calculated as described previously (24): FSR = 100 × (EP-EN)/(EA × t) where t is the incorporation time

expressed in days, EP and EA (atom %) are the ^{13}C enrichments of protein-bound valine and of muscle free valine, respectively. EN (atom %) is the natural ^{13}C enrichment of protein-bound valine which was estimated in rats that were not injected with the flooding dose (2 per group). Absolute synthesis rates (ASR) were calculated from the product of FSR with protein content and expressed in milligrams per day.

Plasma measurements

Plasma glucose was determined by an enzymatic test using an automated system (Olympus AU 400 analyzer, CRI biochemistry platform, France).

Plasma insulin concentrations were measured using a commercially available rat insulin ELISA kit (80-INSRT-E01, Alpco Immunoassays; USA) at each time point, optical densities were determined at 450 nm using a Tecan Infinite M200 Pro plate reader (Tecan US, Inc., NC, USA).

Statistical analysis

Data were analysed using SAS® Studio (SAS Institute Inc) by variance analysis (ANOVA) followed by the Tukey-Kramer test for post hoc comparison of means. We used two class variables, “Protein” with two levels (milk and plant proteins), and “energy content” with two levels (standard and high). When measurements were repeated over time (body weight, food intake, plasma biochemistry, etc.), repeated time ANOVA was performed. For muscle protein metabolism, we added “nutritional state” as class variable with two levels (post absorptive, postprandial). For several analyses, we also added covariates as factors in the variance analysis. To test differences in death rates between groups, nonparametric estimates of the survivor functions were computed to compare survival curves and perform a Log-Rank test. Data are expressed as means ± SE. The level of significance was set at $P < 0.05$.

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

(P=0.02) due to the lower food intake with plant proteins specifically in standard energy fed rats.

Weight slowly increased in all groups during the course of the experiment (P = 0.001), although it plateaued in the last month (Figure 2). Weight increased more in high energy fed animals (P = 0.0001). Once again, due to the lower food intake with plant proteins specifically in standard energy fed rats, there was also a significant time x protein x energy interaction (P = 0.002). However, for a given energy level in the diet, there was never a significant difference between the weight of milk protein fed rats and plant protein fed rats.

Body composition and tissue weight

Before the start of the experiment, body composition was homogenous between rats, and there was no significant difference between groups. The overall mean initial fat mass was 109.3 ± 3.1 g, and the overall mean initial lean mass was 457.4 ± 3.7 g. As shown in figure 3, during the first two months, the fat mass of all rats increased significantly. Animals fed the high-energy diet almost doubled their fat mass. The gain in fat mass was much lower for the standard-energy diet fed rats, but there was a significant difference between MSE rats and PSE rats, with a lower gain for the latter. Thus, the significant effects of variance analysis were energy (P = 0.0001) and protein x energy (P = 0.04). During the same period, lean mass was preserved in high-energy diet fed rats whereas the loss of lean mass of the rats fed the standard-energy diet was significant. Thus, the high-energy diet induced increased lean mass expressed in percent of initial value (P = 0.0003). There was no significant effect of protein type on the evolution of lean mass.

During the last two months, the evolution of body composition was similar in all groups of rats. All groups except PSE rats gained fat mass (+8-10%). Indeed, fat mass, expressed in percent of value at 2 months, was significantly lower in PSE rats than in MSE rats and PHE rats, as reflected by protein x energy interaction (P = 0.05). Regarding lean mass loss, it was significant for all the groups of rats, ~4%, with no differences between groups.

At the end of the experiment, after 4 months of experimental diet, there was no significant difference between the groups regarding muscle mass (Table 2) or muscle total protein mass (data not shown). There was no negative impact of the high-energy diet. In addition, plant proteins allowed the maintenance of muscle mass. In particular, despite a slightly lower food intake in animals fed the standard energy diet, muscle mass was well maintained in PSE rats compared with all the other groups.

Heart weight (Table 2) increased significantly in response to the high-energy diet, as shown by variance analysis. There was no difference in liver weight between groups (Table 2).

