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FOOD & FUNCTION

Diet supplementation with donkey milk upregulates liver mitochondrial uncoupling, reduces energy efficiency and improves antioxidant and antiinflammatory defences in rats

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Dietary PUFA, mainly those of the *n*-3 family, are known to play essential roles in the maintenance of energy balance and in the reduction of body fat deposition through the upregulation of mitochondrial uncoupling that is the main source of reactive oxygen species. We hypothesized that rat supplementation with raw donkey's milk (DM), characterized by low-fat content and higher *n*3:*n*6 ratio, may affect energy balance, lipid metabolism, and prooxidant status as compared to animals treated with cow's milk. In the present study, the effects of drinking raw DM (for 4 weeks) on energy balance, lipid metabolism, antiinflammatory, and antioxidant/detoxifying defences was compared to that produced by rat intake of an iso-energetic amount of raw cow's milk. The hypolipidemic effect produced by DM paralleled with the enhanced mitochondrial activity/proton leakage and with the increased activity or expression of mitochondrial markers namely, carnitine palmitoyl transferase and uncoupling protein 2. The association of decreased energy efficiency with reduced proinflammatory signs (TNF-α and LPS levels) with the significant increase antioxidant (total thiols) and detoxifying enzyme activities (glutathione-*S*-transferase NADH quinone oxidoreductase) in DM-treated animals, indicated that beneficial effects were attributable, at least in part, to the activation of nuclear factor 2 erythroid-related factor 2 pathway.

Keywords:
Donkey milk / Energy expenditure / Inflammatory parameters / Mitochondrial efficiency / Redox status

Milk and milk products contain a number of essential nutrients but, in the western societies, their growing consumption has raised some concerns. Indeed, cow milk (CM) was recognized the leading cause for allergy however the positive association of higher dietary intake of saturated fatty acids, contained in milk and dairy foods, with the development of several human pathologies is still unclear [1]. During the past years, donkey milk (DM) has been indicated as promising food for children affected by cow's milk protein allergy or multiple food intolerance [2, 3] and, although the presence of higher *n*-3 PUFA content or *n*3:*n*6 ratio have been accounted for healthy effects produced by DM intake [4], nevertheless the underlying mechanisms have never been investigated. In this context, as mono and poly-unsaturated have been reported as the better substrates for mitochondrial β-oxidation pathway [5], which is central to the provision of energy and

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Abbreviations: **CM**, cow milk; **CPT**, carnitine palmitoyl transferase; **DM**, donkey milk; **EE**, energy expenditure; **FFA**, free fatty acids; **GSHtot**, total thiols; **GST**, glutathione-*S*-transferase; **ME**, metabolizable energy; **NQO1**, NADH quinone oxidoreductase; **Nrf2**, nuclear factor 2 erythroid-related factor 2; **UCP2**, uncoupling protein 2

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the main source of reactive oxygen species (ROS) [6], thus we decided to use DM as a model of naturally PUFA-rich milk and to compare the effects produced by CM intake on rodents lipid metabolism, redox status, and inflammatory response.

Fat content, fatty acid composition of DM, and CM were preliminarily determined and the obtained values were comparable with those reported in literature [4] (Supporting Information Table S1). Moreover, as both milk treatments were unable to alter cholesterol and alanine aminotransferase levels (marker of hepatic damage) (data not shown), thus their hepatotoxic effect can be excluded.

The energy intake of rats treated with different dietary supplement was first evaluated. Interestingly, despite the higher lipid content (27%) and the lesser carbohydrate content (10%) as provided by CM-supplemented diet, as compared to DM intake, a comparable energy density (isocaloric diet) was provided by the different treatments. In particular, the ingestion of comparable chow and milk amount (87 and 13%, respectively) in supplemented animals resulted in similar energy intake (Supporting Information Table S2). Next, when the effect of milk supplementation on body composition (water, lipid, and protein) was evaluated, increased relative percentage of lipids was observed in CM-treated animals ($p < 0.05$) whereas only unimportant alterations were found in DM-treated rats as compared to controls. Similarly, the significant reduction of serum triglycerids in DM-treated rats (as compared to controls), associated to decreased body weight, lipid gain, and liver lipids as compared to CM-treated animals ($p < 0.01$) (Table 1).

