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FERMENTED GOAT MILK CONSUMPTION IMPROVES DUODENAL EXPRESSION OF IRON HOMEOSTASIS GENES DURING ANEMIA RECOVERY

Jorge Moreno-Fernández, Javier Díaz-Castro, Mario Pulido-Moran, Maria J.M. Alférez, Christine Boesch, Ana Sanchez-Alcover, and Inmaculada López-Aliaga

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EXPRESSION OF IRON HOMEOSTASIS GENES DURING ANEMIA
RECOVERY

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ABSTRACT

In spite of the crucial role of Duodenal cytochrome b (Dcytb), Divalent metal transporter 1 (DMT1), Ferritin light chain (Ftl1), Ferroportin 1 (FPN1), Transferrin receptor 1 (TfR1) and Hepcidin antimicrobial peptide (Hamp) in Fe metabolism, no studies have investigated the modulations of these genes during Fe repletion with fermented milks. Analysis included Fe status markers, gene and protein expression in enterocytes of control and anemic animals fed fermented milks. Fermented goat milk up-regulated enterocyte Dcytb, DMT1, FPN1 and Ftl1, and down-regulated TfR1 and Hamp gene expression in control and anemic animals. Anemia decreased Dcytb, DMT1 and Ftl1, in animals fed fermented cow milk and up-regulated TfR1 and Hamp expression. Fe-overload down-regulated Dcytb and TfR1 in animals fed fermented cow milk, up-regulating DMT1 and FPN1 gene expression. Fermented goat milk increased expression of duodenal Dcytb, DMT1 and FPN1 and decreased Hamp and TfR1, improving Fe metabolism during anemia recovery.

KEY WORDS:

Fermented cow and goat milk; Anemia; Iron metabolism; Gene expression; Fe repletion.
INTRODUCTION

Fe is an essential mineral for life and it is an important cofactor for several enzymatic reactions involved in organism physiology,\(^1\) although if in excess, reacts with oxygen species and generates free radicals, inducing cellular damage to macromolecules and organelles.\(^2\) For these reasons, Fe homeostasis is tightly regulated to avoid both deficiency and toxicity due to Fe excess.

Iron deficiency anemia (IDA) has been described extensively and represents a source of morbidity and mortality worldwide. Recent epidemiological studies suggest that one-third of the world’s population is affected. As a highly prevalent hematological disorder, IDA represents a major public health problem.\(^3\) This pathology is developed usually due to low Fe intake, however, some genetic conditions may also result in anemia by causing defective Fe homeostasis.\(^4\) Several studies have reported noteworthy approach to the molecular mechanisms of Fe metabolism and the way that these processes are being disturbed in this prevalent micronutrient deficiency condition. The above mentioned studies have generated novel physiological approaches, dietary and pharmacological treatments.

Routine clinical tests used to assess Fe transport and storage include serum Fe (representing the Fe available between physiological compartments), total Fe binding capacity (TIBC) (which reflects the organism capacity to bind Fe with transferrin), serum ferritin (the predominant Fe storage protein, reflects the cumulative Fe stores in hematopoietic organs and tissues), and transferrin saturation, which is affected by the rate of Fe absorption in the small bowel as well as the amount of Fe in the stores.\(^5\)
Recently, major scientific efforts have been made to elucidate and understand the molecular mechanism of Fe homeostasis through gastrointestinal absorption, transport, tissue uptake, storage or remobilization from stores, leading to the identification of key proteins on intestinal level, including duodenal cytochrome b (Dcytb), divalent metal transporter 1 (DMT1), ferritin light chain 1 (Ftl1), ferroportin 1 (FPN1), transferrin receptor 1 (TfR1) and hepcidin antimicrobial peptide (Hamp). The orchestrated regulation of these key genes at transcriptional and translational levels regulates Fe uptake and transportation during IDA and during normal Fe metabolism conditions.6

On the other hand, we have previously reported7, 8 that goat milk improves Fe status, because enhances and improves Fe digestive and metabolic utilization, increasing Fe deposits in target organs, favoring the recovery of hematological parameters and minimizing interactions between dietary divalent cations such as Ca and Mg, however the genetic mechanisms controlling these physiological processes during dietary Fe repletion are still not completely elucidated. In spite of the crucial role of Dcytb, DMT1, Ftl1, FPN1, TfR1 and Hamp gene expression in Fe metabolism, to date, no studies have directly tested the modulation of gene expression during Fe repletion with fermented milks. Taking into account all these considerations, the aim of this work was to contribute to a better understanding of the pathophysiology and recovery from IDA, by studying how fermented milk consumption affects Fe absorption and metabolism during Fe repletion. Analysis included Fe status markers in blood and gene expression of Dcytb, DMT1, Ftl1, FPN1, TfR1 and Hamp in duodenal tissue.

