Research Article

Received: 29 March 2016 Revised: 2 June 2016 Accepted article published: 6 July 2016 Published online in Wiley Online Library: 28 July 2016

(wileyonlinelibrary.com) DOI 10.1002/jsfa.7882

Fermented goat milk improves antioxidant status and protects from oxidative damage to biomolecules during anemia recovery

Jorge Moreno-Fernandez,[a,b] Javier Diaz-Castro,[a,b] María JM Alférez,[a,b] Christine Boesch,[c] Teresa Nestares[a,b] and Inmaculada López-Aliaga[a,b*]

Abstract

BACKGROUND: Iron deficiency anemia (IDA) is one of the most common nutritional problems in the world, and it is accepted that reactive oxygen species (ROS) production is altered during IDA. The aim of this study was to assess the influence of fermented goat and cow milks on enzymatic antioxidant activities and gene expression, and their role in protecting from oxidative damage during anemia recovery.

RESULTS: After feeding the fermented milks-based diets (cow or goat), a significant elevation of some antioxidant endogenous enzymes was found, together with an increase in total antioxidant status (TAS), and a decrease in 8-hydroxy-2′-deoxyguanosine (8-OHdG) was recorded in animals consuming fermented goat milk-based diet. In contrast, DNA strand breaks, hydroperoxides, 15-F2t-isoprostanes and protein carbonyl groups were lower in some tissues in animals fed fermented goat milk-based diet, revealing an improvement in both systemic and cellular antioxidant activity of plasma and tissues due to fermented goat milk consumption.

CONCLUSION: Fermented goat milk consumption induces a protective increase in TAS together with lower oxidative damage biomarkers, revealing that the milk protects main cell bioconstituents (lipids, protein, DNA, prostaglandins) from evoked oxidative damage during anemia recovery.

Keywords: fermented milk; oxidative stress; gene expression; anemia

INTRODUCTION

Nutrition is associated with oxidative metabolism, which besides production of energy is involved in a number of vital functions of the host. Under physiological conditions, reactive oxygen species (ROS) play a key role in primary immune defense by phagocytic cells against harmful microorganisms and inflammatory signaling.[1] In this sense, a prolonged excess of ROS is highly damaging for host biomolecules and cells, resulting in dysbalance of the functional antioxidative system of the organism and leading to a substantial increase in pathological inflammatory signaling and oxidative stress.[2] Although the body has an inherently antioxidative system to protect itself from damage caused by peroxidants, this system is not sufficiently effective to totally prevent such damage.[2] Hence there is increasing interest in finding natural antioxidants from food, because it is believed that they can protect from the attack of free radicals and retard the progress of many chronic diseases. Antioxidants from natural sources are likely to be more desirable than those produced chemically, because some synthetic antioxidants have been reported to have side effects.[3]

On the other hand, iron deficiency anemia (IDA) is one of the most common nutritional problems in the world, especially in developing countries. IDA is associated with mortality, hospitalization and health-related quality-of-life measures. There is controversy about the susceptibility to evoked oxidative stress of cells in IDA; however, it is accepted that ROS production plays an important role in hemoglobin (Hb) oxidation and methemoglobin (metHb) production.[4] Therefore the potential oxidative damage generated by the participation of Fe in redox reactions might be due to improper Fe compartmentalization and trafficking during IDA rather than its total accumulation in the body.

Fermented milk constituents have become recognized as functional foods, suggesting that their use has a direct and measurable effect on health outcome. There exist some reports on the beneficial effects of fermented milk on the immune system, metal ion chelation, enzyme inhibition and inhibition of ascorbate autoxidation.[5,6] However, a comprehensive analysis of the influence of fermented milk on antioxidant status and its role against pathogens involved in attacking main biomolecules such as lipids,

* *Correspondence to: I López-Aliaga, Department of Physiology, Faculty of Pharmacy, Campus Universitario de Cartuja, University of Granada, E-18071 Granada, Spain. E-mail: milopez@ugr.es*

a *Department of Physiology, University of Granada, Granada, Spain*

b *Institute of Nutrition and Food Technology 'José Mataix Verdú', University of Granada, Granada, Spain*

c *School of Food Science and Nutrition, University of Leeds, Leeds, UK*

proteins, prostaglandins and DNA is lacking, although fermented milks are dietary sources of natural antioxidants because of the presence of antioxidant peptides. Most identified bioactive peptides were derived from casein and have been shown to exhibit free radical scavenging and inhibit enzymatic and non-enzymatic lipid peroxidation.[7] The antioxidant peptides derived from whey protein are likely the result of the presence of cysteine-rich proteins that aid in the synthesis of glutathione, a potent intracellular antioxidant.[8]

Taking into account all factors mentioned above, the aim of this study was to assess the influence of fermented goat and cow milk-based diets on gene expression and enzymatic antioxidant activities, and their role in protecting main biomolecules such as lipids, proteins and DNA from oxidative damage during anemia recovery, to verify the possible influence of fermented milks on oxidative stress due to modulation of antioxidant defenses.

MATERIALS AND METHODS

Fermentation and dehydration of milks

Fermented milks were prepared according to the method described by Moreno-Fernandez *et al.*[9] Both milk types were inoculated with traditional yoghurt starters *Lactobacillus bulgaricus* subsp. *delbrueckii* and *Streptococcus thermophilus* (initial concentration 1×10^{11} colony-forming units (CFU) mL^{-1}; 10 mL L^{-1} inoculum) and incubated at 37 °C for approximately 24 h. Subsequently, fermented milk samples were subjected to a smooth industrial dehydration process until the final moisture content ranged between 25 and 45 g kg^{-1}.

Total nitrogen, dry matter, ash, total fat, lactose and mineral contents

Total N content was measured using the Kjeldahl method.[10] Dry matter, ash and total fat contents were determined according to AOAC methods.[11] Mineral content in the fermented milks and diets was assessed by multi-elemental analysis using an inductively coupled plasma optical emission spectrometer (ICP-OES). Samples were previously mineralized by the wet method in a sand bath (J.R. Selecta, Barcelona, Spain) using HNO_3 followed by HNO_3 (690 mL L^{-1})/$HClO_4$ (700 mL L^{-1}) (Merck KGaA, Darmstadt, Germany) at a sample/acid ratio of 1:4 (v/v) until the total elimination of organic matter. Analysis of Ca, P, Fe, Zn, Cu, Mg, Na and K was undertaken using an Optima 8300 ICP-OES (PerkinElmer Inc., Waltham, MA, USA) with a segmented-array charge-coupled device (SCD) high-performance detector.

Animals

All animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada (Ref. 11022011) in accordance with European Community guidelines (Declaration of Helsinki, Directive 2010/63/EU for animal experiments). Forty male Wistar albino 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 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 rigorously controlled sanitary and environmental parameters.

During the course of the study, the animals were housed in individual, ventilated, thermo-regulated cages with automatically controlled temperature (22–23 °C), humidity (55–65%) and 12/12 h light (09:00–21:00)/dark cycle. Diet intake was controlled by pair feeding all animals (80% of average intake), and doubly distilled water was available *ad libitum*.