To investigate the mechanism underlying the hypolipidemic effect produced by DM supplement, metabolic parameters were evaluated. Interestingly, although CM and DM treatments resulted in similar increase of metabolizable energy (ME), nevertheless DM had no effect on body weight gain but improved animal energy expenditure (EE) and decreased energy efficiency, as compared to CM treated or to control rats. Moreover, CM supplement associated with higher protein gain, lipid gain, protein gain/ME intake, and lipid gain/ME intake, by contrary, these values were markedly reduced in DM-treated animals ($p < 0.01$). Interestingly, the enhanced energy efficiency found in CM-treated animals was consistent with increased lipid gain and body lipid content (Table 1), in addition, the milk-induced shift from glucose to fat as metabolic substrate milk was indicated by the low respiration quotient. Moreover the increased production of CO_2 and higher O_2 consumption in DM-treated animals were consistent with the increase of daily EE (Supporting Information Table S3). Taken together, these data demonstrated that DM intake improved the ability to utilize fat as metabolic fuel and suggested that, in these animals, almost all of the extra energy was dissipated through an increased metabolic activity.

As liver greatly contributes to whole-body EE and lipid utilization, thus protein mass, oxidative capacity, and energy efficiency were evaluated in mitochondria isolated from the liver of differently treated rats. The increased citrate synthase activity (known mitochondrial marker enzyme) in DM-supplemented animals as compared with controls

Table 1. DM supplement reduces lipid gain, energy efficiency, and triglycerides levels and improves energy expenditure

	Control	CM	DM
Body weight (g)			
Initial body weight	345 ± 5.0[a]	347 ± 4.0[a]	348 ± 6.0[a]
Final body weight	476 ± 2.0[a]	500 ± 1.0[b]	472 ± 5.0[a]
Body weight gain	131 ± 2.2[a]	153 ± 2.0[b]	125 ± 7.0[a]
Body composition (%)			
Water	62.5 ± 0.36[a]	58.6 ± 1.6[b]	63.9 ± 0.14[a]
Lipids	14.8 ± 0.17[a]	18.6 ± 1.1[b]	13.6 ± 0.13[a]
Proteins	14.4 ± 0.67[a]	14.4 ± 2.60[a]	13.5 ± 0.30[a]
Body energy (kJ/g)	9.2 ± 0.20[a]	10.7 ± 0.58[b]	8.5 ± 0.10[a]
Energy balance (kJ)			
ME intake	10148 ± 261[a]	11508 ± 172[b]	11856 ± 231[b]
Body weight gain (kcal intake /bw gain)	77.5 ± 5.5[a]	94.9 ± 6.3[a]	75.2 ± 4.2[b]
Energy efficiency (%)	0.14 ± 0.01[a]	0.23 ± 0.01[b]	0.09 ± 0.01[c]
Protein gain	245 ± 30[a]	552 ± 35[b]	115 ± 23[c]
Lipid gain	1226 ± 39[a]	2113 ± 120[b]	978 ± 66[c]
Protein gain/ME intake (%)	2.5 ± 0.3[a]	4.8 ± 0.5[b]	0.98 ± 0.1[c]
Lipid gain/ME intake (%)	12.1 ± 0.3[a]	18.39 ± 2.0[b]	2.54 ± 0.5[c]
Energy expenditure	8676 ± 293[a]	8843 ± 357[a]	10762 ± 141[b]
Lipid metabolism			
Liver lipids (mg/g)	2.7 ± 0.1[a]	3.3 ± 0.1[b]	2.6 ± 0.1[a]
TG (serum) (mg/dL)	117.6 ± 6.0[a]	130.2 ± 7.9[a]	89.0 ± 7.5[b]

Data are presented as mean ± SE from triplicate analyses on individual samples prepared ($n = 8$) for each experimental group. Differing superscript letters indicate statistically significant differences ($p < 0.05$). TG, triglycerides.