MATERIAL AND METHODS
Fermented cow and goat milks were prepared according to the method described by Moreno-Fernandez et al.\textsuperscript{9} Both milk types were inoculated with traditional yoghurt starters \textit{Lactobacillus bulgaricus sub. delbrueckii} and \textit{Streptococcus thermophiles} and incubated at 37°C for approximately 24 h. Subsequently, both fermented milk samples were evaluated for pH (Crison, Barcelona, Spain) and the fermentation ended when the milks reached pH=4.6. Later, fermented milk samples were subjected to a smooth industrial dehydration process, until the final moisture ranged between 2.5%-4.5%.

Animals

Animal housing, care, handling 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). 80 recently weaned male Wistar albino breed rats, purchased from the University of Granada Laboratory Animal Service (Granada, Spain) were used for the study. To ensure conditions of high biological safety, all the animal assays were carried out in the animal breeding unit of the Centre of Biomedical Research of the University of Granada, with sanitary and environmental controlled parameters in an free of pathogens area. All animals were fed with the same amount of diet (pair feeding with 80% of the average intake) to avoid differences due to the intake and deionized water was available \textit{ad libitum}.

During the study, to ensure welfare and controlled conditions, the animals were housed in individual, ventilated, thermoregulated cages with controlled
temperature (23 ± 2°C), humidity (60 ± 5%) and a circadian rhythm of 12 hours (9:00 to 21:00 h).

**Design of experiment and diets**

At the beginning, the animals were divided into two groups: the control group receiving a normal-Fe diet (44.6 mg/Kg by analysis), 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. On day 40 of the study, two blood samples per rat were collected from the caudal vein (with EDTA to measure the hematological parameters) and the rest of the blood was centrifuged (1500 x g, 4°C, 15 min) without anticoagulant to separate the serum and subsequent analysis of serum Fe, total Fe binding capacity (TIBC), ferritin and serum hepcidin.

After inducing experimentally the Fe-deficiency anemia (day 40 of the study), the rats were placed on an experimental period in which both groups (control and anemic) were fed for 30 days with either 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. Diets were prepared with fermented cow or fermented goat milk powder to provide 20% of protein and 10% of fat (Table 1). During the experimental period, diet intake was also controlled (pair feeding of all the animals with 80% of the average intake) and deionized water was also available *ad libitum*. The Fe content (mg/kg) in the diets by analysis was: normal-Fe diet: 42.7 (fermented cow milk-based diet), 43.5 (fermented goat milk-based diet) and Fe-overload diets: 472.2 (fermented cow milk-based diet) and 472.8 (fermented goat milk-based diet).
At the end of the experimental period (day 70), the animals were anesthetized intraperitoneally with sodium pentobarbital (Sigma-Aldrich Co., St. Louis, MO) and totally bled out by cannulation of the aorta and the hematological parameters were analysed in total blood with anticoagulant (Figure 1). The rest of the blood was centrifuged (1500 x g, 4ºC, 15 min) without anticoagulant to separate the serum and for analysis of serum Fe, TIBC, ferritin and hepcidin. The duodenum was removed and washed repeatedly with ice-cold diethyl pyrocarbonate-treated (DEPC) (Invitrogen, Carlsbad, CA, USA) deionized water until complete elimination of duodenal fluids and bile. Subsequently, duodenal mucosa was scrapped using sterile glass slides and approximately 0.5 g of duodenal mucosa was incubated with RNA-later stabilization solution (Thermo Fisher Scientific, MA USA) overnight at 4ºC. Subsequently, RNA-later solution was removed, the mucosa samples frozen in liquid nitrogen and stored at -80ºC for RNA extraction.

**Hematological tests**

All the hematological parameters studied were measured using an automated haematology analyzer Mythic 22CT (C2 Diagnostics, Grabels, France).

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

Transferrin saturation, serum Fe and TIBC were determined using Sigma Diagnostics Iron and TIBC reagents (Sigma-Aldrich Co., St. Louis, MO). The absorbance of samples was read at 550 nm on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA).

**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).

Serum hepcidin

Hepcidin-25 was determined using a DRG ELISA Kit (DRG Instruments GmbH, Germany). The absorbance was read at 450 nm with a plate reader (Bio-Rad).

