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ORIGINAL CONTRIBUTION

Fermented goat milk consumption during anaemia recovery: ergogenic effect and improvement of skeletal muscle homeostasis

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Abstract
Purpose Anaemia is associated with fatigue and diminished muscular oxygenation, which may affect skeletal muscle (SM). No studies are available about the SM modifications during anaemia recovery; therefore, the aim of this study is to study SM homeostasis during anaemia recovery with fermented milks.
Methods Forty male Wistar rats were placed on a pre-experimental period of 40 days, divided in two groups (control group receiving normal-Fe diet and Fe-deficient group receiving low-Fe diet). Lately, rats were fed with fermented goat or cow milk-based diets, with normal-Fe content during 30 days. After feeding the fermented milks, leptin, adiponectin, non-esterified fatty acids (NEFA) and protein expression (UCP1, PepT1 and irisin) within the SM were assessed.
Results Adiponectin decreased in both groups of animals fed fermented goat milk, while leptin and NEFA increased. UCP1 protein expression increased in control and anaemic animals fed fermented goat milk. UCP1 also increased in both group of anaemic animals fed either fermented cow or goat milk in comparison with their controls. Irisin increased in both group of animals fed fermented goat milk. Finally, PepT1 also showed an increased expression in control and anaemic rats fed fermented goat milk and the anaemia also induced an over-expression of this transporter in animals fed either fermented cow or goat milk.
Conclusion Fermented goat milk consumption during anaemia recovery diminishes adiposity depots and enhances lipolysis, increasing UCP1, PepT1 and irisin protein expression, featuring an ergogenic effect in the SM which is an important endocrine regulator of body metabolism.

Keywords Skeletal muscle · Myokines · Fermented milks · Anaemia · Irisin

Introduction

Muscle is considered a secretory organ, secreting several products. Ectopic expression in skeletal muscle (SM) of uncoupling protein 1 (UCP1) leads to increased energy expenditure, delayed diet-induced obesity, reduced hepatic steatosis, improved glucose homeostasis and increased longevity in SM. This suggests a clear cross-talk between muscle and white adipose tissue mediated by myokines [1, 2], which emphasizes the role of muscles as a source of hormones to modify metabolism and energy expenditure [3].

In addition, it has been suggested that most of the beneficial health effects of exercise could be mediated in part by secretion of myokines by the SM that could counteract harmful effects of pro-inflammatory cytokines released by adipose tissue during obesity or metabolic disorders [4]. In this sense, irisin has a key role on SM mediating some health benefits, increasing energy expenditure and oxidative

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metabolism, stimulating UCP1 and rescuing capabilities in metabolically strained myotubes [5–7]. In addition, protein and amino acids are essential for muscle metabolism, and in this sense, the proton–peptide cotransporter (PepT1) is a member of the larger family of peptide transporters and mediates di- and tripeptide absorption [8], functioning as a nutrient transporter involved in the handling of body nitrogen and, although this transporter is mainly expressed in the enterocyte, we hypothesize that due to the key role of amino acids on muscle fibres synthesis, this transporter could be expressed in SM.

On the other hand, anaemia is a major public health problem and it is associated with loss of physical function, resulting in fatigue and diminished muscular oxygenation, which may affect SM strength and, subsequently, physical performance [9]. However, the recovery of the SM after induced anaemia and the pathway that may negatively affect physical function and muscle bioenergetics has not been studied extensively. Diet plays a key role on anaemia recovery, and dairy products have been reported to modulate energy metabolism, an effect traditionally correlated with the Ca content. A high Ca intake through fermented milk consumption led to a reduction in body fat, increasing lean mass [10]. Zemel et al. [11] developed a hypothesis based on animal and cell models which defends that an increase in Ca intake can mediate intracellular Ca concentrations within adipocytes via reductions in 1,25-dihydroxy-vitamin D_3 (calcitriol) and parathyroid hormone (PTH) concentrations. However, most of these studies have been performed during exercise and under conditions of an adequate nutritional status, not exploring many novel myokines or key proteins involved in energy metabolism during anaemia recovery.

Taking into account everything mentioned above, the present aim of our study is to evaluate the effects of fermented goat or cow milk consumption on target proteins and endocrine regulators involved in muscle metabolism and to investigate a possible ergogenic effect of fermented milks on SM during anaemia recovery.

Materials and methods

Fermentation and dehydration of milks

Fermented milks were prepared according to the method previously described by Moreno-Fernandez et al. [12]. To prepare the fermented milks, raw cow and goat milk was pasteurized at 77 °C for 15 min and cooled to room temperature (25 °C). The milk samples were transferred to sterile Schott flasks inside a laminar flow chamber and stored at 4 °C for 24 h before use. Subsequently, both milks types were inoculated with traditional yoghurt starters *Lactobacillus bulgaricus sub. delbruickii* and *Streptococcus thermophiles* (initial concentration of 1×10^{11} cfu/ml; 1 % inoculum) and incubated at 37 °C for approximately 24 h.

Subsequently, fermented milk samples were subjected to a smooth industrial dehydration process in an air tunnel with internal heaters mounted to the air in independent chamber that allow obtain a uniform distribution of the temperature and a rapid stabilization. In this device, an internal engine turbine with airflow improved the process of dehydration. The fermented milks were dehydrated in a forced-air tunnel (Conterm Selecta, Barcelona, Spain) at 50 ± 3 °C for 24 h, until the final moisture ranged between 2.5 and 4.5 %. These conditions avoid the loss of nutritional properties of the fermented milks.