Table 1. Composition of experimental diets

Component	Content (g kg^{-1} 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
Experimental period, 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 AIN-93G for control rats (45 mg Fe kg^{-1} diet)[12] or with low Fe content (5 mg Fe kg^{-1} diet)[13] for anemic groups.
[b] The constant ingredients consisted of 50 g fiber (micronized cellulose), 100 g sucrose, 2.5 g choline chloride, 2.5 g L-cystine, 35 g mineral premix and 10 g vitamin premix kg^{-1} diet.
[c] Specific vitamin and mineral premix supplements for fermented goat and cow milk-based diets were formulated taking into account the mineral and vitamin contents of the fermented milk powders supplied in order to meet the recommendations of AIN-93G for normal-Fe diets (45 mg Fe kg^{-1} diet).[12]

Experimental design

At the beginning of the study, the 40 rats were divided into two groups: the control group received the AIN-93G diet (44.72 ± 0.98 mg kg^{-1} by analysis);[12] the anemic group received a low-Fe diet (5.91 ± 0.36 mg kg^{-1} by analysis), with anemia being induced experimentally over a period of 40 days by a method developed previously by our research group.[13]

After the induction of anemia (day 40 of the study), the animals were subjected to an experimental period in which the control and anemic groups were fed for a further 30 days with fermented milk-based diets prepared with cow (Holstein breed) or goat (Murciano-Granadina breed) milk powder (200 g protein and 100 g fat kg^{-1}) (Table 1). The Fe contents of the diets were analyzed as 43.98 ± 0.39 and 44.28 ± 0.76 mg kg^{-1} respectively.

On day 70 of the study, urine samples were collected to measure 15-F2t-isoprostanes and 8-hydroxy-2′-deoxyguanosine (8-OHdG). The animals were then anesthetized intraperitoneally with sodium pentobarbital (Sigma Diagnostics, St Louis, MO, USA) and totally bled out by cannulation of the aorta. Blood aliquots with ethylenediaminetetraacetic acid (EDTA) were analyzed to measure hematological parameters, and the rest of the blood was centrifuged (1500 × *g*, 4 °C, 15 min) to measure plasma total antioxidant status (TAS), hydroperoxides, carbonyl groups and 8-OHdG. The remaining blood was centrifuged without anticoagulant to separate the

Table 2. PCR primer sequences and annealing temperatures

Gene	Forward sequence	Reverse sequence	Annealing temperature (°C)
β-Actin	GGGGTGTTGAAGGTCTCAAA	TGTCACCAACTGGGACGATA	57
SOD1	ACACAAGGCTGTACCACTGC	CCTTTCCAGCAGCCACATTG	59
CAT	GTGCATGCATGACAACCAGG	GAATGTCCGCACCTGAGTGA	59
GPx1	CCGTGCAATCAGTTCGGACA	CTCACCATTCACCTCGCACT	59
GR	GTATCACGCTGTGACCACGA	TGGATGCCAACCACCTTCTC	59

red blood cells from the serum for subsequent analysis of Fe, total iron-binding capacity (TIBC), transferrin saturation and ferritin. The liver, brain and duodenum were removed and washed with ice-cold saline solution (9 g L^{-1} NaCl). Liver and duodenal mucosa tissue fractions for quantitative polymerase chain reaction (qPCR) assay were incubated with RNA-later stabilization solution (Thermo Fisher Scientific, Waltham, MA USA) overnight at 4 °C. Subsequently, the RNA-later solution was removed and the tissue samples were frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Erythrocyte, liver, brain and duodenal mucosa cytosolic and membrane fractions were prepared fresh the same day by successive differential centrifugations with hypotonic hemolysis according to the method of Hanahan and Ekholm,[14] preserving these cytosolic fractions at −80 °C for further analyses of antioxidant enzyme activities, hydroperoxides and protein carbonyl groups. Antioxidant enzyme gene expression was assessed in the liver and duodenal mucosa. DNA instability (strand breaks) was measured in isolated rat lymphocytes. Protein contents in the cytosolic fractions were measured following the method described by Lowry *et al.*[15]

Hematological test

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

Serum Fe, 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. The absorbance of samples was read at 550 nm on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The percentage of transferrin saturation was calculated from the equation

$$\text{transferrin saturation (\%)} = [\text{serum Fe concentration } (\mu g\ L^{-1})/\text{TIBC } (\mu g\ L^{-1})] \times 100$$

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 (BioTek, Winooski, VT, USA). The color intensity developed was inversely proportional to the concentration of serum ferritin.

RNA isolation and real-time qPCR

Total RNA was isolated from duodenal mucosa and liver samples using TRIsure lysis reagent (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. RNA quantity and purity were determined by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific) at 260/280 nm. Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) in a 20 μL reaction volume with 1 µg of total RNA following the protocol supplied by the manufacturer.

Real-time qPCR was carried out as two-step procedure in a 20 μL reaction volume using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and SYBR Green detection with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primer sequences for real-time qPCR were designed using standard tools (Spidey, Primer3, NCBI Blast). Primer pairs were obtained from Eurofins MWG Biotech (Ebersberg, Germany). The selected rat genes, detailed in Table 2, were as follows: superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase 1 (GPx1) and glutathione reductase (GR). Measurements were done in duplicate and the expression of the target genes was normalized to the housekeeping gene β-actin which was consistently expressed across the groups. Serial dilutions of control samples were used to determine the efficacy of amplification. Melt curve analysis and gel electrophoresis were used to confirm PCR product size.

Antioxidant enzyme activities

Superoxide dismutase (SOD) activity was determined according to the method of Crapo *et al.*[16] based on its inhibition in the reduction of cytochrome c, measured spectrophotometrically (Thermo Spectronic, Rochester, NY, USA) at 550 nm.

Catalase (CAT) activity was determined following the method described by Aebi[17] by monitoring spectrophotometrically (Thermo Spectronic, Rochester, NY, USA) at 240 nm the decomposition of hydrogen peroxide (H_2O_2) as a consequence of the catalytic activity of CAT.

Glutathione peroxidase (GPx) activity was measured by the method of Flohé and Gunzler[18] based on the instantaneous formation of oxidized glutathione during the reaction catalyzed by GPx. The oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically (Thermo Spectronic) at 340 nm.

Glutathione reductase (GR) activity was determined using the method of Worthington and Rosemeyer[19] based on the oxidation of NADPH to NADP catalyzed by a limiting concentration of GR. In this reaction, oxidized glutathione reduction was determined indirectly by measuring NADPH consumption spectrophotometrically (Thermo Spectronic) at 340 nm.

Total antioxidant status

Freshly thawed batches of plasma were analyzed using the TAS Randox Kit (Randox Laboratories, Ltd, Crumlin, UK). TAS results were expressed in mmol L^{-1} Trolox equivalent. Measurements in duplicate were used to determine the intra-assay variability.

Plasma and membrane lipid hydroperoxides

The peroxide level in test samples was determined by calculation from the known extinction coefficient of the XO–Fe complex or by reference to a standard curve prepared with H_2O_2 solution using

the Pierce™ Quantitative Peroxide Assay Kit (Thermo Scientific, Rockford, IL, USA).