Muscle functionality

Among the >300 parameters of footfalls and locomotion characteristics assessed by the CatWalk XT system, we selected simple integrative parameters, which are related to muscle functionality and easy to interpret (Table 3). Among these parameters, there was a significant effect of time only for the number of steps to perform the run, cadence (steps / s) and swing speed (cm / sec). Cadence significantly decreased with time. It could reflect a deleterious effect of aging. However, the number of steps and swing speed improved with time. The high-energy diet significantly reduced this gain with time in the number of steps and swing speed

(significant interaction time and energy). There were no other significant effects of the high-energy diet. Regarding the protein effect, there was a significant time x protein interaction for run duration, average speed, and step cycle. Compared to plant protein, milk protein improved the performances of rats regarding run duration and step cycle, but reduced their performances regarding average speed. There was also a significant triple interaction (time x protein x energy) for the number of steps and stride length. It was related to lower values in the PHE group.

In addition, we tested the impact of body weight using multivariate models (data not shown). Body weight significantly and independently influenced swing speed and run duration, without affecting the other significant effects. Swing speed and run duration increased when body weight increased. Body weight was also significantly associated with the number of steps, with a size effect similar to that of the effect of the high-energy diet. When body weight was included as a covariate in the statistical model, the interaction time x energy level was no longer significant. Thus, it is likely that the significant effect of a higher energy level on the number of steps was mostly mediated by increases in body weight.

Gastrocnemius muscle protein synthesis

Absolute protein synthesis rates (ASR) were measured in the gastrocnemius muscle in each group, either in the post absorptive or in the postprandial state. The only significant effect was nutritional state (Figure 4). Overall, feeding increased by 13% ASR in the gastrocnemius muscle. ASR could be maintained at the same level whatever the protein type or energy level. Regarding fractional synthesis rates (FSR), there was no significant effect. However, when using a covariate such as gastrocnemius muscle mass or lean body mass, both significantly correlated with

FSR, a significant increase in FSR, independent of feeding, was detected in rats fed plant proteins ($P < 0.001$). Adjusted means ± SE for FSR were 6.5 ± 0.2 % / h for milk proteins and 7.6 ± 0.2 % / h for plant proteins.

Plasma biochemistry

Overall, plasma glucose (Figure 5A) declined over time irrespective of energy level or protein type, even if after two months the energy level tended to increase fasting glycaemia.

There was no clear trend regarding plasma insulin (Figure 5B), probably in relation with heterogeneous values in animals, even before the start of the experiment. Overall, we found a significant time x protein x energy interaction ($P = 0.02$) when analyzing global values, and at 4 months we found lower plasma insulin after feeding with plant proteins compared to milk protein ($P = 0.04$).

DISCUSSION

The aim of this study was to assess the capacity of a mix of purified plant proteins rich in all the indispensable amino acids (IAA) and with relatively high leucine, arginine, and cysteine contents to limit sarcopenia, compared to milk protein. Using aging rats fed a normal diet or a diet rich in fat and sugar for 4 months as a model, we focused our analyses on postprandial protein metabolism and lean muscle mass and function.

We showed that despite a slightly lower food intake in rats fed with the standard energy plant protein diet, lean mass, muscle mass and functionality were maintained

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

Likewise, the response of muscle protein synthesis to feeding was also similar between plant protein and milk protein. This is a key point because we showed that during healthy aging, a defect in the stimulation of muscle protein synthesis during feeding is thought to drive the slow erosion of muscle mass (25). To overcome this resistance of muscle anabolism, one strategy is to improve peripheral amino acid (AA) or leucine availability after feeding (25): 1) Leucine is a key AA for optimizing the stimulation of muscle protein synthesis after feeding in old age (26); 2) Arginine, as a precursor of NO, can increase muscle perfusion and thus the peripheral extraction of nutrients in response to insulin (15, 17). Another strategy would be to mitigate oxidative stress, inflammation, and insulin resistance that increase during aging (9, 27). Because of its presence in glutathione, cysteine can help to prevent the alteration of the redox status observed during aging (18, 19), while long-term supplementation with cysteine was shown to delay loss of muscle mass in aging (20). This was the rationale for designing a protein blend with relatively high amounts of leucine, arginine and cysteine contents and testing its effect in both standard and high fat high sucrose diets.