Animals

All animal care procedures and experimental protocols were approved by the ethics committee (Ref. 11022011) of the University of Granada in accordance with the European Community guidelines (Declaration of Helsinki; Directive 2010/63/EU for animal experiments). Forty male Wistar albino breed rats (21 days of age and weighing about 42 ± 5 g) purchased from the University of Granada Laboratory Animal Service (Granada, Spain) were used during the study. Animal assays were carried out in the animal breeding unit of the Centre of Biomedical Research of the University of Granada in an area certified as free of pathogens, and the animals were kept in conditions of high biological safety, with sanitary and environmental rigorously controlled parameters.

During the course of the study, the animals were housed in individual, ventilated, thermoregulated cages with an automatically controlled temperature (23 ± 2 °C), humidity (60 ± 5 %) and a 12-hour light–dark cycle (9:00 to 21:00 h). Diet intake was controlled, pair feeding all the animals (80 % of the average intake) and deionized water was available ad libitum.

Design of experiment and diets

At the beginning of the study, 40 rats were divided into two groups: the control group receiving a normal-Fe diet (44.6 mg/kg by analysis) [13], and the anaemic group receiving a low-Fe diet (6.2 mg/kg by analysis), induced experimentally during 40 days by a method developed previously by our research group [14]. On day 40 of the study, two blood aliquots per rat were collected from caudal vein (with EDTA to measure the haematological parameters) and the rest of the blood was centrifuged (1500*g*, 4 °C, 15 min) without anticoagulant to separate the serum and subsequent analysis of serum Fe, total Fe-binding capacity (TIBC), ferritin and hepcidin.

Table 1 Composition of the experimental diets

Component	G/Kg diet
Pre-experimental period, standard (non-milk) diet[a]	
Casein	200
Lactose	0
Fat (virgin olive oil)	100
Wheat starch	500
Constant ingredients[b]	200
Experimental period	
Fermented cow milk-based diet[c]	
Protein	205
Lactose	295
Fat	100
Wheat starch	200
Constant ingredients[b]	200
Fermented goat milk-based diet[c]	
Protein	206
Lactose	291
Fat	100
Wheat starch	203
Constant ingredients[b]	200

[a] The diets were prepared according to the recommendations of the AIN-93G for control rats (45 mg Fe/Kg diet) (Reeves et al. [13]), or with low-Fe content (5 mg Fe/Kg diet) (Pallarés et al. [14]), for anaemic groups

[b] The constant ingredients consisted of (g/Kg diet): fibre (micronized cellulose) 50, sucrose 100, choline chloride 2.5, L-cystine 2.5, mineral premix 35 and vitamin premix 10

[c] Specific vitamin and mineral premixes supplements for fermented goat and cow milk-based diets were formulated taking into account the mineral and vitamin contents of the fermented milk powder supplied in order to meet the recommendations of the AIN-93G (Reeves et al. [13])

After the induction of the anaemia (day 40 of the study), the animals were then placed on an experimental period in which the control and anaemic groups were further fed for 30 days with fermented cow milk or fermented goat milk-based diet, with normal-Fe content (45 mg/kg), prepared with fermented cow (Holstein breed) or fermented goat milk (Murciano–granadina breed) powder (20 % of protein and 10 % of fat). The Fe content (mg/kg) in the diets by analysis was 43.7 (cow milk-based diet), 44.2 (goat milk-based diet) (Table 1). Diet intake was also controlled, pair feeding all the animals (80 % of the average intake) and bidistilled water was available ad libitum.

On day 70 of the study, animals were anesthetized intraperitoneally with sodium pentobarbital (Sigma-Aldrich Co., St. Louis, MO), totally bled out by cannulation of the aorta and blood alliquots with EDTA were analysed to measure the haematological parameters and the rest of the blood was centrifuged (1500×*g*, 4 °C, 15 min) without anticoagulant to separate the red blood cells from the serum and subsequent analysis of serum hepcidin, serum Fe, ferritin and TIBC. *Longissimus dorsi* muscles were removed, washed repeatedly with ice-cold deionized water, snap frozen in liquid nitrogen and immediately stored at −80 °C for protein extraction.

Haematological test

All the haematological parameters studied were measured using an automated haematology analyser Sysmex K-1000D (Sysmex, Tokyo, Japan).

Serum iron, total iron binding capacity (TIBC) and transferrin saturation

To calculate the rate of transferrin saturation, serum Fe concentration and TIBC were determined using Sigma Diagnostics Iron and TIBC reagents (Sigma-Aldrich Co., St. Louis, MO). The absorbance of samples was read at 550 nm on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The percentage of transferrin saturation was calculated from the equation:

$$\text{Transferrin saturation (\%)} = \text{serum Fe concentration } (\mu g/L)/\text{TIBC } (\mu g/L) \times 100$$

Serum ferritin

Serum ferritin concentration was determined by using the Rat Ferritin ELISA Kit (Biovendor Gmbh, Heidelberg, Germany). The absorbance of the reaction was read at 450 nm using a microplate reader (Bio-tek, VT, USA). Colour intensity developed was inversely proportional to the concentration of serum ferritin.

Serum hepcidin

Hepcidin-25 was determined using a DRG ELISA Kit (DRG Instruments GmbH, Germany). The microtiter wells are coated with a monoclonal (mouse) antibody directed towards an antigenic site of the Hepcidin-25 molecule. Endogenous Hepcidin-25 of a sample competes with a Hepcidin-25-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate was washed off and a streptavidin-peroxidase enzyme complex was added to each well. After incubation, unbound enzyme complex was washed off and substrate solution is added. The blue colour development was stopped after a short incubation time, turning the colour from blue to yellow. The microplate was read at 450 nm on a microplate reader (Bio-Rad Laboratories Inc., CA, USA), and the intensity of colour developed is reverse proportional to the concentration of hepcidin in the sample.