Protein oxidation (carbonyl groups) measurement

Plasma, liver, brain and duodenal mucosa protein oxidation was measured according to a method based on spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl (PC) to form protein hydrazones.[20]

15-F2t-Isoprostanes

Isoprostanes in urine were measured using the Enzyme Immunoassay for Urinary Isoprostane Kit (Oxford Biomedical Research, Oxford, UK). In this assay, urine samples are mixed with an enhanced dilution buffer that essentially eliminates interference due to non-specific binding. Plates were read spectrophotometrically (BioTek) at 450 nm.

8-Hydroxy-2′-deoxyguanosine

8-OHdG in plasma and urine was measured using the 8OHdG Check Kit (Japan Institute for the Control of Aging, Shizuoka, Japan). To separate interfering substances, ultrafiltration (molecular weight cut-off 10 000 Da) of serum samples was performed. Results were read at 450 nm on a microplate reader (BioTek).

Alkaline single-cell gel electrophoresis (comet assay)

Among the available genotoxicity tests, the comet assay is recognized for its robustness, sensitivity and statistical power to evaluate DNA breaks, which can be considered hallmarks of mutagenicity.[21] We also used the 4′,6-diamidino-2-phenylindole (DAPI) staining method owing to its increased sensitivity of DNA damage detection,[22] making the assay more accurate. DNA instability (strand breaks) was measured in rat lymphocytes on day 70 of the study according to the method described previously.[23] Isolated rat lymphocytes were stained with 20 μL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, St Louis, MO, USA). DAPI-stained nucleoids were scored using a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with computerized image analysis (Komet 3.0, Kinetic Imaging Ltd, Liverpool, UK). The percentage of fluorescence in the comet tail and the olive tail moment (defined as the product of the tail length and the fraction of total DNA in the tail: OTM = [(tail mean − head mean) × tail % DNA]/100) were measured.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS Version 22.0 (SPSS Inc., Chicago, IL, USA). Differences between groups fed normal-Fe-content and low-Fe-content diets during the pre-experimental period (PEP) were tested for statistical significance with Student's *t* test. One-way analysis of variance (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 Tukey's multiple comparison test when main effects and interactions were significant. The level of significance was set at $P < 0.05$.

RESULTS

After Fe deprivation for 40 days, all hematological parameters were consistent with severe induced anemia in the experimental group and different from their counterpart controls ($P < 0.001$), except white blood cells that remained unchanged after severe Fe deprivation (Table 3).

Table 3. Hematological parameters of control and anemic rats (day 40 of PEP)

Parameter	Normal-Fe control group ($n = 20$)	Low-Fe anemic group ($n = 20$)
Total blood		
Hb concentration (g L^{-1})	129.8 ± 3.01	60.01 ± 2.94*
RBCs (10^{12} L^{-1})	7.14 ± 0.17	3.10 ± 0.21*
Hematocrit (%)	40.01 ± 1.15	11.45 ± 1.23*
MCV (fL)	56.41 ± 0.57	37.90 ± 0.37*
MCH (pg)	19.81 ± 0.13	14.12 ± 0.69*
MCHC (g dL^{-1})	35.41 ± 0.37	29.97 ± 0.85*
RDW (%)	16.86 ± 0.33	19.31 ± 0.39*
Platelets (10^9 L^{-1})	763 ± 72.12	2197 ± 120*
WBCs (10^9 L^{-1})	8.90 ± 0.37	8.41 ± 0.96
Serum		
Fe (μg L^{-1})	1342 ± 115	599 ± 57.22*
TIBC (μg L^{-1})	2750 ± 195	18031 ± 663*
Transferrin saturation (%)	48.66 ± 6.51	3.77 ± 0.38*
Ferritin (μg L^{-1})	81.01 ± 2.22	49.24 ± 1.47*

Data are shown as mean ± SEM.
*Significantly different from control group ($P < 0.001$, Student's *t* test).
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 iron-binding capacity.

SOD gene expression increased in liver of control animals and CAT gene expression increased in liver of control and anemic animals fed fermented goat milk ($P < 0.001$) (Figs 1A and 1B). Expression of these enzymes decreased in duodenal mucosa of both groups of animals fed either fermented goat or cow milk ($P < 0.001$) (Figs 2A and 2B). GPx mRNA increased in liver of control animals ($P < 0.001$) (Fig. 1C) and in duodenal mucosa of both groups of animals ($P < 0.001$) (Fig. 2C) fed fermented goat milk. GR gene expression increased in liver and duodenal mucosa of control animals fed fermented goat milk ($P < 0.001$). Anemia decreased GR gene expression in liver and duodenal mucosa of rats fed fermented goat milk ($P < 0.001$) (Figs 1D and 2D).

The antioxidant enzyme activities are shown in Table 4. SOD activity increased in liver ($P < 0.001$), erythrocyte ($P < 0.001$) and plasma ($P < 0.001$) of both groups fed fermented goat milk. Anemia increased SOD activity in duodenal mucosa ($P < 0.001$) and decreased it in erythrocyte ($P < 0.001$) of rats fed both fermented milks. CAT activity increased in duodenal mucosa ($P < 0.001$) of control rats and brain ($P < 0.01$) of control and anemics rats fed fermented goat milk. Anemia decreased CAT activity in duodenal mucosa of rats fed both fermented milks ($P < 0.001$) and in erythrocyte of rats fed fermented cow milk ($P < 0.001$) and increased in erythrocyte of rats fed fermented goat milk ($P < 0.001$). GPx activity increased in liver ($P < 0.05$) and brain ($P < 0.001$) of control animals and duodenal mucosa of control and anemic animals fed fermented goat milk ($P < 0.001$) and in erythrocyte of anemic rats fed fermented goat milk ($P < 0.001$). Anemia decreased GPx activity in duodenal mucosa and erythrocyte ($P < 0.001$) of rats fed fermented cow milk and in liver ($P < 0.01$) of animals fed fermented goat milk. GR activity increased in liver, duodenal