The possibility that our plant protein blend could stimulate muscle protein synthesis independently of feeding, as suggested by our results on adjusted FSR, was a surprise. The fact that muscle mass was not increased implies a corresponding increase in muscle proteolysis, and thus a higher turnover. To the best of our knowledge, such a result has not been described previously.

Currently, numerous studies are performed to assess the suitability of plant proteins to partially replace animal proteins. Single proteins and mixtures of proteins are

tested, as are crude food products (lentils for instance) and purified fractions. Plant crude products with a high protein content are more likely to have low digestibility (presence of anti-nutritional factors), and to be marginally deficient in certain IAA (lysine in cereals, sulfur AA in legumes).

Our approach was to mix numerous highly purified plant proteins, using industrial co-products when possible, thus lowering the risk of poor digestibility, mainly through the inactivation of trypsin inhibitors.

Blending sources also offer the opportunity to tailor AA composition. It was easy to find a blend with an excellent AA balance (5). A deficiency in one AA, especially in the long term, can affect tissue protein anabolism. However, another additional effect could occur. Indeed, studies using soy and wheat as protein sources observed an increase in urea synthesis (28). This was attributed to poor utilization of dietary AA in the intestine, leading in turn to a marked increase in free AA content in the portal vein, which could constitute a signal for the liver to increase urea synthesis, thus further limiting the availability of dietary AA for peripheral protein synthesis (28). The reason for the poor utilization of dietary AA after the ingestion of wheat or soy is not completely clear. It could result from a suboptimal IAA balance (for instance lysine in wheat) and / or in the lower proportion of total IAA among total AA (38% for soy *vs* 43% for milk proteins). It was also proposed that this phenomenon could result from modifications in digestion kinetics (29). Thus, it was shown that soy proteins could not stimulate muscle proteins in old rats to the same extent as whey (30) .

In our plant blend, the content of all the IAA was similar to or even slightly higher than that of milk proteins, except for lysine. Lysine content was 56% of milk protein content but 163% of wheat protein content and allowed a lysine intake much higher than the requirement of mature rats (31). However, the proportion of IAA was 38% in

our plant protein blend, likewise for soy. Given the excellent results we obtained with our plant blend regarding the long-term effect on muscle mass and the short-term effect on muscle protein synthesis, this suggests that the stimulation of urea synthesis observed with soy (29) is not related to the slightly lower IAA proportion but to possible specific digestion kinetics issues.

To the best of our knowledge, no similar plant protein mix has been studied in the long term in the context of muscle aging. In previous studies, combinations of cereals and legumes were studied, showing most of the time that protein retention was better with the mix than with cereals or legumes alone, but still lower than with animal proteins (32). Recently, Salles et al. (33) concluded that nitrogen balance, lean body mass, muscle mass and post-absorptive protein synthesis could be maintained during aging at the same level after two months of feeding with purified pea proteins compared with casein or whey. The same team compared casein, whey and legume enriched pasta (lentil, split pea, and faba bean), and showed that after 6 weeks in 22 month-old rats, despite lower digestibility and leucine content, legume enriched pasta allowed maintaining lean body mass, nitrogen balance and post-absorptive muscle protein synthesis (34). The authors argued that higher post-prandial insulin secretion and high glycine and arginine content could have been contributive factors. Our plant protein mix had similar glycine and arginine levels to that of legume enriched pasta (34), and higher levels of leucine and cysteine than pea proteins (33). If the results on muscle mass and function during aging were indeed mediated by these AA, our findings would confirm those of Salles et al. (33).

Another unexpected result of our study was that the high-energy diet (rich in fat and sucrose) seemed to have protective effects regarding lean body mass, independently of the type of ingested protein. Indeed, the high-energy diet prevented a decrease in

lean body mass during the first two months of the experiment. Our initial objective was to challenge the metabolism of aging rats by inducing insulin resistance to test the capacity of our plant protein mix with high arginine and cysteine contents to counteract the potential deleterious effects of the high-energy diet. It turned out that insulin and glucose levels were not affected by energy, although there was a marked gain in body weight and fat mass. Previously, we showed that replacing all starch by sucrose induced insulin resistance in old rats (27). However, such changes are not always obtained after high fat high sucrose diets in rats (23). In the present study, food intake remained moderate and decreased during aging, in particular in rats fed the high-energy diet. This may have attenuated the negative impacts of the high-energy diet, in particular on the regulation of lean body mass.