RNA isolation and real time qPCR

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

Quantitative real time PCR was carried out as two step procedure in a total reaction volume of 20 µl using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and SYBR Green detection using Sso Advanced Universal SYBR Green Supermix (Bio-Rad). Primer sequences for quantitative real-time PCR were designed by use of 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: duodenal cytochrome
b (Dcytb), divalent metal transporter 1 (DMT1), ferritin (Ftl1), Ferroportin 1 (FPN1), transferrin receptor 1 (TfR1) and hepcidin antimicrobial peptide (Hamp). 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.

**Western blotting and immunocytochemistry**

Finely chopped mucosa samples were obtained using Potter–Elvehjem homogenizer apparatus on ice, and whole cell proteins were extracted using T-PER tissue extraction reagent (Thermo Scientific Inc., Hanover Park, IL, USA). Protease inhibitor (1:200 dilution; Sigma-Aldrich, St. Louis, MO, USA) was incorporated. Protease inhibitor mixture (1:200 dilution (Sigma-Aldrich, St. Louis, MO) was added to avoid protein degradation. Total protein concentration was determined in the extracts using a Pierce BCA Protein Assay Kit (Thermo Scientific). Twelve µg of total protein were separated on 4–20% Criterion TGX (Tris-Glycine extended) gels (Mini-PROTEAN TGX Precast Gels, 15 µL; 15 wells; Bio-Rad). An electrophoretic separation was performed at 250 V in a vertical electrophoresis tank (Mini-PROTEAN System; Bio-Rad) for 20 min. Fermentas PageRuler Plus Prestained Protein Ladder was employed as molecular weight marker (Thermo Scientific). Thereafter, proteins were transferred from gel onto a PVDF membrane (Bio-Rad) by wet transfer for 60 min at 120V with transfer buffer comprising 250 mM Trizma HCl, 200 mM glycine, and 6% methanol, pH 8.3 (Sigma-Aldrich). After transfer, the membranes were blocked
with 5% dry milk in Tris-buffered saline (TBS) plus Tween-20 (TTBS) (Bio-Rad) solution for 1 h at room temperature. The blots were then washed 3 times in TBS, and incubated with rabbit anti DMT1 polyclonal antibody [(Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (dilution 1:400)], rabbit anti SLC40A1 polyclonal antibody (FPN1) [Abcam, UK (dilution 1:800)] and mouse anti β-actin monoclonal antibody [Abcam, UK (dilution 1:1000)] as primary antibodies, in 5% dry milk in TTBS overnight at 4 °C with shaking. β-actin was used as loading control.

Blots were then washed 3 times for 5 min each in TTBS and incubated with the appropriate secondary conjugated antibody [ImmunStar Goat Anti-Mouse (GAM)-HRP; 1:80,000 and Immun-Star Goat Anti-Rabbit (GAR)-HRP; Bio-Rad Laboratories; 1:40,000] in TTBS for 1 h at room temperature. The bands were visualised with Luminata forte western HRP Substrate (Merck KGaA, Darmstadt, Germany). Signal quantification and recording densitometry of each band were performed with chemiluminescence in ImageQuant LAS 4000 (Fujifilm Life Science Corporation, USA). All results were analysed with Image J software.

**Statistical analysis**

Data are reported as means ± standard error of the mean (SEM) of 40 animals per group during the pre-experimental period (anemia induction) and 10 animals per group during the experimental period (anemia recovery). Statistical analyses were performed using the SPSS computer program (version 22.0, 2013, SPSS Inc., Chicago, IL). Data were analysed by 2-way ANOVA to determine the effects of anemia, type of diet and dietary Fe content. Differences were considered significant at P<0.05. Differences between groups (normal-Fe vs. low-Fe 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. A power
analysis was performed to estimate the number of rats needed to obtain 80%
power at a confidence level of 95%, and although eight animals would be required
to obtain significant differences in hematological parameters and seven animals
per group would be required to obtain significant differences in gene expression
patterns, to ensure a power calculation, 10 rats per groups were used.

RESULTS

Hematological parameters

After anemia induction with Fe deprivation during 40 d, all the
hematological parameters in the IDA group were different from the controls \( P < 0.001 \), except white blood cells that remained unchanged after anemia induction
(Table 3). After 30 d feeding the fermented milk-based diets, the hematological
parameters were recovered with both milk-based diets, either with normal-Fe or
Fe-overload. Serum hepcidin was higher in control and anemic animals fed
fermented goat milk either with normal-Fe or Fe-overload in comparison with
fermented cow milk \( P < 0.001 \). As expected, serum Fe was higher in the Fe-
overload groups \( P < 0.01 \). Fe-overload also increased hemoglobin \( P < 0.001 \),
serum ferritin \( P < 0.01 \), transferrin saturation \( P < 0.01 \) and TIBC \( P < 0.01 \)
(Table 4).