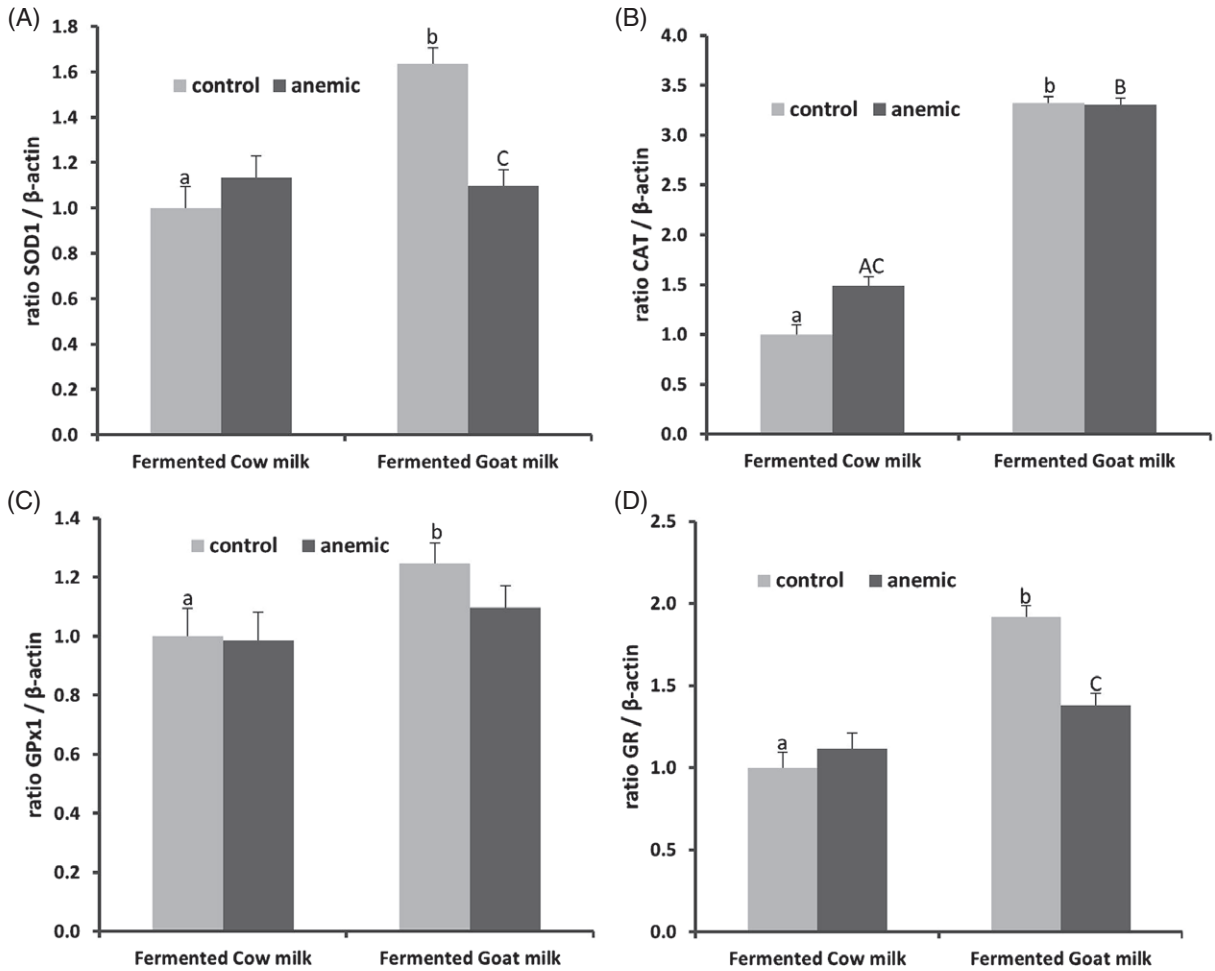

Figure 1. Effect of fermented cow and goat milk in control and anemic rats on liver mRNA levels of (A) SOD1, (B) CAT, (C) GPx1 and (D) GR. Data are mean with SEM of ten animals per group. [a,b]Mean values among groups of controls rats with different letters are significantly different ($P < 0.05$, Tukey's test). [A,B]Mean values among groups of anemic rats with different letters are significantly different ($P < 0.05$, Tukey's test). [C]Mean values from the corresponding group of control rats are significantly different ($P < 0.05$, Student's *t* test).

mucosa and brain in both groups of animals fed fermented goat milk ($P < 0.001$), while no differences were recorded in erythrocyte. Anemia increased GR activity in duodenal mucosa and brain with both fermented milks ($P < 0.001$) and decreased in liver of animals fed fermented goat milk ($P < 0.001$).

Table 5 shows that after 30 days of feeding the fermented milk-based diets (day 70 of the study), TAS was higher ($P < 0.01$ for control and anemic), plasma 8-OHdG was lower ($P < 0.001$ for control and $P < 0.01$ for anemic) and urine 8-OHdG and 15-F2t-isoprostanes were lower ($P < 0.001$ for control and anemic) in animals fed fermented goat milk in comparison with fermented cow milk. In general, anemia did not show effects on TAS, plasma and urine 8-OHdG and urine15-F2t-isoprostanes.

With regard to oxidative stress-mediated damage to lipids (Table 6), hydroperoxide levels were higher in plasma, erythrocyte ($P < 0.001$) and liver ($P < 0.01$) for control and anemic animals fed fermented cow milk-based diet, showing no effect in brain and duodenal mucosa. In general, anemia increased susceptibility to lipid oxidation in all tissues of animals fed fermented goat or cow milk ($P < 0.001$ for erythrocyte, liver and brain; $P < 0.01$ for duodenal mucosa).

Protein oxidative damage is shown in Table 7. Fermented goat milk consumption reduced protein carbonyl levels in plasma ($P < 0.05$) and duodenal mucosa ($P < 0.01$) of control and anemic rats, while no statistical differences were recorded in liver and brain. Anemia had no effect on protein carbonyl values in the tissues studied.

DNA oxidative damage (Table 8) was lower when fermented goat milk was supplied, as revealed by the percentage of DNA in tail and OTM ($P < 0.001$), compared with those rats that consumed fermented cow milk. Anemia had no effect on tail DNA, but it increased OTM in animals fed fermented cow milk ($P < 0.001$) and decreased this parameter in animals fed fermented goat milk ($P < 0.05$).

DISCUSSION

The underlying research hypothesis of this study was that fermented cow or goat milk, because they are dietary sources of natural antioxidants, would influence oxidative/antioxidant status during anemia recovery. The findings amply confirm the hypothesis, revealing significant elevation of TAS, SOD, CAT, GPx and GR (hepatic gene expression), SOD, GPx, GR (hepatic activity), together with a decrease in isoprostanes, DNA strand breaks and 8-OHdG, and the repression of lipid (plasma, erythrocyte and liver) and protein (plasma and duodenal mucosa) oxidation in rats