In situations where higher energy intake does not lower insulin action, it is considered favorable to protein metabolism, through classical sparing effects on intermediary metabolism (35). In addition, the high-energy diet increased the weight of animals, which then had to use more force to move in the cage, perhaps also explaining the better maintenance of lean body mass during the first two months of the experiment. A similar or slightly higher lean mass was also observed in other animal studies using high fat diets during aging (36-38). In humans, obesity is often associated with a higher lean body mass, and it was suggested that “carrying greater weight might have physical training-like effects” (39).

However, these “training effects” on lean body mass did not translate into improvements in muscle functionality. Indeed, two measurements obtained with the CatWalk XT system, the number of steps to perform the run and the swing speed, improved with time, but the high-energy diet significantly reduced this improvement.

It was surprising to observe an improvement with time in muscle functionality. Recently, comparing 6 and 24-month-old Wistar rats, Le Bacquer et al. (40) obtained a higher average speed, lower step number, and higher cadence in adult rats compared to older rats. This is what could be expected in response to the deleterious effect of aging. For the same parameters, we saw no direct effect of time on average speed, a contrary result for step number, and a similar result for cadence. Our studies are different because there was a large age difference in the study by Le Bacquer et al. (40), while in our study the measurements were repeated for the same animals. That is why we postulate that the improvements that we saw with time may have been due to habituation. Animals were more familiar with the task they were required to perform the second time. It makes sense that, despite habituation, high-energy fed animals would have greater difficulty in performing the task due to the fact that they were heavier the second time.

Besides energy, we found other significant effects of the type of protein with the CatWalk XT system. Compared to milk protein, plant proteins reduced the performances of rats regarding run duration and step cycle but improved their performances regarding average speed. These results are difficult to interpret, because 1) there was no effect of time alone; 2) they cannot be clearly linked to differences in body weight, lean body mass or muscle mass; 3) the same type of protein had positive and negative effects. Analyzing footfalls and locomotion characteristics by the CatWalk XT system is a complex process, probably involving not only neuro-muscular function but also brain function (memory, learning capacity, confidence). It is possible that these differences observed between animal and plant proteins could result from effects on brain function. Indeed, the type of protein ingested can affect brain function by changing the blood concentration of large

neutral AA and hence serotonine and catecholamine synthesis. Specific peptides generated during protein digestion could also influence brain function and some are found in dairy products (41). However, the tryptophan, tyrosine, and branched chain AA content was similar between milk proteins and our mix of plant proteins, and the amount and nature of bioactive peptides generated after the ingestion of milk proteins or our blend of plant protein remains poorly understood.

In conclusion, in this aging rat model, we showed that a tailored blend of plant protein can adequately replace milk proteins in the long term, as it resulted in no difference in the response of muscle protein synthesis to feeding, or in the evolution of lean body mass and muscle mass during aging. We could not test the capacity of these plant proteins to protect muscle in a situation of altered insulin sensitivity because the high fat high sucrose diet only induced a marked increase in body fat without obvious deleterious effects on insulin sensitivity. Many other aspects of substituting animal proteins with plant protein need to be examined in other studies, but the present findings offer an important proof of concept from the nutritional standpoint that appropriately blended plant proteins can have a high nutritional value even in demanding situations such as protein metabolism and sarcopenia in the elderly.

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LD, FM, DR, DH, LM designed the research. LD, JL, VM, MJ, LM conducted the research. LD, CLB, FM provided essential reagents. LD, FM, DR, LM analyzed data or performed statistical analysis. LD, FM, LM wrote the paper. LM had primary responsibility for the final content. All the authors have read and approved the final

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

Figure Legends

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

18-month-old rats were fed for 4 months with a diet containing as protein source either milk proteins or a plant protein mix combined with either standard energy content or high energy content with a high level of fat and sucrose: MSE = milk protein standard energy, PSE = plant protein standard energy, MHE = milk protein high energy, PHE = plant protein high energy. Rats ate *ad libitum*. Significant effects (SE) of repeated time variance analysis are given and the results showed that the time effect was significant, as were all the interactions (time x protein, time x energy, time x protein x energy). *: at this time point, there was a significant difference between MSE rats and PSE rats, $P < 0.05$. #: at this time point there was a significant difference between rats fed standard energy diets and rats fed high energy diets, $P < 0.05$. Values are means ± SE (n=18-20 per group).