Assessment of body composition

Whole body composition (fat and lean tissues) was determined using quantitative magnetic resonance (QMR) with an Echo MRI Analyzer system by Echo Medical Systems (Houston, TX) (Tang et al. 2000). All QMR measurements were made during the light phase (09:00 a.m.–6:00 p.m.). Scans were performed by placing animals into a thin-walled plastic cylinder (3 mm thick, 6.5 cm inner diameter), with a cylindrical plastic insert added to limit movement. While in the tube, animals were briefly subjected to a low-intensity (0.05 T) electromagnetic field to measure fat, lean mass, free water and total body water. Briefly, this system generates a signal that modifies the spin patterns of hydrogen atoms within the subject and uses an algorithm to evaluate the four components measured—fat mass, lean muscle mass equivalent, total body water and free water. QMR scans were performed with accumulation times of 2 min.

Thyroid hormones, insulin, leptin and adiponectin measurement

Triiodothyronine (T_3), thyroxine (T_4) and thyroid-stimulating hormone (TSH), were determined using the RTHYMAG-30 K Milliplex MAP Rat Thyroid Magnetic Bead Panel; insulin and leptin were determined using the RMHMAG-84 K Milliplex MAP Rat Metabolic Hormone Magnetic Bead Panel; and adiponectin levels were measured using the RADPCMAG-82 K Milliplex MAP Rat Adipocyte Panel Metabolism Assay (Millipore Corporation, MI, USA), based on immunoassays on the surface of fluorescent-coded beads (microspheres), following the specifications of the manufacturer (50 events per bead, 50 µl sample, gate settings: 8000–15,000, time out 60 s, melatonin bead set: 34). Plate was read on LABScan 100 analyzer (Luminex Corporation, TX, USA) with xPONENT software for data acquisition. Average values for each set of duplicate samples or standards were within 15 % of the mean. Thyroid hormones, insulin, leptin and adiponectin concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

Non-esterified fatty acids (NEFA)

Non-esterified fatty acids (NEFA) are molecules released from triglycerides by the action of the enzyme lipase and are transported in the blood bound to albumin. They contribute only a small proportion of the body's fat; however, they provide a large part of the body's energy. NEFA were measured using a commercial kit (Randox Laboratories Ltd., Crumlin, UK). 50 µl of standards or serum samples were pipetted into Eppendorf tubes. Thereafter, 1 ml of the solution R1 was added to all tubes. The mixtures were vortexed for 5–10 s and incubated at 37 °C for 10 min. This was followed by adding 2 ml of solution R2; the tube was mixed and re-incubated at 37 °C. After 10 min, the mixture was measured for the optical density at 550 nm in a spectrophotometer (Bio-tek, VT, USA). In addition, all the standards, serum samples and assay-control were analysed in duplicate. The calculation of sample NEFA concentration was fitted by using the following equation:

$$\text{NEFA mmol/L} = (\text{Absorbance of sample}/\text{Absorbance of standard}) \times \text{concentration of standard.}$$

Western blot analysis and immunohistochemistry

Fifty milligram of *longissimus dorsi* muscle samples were added to a glass Potter–Elvehjem apparatus on ice, and whole cell extracts were obtained from the tissue by homogenization in 1:20 (w/v) of tissue to T-PER Reagent (Thermo Scientific Inc., Hanover Park, IL, USA). It was centrifuged to pellet tissue debris. Protease inhibitor (1:200 dilution; Sigma-Aldrich, St. Louis, MO, USA) was added, avoiding protein degradation. Total protein concentration was determined in extract using a Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Scientific Inc., Hanover Park, IL, USA). 12 µg of total protein from the extract was loaded in 4–20 % Criterion TGX (Tris–Glycine extended) gels (Mini-Protean TGX Precast Gels, 15 µL; 15 wells; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The electrophoresis was carried out at 250 V in a vertical electrophoresis tank (Mini-PROTEAN® System; Bio-Rad Laboratories, Inc.) for 20 min.

Fermentas PageRuler Plus Prestained Protein Ladder was used as molecular weight marker (Thermo Scientific Inc., Hanover Park, IL, USA). Subsequently, proteins were transferred from gel onto PVDF blots (Bio-Rad Laboratories, Inc.) by wet transfer for 60 min at 120 V with transfer buffer comprising 250 mM Trizma HCl, 200 mM glycine and 6 % methanol, pH 8.3 (Sigma-Aldrich). After transfer, the blots were blocked with 5 % dry milk in Tris-buffered saline (TBS) plus Tween-20 (TTBS) [0.8 % ClNa and 0.242 % 2-amino- 2-(hydroxymethyl)-1,3-propanediol] and 1 % Tween-20 (Bio-Rad Laboratories, Inc.) solution for 1 h at room temperature. Membranes were then washed three times in TBS, and finally incubated with rabbit polyclonal to anti-UCP1 antibody [(Abcam, UK (dilution 1:1000)], rabbit monoclonal anti-FNDC5 RabMAb (Irisin) antibody [Abcam, UK (dilution 1:800)], PepT1[Abcam, UK (dilution 1:250)] and mouse monoclonal to beta Actin antibody [Abcam, UK (dilution 1:1000)] as primary antibodies, in 5 % dry milk in TTBS overnight at 4 °C with shaking. β-actin was used as a control for total protein loaded.