Effect of fermented milks on Fe metabolism and uptake related genes and
proteins
The relative expression pattern of genes relevant to Fe metabolism is shown in Figure 2A-F. Fermented goat milk up-regulated enterocyte Dcytb gene expression in both groups of animals either with normal-Fe or Fe-overload (P< 0.001), previously induced anemia decreased Dcytb expression in the animals fed fermented cow milk with normal-Fe content (P< 0.01) and Fe-overload down-regulated Dcytb expression in both groups (control and anemic rats) fed fermented cow milk with normal-Fe content (P< 0.001) and in the anemic group fed fermented goat milk (P< 0.001) (Figure 2A).

Expression of DMT1 increased in control and anemic animals fed fermented goat either with normal-Fe or Fe-overload (P< 0.001). Enterocyte DMT1 expression was down-regulated in the anemic group fed fermented cow milk with Fe-overload (P< 0.05), however, the induction was much higher in the animals fed fermented goat milk with normal-Fe content (P< 0.001). Fe-overload increased DMT1 gene expression in the control group fed fermented goat milk (P< 0.001), but decreased its expression in the anemic animals fed the same diet (P< 0.001) (Figure 2B).

Similarly, DMT1 relative protein expression was higher in the animals fed fermented goat milk either with normal-Fe (P< 0.001) or Fe-overload (P< 0.01). Anemia increased DMT1 protein expression in animals fed fermented cow milk with normal Fe content (P< 0.05) and decreased this protein expression in animals fed fermented goat milk with normal-Fe content (P< 0.001). Fe-overload decreased DMT1 protein expression in all animals fed fermented goat milk in comparison with normal-Fe content groups (P< 0.001 for control animals and P< 0.05 for anemic animals) (Figures 3A and 3C).
Fermented goat milk induced an up-regulation of Ftl1 expression in control and anemic animals, especially in the anemic groups either with normal-Fe or Fe-overload (P< 0.05). Anemia up-regulated enterocyte Ftl1 expression in the animals fed both fermented milks with Fe-overload (P< 0.01). Fe-overload up-regulated Ftl1 expression in the anemic groups fed fermented cow milk (P< 0.05) and fermented goat milk (P< 0.01) (Figure 2C).

Fermented goat milk consumption induced a marked up-regulation of FPN1 mRNA in control and anemic rats fed with normal-Fe or Fe-overload (P< 0.001). Anemia increased FPN1 expression in all the groups of rats fed fermented milks (P< 0.001), except in those fed fermented goat milk diet with Fe-overload, which recorded a down-regulation of this gene (P< 0.001). Fe-overload in animals fed fermented cow milk down-regulated FPN1 gene expression in control rats (P< 0.05) and up-regulated this gene expression in anemic animals (P< 0.05), however in the animals fed fermented goat milk, Fe-overload up-regulated FPN1 in control animals (P< 0.001) and down-regulated this gene in anemic rats (P< 0.01) (Figure 2D).

With regard to FPN1 protein expression, a higher expression of this protein was recorded in control animals fed fermented goat milk with normal-Fe content in comparison with fermented cow milk (P< 0.001). Anemia increased FPN1 protein expression in the animals fed fermented cow milk with normal-Fe content (P< 0.001) and reduced this protein expression in the animals fed fermented goat milk with normal-Fe content (P< 0.001). Fe-overload increased FPN1 protein expression in control rats fed fermented cow milk (P< 0.001) and reduced this protein expression in control (P< 0.001) and anemic (P< 0.05) rats fed fermented goat milk (Figures 3B and 3C).
TfR1 was down-regulated in control and anemic rats fed fermented goat milk with normal-Fe content (P< 0.001) and in the anemic rats fed the same diet with Fe-overload (P< 0.001). Anemia induced a clear up-regulation of TfR1 gene expression in the anemic animals fed fermented goat milk with normal-Fe (P< 0.001). Fe-overload diminished TfR1 expression in all the experimental groups (P< 0.001), except in the control rats fed fermented goat milk (Figure 2E).

Hamp enterocyte expression was down-regulated in all the experimental groups (control and anemic) fed fermented goat milk (either with normal-Fe or Fe-overload) (P< 0.001). Anemia increased Hamp gene expression in the animals fed fermented goat milk with Fe-overload (P< 0.01). Fe-overload only showed an effect in the control group fed fermented goat milk, where a decrease in the expression of Hamp was recorded (P< 0.001) (Figure 2F).