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

See figure 1 for group description. Significant effects of repeated time variance analysis are given and the results showed that the time effect was significant, as were time x energy and time x protein x energy interactions. # : at this time point, there was a significant difference between rats fed standard energy diets and rats fed high energy diets, $P < 0.05$. Values are means ± SE (n=18-20 per group)

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

See figure 1 for group description. Body composition was measured before and after 2 and 4 months of the experimental diet. Fat mass and lean mass measurements are expressed in percent of the initial measurement for the first 2 months, and in percent of the value at 2 months for the last 2 months. Significant effects (SE) of variance analysis are given. a, b: means assigned with the same letter were not significantly different. *: significantly different from 0 ($P < 0.05$). Values are means ± SE (n=18-20)

Figure 4 Absolute synthesis rates (ASR) in the gastrocnemius muscle after 4 months of experimental diets.

See Figure 1 for group description. Absolute protein synthesis rates were measured in the gastrocnemius muscle either in the post absorptive state (PA) or in the post-prandial state (PP) in MSE, PSE, MHE and PHE rats, and are expressed in mg protein synthetized per day. Bigger red dots represent mean values, and smaller blue dots individual values (n= 5-9 per group). SE: variance analysis significant effects. Only nutritional state had a significant effect ($P = 0.02$).

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

See figure 1 for group description. Plasma glucose (A) and insulin (B) levels were measured in the post- absorptive state before and after 2 and 4 months of experimental diets. SE: significant effect of repeated time variance analysis. *: Variance analysis performed at this time point detected a significant effect of energy ($P = 0.01$); $: Variance analysis performed at this time point detected a significant effect of protein ($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:

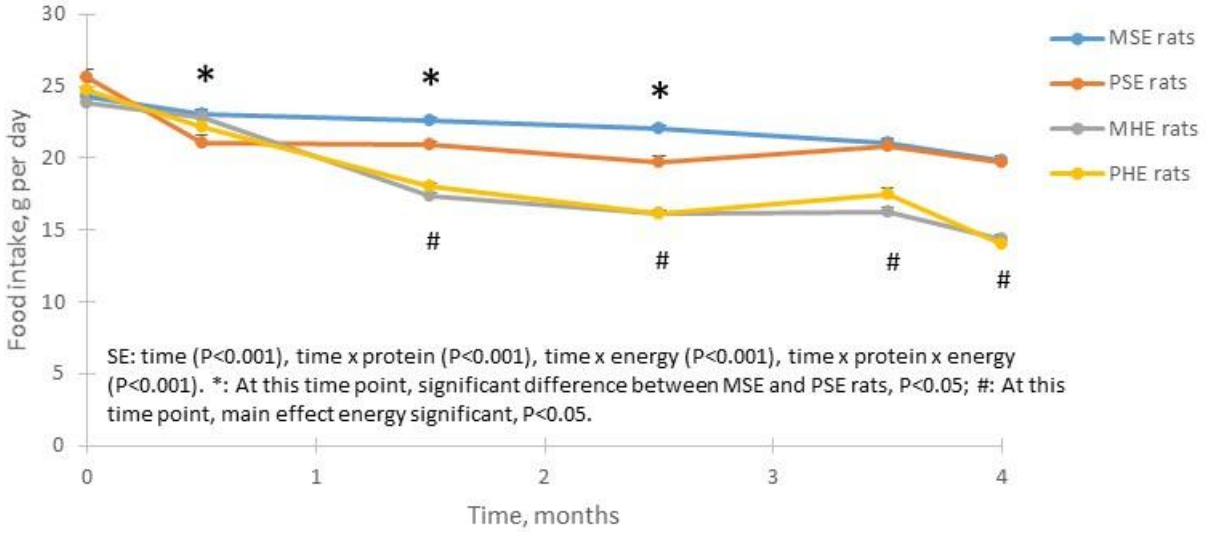

30
25
20
15
10
5
0
Food intake, g per day
0
1
2
3
4
Time, months
MSE rats
PSE rats
MHE rats
PHE rats
*
*
*
#
#
#
#
SE: time (P<0.001), time x protein (P<0.001), time x energy (P<0.001), time x protein x energy (P<0.001). *: At this time point, significant difference between MSE and PSE rats, P<0.05; #: At this time point, main effect energy significant, P<0.05.