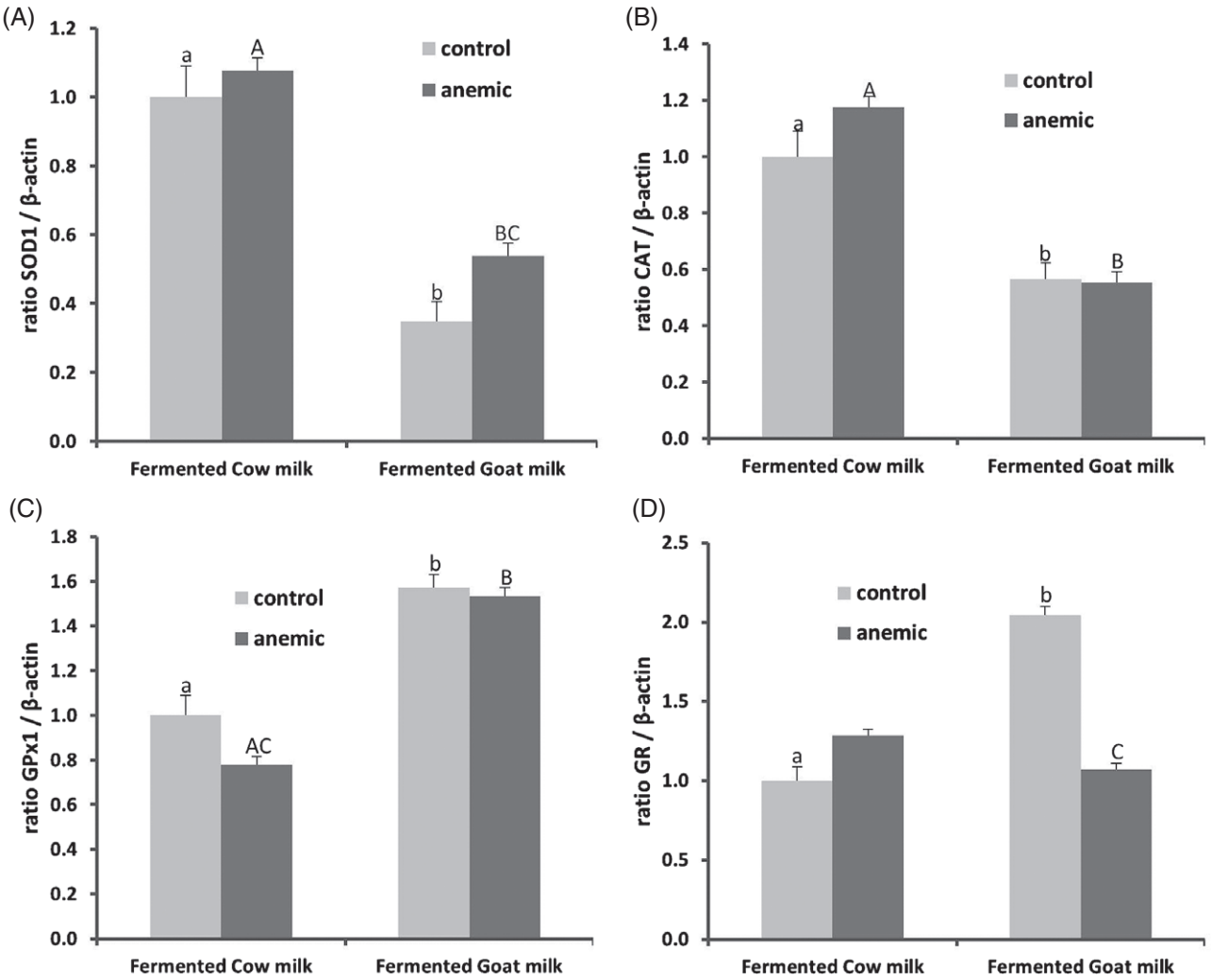

Figure 2. Effect of fermented cow and goat milk in control and anemic rats on duodenal mucosa mRNA levels of (A) SOD1, (B) CAT, (C) GPx1 and (D) GR. Data are mean with SEM of ten animals per group. [a,b]Mean values among groups of controls rats with different letters are significantly different ($P < 0.05$, Tukey's test). [A,B]Mean values among groups of anemic rats with different letters are significantly different ($P < 0.05$, Tukey's test). [C]Mean values from the corresponding group of control rats are significantly different ($P < 0.05$, Student's *t* test).

consuming fermented goat milk highlights the improvement in both systemic and cellular antioxidant activity of plasma due to fermented goat milk consumption.

In agreement with previous reports,[24] this study shows a significantly higher level of lipid oxidation biomarkers (hydroperoxides) in plasma, erythrocyte and liver of IDA compared with control rats. This finding can be attributed to permanent structural membrane alterations in cells during Fe deficiency;[25,26] however, in the current study, this oxidative damage seems to be lipid-specific, because no increase in oxidative damage to proteins, prostaglandins or DNA due to Fe deficiency has been recorded in the tissues studied, which is in agreement with previous results.[23]

It has been previously reported that SOD and CAT levels were significantly lower in anemia situations;[27] however, in our study, this reduction in SOD and CAT activities was recorded especially when fermented cow milk was consumed. Previous studies reported conflicting results regarding the levels of these antioxidant enzymes in IDA. While Adelakun *et al.*[28] did not find significant differences in SOD and CAT, Hundekar *et al.*[29] reported an increased SOD level and other studies have reported decreased levels of SOD and CAT in situations of anemia.[30,31] Reductions in SOD and CAT levels are related to the severity of oxidative stress;[30,31] therefore the results of the current study reveal that oxidative stress is lower when fermented goat milk is supplied during anemia recovery.

Fermented goat milk contains casein together with short-chain and medium-chain fatty acids (capric, caprylic and caproic acids).[9] The antioxidative enzymes, so-called primary scavengers, in red blood cells and serum include SOD, CAT, GPx and GR. Highly aggressive and toxic hydroxyl radicals have been proposed to play important pathogenic roles in attacking main biomolecules such as lipids, proteins and DNA. SOD is considered the first intracellular defense against reactive oxygen molecules. This enzyme causes dismutation of the superoxide anion radical to H_2O_2,[32] which is further degraded by CAT and peroxidase actions. However, CAT conjugated to SOD represses the conversion of H_2O_2 to hydroxyl radicals. Superoxide anion radicals are dismuted by SOD to H_2O_2 and oxygen. Under physiological conditions, the conjugation of CAT to SOD ensures that, as soon as a superoxide dismutation reaction occurs, the resultant H_2O_2 is removed by the immediate proximity of the CAT molecule.[33] A high level of lipid peroxides certainly leads to both a decreased activity of

Table 4. Antioxidant enzyme activities (U mg^{-1} protein) in plasma and cytosolic fractions of erythrocyte, liver, brain and duodenal mucosa from control and anemic rats fed for 30 days with fermented cow or goat milk-based diet

	Fermented cow milk-based diet		Fermented goat milk-based diet	
Enzyme/location	Control group	Anemic group	Control group	Anemic group
SOD				
Erythrocyte	0.678 ± 0.44^{a}	0.342 ± 0.033^{AC}	1.338 ± 0.067^{b}	0.807 ± 0.047^{BC}
Liver	1.206 ± 0.107^{a}	0.970 ± 0.065^{A}	1.780 ± 0.104^{b}	1.551 ± 0.111^{B}
Brain	1.051 ± 0.051	0.740 ± 0.096^{C}	1.085 ± 0.091	0.908 ± 0.089
Duodenal mucosa	2.278 ± 0.193	3.289 ± 0.144^{C}	2.119 ± 0.103	3.281 ± 0.163^{C}
Plasma	20.057 ± 0.298^{a}	19.385 ± 0.420^{A}	23.679 ± 0.993^{b}	22.564 ± 0.710^{B}
CAT				
Erythrocyte	2.607 ± 0.595	0.840 ± 0.086^{AC}	2.378 ± 0.346	3.493 ± 0.512^{BC}
Liver	10.285 ± 0.758	10.672 ± 0.477^{A}	12.858 ± 0.872	13.241 ± 1.011^{B}
Brain	0.570 ± 0.062^{a}	0.599 ± 0.024^{A}	0.811 ± 0.070^{b}	0.933 ± 0.097^{B}
Duodenal mucosa	47.440 ± 2.753^{a}	33.425 ± 2.344^{C}	95.537 ± 7.643^{b}	36.622 ± 1.128^{C}
GPx				
Erythrocyte	0.533 ± 0.094	0.385 ± 0.064^{AC}	0.699 ± 0.062	0.706 ± 0.077^{B}
Liver	0.820 ± 0.064^{a}	0.697 ± 0.038	1.076 ± 0.063^{b}	0.736 ± 0.088^{C}
Brain	0.070 ± 0.016^{a}	0.088 ± 0.009	0.134 ± 0.014^{b}	0.123 ± 0.039
Duodenal mucosa	0.149 ± 0.042^{a}	0.050 ± 0.007^{AC}	0.320 ± 0.033^{b}	0.250 ± 0.051^{B}
GR				
Erythrocyte	18.940 ± 1.142	22.534 ± 1.739	15.080 ± 1.052	19.132 ± 1.049
Liver	27.445 ± 0.886^{a}	26.488 ± 0.871^{A}	49.305 ± 1.313^{b}	36.928 ± 1.667^{BC}
Brain	20.479 ± 1.123^{a}	33.570 ± 1.820^{AC}	35.813 ± 1.377^{b}	43.051 ± 1.825^{BC}
Duodenal mucosa	27.954 ± 0.634^{a}	80.893 ± 1.903^{AC}	38.989 ± 1.040^{b}	129.996 ± 3.822^{BC}

Data are shown as mean ± SEM for ten animals per group. ^{a,b}Mean values within a row and within control groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{A,B}Mean values within a row and within anemic groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{C}Indicates difference ($P < 0.05$) for control *versus* anemic group within a diet by Tukey's test.