Blots were then washed three times for 5 min each in TTBS and incubated with the appropriate secondary conjugated antibody [ImmunStar Goat Anti-Mouse (GAM)-HRP; 1:80,000 and Immun-Star Goat Anti-Rabbit (GAR)-HRP; Bio-Rad Laboratories Inc.; 1:50,000] in TTBS for 1 h at room temperature. The membranes were visualized with Luminata forte western HRP Substrate (Merck KGaA, Darmstadt, Germany). Signal quantification and recording densitometry of each band were performed with chemiluminescence in ImageQuant LAS 4000 (Fujifilm Life Science Corporation, USA). All results were analysed with Image J software.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using the SPSS computer program (version 22.0, 2013, SPSS Inc., Chicago, IL). All data are reported as mean values with their standard errors. Differences between groups fed normal-Fe- or low-Fe-content diets during the PEP were tested for statistical significance with Student's *t* test. Variance analysis by one-way ANOVA was used to compare the different diets supplied to the animals. Following a significant *F* test ($P < 0.05$), individual means were tested by pairwise comparison with the Tukey's multiple comparison tests, when main effects and interactions were significant. The level of significance was set at $P < 0.05$. A power analysis was performed to estimate the number of rats needed to obtain 80 % power at a confidence level of 95 %. Eight animals would be required to obtain 8 % differences in haematological parameters among diets. Similarly, seven animals per group would be required to obtain 10 % differences in gene expression patterns between diets. To ensure a power calculation, ten rats per groups were used.

Results

After Fe deprivation (5 mg/kg of diet) during 40 days, all the haematological parameters in the IDA group were different from those of the counterpart controls. All these parameters were statistically different between controls and anaemic rats ($P < 0.001$) (Table 2).

At the end of the EP, all the haematological parameters were recovered with both fermented milk-based diets; however, the platelet concentrations still were slightly elevated ($P < 0.05$) and serum Fe was lower ($P < 0.05$) in the

Table 2 Hematological parameters in control and anaemic rats fed either normal-Fe or low-Fe diets (PEP) or fermented cow or goat milk diets (EP)

	PEP		EP			
	AIN 93G diet		Fermented cow milk diet		Fermented goat milk diet	
	Normal-Fe control group ($n = 20$)	Low-Fe anaemic group ($n = 20$)	Control group ($n = 10$)	Anaemic group ($n = 10$)	Control group ($n = 10$)	Anaemic group ($n = 10$)
Total blood						
Hb concentration (g/L)	121.42 ± 2.65	59.97 ± 2.47 *	127.97 ± 2.65^{a}	130.25 ± 2.63^{A}	132.07 ± 2.68^{a}	129.01 ± 2.53^{A}
RBCs (10^{12}/L)	6.98 ± 0.22	3.05 ± 0.31*	7.14 ± 0.16^{a}	7.25 ± 0.25^{A}	7.36 ± 0.20^{a}	7.54 ± 0.27^{A}
Haematocrit (%)	42.09 ± 1.07	13.45 ± 1.47*	39.45 ± 1.31^{a}	40.21 ± 1.32^{A}	41.88 ± 1.11^{b}	42.05 ± 1.10^{B}
Platelets (10^{9}/L)	757 ± 73.54	2120 ± 117*	939.25 ± 69.67^{a}	973.25 ± 67.41^{A}	928.00 ± 77.84^{a}	941.67 ± 70.65^{A}
Serum						
Fe (µg/L)	1225 ± 97.51	617 ± 54.56 *	1341 ± 97.38^{a}	1230 ± 111.22^{A,C}	1359 ± 99.88^{a}	1348 ± 95.36^{B}
TIBC (µg/L)	2571 ± 153	17,641 ± 575 *	2841 ± 186^{a}	2599 ± 182^{A}	2787 ± 183^{a}	2753 ± 197^{A}
Transferrin saturation (%)	46.95 ± 3.75	4.18 ± 0.50 *	44.83 ± 3.84^{a}	44.29 ± 4.51^{A}	46.32 ± 4.65^{a}	46.94 ± 5.11^{A}
Ferritin (µg/L)	77.87 ± 2.10	47.98 ± 1.63 *	82.35 ± 2.87^{a}	81.97 ± 2.37^{A}	84.33 ± 2.33^{a}	82.34 ± 2.65^{A}
Hepcidin (ng/mL)	16.91 ± 0.62	13.41 ± 0.69*	14.50 ± 0.51^{a}	14.58 ± 0.49^{A}	16.67 ± 0.51^{b}	16.32 ± 0.60^{B}

Data are shown as the mean values ± SEM

Hb haemoglobin, *RBCs* red blood cells, *MCV* mean corpuscular volume, *MCH* mean corpuscular Hb, *MCHC* mean corpuscular Hb concentration, *RDW* red cell distribution width, *WBCs* white blood cells, *TIBC* total Fe-binding capacity

* Significantly different from the control group ($P < 0.001$, Student's *t* test)

[a,b] Mean values within a row and within control groups with different superscript lowercase letters differ ($P < 0.05$) by Student's *t* test

[A,B] Mean values within a row and within anaemic groups with different superscript capital letters differ ($P < 0.05$) by Student's *t* test

[C] Indicates difference ($P < 0.05$) for control versus anaemic group within a diet by the Tukey's test

Table 3 Body composition in control and anaemic rats fed either normal-Fe or low-Fe diets (PEP) or fermented cow or goat milk diets (EP)

