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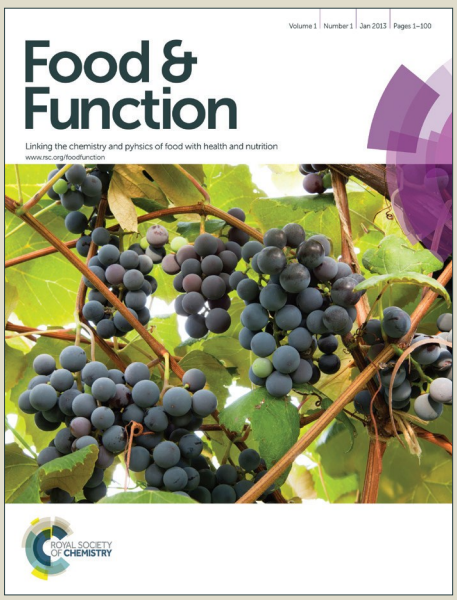


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ARTICLE

# Fermented goat milk consumption improves melatonin levels and influences positively antioxidant status during nutritional ferropenic anemia recovery.

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Jorge Moreno-Fernandez[a,b], Javier Diaz-Castro[a,b], Mª José M. Alférez[a,b], Teresa Nestares[a,b], Julio J. Ochoa[a,b], Ana Sánchez-Alcover[a,b] and Inmaculada López-Aliaga[a,b,†]

The aim of the current study was to asses the influence of fermented goat or cow milk on melatonin levels and antioxidant status and during anemia recovery. Eighty male Wistar rats were placed on a pre-experimental period of 40 days and randomly divided in two groups, a control group receiving normal-Fe diet (45 mg/kg) and the Fe-deficient group receiving low-Fe diet (5 mg/kg). Lately, the rats were fed with fermented goat or cow milk-based diets, with normal-Fe content or Fe-overload (450 mg/kg) during 30 days. After 30 days of feeding the fermented milks, total antioxidant status (TAS) was higher in both groups of animals fed fermented goat milk with normal-Fe content. Plasma and urine 8-OHdG were lower in control and anemic rats fed fermented goat milk. Melatonin and corticosterone increased in the anemic groups during Fe replenishment with both fermented milks. Urine isoprostanes were lower in both groups fed fermented goat milk. Lipids and protein oxidative damage were higher in all tissues with fermented cow milk. During anemia instauration, an increase in melatonin was observed, fact that would improve the energy metabolism and impaired inflammatory signaling, however, during anemia recovery, fermented goat milk had positive effects on melatonin and TAS, even in situation of Fe-overload, limiting the evoked oxidative damage.

## Introduction

Although Fe is relatively an abundant mineral in normal diets, it has low bioavailability, and Fe deficiency represents one of the most common nutritional deficiencies worldwide, with significant implications to public health [1]. Total body Fe content is maintained by balancing loss of Fe with assimilation via nutritional intake. The most obvious manifestation of Fe deficiency is anemia, but lack of Fe can also have an adverse effect on the immune system [2] and cognitive development [3]. Fe is an essential nutrient because Fe cofactors, including heme and iron-sulfur clusters, are required for the activity of a number of enzymes involved in a range of cellular processes. Furthermore, Fe is also an important component of molecules that undergo redox reactions in cells [4].

Additionally, Fe overload is common during anemia recovery, especially in industrialized countries where red meat consumption and the use of Fe fortification products are widespread [5]. Hepatotoxicity and spleen dysfunction are pathological findings in patients with Fe overload. The aetiology of these multiple organ dysfunctions could be attributed to the presence of excess free Fe released through the breakdown of heme by heme oxygenase, which is ubiquitous abundant in such reticuloendothelial organs [6]. This free Fe increases oxidative stress via generation of reactive oxygen species (ROS) [7] as well as depletes cellular stores of antioxidants [4]. Consequently, it is important to maintain Fe homeostasis via ensuring proper iron supply while preventing accumulation of excess Fe during anemia recovery [8], fact that would lead to an adequate antioxidant status.

On the other hand, fermented milks are traditionally used foods, and the use of goat's and cow's milks as raw materials is well established in the modern dairy industry. Beyond the simple nutritional value of milk compounds, other components, in fermented milks includes glycoproteins, antibodies and oligosaccharides, which have a key role reducing the number of pathogen infections and promoting the development of the intestinal epithelium. Therefore, fermented milk is more than a simple source of essential nutrients [9].