Table 5. Oxidative/antioxidant biomarkers in plasma and urine from control and anemic rats fed for 30 days with fermented cow or goat milk-based diet

	Fermented cow milk-based diet		Fermented goat milk-based diet	
Biomarker	Control group	Anemic group	Control group	Anemic group
Plasma TAS (mmol L^{-1} Trolox equivalent mL^{-1})	0.490 ± 0.009^{a}	0.464 ± 0.003^{A}	0.559 ± 0.001^{b}	0.510 ± 0.007^{B}
Plasma 8-OHdG (ng mL^{-1})	1.051 ± 0.010^{a}	1.184 ± 0.060^{A}	0.871 ± 0.070^{b}	0.961 ± 0.065^{B}
Urine 8-OHdG (ng mL^{-1})	12.411 ± 0.274^{a}	11.105 ± 0.502^{A}	6.109 ± 0.269^{b}	5.713 ± 0.372^{B}
Urine isoprostanes (ng mL^{-1})	6.321 ± 0.432^{a}	3.370 ± 0.418^{AC}	0.921 ± 0.093^{b}	0.884 ± 0.167^{B}

Data are shown as mean ± SEM for ten animals per group. ^{a,b}Mean values within a row and within control groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{A,B}Mean values within a row and within anemic groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{C}Indicates difference ($P < 0.05$) for control *versus* anemic group within a diet by Tukey's test.

antioxidative mechanisms and an excess of substrate, which can undergo abnormal oxidative modifications.[34] Goat milk has a better lipid quality than cow milk,[35] therefore the generation of free radicals is lower. This trend to prevent lipid peroxidation in animals fed fermented goat milk can be due to its beneficial nutritional character, which provides a lower substrate for lipid peroxidation and consequently decreases the generation of free radicals.[35] Additionally, some milk proteins can act as antioxidants.[36] The ability of peptides to inhibit deleterious changes caused by lipid oxidation appears to be related to certain amino acid residues in the peptides, such as tyrosine, methionine, histidine, lysine and tryptophan, which are capable of chelating pro-oxidative metal ions.[37] In this sense, as previously reported,[9] fermented goat milk has higher amounts of tyrosine, methionine, histidine, lysine and tryptophan; therefore peptides deriving from fermented goat milk could inhibit lipid oxidation, reducing the hydroperoxide output and contributing to the improvement in TAS, limiting oxidative damage to the biomolecules.

In addition, fermented goat milk has higher amounts of antioxidant minerals (especially Mg, Zn and Se),[9] which play a positive role in antioxidant status,[38,39] and higher amounts of vitamin A,[9] which can be incorporated into plasma and contribute to plasma antioxidative capacity.[40] Finally, goat milk has bioactive peptides,[41,42] which also have some antioxidative properties, explaining the lower rate of 8-OHdG, carbonyl groups and isoprostanes achieved in the current study with fermented goat milk.

On the other hand, fermented goat milk has higher amounts of vitamin D,[9,43] which is a membrane antioxidant with the potential

Table 6. Hydroperoxides (nmol mg^{-1} protein) in plasma and cytosolic fractions of erythrocyte, liver, brain and duodenal mucosa from control and anemic rats fed for 30 days with fermented cow or goat milk-based diet

	Fermented cow milk-based diet		Fermented goat milk-based diet	
Location	Control group	Anemic group	Control group	Anemic group
Plasma	11.191 ± 0.468^{a}	10.319 ± 0.361^{A}	5.368 ± 0.459^{b}	2.168 ± 0.439^{BC}
Erythrocyte	78.551 ± 4.878^{a}	179.362 ± 7.468^{AC}	50.962 ± 5.319^{b}	106.251 ± 7.022^{BC}
Liver	282.523 ± 17.609^{a}	346.588 ± 17.682^{AC}	194.351 ± 12.889^{b}	286.052 ± 14.632^{BC}
Brain	16.553 ± 1.002	12.569 ± 0.519^{C}	19.161 ± 1.202	12.051 ± 0.959^{C}
Duodenal mucosa	11.941 ± 1.112	15.373 ± 0.949^{C}	12.381 ± 1.112	15.731 ± 0.920^{C}

Data are shown as mean ± SEM for ten animals per group. ^{a,b}Mean values within a row and within control groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{A,B}Mean values within a row and within anemic groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{C}Indicates difference ($P < 0.05$) for control *versus* anemic group within a diet by Tukey's test.

Table 7. Protein carbonyl groups (nmol mg^{-1} protein) in plasma and cytosolic fractions of liver, brain and duodenal mucosa from control and anemic rats fed for 30 days with fermented cow or goat milk-based diet

	Fermented cow milk-based diet		Fermented goat milk-based diet	
Location	Control group	Anemic group	Control group	Anemic group
Plasma	10.802 ± 1.339^{a}	8.278 ± 1.251^{A}	7.212 ± 1.183^{b}	5.421 ± 1.312^{B}
Liver	3.868 ± 0.709	2.572 ± 0.601	3.167 ± 0.688	2.062 ± 0.501
Brain	4.422 ± 0.711	4.868 ± 0.639	3.672 ± 0.791	4.311 ± 0.702
Duodenal mucosa	6.012 ± 0.768^{a}	5.901 ± 0.689^{A}	3.612 ± 0.403^{b}	3.379 ± 0.601^{B}

Data are shown as mean ± SEM for ten animals per group. ^{a,b}Mean values within a row and within control groups with different letters differ ($P < 0.05$) by Student's *t* test. ^{A,B}Mean values within a row and within anemic groups with different letters differ ($P < 0.05$) by Student's *t* test.

to inhibit lipid peroxidation. In addition, vitamin D administration to rats resulted in a marked elevation of SOD activity in hepatic and renal tissues.[44] Vitamin D reduces oxidative stress by up-regulating antioxidative molecules, including glutathione, GPx and SOD, and this effect may be comparable to that of vitamin E.[45] Cytokines also have a regulatory role over circulating SOD. For example, tumor necrosis factor-α down-regulates extracellular SOD expression, whereas interferon-γ and interleukin-4 up-regulate extracellular SOD expression.[46] Vitamin D, by decreasing tumor necrosis factor-α and increasing interleukin-4 secretion, up-regulates SOD activity.[47] The higher amount of vitamin D in fermented goat milk also explains the improvement in TAS, SOD and GPx over-expression.