	PEP		EP			
	AIN 93G diet		Fermented cow milk diet		Fermented goat milk diet	
	Normal-Fe control group ($n = 20$)	Low-Fe anaemic group ($n = 20$)	Control group ($n = 10$)	Anaemic group ($n = 10$)	Control group ($n = 10$)	Anaemic group ($n = 10$)
Weight (g)	243.71 ± 4.62	200.83 ± 2.61**	361.10 ± 9.48^{a}	346.51 ± 8.42^{A,C}	286.51 ± 6.23^{b}	263.45 ± 3.68^{B,C}
Fat (%)	7.45 ± 0.35	6.27 ± 0.39**	8.61 ± 0.70^{a}	8.96 ± 0.89^{A}	7.75 ± 0.84^{a}	6.98 ± 0.62^{B}
Lean mass (%)	90.56 ± 0.45	92.39 ± 0.61*	86.96 ± 0.73^{a}	86.66 ± 0.87^{A}	92.21 ± 1.90^{b}	92.10 ± 0.81^{B}
Free water (%)	0.41 ± 0.04	0.73 ± 0.09**	0.25 ± 0.03^{a}	0.22 ± 0.02^{A}	0.41 ± 0.07^{b}	0.42 ± 0.08^{B}
Total water (%)	76.75 ± 0.56	79.91 ± 0.48**	73.62 ± 0.58^{a}	73.58 ± 0.65^{A}	78.12 ± 1.48^{b}	78.51 ± 0.61^{B}

* Significantly different ($P < 0.01$) from the control group by Student's *t* test

** Significantly different ($P < 0.001$) from the control group by Student's *t* test

^{a,b} Mean values within a row and within control groups with different superscript lowercase letters differ ($P < 0.05$) by Student's *t* test

^{A,B} Mean values within a row and within anaemic groups with different superscript capital letters differ ($P < 0.05$) by Student's *t* test

^{C} Indicates difference ($P < 0.05$) for control versus anaemic group within a diet by the Tukey's test

Table 4 Plasma concentration of hormones affecting the basal metabolic rate and NEFA in control and anaemic rats fed either normal-Fe or low-Fe diets (PEP) or fermented cow or goat milk diets (EP)

	PEP		EP			
	AIN 93G diet		Fermented cow milk		Fermented goat milk	
	Normal-Fe control group ($n = 20$)	Low-Fe anaemic group ($n = 20$)	Control group ($n = 10$)	Anaemic group ($n = 10$)	Control group ($n = 10$)	Anaemic group ($n = 10$)
TSH (pg/mL)	30.23 ± 3.75	52.61 ± 5.62**	32.54 ± 2.23^{a}	31.67 ± 2.92^{A}	31.89 ± 1.88^{a}	31.97 ± 2.32^{A}
T_3 (pg/mL)	15,342 ± 429	10,164 ± 339**	13,213 ± 415 ^{a}	13,285 ± 387^{A}	13,725 ± 279^{a}	13,422 ± 416^{A}
T_4 (pg/mL)	1441 ± 96.74	1038 ± 93.32**	1378 ± 88.21^{a}	1340 ± 73.59^{A}	1298 ± 111^{a}	1355 ± 93.31^{A}
Insulin (pg/mL)	731.40 ± 31.55	859.37 ± 29.21*	735.25 ± 34.26^{a}	729.65 ± 44.61^{A}	740.53 ± 53.12^{a}	778.91 ± 83.91^{A}
Adiponectin (ng/mL)	1679 ± 139	1225 ± 107*	1426 ± 127^{a}	1484 ± 112^{A}	1054 ± 115^{b}	1023 ± 105^{B}
Leptin (pg/mL)	1131 ± 119	1635 ± 133**	1547 ± 75.88^{a}	1802 ± 85.81^{A}	2375 ± 132^{b}	2556 ± 113^{B}
NEFA (mmol/L)	0.40 ± 0.09	0.39 ± 0.07	0.47 ± 0.08^{a}	0.43 ± 0.07^{A}	0.63 ± 0.09^{b}	0.65 ± 0.08^{B}

*Significantly different ($P < 0.01$) from the control group by Student's *t* test

**Significantly different ($P < 0.001$) from the control group by Student's *t* test

^{a,b} Mean values within a row and within control groups with different superscript lowercase letters differ ($P < 0.05$) by Student's *t* test

^{A,B} Mean values within a row and within anaemic groups with different superscript capital letters differ ($P < 0.05$) by Student's *t* test

anaemic group fed the fermented cow milk compared with its controls, revealing that Fe repletion was more efficient with fermented goat milk (Table 2).

Body composition parameters showed differences between both experimental groups in the PEP. Weight and body fat were lower in the anaemic group compared with the control group ($P < 0.001$ for both). In contrast, lean mass, free water and total water were higher in the Fe-deficient group ($P < 0.01$ for lean mass and $P < 0.001$ for free and total water). During the EP, body weight was lower in control and anaemic animals fed fermented goat milk compared with those fed fermented cow milk ($P < 0.001$). Lean mass and total water were higher in both groups of animals fed fermented goat milk ($P < 0.001$) (Table 3).