Goat milk is considered a healthy food and its consumption has positive influence on Fe metabolism [10], bone turnover 1[1] and enzymatic antioxidant defence [7, 12]. Taken together, anemia pathophysiologic complexity intimately related with a hyperoxidative status induced by Fe-overload during anemia

[a]Department of Physiology, University of Granada, Granada, Spain.
[b]Institute of Nutrition and Food Technology "José Mataix Verdú", University of Granada, Granada, Spain.
† Corresponding author:
Mª Inmaculada López-Aliaga, Department of Physiology, Faculty of Pharmacy, Campus Universitario de Cartuja, University of Granada, 18071, Granada, Spain. Tel: 34-958-243880, Fax: 34-958-248959, E-mail address: milopez@ugr.es

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recovery, this study aimed to assess the influence of fermented goat or cow milk on antioxidant status and melatonin levels, because this hormone takes part in a variety of molecular pathways, such as apoptotic, anti-proliferative, anti-metastasis, anti-angiogenesis, anti-inflammatory and due to its potent anti-oxidative activity, it has been recognized as an organ protective and anti-ageing agent. Total antioxidant status and the oxidative stress-mediated damage to the main biomolecules (lipids, protein, DNA and prostaglandins), were measured to verify the possible influence of fermented milks on melatonin levels and the indirect action of this hormone in oxidative stress due to modulation of the antioxidant defenses.

## Materials and Methods

### Fermentation and dehydration of the milks

To prepare the fermented milks, raw cow and goat milk was pasteurized at 77°C for 15 min and cooled to room temperature (25ºC). 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 1×1011 cfu/ml; 1 % inoculum) and incubated at 37°C for approximately 24 h. At the end of fermentation, the fermented milks were cooled to 15°C in an ice bath, and the clot was broken up with a stainless steel perforated disk with up and down movements for approximately 1 min. The fermented milk samples were evaluated for pH) using digital pH meter (Crison, Barcelona, Spain) and the fermentation process ended when the milks reached pH=4.6. All the experiments were carried out in triplicates.

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 air flow improved the process of dehydration. The fermented milks were dehydrated in a forced-air tunnel (Conterm Selecta, Barcelona, Spain) at 50 ± 3ºC 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.

### Total nitrogen, dry matter, ash, total fat, lactose and minerals content

The N content was measured using the Kjeldahl method [13]. Protein N content was calculated as the difference between total N and non-protein N. Protein, casein and whey-protein N values were converted to protein, casein and whey-protein by multiplying by a factor of 6.38. Dry matter, ash and total fat were determined according to the methods described by AOAC [14]. Milk lactose was calculated as the difference between the amount of total solids and protein + fat + total ash. The ash content was determined by incineration in an electric muffle furnace at 550°C. Mineral content in the fermented milks and diets was assessed by multi-elemental analysis by inductively coupled plasma-optical emission spectrometer (ICP-OES). Samples were previously mineralized by wet method in a sand bath (J.R. Selecta, Barcelona, Spain) using $HNO_3$ followed by a mixture of $HNO_3$:$HClO_4$ (69%:70%, v/v; Merck KGaA, Darmstadt, Germany; ratio 1:4, v/v) until the total elimination of organic matter. Ca, P, Fe, Zn, Cu, Mg, Na and K analysis was undertaken using an ICP-OES Optima 8300 (PerkinElmer Inc. Waltham, USA) with a segmented-array charge-coupled Device (SCD) high-performance detector. Elements were analysed according to compatibility under optimised set of conditions. Five different analytical methods were used where either sample preparation or instrument optimisation were consistent on specifications suitable for each element being analysed. For the calibration of the apparatus, multi elemental Astasol calibration solutions (Analytika, Khodlova, Prague) were used. For the calibration curve, the following working dilutions of the analytical standard were prepared: 0.1, 0.5, 1.0, 10, 50 mg $L^{-1}$. An internal standard solution of 10 mg L–1 was used after each series of 5 samples. The acceptable result was assessed as 10%. The samples were measured in three replicates.

### Animals

All animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada (Ref. 11022011) in accordance with the European Community guidelines (Declaration of Helsinki; Directive 2010/63/EU for animal experiments). 80 male Wistar albino breed rats (21 d of age and weighing about 42 ± 5 g), purchased from the University of Granada Laboratory Animal Service (Granada, Spain) were used during the study. Animals 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 (22-23°C), humidity (55-65%) and a 12-hour light-dark cycle (9:00 to 21:00). Diet intake was controlled, pair feeding all the animals (80% of the average intake) and bidistilled water was available *ad libitum*.

### Experimental design

At the beginning of the study 80 rats were divided into two groups: the control group receiving a normal-Fe diet (44.6 mg/kg by analysis) [15], and the anemic group receiving a low-Fe diet (6.2 mg/kg by analysis), induced experimentally during 40 d by a method developed previously by our research group [16].

After the induction of the anemia (day 40 of the study), the animals were then placed on an experimental period in which

the control and anemic groups were further fed for 30 days with fermented cow milk or fermented goat milk-based diet, with normal-Fe content (45 mg/kg) or Fe-overloaded (450 mg/kg) to induce chronic Fe-overload [17], 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 were: normal-Fe diet: 42.7 (cow milk-based diet), 43.5 (goat milk-based diet) and Fe-overload diets: 472.2 (cow milk-based diet) and 472.8 (goat milk-based diet) (Table 1).