It has also been previously reported that fermented goat milk consumption increased plasma levels of melatonin.[48] This tryptophan derivative is a potent, endogenously produced and diet-derived free radical scavenger and broad-spectrum antioxidant, and its protective role in reducing oxidative stress and lipid peroxidation has been reported in various experimental models.[49] Melatonin has also been reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism, including the activities of SOD, GR, GPx and nitric oxide synthase.[50] The increase in melatonin levels could also explain the increase in TAS and antioxidant enzyme activities in the different tissues studied, protecting cell biomolecules from oxidation.

We have assessed the three measures of DNA migration which are commonly used: percentage of DNA in tail and head (tail and head intensity) and OTM. OTM appears the most representative measurement, because it incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).[51] In addition, 8-OHdG, an oxidized nucleoside of DNA, excised during the repair of oxidative damage to deoxyguanosine sites in DNA, was recorded as an additional measurement of DNA damage. In the current study, a positive role of fermented goat milk in genomic stability has been recorded, reducing strand breaks and 8-OHdG. These results are in agreement with those reported previously by our research group[38,39] and can be explained by the high bioavailability of Mg and Zn from goat milk. The improvement in Mg metabolism enhances genomic stability, acting as an essential cofactor in several pathways and for double-strand break repair. On the other hand, Zn can reduce the Fe-mediated oxidation of lipids, proteins and DNA, improving DNA stability.[39] In addition, as mentioned above, fermented goat milk consumption has positive effects on enzymatic TAS, limiting the generation of free radicals and the oxidative-induced damage to DNA.

CONCLUSIONS

The antioxidant status is associated with biomarkers of oxidative stress such as hydroperoxides, isoprostanes, 8-OHdG and DNA strand breaks during nutritional ferropenic anemia recovery. While fermented goat milk consumption reduced GPx1 expression in the duodenal mucosa, it induced a protective effect in the tissues studied, increasing TAS and decreasing the oxidative damage biomarkers, which directly correlates with the expression/activity of antioxidant enzymes in the liver, revealing that the milk protects main cell bioconstituents (lipids, protein, prostaglandins and DNA) from evoked oxidative damage during anemia recovery.

Table 8. DNA damage in lymphocytes of peripheral blood of control and anemic rats fed for 30 days with fermented cow or goat milk-based diet

	Fermented cow milk-based diet		Fermented goat milk-based diet	
Parameter	Control group	Anemic group	Control group	Anemic group
Tail DNA (%)	12.189 ± 0.701^{a}	13.302 ± 0.669^{A}	3.879 ± 0.382^{b}	3.061 ± 0.182^{B}
Olive tail moment (OTM)	0.212 ± 0.011^{a}	0.384 ± 0.015^{AC}	0.054 ± 0.002^{b}	0.030 ± 0.002^{BC}

Data are shown as mean ± SEM for ten animals per group. [a,b]Mean values within a row and within control groups with different letters differ ($P < 0.05$) by Student's *t* test. [A,B]Mean values within a row and within anemic groups with different letters differ ($P < 0.05$) by Student's *t* test. [C]Indicates difference ($P < 0.05$) for control *versus* anemic group within a diet by Tukey's test.

ACKNOWLEDGEMENTS

This work was supported by the Andalusian Government, Excellence Research Project No. P11-AGR-7648. Jorge Moreno-Fernandez was supported by a grant from the University of Granada and International Excellence Campus (CEI-Biotic) during his stay at the University of Leeds and is grateful to the Excellence PhD Program 'Nutrición y Ciencias de los Alimentos' from the University of Granada.

REFERENCES

1 Halliwell B and Gutteridge JM, Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* **246**:501–514 (1986).
2 Petrof EO, Kojima K, Ropeleski MJ, Musch MW, Tao Y, De Simone C, *et al.*, Probiotics inhibit nuclear factor-*k*B and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology* **127**:1474–1487 (2004).
3 Osuntoki A and Korie I, Antioxidant activity of whey from milk fermented with *Lactobacillus* species isolated from Nigerian fermented foods. *Food Technol Biotechnol* **48**:505–511 (2010).
4 Ansarihadipour H and Dorostkar H, Comparison of plasma oxidative biomarkers and conformational modifications of hemoglobin in patients with diabetes on hemodialysis. *Iran Red Crescent Med J* **16**:e22045 (2014).
5 Lin MY and Yen CL, Antioxidative ability of lactic acid bacteria. *J Agric Food Chem* **47**:1460–1466 (1999).
6 Sharma R, Kapila R and Kapila S, Probiotics as anti-immunosenescence agents. *Food Rev Int* **29**:201–216 (2013).
7 Korhonen H and Pihlanto A, Bioactive peptides: production and functionality. *Int Dairy J* **16**:945–960 (2006).
8 Hayes M, Stanton C, Fitzgerald GF and Ross RP, Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnol J* **2**:435–449 (2007).
9 Moreno-Fernandez J, Diaz-Castro J, Alferez MJ, Hijano S, Nestares T and Lopez-Aliaga I, Production and chemical composition of two dehydrated fermented dairy products based on cow or goat milk. *J Dairy Res* **83**:81–88 (2016).
10 AOAC, Official Methods of Analysis of AOAC International *(18th edn)*. Association of Official Analytical Chemists, Arlington, VA (2005).
11 AOAC, Official Methods of Analysis of AOAC International *(16th edn)*. Association of Official Analytical Chemists, Arlington, VA (1995)
12 Reeves PG, Nielsen FH and Fahey Jr GC, AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**:1939–1951 (1993).
13 Pallares I, Lisbona F, Aliaga IL, Barrionuevo M, Alferez MJ and Campos MS, Effect of iron deficiency on the digestive utilization of iron, phosphorus, calcium and magnesium in rats. *Br J Nutr* **70**:609–620 (1993).
14 Hanahan DJ and Ekholm JE, The preparation of red cell ghosts (membranes). *Meth Enzymol* **31**:168–172 (1974).
15 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275 (1951).
16 Crapo JD, McCord JM and Fridovich I, Preparation and assay of superoxide dismutases. *Meth Enzymol* **53**:382–393 (1978).
17 Aebi H, Catalase *in vitro*. *Meth Enzymol* **105**:121–126 (1984).
18 Flohé L and Gunzler WA, Assays of glutathione peroxidase. *Meth Enzymol* **105**:114–121 (1984).
19 Worthington DJ and Rosemeyer MA, Human glutathione reductase: purification of the crystalline enzyme from erythrocytes. *Eur J Biochem* **48**:167–177 (1974).
20 Reznick AZ and Packer L, Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Meth Enzymol* **233**:357–363 (1994).
21 Heuser VD, de Andrade VM, Peres A, Gomes de Macedo Braga LM and Bogo Chies JA, Influence of age and sex on the spontaneous DNA damage detected by micronucleus test and comet assay in mice peripheral blood cells. *Cell Biol Int* **32**:1223–1229 (2008).
22 Rojas E, Lopez MC and Valverde M, Single cell gel electrophoresis assay: methodology and applications. *J Chromatogr B* **722**:225–254 (1999).
23 Diaz-Castro J, Alferez MJ, Lopez-Aliaga I, Nestares T, Granados S, Barrionuevo M, *et al.*, Influence of nutritional iron deficiency anemia on DNA stability and lipid peroxidation in rats. *Nutrition* **24**:1167–1173 (2008).
24 Hundekar P, Suryakar A, Karnik A, Ghone R and Vasaikar M, Antioxidant status and lipid peroxidation in sickle cell anaemia. *Biomed Res India* **21**:461–464 (2010).
25 Kumerova A, Lece A, Skesters A, Silova A and Petuhovs V, Anaemia and antioxidant defence of the red blood cells. *Mater Med Pol* **30**:12–15 (1998).
26 Tamer L, Polat G, Yücebilgiç G and Güvenç B, The levels of sera malondialdehyde, erythrocyte membrane Na^{+}-K^{+}/Mg^{++} and Ca^{++}/Mg^{++} adenosine 5′ triphosphatase in patients with sickle cell anemia. *Turk J Hematol* **17**:23–26 (2000).
27 Al-Naama LM, Hassan MK and Mehdi JK, Association of erythrocytes antioxidant enzymes and their cofactors with markers of oxidative stress in patients with sickle cell anemia. *Qatar Med J* **2015**(2):14 (2015).
28 Adelakun A, Ajani O, Ogunleye T, Disu E, Kosoko A and Arinola G, Respiratory burst enzymes and oxidant-antioxidant status in Nigerian children with sickle cell disease. *Br Biotechnol J* **4**:270–278 (2014).
29 Hundekar P, Suryakar A, Karnik A, Valvi R, Ghone R and Bhagat S, The effect of antioxidant supplementation on the oxidant and antioxidant status in sickle cell anemia. *J Clin Diagn Res* **57**:1339–1342 (2011).
30 Gizi A, Papassotiriou I, Apostolakou F, Lazaropoulou C, Papastamataki M, Kanavaki I, *et al.*, Assessment of oxidative stress in patients with sickle cell disease: the glutathione system and the oxidant–antioxidant status. *Blood Cells Mol Dis* **46**:220–225 (2011).
31 Alsultan AI, Seif MA, Amin TT, Naboli M and Alsuliman AM, Relationship between oxidative stress, ferritin and insulin resistance in sickle cell disease. *Eur Rev Med Pharmacol Sci* **14**:527–538 (2010).
32 Vasconcelos SML, Goulart MOF, Moura JBDF, Manfredini V, Benfato MDS and Kubota LT, Reactive oxygen and nitrogen species, antioxidants and markers of oxidative damage in human blood: main analytical methods for their determination. *Quim Nova* **30**:1323–1338 (2007).
33 Liczmanski AE, Oxygen toxicity. *II. Defense mechanisms. Postepy Biochem* **34**:293–310 (1988) (in Polish).
34 Jessup W, Dean RT, de Whalley CV, Rankin SM and Leake DS, The role of oxidative modification and antioxidants in LDL metabolism and atherosclerosis. *Adv Exp Med Biol* **264**:139–142 (1990).
35 Diaz-Castro J, Perez-Sanchez LJ, Ramirez Lopez-Frias M, Lopez-Aliaga I, Nestares T, Alferez MJM, *et al.*, Influence of cow or goat milk