**DISCUSSION**

Dietary fermented goat milk was found to be more beneficial in overcoming the effects of Fe deficiency, when compared with fermented cow milk. Among the studied genes, up-regulations in relative expression of enterocyte Dcytb, DMT1 and FPN1 mRNA were most significant, thereby confirming our research hypothesis (fermented goat milk improves Fe homeostasis) and previous results indicating better anemia recovery with goat milk consumption,7, 8 but in this case at the gene expression level.

It has been previously reported that a low vitamin A content in the diet, led to a reduced DMT1 protein expression by post-transcriptional regulation due either to a decreased translation or increased degradation, affecting negatively Fe metabolism.13 In addition, intracellular Fe sequestration is related to inflammatory
signaling. In a study conducted by Katz et al., vitamin A attenuated inflammatory signaling, and induced a recovery of the intracellular Fe-related proteins abnormalities, as well as alleviated intracellular Fe sequestration in duodenal cells. These results were serum hepcidin independent, thus they reflects the local intestinal compartmental events (at enterocyte level) and suggest that by applying anti-inflammatory compounds, less Fe is locked in inflamed intestinal epithelial cells, leading to its increased bioavailability by up-regulating the genes involved in Fe homeostasis. Taking into account that goat milk contains more vitamin A than cow milk, this vitamin might be contributing to the increased expression of Fe-status related genes.

In addition, goat milk fat is richer in medium chain triglycerides (MCT), which are able to pass through the mitochondrial membrane independently of carnitine, do not need re-esterification and are oxidized in the mitochondria, therefore providing fast energy discharge available for several metabolic pathways, conferring metabolic and energetic advantages to the enterocytes. Through goat milk consumption, the higher amount of MCT would provide more energy, inducing a trophic effect in the enterocyte and contribute to the up-regulation of the genes studied and hence improving duodenal Fe absorption.

Regulation of Fe uptake in the organism occurs in the enterocyte, which determines how much Fe is acquired in the intestinal lumen. Subsequent to reduction of dietary ferric iron (Fe$^{3+}$) by Dcytb, ferrous Fe (Fe$^{2+}$) is transported across the brush-border membrane of enterocytes via DMT1. In our case an up-regulation of Dcytb was recorded in all the groups fed fermented goat milk, revealing a higher capacity of dietary Fe reduction and thus conferring more substrate to DMT1, improving therefore the bioavailability by the enterocyte.
Anemia induced an over-expression of Dcytb mRNA in the groups fed fermented goat milk with normal-Fe content, however this trend was inverse in the case of rats fed fermented cow milk with normal-Fe content, revealing that Fe bioavailability by DMT1 would be lower in the case of cow milk. An up-regulation of Dcytb and DMT1, has been previously reported in situation of Fe-deficiency, as a compensatory mechanism to promote Fe absorption, findings in agreement with those obtained in the current study.

Furthermore, present results clearly show an up-regulation of DMT1 expression in animals consuming fermented goat milk in comparison with fermented cow milk. IDA did not show any effect on enterocyte DMT1 expression in the animals fed fermented cow milk, whereas increased DMT1 mRNA was observed in animals consuming fermented goat milk with normal-Fe content, however a significant reduction was observed in the anemic animals fed goat milk with Fe-overload, in a protective mechanism to avoid excessive Fe storage and subsequent Fe mediated damage in the tissues. DMT1 is a multipass, transmembrane protein that mediates the uptake of divalent cations, especially ferrous iron (Fe$^{2+}$). Physiological data reveal the essential role of DMT1 as an essential intestinal Fe importer, fact that can be confirmed because severe IDA that results from deletion$^{19}$ or mutation$^{20}$ of the gene in rodents. The up-regulation of DMT1 in the animals consuming goat milk confirms the better Fe absorption, fact that influences positively the recovery of the hematological parameters and confirms previous findings, on improved Fe digestive and metabolic utilization after goat milk consumption (non fermented)$^{7,8}$ at the level of gene expression.

Fe$^{2+}$ leaves enterocytes via FPN1-mediated transport, being the only protein capable to export ferrous Fe in mammals.$^{21}$ FPN1 is highly expressed
various types of cells that play critical roles in Fe homeostasis, such as enterocytes, macrophages and hepatocytes, consistent with its roles in Fe absorption and recycling. The key role of FPN1 can be exemplified by knockout of the gene in mice, which leads to a severe IDA. FPN1 gene mutations have been reported, and, although they are not common, they represent an important group of Fe-loading disorders. Subjects affected feature several phenotypes depending upon how the mutations alter FPN1 export function. It is noteworthy that these reports clearly feature the critical, non-redundant role of FPN1 in Fe absorption. Similarly to Dcytb and DMT1, an up-regulation in relative expression of FPN1 mRNA was observed in animals consuming fermented goat milk either with normal-Fe or Fe-overload. As previously reported, anemia induces an up-regulation of FPN1 as a compensatory response to systemic Fe depletion, to adjust the rates of Fe export, results in agreement with those obtained in the current study.