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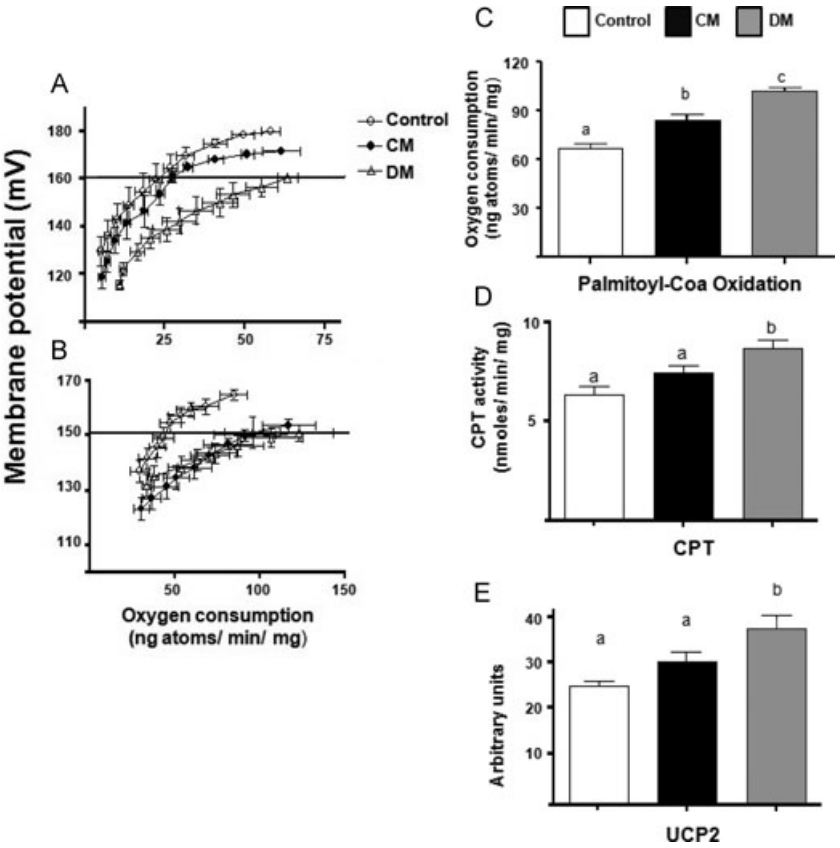

Figure 1. Dietary supplement with DM increases mitochondrial proton leakage. Respiration rates of mitochondria, isolated from the liver of CM-, DM-treated, or control animals were examined by interpolation at 160 mV (A) and 150 mV (B) for basal and palmitate-induced proton leaks, respectively. β-Oxidation rate (C), CPT activity (D) and UCP2 expression (E) in liver samples from milk supplemented and control animals were evaluated. Data are mean values ± SE from triplicate analysis on individual samples ($n = 24$). Differing superscript letters indicate statistically significant differences ($p < 0.05$).

(10.88 ± 1.14 or 7.18 ± 0.67 μmoles/min/g wet liver, respectively) was indicative of an increased mitochondrial protein mass. Next, the significantly higher State 3 and State 4 respiration rates, using succinate as a substrate, were found in CM-treated and DM-treated rats ($p < 0.05$). When glutamate was used as substrate, mitochondria from DM-treated rats showed higher State 3 and State 4 respiration rates (36 and 26%, respectively as compared to controls or to CM-treated rats (Supporting Information Table S4). As glutamate or succinate oxidation involves NADH or $FADH_2$-linked pathways, respectively, presented data demonstrated for the first time the ability of dietary DM to increase mitochondrial capacities in both NADH-linked and $FADH_2$-linked respiration pathways. Of note, as improved β-oxidation respiratory rate (State 3, using palmitoyl-carnitine as a substrate) in CM-treated rats was further increased in rats supplemented with DM (Fig. 1) thus we decided to investigate the effects produced by the different milk intake on the efficiency of free fatty acids (FFA) oxidation pathway.