On day 70 of the study, urine samples were collected to measure 15-F2t-isoprostanes and 8-hydroxy-2´-deoxyguanosine (8-OHdG) and animals were anesthetized intraperitoneally with sodium pentobarbital (Sigma Diagnostics, St Louis, MO), totally bled out by cannulation of the aorta and blood aliquots with EDTA were analysed to measure the hematological parameters and the rest of the blood was centrifuged (1500g, 4ºC, 15 min) to measure plasma thiobarbituric acid–reactive substances (TBARS), carbonyl groups, 8-OHdG, total antioxidant status (TAS), melatonin and corticosterone. The remaining blood was centrifuged without anticoagulant to separate the red blood cells from the serum and subsequent analysis of Fe, ferritin and total iron binding capacity (TIBC). The liver, brain and duodenum were removed and washed with ice-cold saline solution (0.9% w/v, NaCl). Brain, liver, duodenal mucosa and erythrocyte cytosolic fractions were prepared fresh the same day by successive differential centrifugations with hypotonic hemolysis according to the method of Hanahan and Ekholm [18], preserving these cytosolic fractions at -80°C for further analyses of protein oxidation (carbonyl groups) and thiobarbituric acid reactive substances (TBARS). Protein contents in the cytosolic fractions were measured following the method described by Lowry et al., [19].

**Hematological test**

All the hematological parameters studied were measured using an automated hematology analyzer Sysmex K-1000D (Sysmex, Tokyo, Japan).

**Serum ferritin**

Serum ferritin concentration was determined 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, Vermont, USA). Colour intensity developed was inversely proportional to the concentration of serum ferritin.

**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 Diagnostics). 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:

Transferrin saturation (%) = serum Fe concentration (µg/L)/TIBC (µg/L) x 100

**Thiobarbituric acid reactive substances (TBARS) measurement**

The lipid peroxidation was evaluated on plasma, liver, brain, duodenal mucosa and erythrocyte cytosolic fractions by measuring the concentration of TBARS according to the methods of Yagi [20] and Ohkawa et al., [21]. The reaction product was measured by spectrophotometric analysis (Bio-tek,Vermont, USA) at 532 nm. The assay was calibrated using tetraethoxypropanone (Sigma-Aldrich, Taufkirchen, Germany) as a malondialdehyde source.

**Protein oxidation (carbonyl groups) measurement**

The plasma, liver, brain and duodenal mucosa protein oxidation was measured according to a method based on the spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl (PC) to form protein hydrazones [22]. Each sample of extracted protein was treated either with 10 mM DNPH in 2.5 M HCl and 2.5 M HCl. The final precipitates were dissolved in 2 ml of 6 M guanidine hydrochloride solution and were left for 10 min at 37º C. Because about 10-15% of proteins were lost in the various washing steps, a procedure was adopted to determine the protein levels in the actual final pellets after all washings were finished. The pellets derived from the 2.5 M HC1 treated samples were dissolved in 6 M guanidine hydrochloride, and the proteins were quantified by reading the absorption at 280 mm (Bio-tek, Vermont, USA). The level of PC was calculated at the maximum absorbance (366 nm) of the DNPH-treated samples.

**8-hydroxy-2´-deoxyguanosine (8-OHdG)**

8-OHdG is an oxidized nucleoside of DNA, excised during the repair of oxidative damage to deoxyguanosine sites in DNA and it has been widely used as a biomarker for DNA oxidative damage. 8-OHdG in plasma and urine was measured using a commercial kit (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan). The 8-OHdG Check is a competitive in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative detection of the oxidative DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG). To separate interfering substances, filtration of serum using an ultra filter (cut off molecular weight 10000) was done. Results were read at 450 nm on a microplate reader (Bio-tek, Vermont, USA).

**15-F2t-isoprostanes**

The isoprostanes are prostaglandin-like compounds formed in vivo from the free radical-catalyzed peroxidation of essential fatty acids. Isoprostanes in urine were measured using a commercial kit Enzyme Immunoassay for Urinary Isoprostane (Oxford Biomedical Research, Oxford, England), a competitive enzyme-linked immunoassay (ELISA) for determining levels of 15-F2t-Isoprostane (the best characterized isoprostane) in urine samples. Urine samples are mixed with an enhanced dilution buffer that essentially eliminates interference due to non-specific binding. The 15-F2t-Isoprostane in the samples or standards competes with 15-F2t-Isoprostane conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-F2t-Isoprostane coated on the microplate. The HRP activity results in colour development when substrate is added, with the intensity of the colour proportional to the amount of 15-F2t-Isoprostane-HRP bound and inversely proportional to the amount of unconjugated 15-F2t-Isoprostane in the samples or standards. Plate was read spectrophotometrically (Bio-tek, Vermont, USA) at 450 nm.