Fe homeostasis in the enterocytes is regulated towards the FPN1-Hamp axis, which involves specific physiological adaptations that have evolved to maximize Fe uptake from the diet when the Fe requirements increase. These adaptations include direct effects on duodenal cells gene transcription, posttranscriptional control of mRNA stability, and morphological remodeling of the duodenal epithelium, probably all in response to alterations in intracellular Fe levels in enterocytes. Hepcidin plays a central role in Fe absorption by the duodenal epithelial cells (enterocytes). Hamp expression was found to be decreased in rats consuming fermented goat milk in comparison with rats consuming cow milk. This down-regulation of enterocyte Hamp would increase Fe efflux from the duodenal cells, because enterocyte Hamp mRNA expression
correlates inversely with the activity of FPN1 and Fe absorption.\textsuperscript{26} Once more these data indicate the better Fe status recovery and absorption with fermented goat milk.

In contrast, serum hepcidin followed a different pattern during Fe repletion with fermented milks, increasing in the animals fed fermented goat milk, finding in agreement with previous results.\textsuperscript{27} As previously mentioned, hepcidin intervenes in regulating Fe storage in the enterocytes and liver (attenuating both the intestinal Fe absorption and the liberation of the Fe of the macrophages and hepatocytes), being a key factor in the erythroid regulation, recording a diminished expression of this peptide in response to the anemic hypoxia.\textsuperscript{28} Therefore, the increase in serum hepcidin recorded during Fe repletion with fermented goat milk, can be explained by the induction of erythropoiesis,\textsuperscript{29} due to increase of duodenal Fe absorption. The higher levels of this peptide hormone compared with fermented cow milk diet would decrease Fe export from hepatocytes, increasing its storage in liver and revealing that Fe stores were replete.\textsuperscript{27}

It is known that duodenal Fe absorption correlates with Fe status of the body. In the present study, most of the genes showed significantly higher mRNA abundance during Fe depletion with fermented goat milk compared with cow milk. This finding suggests that up-regulation of genes coding for proteins involved in Fe absorption, such as Dcytb, DMT1 and FPN1, play a key role in enhanced absorption of Fe during IDA recovery. In addition, enhanced expression of TfR1 has been reported in IDA\textsuperscript{30, 31} therefore the down-regulation of this gene in the animals consuming fermented goat milk reveals, once again, the adapted genetic regulation of Fe metabolism during Fe repletion.
Ft1 is an intracellular protein that stores and releases Fe in a strictly controlled way. Ferritin gene is up-regulated in situation of Fe overload, to ensure higher Fe sequestration, which prevents Fe-mediated oxidative damage to the main biomolecules. In the current study, the expression of enterocyte ferritin mRNA is higher in the anemic group fed goat milk with normal-Fe content. In addition, Fe-overload increased ferritin gene expression in control group fed fermented cow milk and anemic group fed fermented goat milk, probably as a compensatory mechanism to avoid the oxidative damage induced by Fe-overload.

In conclusion, consumption of fermented goat milk was found to be more beneficial compared to fermented cow milk in overcoming the effects of IDA. Effects due to fermented goat milk can be explained on molecular level as changes in key proteins of intestinal Fe metabolism showing increased expression of duodenal Dcytb, DMT1 and FPN1. In addition, Hamp and TfR1 showed significantly lower or equal mRNA abundance during Fe repletion with fermented goat milk, compared with fermented cow milk, revealing that changes in relative expression of enterocyte Fe-related genes are sensitive molecular biomarkers for IDA recovery with fermented goat milk consumption. The results also suggest that fermented goat milk promotes Fe-related genes expressions mechanisms, increasing Fe bioavailability and Fe repletion after induced anemia, a fact that can have significant implications in populations suffering nutritional deficiencies to alleviate IDA.
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Notes

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

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<thead>
<tr>
<th>Component</th>
<th>g/Kg diet</th>
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<td>Pre-experimental period, Standard (non-milk) diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
</tr>
<tr>
<td>Lactose</td>
<td>0</td>
</tr>
<tr>
<td>Fat (virgin olive oil)</td>
<td>100</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>500</td>
</tr>
<tr>
<td>Constant ingredients&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experimental period