Endocrine regulators of basal metabolism during the PEP also showed differences between both experimental groups (Table 4). Triiodothyronine (T_3) and thyroxine (T_4) decreased ($P < 0.001$), while thyroid-stimulating hormone (TSH) increased ($P < 0.001$) in the anaemic group. Adiponectin showed a decrease ($P < 0.001$ for ghrelin and $P < 0.01$ for adiponectin) in the anaemic group, while insulin and leptin increased in the Fe-deficient group ($P < 0.01$ for insulin and $P < 0.001$ for leptin). Fermented milks also induced changes in the endocrine regulators of basal

metabolism. No differences were recorded in thyroid hormones or insulin; however, adiponectin decreased in both groups of animals fed fermented goat milk ($P < 0.001$), while leptin and NEFA increased ($P < 0.001$). Plasma irisin (Fig. 1) decreased in both groups of anaemic animals ($P < 0.001$) and increased in both groups of animals fed fermented goat milk in comparison with fermented cow milk ($P < 0.001$).

With regard to target proteins studied involved in muscle metabolism, UCP1 protein expression increased in control and anaemic animals fed fermented goat milk in comparison with those fed fermented cow milk ($P < 0.001$). In addition, UCP1 also increased in both group of anaemic animals fed either fermented cow or goat milk in comparison with their counterpart controls ($P < 0.05$) (Figs. 2a, 3). PepT1 also showed an increased expression in control and anaemic rats fed fermented goat milk ($P < 0.001$), and the anaemia also induced an over-expression of this transporter in animals fed either fermented cow or goat milk ($P < 0.01$ for fermented cow milk and $P < 0.05$ for fermented goat milk) (Figs. 2b, 3). Finally, irisin increased in both group of animals (control and anaemic) fed fermented goat milk ($P < 0.001$) and decreased in anaemic animals fed fermented goat milk in comparison with controls ($P < 0.01$) (Figs. 2c, 3).

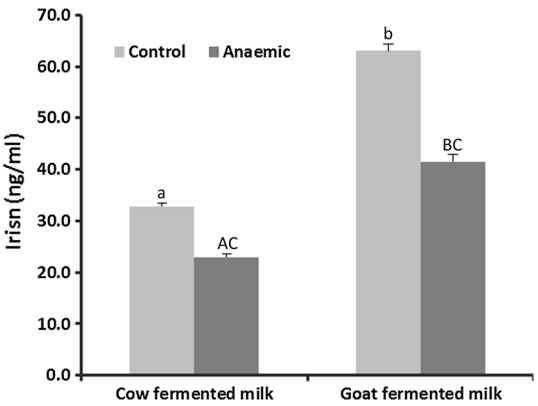

Fig. 1 Effect of fermented goat or cow milk-based diet consumption on plasma irisin levels in control and anaemic rats. Values are mean ± SEM ($n = 10$). [a,b]Mean values among groups of controls rats with *different superscript letters* in the same row were significantly different ($P < 0.05$, Tukey's test). [A,B]Mean values among groups of anaemic rats with *different upper case superscript letters* in the same row were significantly different ($P < 0.05$, Tukey's test). [C]Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's *t* test)

Discussion

After dietary Fe deprivation (PEP), all the haematological parameters of both experimental groups were drastically different from their controls. In the control rats, all haematological parameters studied were within normal limits for this species [15]. The most widely used screening

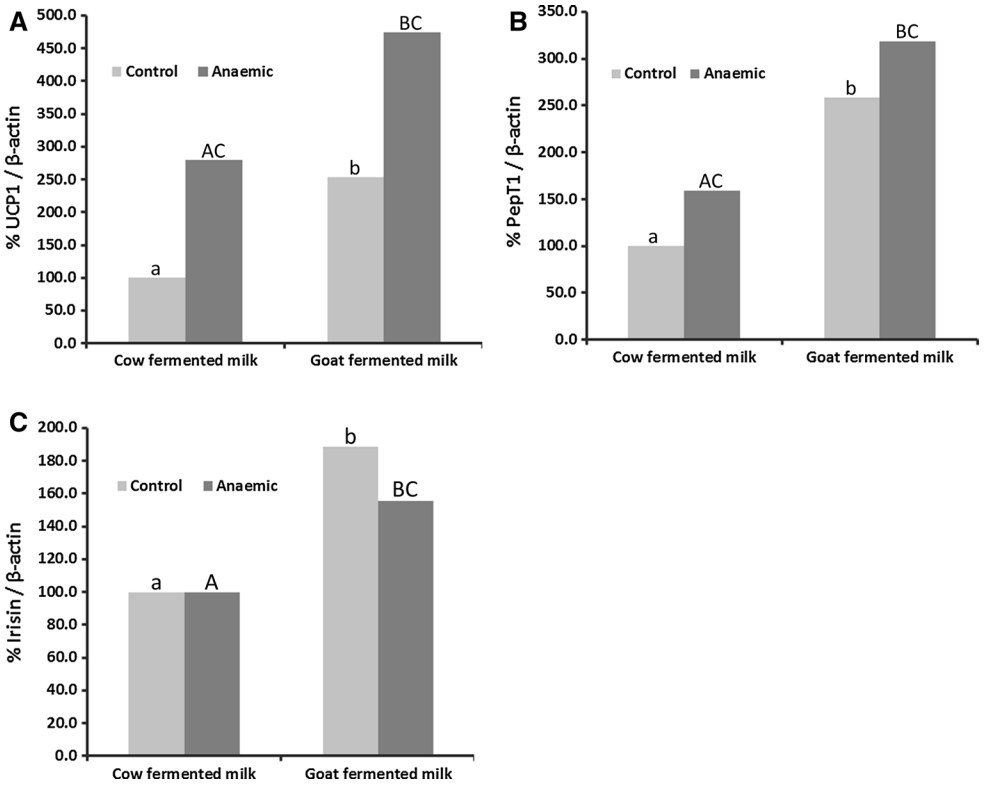

Fig. 2 **a–c** Effect of fermented goat or cow milk-based diet consumption on skeletal muscle expression of UCP1, PepT1 and irisin in control and anaemic rats. **a** Values are mean ± SEM ($n = 10$). **b** [a,b]Mean values among groups of control rats with *different superscript letters* in the same row were significantly different ($P < 0.05$, Tukey's test). [A,B]Mean values among groups of anaemic rats with *different upper case superscript letters* in the same row were significantly different ($P < 0.05$, Tukey's test). [C]Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's *t* test)