**Total antioxidant status (TAS)**

To determine plasma TAS levels, peripheral blood was placed in pre-cooled test tubes on the examination day. Plasma was immediately separated in refrigerated centrifuge aliquoted and stored at –20°C until further use. Freshly thawed batches of plasma were analysed using TAS Randox kit (Randox laboratories, Ltd, Crumlin, UK). Results were expressed in mM of Trolox equivalents. The reference range for human blood plasma is given by the manufacturer as 1.30–1.77 mmol/L. The linearity of calibration extends to 2.5 mmol/L of Trolox. Measurements in duplicate were used to determine intra-assay variability.

**Melatonin and corticosterone measurement**

Melatonin and corticosterone plasma levels were determined using the RSHMAG-69K Milliplex MAP Rat Stress Hormone Magnetic Bead Panel (Millipore Corporation, Missouri, 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: 8000-15000, time out 60 seconds, melatonin bead set: 34). Plate was read on LABScan 100 analyzer (Luminex Corporation, Texas, USA) with xPONENT software for data acquisition. Average values for each set of duplicate samples or standards were within 15% of the mean. Melatonin and corticosterone concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

**Statistical analysis**

Data are reported as means ± standard error of the mean (SEM). Statistical analyses were performed using the SPSS computer program (version 22.0, 2013, SPSS Inc., Chicago, IL). Differences between groups (control vs. anemic and normal-Fe vs. Fe-overload) were tested for statistical significance with Student's t test. Individual means were tested by pairwise comparison with Tukey's multiple comparison test when main effects and interactions were significant. Data were analysed by 2-way ANOVA to determine the effects of anemia, type of diet and Fe content in the diet. Differences were considered significant at $P<0.05$.

## Results

After Fe deprivation (5 mg/kg of diet) during 40 d, all the hematological parameters in the experimental group were different from those of the counterpart controls. All these parameters were statistically different between controls and anemic rats ($P < 0.001$), except white blood cells that remained unchanged after severe Fe deprivation. In addition, corticosterone and melatonin levels increased during anemia induction ($P < 0.001$) (Table 2).

Table 3 shows that after 30 days of feeding the milk-based diets (day 70 of the study), TAS was higher in both groups of animals (control and anemic) fed fermented goat milk with normal-Fe content with respect to fermented cow milk ($P < 0.01$). Plasma 8-OHdG was lower in the control animals fed normal-Fe fermented goat milk ($P < 0.001$) in comparison with fermented cow milk and was also lower in both groups (control and anemic) fed Fe-overloaded fermented goat milk ($P < 0.001$). Plasma melatonin levels were higher in control and anemic animals fed fermented goat milk either with normal-Fe or Fe-overload ($P < 0.001$). Plasma corticosterone levels was lower in all the groups fed fermented goat milk, either with normal-Fe ($P < 0.001$ for control rats with normal-Fe; $P < 0.05$ for anemic rats) or Fe-overload ($P < 0.001$ for both groups of animals). Melatonin and corticosterone levels also increased in the all anemic groups during Fe replenishment with both fermented milks either with normal-Fe or Fe-overload ($P < 0.001$). Urine 8-OHdG and 15-F2t-isoprostanes were lower in both groups of animals fed fermented goat milk either with normal-Fe or Fe-overload ($P < 0.001$).

With regard to the oxidative stress-mediated damage to lipids (Table 4), in general, TBARs levels are higher in plasma, brain, duodenal mucosa and erythrocyte for control and anemic animals fed with fermented cow milk-based diet either with normal-Fe or Fe overload ($P < 0.001$). In general, anemia had no effect on TBARs levels in all tissues studied under the different experimental conditions. Fe overload in the diet had a negative effect in the rats fed fermented cow milk on lipid peroxidation in plasma of anemic rats ($P < 0.05$), and liver, mucosa duodenal and erythrocyte of control and anemic rats ($P < 0.05$ in all the cases), showing no effect on brain.

Protein oxidative damage is shown in Table 5. In plasma, fermented goat milk consumption reduced protein carbonyl levels in control and anemic rats either with normal-Fe or Fe overload ($P < 0.05$). In liver, fermented goat milk had a protective effect on proteins in the control and anemic Fe-overloaded groups ($P < 0.05$). In duodenal mucosa, fermented goat milk reduced oxidative damage to proteins in control and anemic rats either with normal-Fe ($P < 0.01$) or Fe overload ($P < 0.001$). Anemia had no effect on protein carbonyl values in the tissues studied under the different experimental conditions. Fe-overload showed a negative effect in plasma and liver of control rats consuming fermented cow milk ($P < 0.05$) and in duodenal mucosa of anemic rats consuming the same diet ($P < 0.05$). Once again, brain was unaffected by the diet, anemia or Fe content.