Mitochondrial efficiency is mainly due to basal (i.e. in the absence of FFA) and to FFA-induced proton leakage. Basal proton leakage occurred in a variety of cells and tissues [7] and its key role in resting EE has been demonstrated [8]. To determine the effect of DM supplement on mitochondrial leakage, both basal and FFA-induced proton leakage was measured in differently treated animals. When basal proton leakage was evaluated in mitochondria prepared from milk-treated animals, DM-treated mitochondria showed the higher oxygen consumption to maintain the same membrane potential, as compared to untreated or CM-treated rats (Fig. 1A). By contrary, mitochondria from control animals (in FFA-acid-induced conditions) consumed less oxygen as compared to milk-supplemented animals that exhibited overlapping kinetic curves (Fig. 1B). The activity of carnitine palmitoyl transferase (CPT), the rate-limiting enzymes in the β-oxidation pathway [9] and the expression of uncoupling protein 2 (UCP2), which modulates the EE and FFA metabolism [10], were next determined. The significant increase of CPT and UCP2 levels further confirmed the DM ability to improve the FFA β-oxidation pathway (Fig. 1C and D). Taken together these results showed that dietary supplementation with DM associated with increased mitochondrial mass and activity (with both NADH-linked and $FADH_2$-linked substrates) and with reduced energy efficiency/higher proton leakage.

The protective role played by β-oxidation against oxidative stress along with the effect produced by mitochondrial ROS on UCP2 activity has been reported [11]. Herein, the intracellular concentration of the major redox buffers (total thiols (GSHtot)) and activity of an intramitochondrial sensor of redox status (aconitase) [12], were measured to investigate the effects produced by DM-induced mitochondrial leakage

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on animal antioxidant status. Interestingly, the beneficial effects produced by DM supplementation was indicated by the higher GSHtot concentration or the improved aconitase activity ($p = 0.0004$ or $p < 0.001$, respectively) in liver (Fig. 2A and B). To further investigate the mechanism underlying the anti-oxidant effect elicited by DM intake, activity of NADH quinone oxidoreductase (NQO1), and glutathione-*S*-transferase (GST) were measured. In response to mild oxidative stress the basic leucine-zipper transcription factor nuclear factor 2 erythroid-related factor 2 (Nrf2) is released from its inhibitor (Keap1) and triggers the activation of distinct set of genes encoding detoxifying enzymes including NQO1 and GST [13]. Thus the significant increase of GST or NQO1 activities in the liver of animals supplemented with DM ($p = 0.001$ or $p = 0.02$, respectively) indicated that the improved cytoprotective defenses resulting from DM intake were attributable, at least in part, to activation of Nrf2–ARE pathway.

Finally, the reported association of enhanced antiinflammatory defenses with Nrf2 signaling [14], prompted us to evaluate the consequences of DM intake on the levels of several proinflammatory indicators. The beneficial effects produced by DM intake was indicated by lower TNF-α concentration in DM-treated rats (Fig. 2C) and the LPS reduction (marker of metabolic and inflammatory diseases) (Fig. 2D) [15]. The immune-modulatory effect produced by DM intake was further supported by immune-histological evaluation of rodent livers (Supporting Information Fig. S1), and the lower concentration of serum TNF-α, as compared to liver, was indicative of an improvement of body antiinflammatory status.

Presented results demonstrate, for the first time, that dietary supplementation with DM milk increases EE and decreases body lipid accumulation via the mild augmentation of mitochondrial uncoupling pathway which associated to chemo-protective and antiinflammatory effects in rodents.