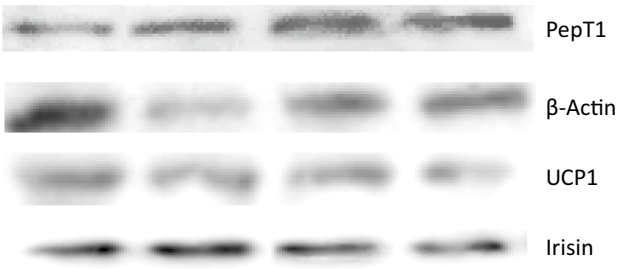

Fig. 3 Representative immunoblots of UCP1, PepT1, irisin and β-actin are shown

measurement in Fe status is Fe saturation of plasma transferrin, which is low in this pathology [16]. In addition, serum ferritin has been also one of the most useful laboratory measure of Fe status because is a well-standardized measurement that offers important advantages over bone-marrow examination for identifying Fe deficiency, because its concentration is directly proportional to body Fe stores, and finally, serum hepcidin levels are also dramatically reduced in the anaemic group, reflecting the impairment of Fe status, because its reduction is a really accurate indicator of Fe deficiency. At the end of the EP, after fed the animals with fermented cow or goat milk, all the haematological parameters were recovered, indicating that a normal-Fe status was established again in all the animals.

In the current study, although lean mass was not decreased by the induction of the anaemia, it is well known that Fe deficiency results in fatigue and diminished muscular oxygenation, which may affect muscle strength and quality and, subsequently, physical performance [9]. In our case, the degree of Fe deficiency produced by the Fe-restrictive diet is severe enough to impair the body weight, which coincides with the results of other authors [17] and it is possible to attribute this fact to the lower levels of thyroid hormones found in this pathology in the current study. Fe deficiency-induced alterations in central nervous system control of thermoregulatory responses are responsible for the lower thyroid hormone response, and the overall failure to properly thermoregulate that characterize Fe deficiency status, diminishing basal metabolic rate and weight gain. The increase in TSH would be a compensatory mechanism to induce T_3 and T_4 production, diminished by the Fe deficiency.

In the current study, we demonstrated that after supplying the fermented milk diets, body fat percentage was negatively associated with adiponectin levels, suggesting that this messenger is strictly associated with the fat mass reduction. A substantial amount of theory supporting the beneficial role of Ca intake in both SM and adipocyte fat metabolism is available on the scientific literature. Acute effects of Ca may result from an increase in adipocyte lipolysis, raising plasma NEFA availability and reduced inhibition of carnitine and long-chain fatty acid esterification. Chronic Ca intake may result in increased mitochondrial density, although all these effects are yet to be seen in human subjects during exercise, which itself produces shifts in calciotropic hormone concentrations [18]. Data from the present study are in agreement with a number of other studies which have shown beneficial effects of dairy product consumption on body composition [19, 20]; however, these studies were performed with dairy products from cow milk and in conditions of normal-Fe status. Previous research has shown that Ca may increase faecal excretion of fatty acids, including saturated fat and bile acids, minimizing effects on serum cholesterol and increasing energy loss which may impact on measures of adiposity [21] and also influences energy partitioning through lipogenesis and lipolysis. In this sense, as we have previously reported, goat milk increases Ca bioavailability [22], due to the higher content of vitamin D, which favours Ca energy-dependent transcellular saturable transport; therefore, this can be one reason to explain the effect of fermented goat milk effect diminishing adiposity depots and enhancing lipolysis in adipose depots. In this sense, one of the major determinants of endurance capacity and muscle function is increased fat oxidation, which leads to the sparing of glycogen consumption in SM during physical activity [23], and in this sense, serum levels of NEFA are one of the major fuels available when glycogen and glucose are used for oxidative metabolism during physical activity [24]. Therefore, the increase in NEFA levels in the animals consuming fermented goat milk indicates enhanced lipolysis, featuring an ergogenic effect due to the mentioned relationship between NEFA and physical performance.

We have found a noticeably over-expression of uncoupling protein 1 (UCP1) in SM of anaemic animals. This finding can be explained due to the fact that Fe deficiency is related to increased heat loss and reduced thermogenesis [25], and the over-expression of UCP1 in SM in both groups of anaemic animals obtained in the current study would be a compensatory mechanism to re-establish altered thermogenesis during the induction of the anaemia in the PEP. In this sense, heat production takes place through the uncoupling process, which uncouples respiration from ATP synthesis thereby dissipating chemical energy as heat, a process mediated by UCP1, a unique inner-membrane mitochondrial protein [26]; therefore, the increased expression of UCP1 in the anaemic groups would lead to an increased thermogenesis and heat production in these groups of animals. On the other hand, in the view of the current results, we can affirm that increased UCP1 protein expression in the animals fermented goat milk would lead to an increased thermogenesis, indicating once more enhanced muscle metabolism and corroborating

the ergogenic effect of fermented goat milk. This finding also suggests that the SM cells are well equipped in other thermogenic transcription factors, with good levels of mitochondria and indicates an improvement in fatty acids degradation, representing a positive growth advantage of SM cells with uncoupled respiration [27].