## Discussion

After Fe deprivation (5 mg/kg of diet) during 40 d, all the hematological parameters in the experimental group were different from those of the counterpart controls, due to progressive Fe depletion from body stores. All these parameters were statistically different between controls and anemic rats, except white blood cells that remained unchanged after severe Fe deprivation. Taking into account the long term Fe restriction in the diet, all these findings were expected and reflect the degree of severe Fe-deficiency induced. In the anemic group, an increase in the number of platelets and also the cortisol in serum were recorded. This can be explained for the reduction in the activity of monoaminooxidase in anemic rats, and the low level of aldehyde oxidase activity. These enzymatic changes produce an increase in the endogenous levels of circulating catecholamines, which are known to increase the release rate of adrenocorticotrophic hormone, which in turn produces an increase in the production of glucocorticoids [23]. In addition, anemia, can be considered a pro-inflammatory state, arising in part from a defect in the normal compensatory production of erythropoietin in response to a declining hemoglobin concentration [24]. In addition, inflammatory cytokines may negatively influence Fe absorption and recycling, thereby interfering with hemoglobin synthesis [25]. Therefore the increase in corticosterone could be a compensatory mechanism to alleviate, at least partly, the anemia-induced inflammatory state, because Glucocorticoids protect endothelial and epithelial cells from stress-induced apoptosis, exhibiting potent anti-inflammatory effects [26].

In addition, in the anemic animals, an increase in the melatonin secretion was observed. This can be explained by the increased corticosterone levels recorded in the anemic groups. It is well known that glucocorticoids upregulate the gene expression for melatonin synthesis. A reasonable explanation for upregulation of gene expression for melatonin and its enhanced production induced by stress is that the elevated melatonin would protect organisms against the potential damages caused by the stresses; alternatively, these responses may precondition organisms to readily cope with the stressors such ischemia or hypoxia [27]. In addition, melatonin increased synthesis in the anemic group can be also due to the fact that this hormone displays an exceptional multiplicity of actions. Melatonin is involved in sleep initiation, vasomotor control, adrenal function, anti-excitatory actions, immunomodulation including anti-inflammatory properties, direct and indirect antioxidant actions, and energy metabolism [28, 29]. As previously mentioned, anemia is considered a pro-inflammatory state, Fe-deficiency provokes a depletion of the Fe stores due to the decrease of the hepcidin, affecting negatively the metabolism and growth, since the hypoxia induced by the lack of Fe limits ATP production [8], therefore increased melatonin synthesis in the anemic animals would be also a compensatory mechanism to improve the energy metabolism and inflammatory signalling impaired during the anemia establishment.

**Melatonin and corticosterone levels during chronic Fe-repletion with fermented milk-based diets**

Due to hormone-mediated modulations of different cellular antioxidant systems have been previously demonstrated, including melatonin itself [30], we have measured different total antioxidant status (TAS) and the oxidative stress-mediated damage to the main biomolecules (lipids, protein, DNA, and prostaglandins) in order to verify a possible melatonin-mediated modulation on cell responses to oxidative stress, thus allowing a better attribution of direct melatonin antioxidant effects.

As milk of both species was obtained under similar welfare and conditions of photoperiodicity, we can affirm that circadian rhythm-related variations are not responsible of melatonin differences between both fermented milks. Melatonin a tryptophan derivative is a potent, endogenously produced and diet-derived free radical scavenger and broad-spectrum antioxidant [31]. In this sense, as previously reported, goat milk fermentation release high amounts of tryptophan and tryptophan-lactokinin [32], improving the substrate for melatonin biosynthesis in the pineal gland.

During melatonin synthesis, two enzymes are believed to play important roles in its production; they are arylalkylamine N-acetyltransferase (AA-NAT) and acetylserotonin O-Methyltransferase (ASMT). AA-NAT is often stated to be the rate-limiting enzyme in melatonin biosynthesis. Even at high AA-NAT activity, any change in ASMT activity would result in a similar modification in melatonin production [33]. A phenomenon of dissociation between the AA-NAT gene expression and melatonin production is also observed in the pineal gland of bovines [34], reason why we could postulate that melatonin production would be higher in the goat. In addition, adaptation to stress in mammals involves primarily the adrenal gland and glucocorticoids. Activation of the hypothalamus-pituitary-adrenal axis by specific cytokines increases the release of corticosterone, which in turn feeds back and suppresses the immune reaction [35]. In the current study, stress conditions can be related to higher concentrations of corticosterone in plasma in the animals fed cow milk; these findings have been previously found in situations of stress in sheep 36 and goats [37]. The results of the current study provide direct evidence that melatonin modulates the activity of the hypothalamus-pituitary-adrenal axis, fact reported previously in rats [38], goats [37], and humans [39].