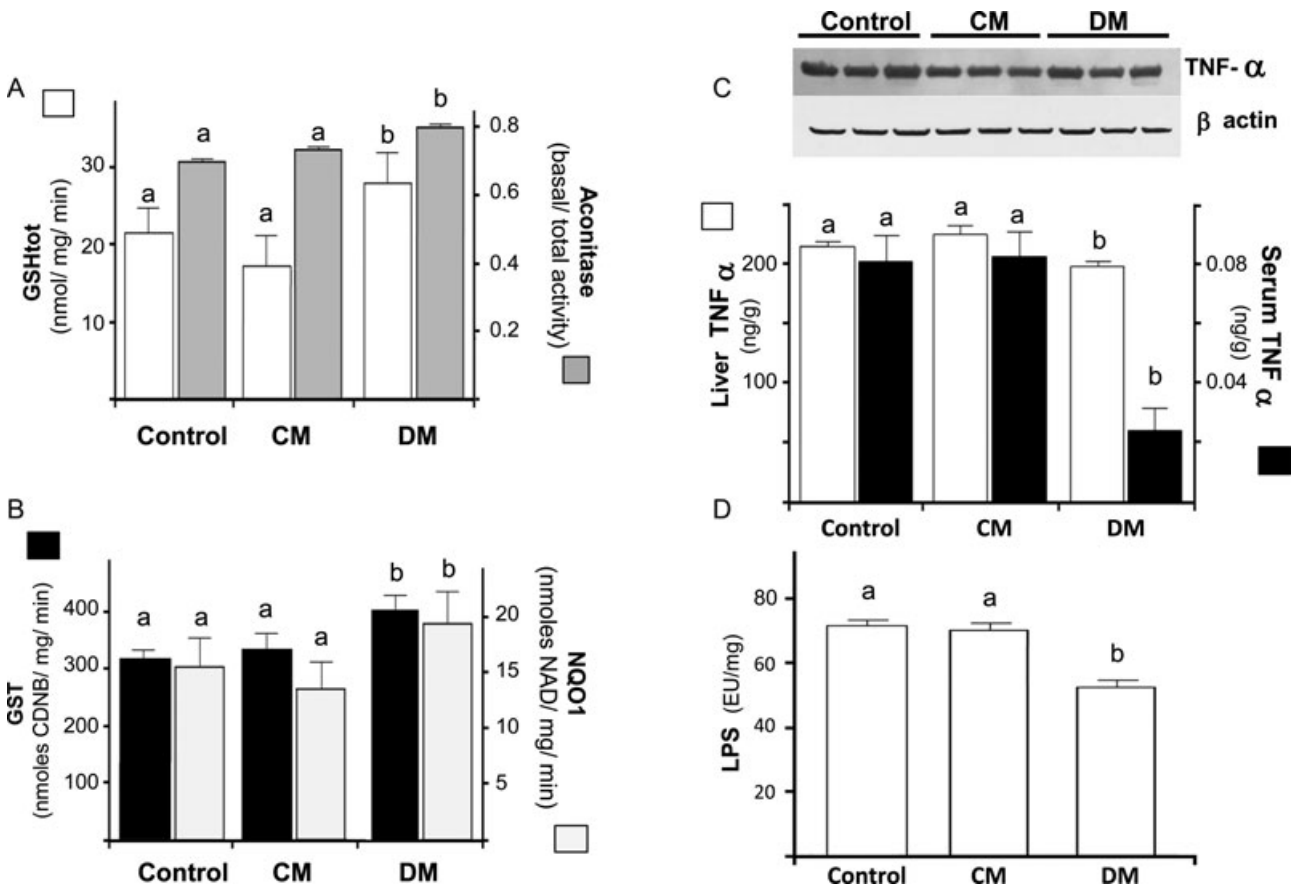

Figure 2. Effects of different milk supplement on anti-oxidant/detoxifying and on antiinflammatory status of rats. GSHtot content and aconitase activity (A), were measured in rat liver to examine the effects of the different treatments on animal redox status and their values were finally expressed as nmoles/mg/min or basal/total activity, respectively. GST and NQO1 activities (B) in liver were evaluated to determine the influence of the different milk supplement on detoxifying enzymes activity (expressed as nmoles chloro-dinitro benzene; CDNB/mg/min or nmoles NAD/mg/min, respectively). Hepatic expression of TNF-α, its concentration in blood or liver (C) and LPS levels in blood (D) were used to determine the effects of the different treatments on animal inflammatory status. TNF-α and LPS concentrations were expressed as ng/g protein or EU/mg protein, respectively. Representative Western immunoblot is shown (insert) and β-actin was used as loading control. Data are presented as mean values ± SE from triplicate analyses on individual samples ($n = 24$). Differing superscript letters indicate statistically significant differences ($p < 0.05$).

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In particular, metabolic shift associated to DM intake is not consistent with literature data produced by increased dietary fat/carbohydrate ratio [16, 17]. On the other hand as antiinflammatory effects associated to DM supplement resembles those resulting from *n*-3 PUFA intake [18], thus studies aimed at better understanding of *n*3 and *n*6 PUFA role, as well as Peroxisome proliferator-activated receptor (PPAR)α involvement, in the beneficial effects produced by DM consumption are in progress.

The authors have declared no conflict of interest.

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