PepT1 has been shown to be regulated by hormones such as insulin, thyroxin, and mainly leptin that influence energy balance, food consumption, and appetite [28–30]. Between the physiological regulators of PepT1, leptin is the key regulator of PepT1 expression and activity and an increase in leptin plasma levels is associated with enhanced PepT1 function, which physiological relevance increasing peptide absorption. Leptin exerts a dual (short term and long term) effect on PepT1 function: increasing translocation of PepT1 molecules from cytoplasmic pool to apical membrane and activation of the transcription of PepT1 gene and/or enhanced of PepT1 mRNA stability to reconstitute cytoplasmic pool of PepT1 transporter [28]. In this sense, fermented goat milk increased leptinemia in both groups of animals, explaining the higher protein expression of PepT1 in SM. To our knowledge, this is the first study reporting an expression of PepT1 in SM, because most of the studies performed have reported that this peptide is mainly expressed in the enterocyte [28–30], and taking into account, the physiological role of PepT1 in peptide uptake, the increased expression of this transporter in the SM could represent an ergogenic effect, favouring muscle fibre synthesis and regeneration in the animas fed fermented goat milk and also explaining the higher amount of lean mass in these animals.

Fermented goat milk consumption greatly increased irisin levels in plasma and expression in the SM. Irisin is a myokine, which triggers the conversion of white adipose tissue to beige and/or brown adipose tissue (browning), leading to increased energy expenditure and, subsequently to improved tissue metabolic profile, by promoting weight loss, improved glucose tolerance, insulin sensitization and muscle performance [5, 31]. Under physiological conditions, physical activity stimulates irisin mRNA expression, revealing its important role in muscle fibres differentiation and functioning [32], fact that indicates once more the beneficial effect of fermented goat milk in SM function. The increased expression of irisin can also explain the enhanced lipolysis in adipose depots, the increased concentrations of NEFA in plasma, significant reductions in the visceral adipose tissue mass and the increased lean mass in animals fed fermented goat milk.

Conclusion

Fermented goat milk consumption during anaemia recovery enhances lipolysis and thermogenesis in the SM, which is an important endocrine regulator of body metabolism. In addition, a noticeably over-expression of UCP1 was found in SM during Fe deficiency recovery with fermented milks, finding that would be a compensatory mechanism to re-establish altered thermogenesis during the induction of the anaemia. UCP1 protein expression increased in the animals fermented goat milk, indicating enhanced muscle metabolism, increased thermogenesis, good levels of mitochondria and an improvement in fatty acids degradation, representing a positive growth advantage of SM cells. The increased expression of PepT1 in the SM of animals fed fermented goat milk could favour muscle fibre synthesis and regeneration, explaining also the higher amount of lean mass in these animals. Irisin up-regulation in SM of animals fed fermented goat milk contributes to a favourable

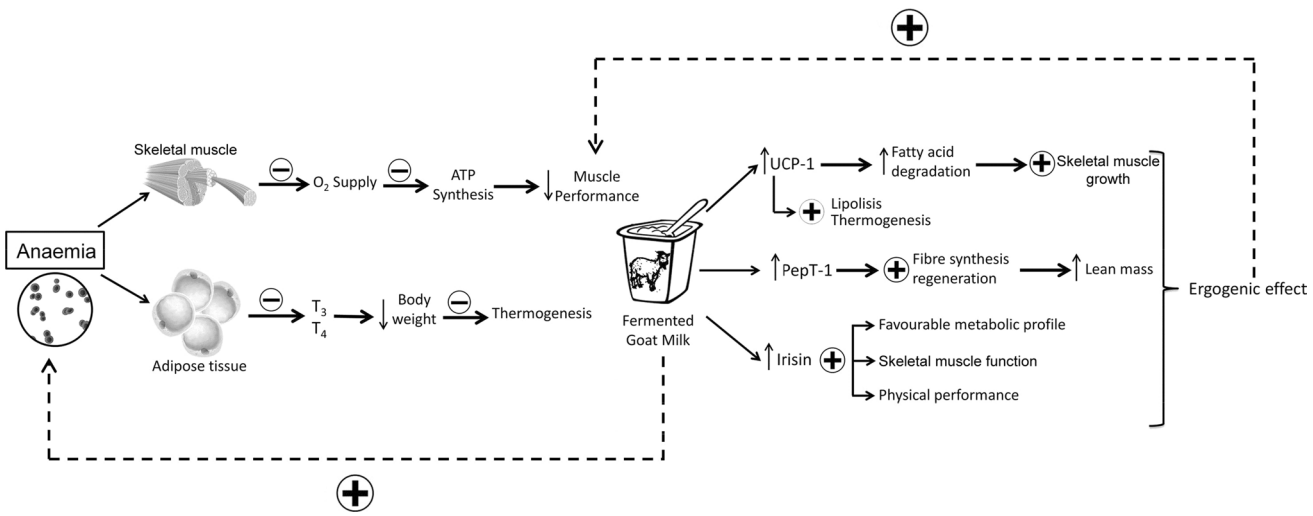

Fig. 4 Schematic effects of anaemia and fermented goat milk on skeletal muscle metabolism and UCP1, PepT1 and irisin protein expression

metabolic profile and SM function, improving also physical performance. These findings indicate an ergogenic effect of fermented goat milk consumption in the SM that could have important translational consequences, since the SM is implicated in many metabolic pathways, and fermented goat milk consumption improves SM homeostasis (Fig. 4).

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