**Antioxidant status**

Melatonin and its metabolites function as potent antioxidants and scavenge hydroxyl free radicals and many related reactants [40, 41]. Their protective roles have been reported in various experimental models by reducing oxidative stress and lipid peroxidation [42]. Melatonin has also been reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism, i.e. the activities of superoxide dismutase, glutathione reductase, glutathione peroxidase, glucose 6-phosphate dehydrogenase, and nitric oxide synthase [43]. The increase in plasma melatonin levels in the groups fed fermented goat milk could explain the increase in total antioxidant status (TAS) recorded in the current study, however there are other nutritional characteristics of goat milk that contributes to the action of melatonin in ameliorating oxidative stress due to modulation of antioxidant defenses. In this sense, previous studies of our research group have shown that goat milk has a better lipidic quality compared with cow milk, improving the nutritive utilization of fat and diminishing plasma total cholesterol [7, 44, 45]. The better nutritive utilization of goat milk fat and the healthy lipid plasma profile provides a lower substrate for lipid peroxidation and consequently decreases the generation of free radicals avoiding once more the oxidative damage to the biomolecules [7]. In addition, habitual goat milk consumption has positive effects on enzymatic antioxidant defence (catalase, superoxide dismutase and glutathione peroxidase), even in a situation of Fe overload, limiting the generation of free radicals [12]. On the other hand, as previously reported brain is relatively independent of the Fe variations in the organism because is little affected both by the Fe-overload and by the anemia, because during anemia induction or recovery, unaltered activities of antioxidant enzymes has been previously reported [12], reason that explain the absence of oxidative damage to lipids and proteins in the nervous tissue due to Fe changes in the organism.

Another interesting result of the present study is the positive role on DNA stability of the fermented goat milk consumption, even during Fe overloading, results which are in agreement with those reported previously by us [46]. Melatonin protects DNA, proteins, and biological membrane lipids from the deleterious effects of free radicals, without the need for a specific receptor on the cells [47]. Although the activity of melatonin is in part related to its direct antioxidant effect due to its hydrophilic and lipophilic nature of the hormone, allowing melatonin to move freely across all cellular barrier, there are several studies suggesting that its activity might be associated with an indirect antioxidant activity by influencing gene expression and regulation of oxidant and antioxidant enzymes [48, 49]. In the case of fermented goat milk another positive factor that could enhance genomic stability could be the high bioavailability of Mg from goat milk [10]. The improvement of Mg metabolism enhances genomic stability, acting as an essential cofactor in several pathways, and moreover, Mg is also required for double-strand break repair [7].

## Conclusions

In summary, during anemia instauration, the increase in the melatonin secretion and corticosterone levels, could be a compensatory mechanism to alleviate, at least partly, the anemia-induced inflammatory state, improving the energy metabolism and inflammatory signalling impaired in the course of this pathology. Fermented goat milk has positive effects on anemia recovery, increasing melatonin and total antioxidant status, even in situation of Fe-overload, fact that limits the oxidative damage to the main biomolecules (lipids, protein DNA, prostaglandins). The knowledge gained from these findings provide a basis to recommend the inclusion of fermented goat milk in the diet of people affected by nutritional Fe-deficiency anemia, especially in those consuming oral supplements of Fe.

## Conflicts of interest

The authors declare no conflict of interest. The funding sponsor had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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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] | Fermented goat milk-based diet[c] |
| Protein | 205 | 206 |
| Lactose | 295 | 291 |
| Fat | 100 | 100 |
| Wheat starch | 200 | 203 |
| Constant ingredients[b] | 200 | 200 |

[a] The diets were prepared according to the recommendations of the AIN-93G for control rats (45 mg Fe/Kg diet)[15], or with low Fe content (5 mg Fe/Kg diet)[16], for anemic 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, 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 for normal-Fe diets (45 mg Fe/Kg diet)[15] or Fe-overload (450 mg Fe/Kg diet)[17].

Table 2- Hematological parameters of control and anemic rats (day 40 of the study, pre-experimental period).