Fermented cow milk-based diet<sup>c</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>g/Kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>205</td>
</tr>
<tr>
<td>Lactose</td>
<td>295</td>
</tr>
<tr>
<td>Fat</td>
<td>100</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>200</td>
</tr>
<tr>
<td>Constant ingredients&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200</td>
</tr>
</tbody>
</table>

Fermented goat milk-based diet<sup>c</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>g/Kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>206</td>
</tr>
<tr>
<td>Lactose</td>
<td>291</td>
</tr>
<tr>
<td>Fat</td>
<td>100</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>203</td>
</tr>
<tr>
<td>Constant ingredients&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200</td>
</tr>
</tbody>
</table>

<sup>a</sup> The diets were prepared according to the recommendations of the AIN-93G for control rats (45 mg Fe/Kg diet) (Reeves et al., 1993), or with low Fe content (5 mg Fe/Kg diet) (Pallarés et al., 1993), for anaemic groups.

<sup>b</sup> 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.

<sup>c</sup> 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) (Reeves et al., 1993) or Fe-overload (450 mg Fe/Kg diet) (Raja et al., 1994).
**Table 2.** PCR primer sequences and annealing temperatures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>GGGTGTTGAAGGTCTCTAAA</td>
<td>TGTCACCAACTGGGACGATA</td>
<td>57</td>
</tr>
<tr>
<td>Dcytb</td>
<td>AGATTGCCCATTGGACCTGGAA</td>
<td>GAGCCCATGGAAGCAGAAAG</td>
<td>57</td>
</tr>
<tr>
<td>DMT1</td>
<td>GGCAATGTGGCACTGTATGTG</td>
<td>CCGCTGTATCTTCGCTCAG</td>
<td>59</td>
</tr>
<tr>
<td>Ftl1</td>
<td>GCCCTGGAGAAGAACCTGAA</td>
<td>AGTCGTGCTTCAGAGTGAGG</td>
<td>59</td>
</tr>
<tr>
<td>FPN1</td>
<td>GAACAAGACCCACCTGTGC</td>
<td>AGGATGGAACCACTCAGTCC</td>
<td>57</td>
</tr>
<tr>
<td>TfiR1</td>
<td>CAAATGGTTCTGATACAGGCACG</td>
<td>CTCCACGAGCAGAATACAGC</td>
<td>59</td>
</tr>
<tr>
<td>Hamp</td>
<td>CCTATCTCCGGCAACAGCG</td>
<td>GGAAGTTGGTGTCGCTT</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Normal-Fe Control group ((n = 40))</td>
<td>Low-Fe Anemic group ((n = 40))</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------------------</td>
<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Total blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb concentration (g/L)</td>
<td>133.88 ± 2.98</td>
<td>60.22 ± 2.87*</td>
<td></td>
</tr>
<tr>
<td>RBCs ((10^{12}/L))</td>
<td>7.14 ± 0.20</td>
<td>3.08 ± 0.24*</td>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40.01 ± 1.13</td>
<td>12.76 ± 1.33*</td>
<td></td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>55.76 ± 0.53</td>
<td>36.91 ± 0.37*</td>
<td></td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.47 ± 0.15</td>
<td>14.10 ± 0.63*</td>
<td></td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>35.54 ± 0.36</td>
<td>30.21 ± 0.82*</td>
<td></td>
</tr>
<tr>
<td>RDW (%)</td>
<td>16.23 ± 0.35</td>
<td>19.16 ± 0.39*</td>
<td></td>
</tr>
<tr>
<td>Platelets ((10^9/L))</td>
<td>733 ± 72.15</td>
<td>2123 ± 119*</td>
<td></td>
</tr>
<tr>
<td>WBCs ((10^9/L))</td>
<td>8.91 ± 0.39</td>
<td>8.53 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes ((10^6/ml))</td>
<td>7.98 ± 0.58</td>
<td>5.76 ± 0.84*</td>
<td></td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe (µg/L)</td>
<td>1336 ± 99.12</td>
<td>601 ± 55.98*</td>
<td></td>
</tr>
<tr>
<td>TIBC (µg/L)</td>
<td>2675 ± 189</td>
<td>17935 ± 598*</td>
<td></td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>49.02 ± 5.87</td>
<td>3.93 ± 0.41*</td>
<td></td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>79.74 ± 2.12</td>
<td>49.65 ± 1.64*</td>
<td></td>
</tr>
<tr>
<td>Hepcidin, ng/mL</td>
<td>16.87 ± 0.46</td>
<td>13.48 ± 0.62*</td>
<td></td>
</tr>
</tbody>
</table>

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.
Table 4. Haematological parameters from control and anemic rats fed for 30 days with fermented cow or goat milk-based diets with normal-Fe content or Fe-overload (experimental period)