Figure 2 :

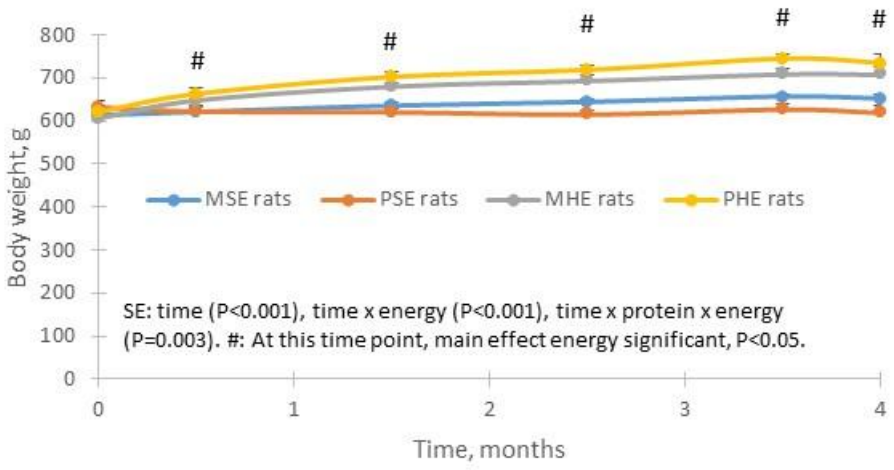
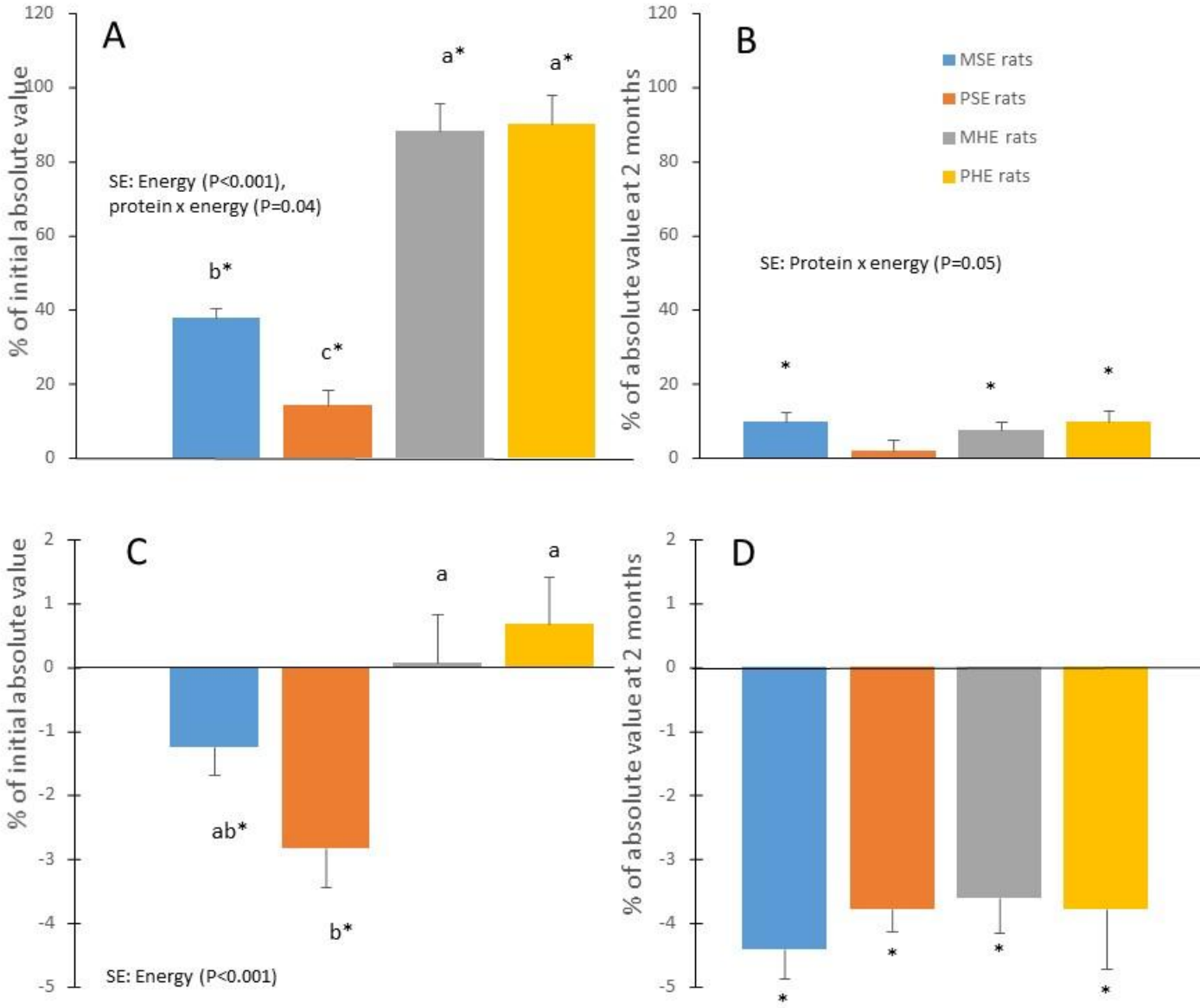