consumption on antioxidant defence and lipid peroxidation during chronic iron repletion. *Br J Nutr* **108**:1–8 (2012).
36 Hattori M, Yamaji-Tsukamoto K, Kumagai H, Feng Y and Takahashi K, Antioxidative activity of soluble elastin peptides. *J Agric Food Chem* **46**:2167–2170 (1998).
37 Karel M, Tannenbaum SR, Wallace DH and Maloney H, Autoxidation of methyl linoleate in freeze-dried model systems. *III. Effects of added amino acids. J Food Sci* **31**:892–896 (1966).
38 Díaz-Castro J, Hijano S, Alférez MJM, López-Aliaga I, Nestares T, López-Frías M, *et al.*, Goat milk consumption protects DNA against damage induced by chronic iron overload in anaemic rats. *Int Dairy J* **20**:495–499 (2010).
39 Diaz-Castro J, Sanchez-Alcover A, Hijano S, Alferez MJ, Nestares T, Moreno M, *et al.*, Goat milk supplemented with folic acid protects cell biomolecules from oxidative stress-mediated damage after anaemia recovery in comparison with cow milk. *Eur J Nutr* **53**:1165–1175 (2014).
40 Banala RR and Karnati PR, Vitamin A deficiency: an oxidative stress marker in sodium fluoride (NaF) induced oxidative damage in developing rat brain. *Int J Dev Neurosci* **47B**:298–303 (2015).
41 Epand RM and Vogel HJ, Diversity of antimicrobial peptides and their mechanisms of action. *Biochim Biophys Acta* **1462**:11–28 (1999).
42 Hancock RE and Diamond G, The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* **8**:402–410 (2000).
43 Diaz-Castro J, Ramirez Lopez-Frias M, Campos MS, Lopez-Frias M, Alferez MJ, Nestares T, *et al.*, Goat milk during iron repletion improves bone turnover impaired by severe iron deficiency. *J Dairy Sci* **94**:2752–2761 (2011).
44 Hamden K, Carreau S, Jamoussi K, Miladi S, Lajmi S, Aloulou D, *et al.*, 1α,25 Dihydroxyvitamin D3: therapeutic and preventive effects against oxidative stress, hepatic, pancreatic and renal injury in alloxan-induced diabetes in rats. *J Nutr Sci Vitaminol* **55**:215–222 (2009).
45 Sardar S, Chakraborty A and Chatterjee M, Comparative effectiveness of vitamin D3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague–Dawley rats. *Int J Vitam Nutr Res* **66**:39–45 (1996).
46 Stralin P and Marklund SL, Multiple cytokines regulate the expression of extracellular superoxide dismutase in human vascular smooth muscle cells. *Atherosclerosis* **151**:433–441 (2000).
47 Nikooyeh B, Neyestani TR, Tayebinejad N, Alavi-Majd H, Shariatzadeh N, Kalayi A, *et al.*, Daily intake of vitamin D- or calcium-vitamin D-fortified Persian yogurt drink (*doogh*) attenuates diabetes-induced oxidative stress: evidence for antioxidative properties of vitamin D. *J Hum Nutr Diet* **27**(Suppl. s2):276–283 (2014).
48 Moreno-Fernandez J, Diaz-Castro J, Alferez MJ, Nestares T, Ochoa JJ, Sanchez-Alcover A, *et al.*, Fermented goat milk consumption improves melatonin levels and influences positively the antioxidant status during nutritional ferropenic anemia recovery. *Food Funct* **7**:834–842 (2016).
49 Ghosh G, De K, Maity S, Bandyopadhyay D, Bhattacharya S, Reiter RJ, *et al.*, Melatonin protects against oxidative damage and restores expression of GLUT4 gene in the hyperthyroid rat heart. *J Pineal Res* **42**:71–82 (2007).
50 Ciftci M, Bilici D and Kufrevioglu OI, Effects of melatonin on enzyme activities of glucose-6-phosphate dehydrogenase from human erythrocytes *in vitro* and from rat erythrocytes *in vivo*. *Pharmacol Res* **44**:7–11 (2001).
51 Kumaravel TS and Jha AN, Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat Res* **605**:7–16 (2006).