<table>
<thead>
<tr>
<th></th>
<th>Fermented cow milk</th>
<th>Fermented goat milk</th>
<th>2-WAY ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe content</td>
<td>Control group</td>
<td>Anemic group</td>
</tr>
<tr>
<td>Hb concentration (g/l)</td>
<td>Normal</td>
<td>129.55 ± 2.89</td>
<td>129.33 ± 2.61</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>142.63 ± 2.63C</td>
<td>141.10 ± 2.92AC</td>
</tr>
<tr>
<td>RBCs (10^{12}/l)</td>
<td>Normal</td>
<td>7.06 ± 0.18</td>
<td>7.08 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>6.94 ± 0.17a</td>
<td>7.19 ± 0.24</td>
</tr>
<tr>
<td>Haematocrit %</td>
<td>Normal</td>
<td>40.02 ± 1.19a</td>
<td>39.01 ± 0.97A</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>39.37 ± 1.33a</td>
<td>44.89 ± 2.76C</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>Normal</td>
<td>57.68 ± 0.54</td>
<td>55.34 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>56.79 ± 0.58</td>
<td>53.18± 0.55A</td>
</tr>
<tr>
<td>Platelets (10^{9}/l)</td>
<td>Normal</td>
<td>933.00 ± 70.32</td>
<td>963.00 ± 66.45</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>939.67 ± 71.37</td>
<td>965.50 ± 72.22</td>
</tr>
<tr>
<td>Serum Fe (µg/l)</td>
<td>Normal</td>
<td>1346 ± 86.18</td>
<td>1355 ± 86.35</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>1591± 100C</td>
<td>1587 ± 102C</td>
</tr>
<tr>
<td>TIBC (µg/l)</td>
<td>Normal</td>
<td>2787 ± 158</td>
<td>2798 ± 137</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>3145 ± 177C</td>
<td>3254 ± 175C</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>Normal</td>
<td>45.98 ± 0.91</td>
<td>45.32 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>47.76 ± 1.32C</td>
<td>47.88± 1.02C</td>
</tr>
<tr>
<td>Serum ferritin (µg/l)</td>
<td>Normal</td>
<td>83.25 ± 1.75</td>
<td>82.97 ± 1.68</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>87.73 ± 1.87C</td>
<td>86.87± 1.91C</td>
</tr>
<tr>
<td>Serum hepcidin (ng/mL)</td>
<td>Normal</td>
<td>14.25 ± 0.59a</td>
<td>14.42± 0.49A</td>
</tr>
<tr>
<td></td>
<td>Overload</td>
<td>15.75 ± 0.62aC</td>
<td>14.98± 0.58A</td>
</tr>
</tbody>
</table>

<sup>1</sup>NS, not significant.

<sup>a,b</sup>Mean values among groups of controls rats fed with different diet and different lower case letters in the same row indicated significant difference by 2-way ANOVA (Tukey’s test).

<sup>A,B</sup>Mean values among groups of anaemic rats fed with different diet and different upper case letters in the same row indicated significant difference by 2-way ANOVA (Tukey’s test).

<sup>c</sup>Mean values were significantly different from the corresponding group of rats fed with normal Fe content at P < 0.05 by Student’s t test.
Figure 1. Experimental design of the study.
**Figure 2.** Effect of fermented cow and goat milk in control and anemic rats under normal and Fe overload conditions on mRNA levels of duodenal Dcytb (A), DMT1 (B), Ftl1 (C), FPN1 (D), Tfr1 (E) and Hamp (F). Data are means with SEM of 10 animals per group.

a,b Means values among groups of controls rats with different superscript letters were significantly different (P < 0.05, Tukey’s test).

A,B Means values among groups of anemic rats with different upper case superscript letters were significantly different (P < 0.05, Tukey’s test).

C Means values from the corresponding group of control rats were significantly different (P < 0.05, Student’s t test).

D Means values from the corresponding group of rats fed with normal-Fe content were significantly different (P < 0.05, Student’s t test).
Figure 3. Effect of fermented cow and goat milk in control and anemic rats under normal and Fe overload conditions on expression of DMT1 (A) and FPN1 (B) protein. Data are mean with SEM of 10 animals per group. Representative immunoblots of DMT1, FPN1 and β-actin are shown (C).

a, b Means values among groups of controls rats with different superscript letters were significantly different (P < 0.05, Tukey’s test).

A, B Means values among groups of anemic rats with different upper case superscript letters were significantly different (P < 0.05, Tukey’s test).

C Means values from the corresponding group of control rats were significantly different (P < 0.05, Student’s t test).

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