| | Normal-Fe Control group ($n$ = 40) | Low-Fe Anemic group ($n$ = 40) |
|---|---|---|
| Total blood | | |
| Hb concentration (g/L) | 136.94 ± 3.11 | 59.89 ± 2.92 * |
| RBCs ($10^{12}$/L) | 7.035 ± 0.18 | 3.04 ± 0.23 * |
| Hematocrit (%) | 39.78 ± 1.15 | 11.54 ± 1.31 * |
| MCV (fL) | 56.33 ± 0.56 | 37.87 ± 0.36 * |
| MCH (pg) | 19.90 ± 0.14 | 14.21 ± 0.67 * |
| MCHC (g/dl) | 35.22 ± 0.37 | 30.73 ± 0.86* |
| RDW (%) | 16.68 ± 0.34 | 19.22 ± 0.41 * |
| Platelets ($10^9$/L) | 744 ± 73.21 | 2249 ± 117 * |
| WBCs ($10^9$/L) | 8.87 ± 0.38 | 8.42 ± 0.97 |
| Lymphocytes ($10^6$/ml) | 8.01 ± 0.61 | 5.88 ± 0.85* |
| Serum | | |
| Fe (µg/L) | 1352 ± 105 | 597 ± 57.12 * |
| TIBC (µg/L) | 2749 ± 197 | 18132 ± 673 * |
| Transferrin saturation (%) | 48.56 ± 6.49 | 3.72 ± 0.38 * |
| Ferritin (µg/L) | 80.66 ± 2.21 | 49.14 ± 1.45 * |
| Plasma | | |
| Melatonin (pg/ml) | 11.56 ± 0.29 | 14.68 ± 0.34 * |
| Corticosterone (ng/ml) | 178.79 ± 28.53 | 342.79 ± 44.25 * |

Data are shown as the mean values ± SEM.

Hb, hemoglobin; 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).

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Table 3- Oxidative/antioxidant biomarkers in plasma and urine from control and anemic rats fed for 30 days with fermented cow or goat milk-based diets with normal-Fe content or Fe-overload[1]

| | Fermented cow milk-based diet | | Fermented goat milk-based diet | | *P*-value (2-way ANOVA) | | |
|---|---|---|---|---|---|---|---|
| | Control group | Anemic group | Control group | Anemic group | Diet | Anemia | Fe-overload |
| Plasma TAS (mM Trolox eq/ml) | | | | | | | |
| Fe-normal | 0.486 ± 0.009a | 0.486 ± 0.004A | 0.518 ± 0.002b | 0.510 ± 0.006B | <0.01 | NS[2] | <0.05 |
| Fe-overload | 0.485 ± 0.003 | 0.497 ± 0.003 | 0.495 ± 0.005D | 0.495 ± 0.007 | NS | NS | |
| Plasma 8-OHdG (ng/ml) | | | | | | | |
| Fe-normal | 1.048 ± 0.098a | 1.186 ± 0.059A | 0.869 ± 0.066b | 0.952 ± 0.063B | <0.001 | NS | <0.01 |
| Fe-overload | 1.690 ± 0.158aD | 1.434 ± 0.128AD | 0.862 ± 0.073b | 0.744 ± 0.082BD | <0.001 | NS | |
| Plasma melatonin (pg/ml) | | | | | | | |
| Fe-normal | 12.539 ± 0.331a | 14.410 ± 0.322AC | 15.742 ± 0.322b | 18.862 ± 0.789BC | <0.001 | <0.001 | <0.01 |
| Fe-overload | 12.911 ± 0.342a | 20.353 ± 0.968ACD | 15.081 ± 0.409b | 25.821 ± 0.931BCD | <0.001 | <0.001 | |
| Plasma corticosterone (ng/ml) | | | | | | | |
| Fe-normal | 133.06 ± 9.06a | 160.95 ± 5.78AC | 87.06 ± 8.79b | 135.56 ± 5.54BC | <0.01 | <0.001 | <0.01 |
| Fe-overload | 141.72 ± 9.23a | 188.36 ± 9.77ACD | 128.59 ± 7.61bD | 136.28 ± 9.87BC | <0.001 | <0.001 | |
| Urine 8-OHdG (ng/ml) | | | | | | | |
| Fe-normal | 12.312 ± 0.264a | 11.055 ± 0.492A | 6.005 ± 0.271b | 5.706 ± 0.369B | <0.001 | NS | <0.05 |
| Fe-overload) | 11.573 ± 0.413a | 12.237 ± 0.355A | 4.938 ± 0.354bD | 5.460 ± 0.309B | <0.001 | NS | |
| Urine isoprostanes (ng/ml) | | | | | | | |
| Fe-normal | 6.312 ± 0.423a | 3.362 ± 0.417AC | 0.912 ± 0.091b | 0.896 ± 0.162B | <0.001 | <0.05 | NS |
| Fe-overload | 6.107 ± 0.646a | 4.042 ± 0.391AC | 1.394 ± 0.251b | 1.104 ± 0.161B | <0.001 | <0.05 | |

TAS, total antioxidant status; 8-OHdG, 8-hidroxy guanosine

[1]Data are shown as the mean ± SEM for ten animals per group

[2] Non significant.

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 anaemics 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).

D Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's *t* test).