800
700
600
500
400
300
200
100
0
Body weight, g
#
#
#
#
#
MSE rats
PSE rats
MHE rats
PHE rats
SE: time (P<0.001), time x energy (P<0.001), time x protein x energy (P=0.003). #: At this time point, main effect energy significant, P<0.05.
0
1
2
3
4
Time, months

Figure 3:



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

Figure 4:

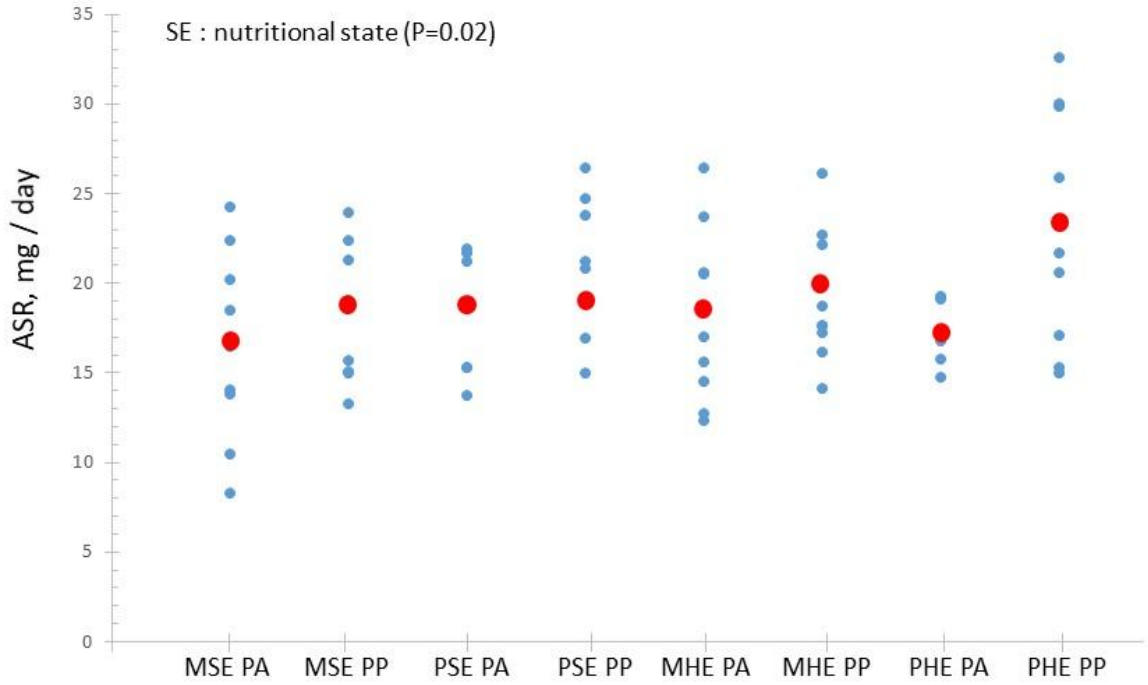
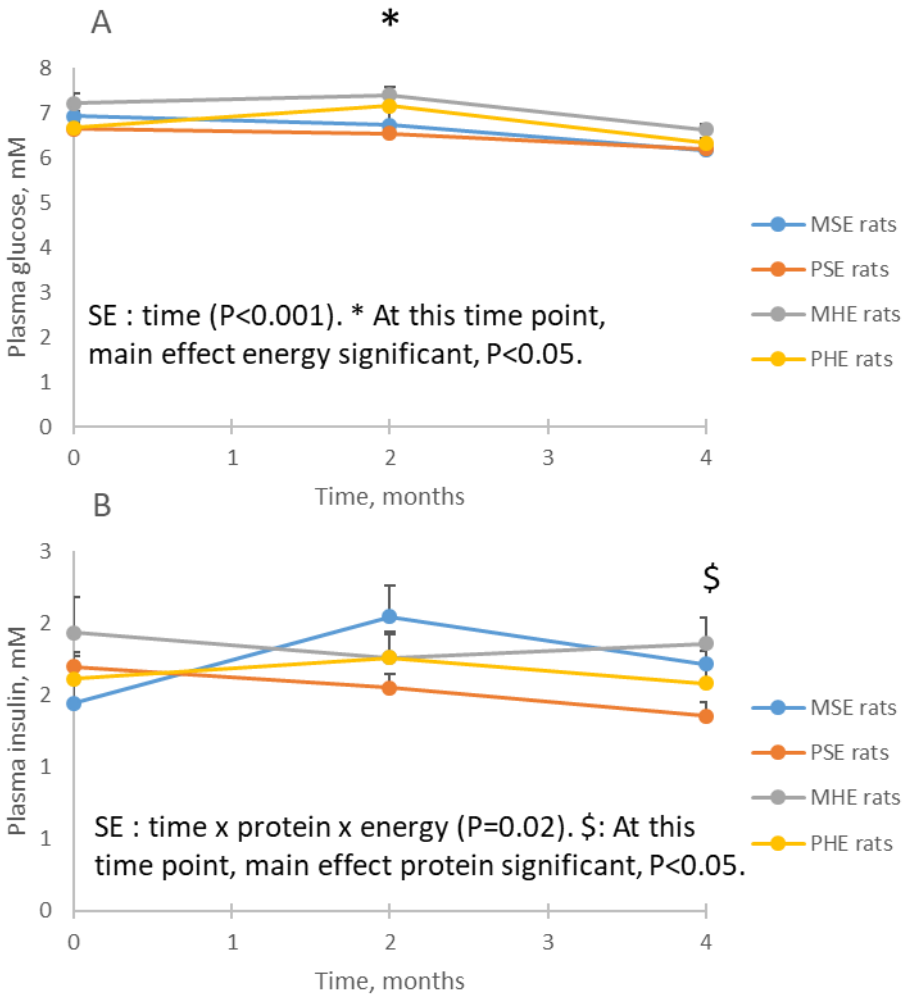

SE : nutritional state (P=0.02)
ASR, mg / day
35
30
25
20
15
10
5
0
MSE PA
MSE PP
PSE PA
PSE PP
MHE PA
MHE PP
PHE PA
PHE PP

Figure 5 :


A
*
Plasma glucose, mM
8
7
6
5
4
3
2
1
0
SE : time (P<0.001). * At this time point, main effect energy significant, P<0.05.
0
1
2
3
4
Time, months
MSE rats
PSE rats
MHE rats
PHE rats
B
$
Plasma insulin, mM
3
2
2
1
1
0
SE : time x protein x energy (P=0.02). $: At this time point, main effect protein significant, P<0.05.
0
1
2
3
4
Time, months
MSE rats
PSE rats
MHE rats
PHE rats