Table 4- Thiobarbituric acid-reactive substances (nmol/mg protein) in plasma, cytosolic fractions of liver, brain, duodenal mucosa and erythrocyte from control and anemic rats fed for 30 days with fermented cow or goat milk-based diets with normal-Fe content or Fe-overload [1]

| | Fermented cow milk-based diet | | Fermented goat milk-based diet | | *P*-value (2-way ANOVA) | | |
|---|---|---|---|---|---|---|---|
| | Control group | Anemic group | Control group | Anemic group | Diet | Anemia | Fe-overload |
| Plasma | | | | | | | |
| Fe-normal | 10.67 ± 1.46a | 8.180 ± 1.05A | 3.824 ±0.81b | 4.295 ± 0.80B | <0.001 | NS[2] | <0.05 |
| Fe-overload | 13.72 ± 1.66a | 12.625 ± 1.40AD | 4.713 ± 0.92b | 5.175 ± 0.96B | <0.001 | NS | |
| Liver | | | | | | | |
| Fe-normal | 3.65 ± 0.79 | 2.69 ± 0.58 | 3.25 ± 0.63 | 2.14 ± 0.61 | NS | NS | <0.05 |
| Fe-overload | 5.97 ± 0.88aD | 4.66 ± 0.76AD | 3.56 ± 0.27b | 2.56 ± 0.69B | <0.001 | NS | |
| Brain | | | | | | | |
| Fe-normal | 14.30 ± 0.87a | 13.41 ± 0.85A | 10.41 ± 0.98b | 9.42 ± 0.77B | <0.001 | NS | NS |
| Fe-overload | 14.54 ± 0.92a | 14.73 ± 1.32ª | 11.72 ± 0.92b | 10.44 ± 1.15B | <0.001 | NS | |
| Duodenal mucosa | | | | | | | |
| Fe-normal | 6.04 ± 0.61a | 6.86 ± 0.69A | 4.18 ± 0.48b | 4.07 ± 0.21B | <0.001 | NS | <0.05 |
| Fe-overload | 8.36 ± 0.49aD | 8.32 ± 0.55AD | 4.62 ± 0.59b | 4.94 ± 0.51B | <0.001 | NS | |
| Erythrocyte | | | | | | | |
| Fe-normal | 11.32 ± 1.89a | 9.77 ± 0.89AC | 6.51 ± 0.74b | 6.46 ± 0.77B | <0.001 | <0.05 | <0.05 |
| Fe-overload | 15.65 ± 1.96aD | 14.43 ± 1.84AD | 7.11 ± 0.96b | 6.97 ± 0.79B | <0.001 | NS | |

[1]Data are shown as the mean ± SEM for ten animals per group
[2] Non significant.
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 anaemics 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).
D Mean values from the corresponding group of rats fed with normal-Fe content were significantly different (*P < 0.05*, Student's *t* test).

Table 5- Protein carbonyl groups (nmol/mg protein) in plasma, cytosolic fractions of liver, brain and duodenal mucosa from control and anemic rats fed for 30 days with fermented cow or goat milk-based diets with normal-Fe content or Fe-overload[1]

| | Fermented cow milk-based diet | | Fermented goat milk-based diet | | *P*-value (2-way ANOVA) | | |
|---|---|---|---|---|---|---|---|
| | Control group | Anemic group | Control group | Anemic group | Diet | Anemia | Fe-overload |
| Plasma | | | | | | | |
| Fe-normal | 10.67 ± 1.36a | 8.18 ± 1.35A | 7.14 ± 1.30b | 5.54 ± 1.43B | <0.05 | NS[2] | <0.05 |
| Fe-overload | 14.72 ± 1.46aD | 10.62 ± 1.20AC | 9.49 ± 1.22b | 7.53 ± 1.51B | <0.05 | <0.05 | |
| Liver | | | | | | | |
| Fe-normal | 3.65 ± 0.69 | 2.69 ± 0.58 | 3.25 ± 0.73 | 2.14 ± 0.60 | NS | NS | <0.05 |
| Fe-overload | 4.97 ± 0.78aD | 3.96 ± 0.66AD | 3.26 ± 0.62b | 2.36 ± 0.58B | <0.05 | NS | |
| Brain | | | | | | | |
| Fe-normal | 4.31 ± 0.73 | 4.85 ± 0.77 | 3.82 ± 0.80 | 4.29 ± 0.80 | NS | NS | NS |
| Fe-overload | 5.47 ± 1.09 | 5.97 ± 1.14 | 4.71 ± 0.91 | 5.07 ± 1.06 | NS | NS | |
| Duodenal mucosa | | | | | | | |
| Fe-normal | 5.97 ± 0.80a | 5.88 ± 0.76A | 3.59 ± 0.44b | 3.40 ± 0.58B | <0.01 | NS | <0.05 |
| Fe-overload | 7.86 ± 0.93a | 8.02 ± 1.07AD | 4.16 ± 0.73b | 4.48 ± 0.81B | <0.001 | NS | |

[1]Data are shown as the mean ± SEM for ten animals per group

[2] Non significant.

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 anaemics 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).

D Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's *t* test).