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Article

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FERMENTED GOAT MILK CONSUMPTION IMPROVES DUODENAL 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 reported[7, 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

Fermentation and dehydration of the milks

Fermented cow and goat 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 sub. delbruickii* and *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 *ad libitum*.

During the study, to ensure welfare and controlled conditions, the animals were housed in individual, ventilated, thermoregulated cages with controlled

119 temperature (23 ± 2ºC), humidity (60 ± 5%) and a circadian rhythm of 12 hours
120 (9:00 to 21:00 h).
121
122 **Design of experiment and diets**
123 At the beginning, the animals were divided into two groups: the control group
124 receiving a normal-Fe diet (44.6 mg/Kg by analysis) [10], and the anemic group
125 receiving a low-Fe diet (6.2 mg/Kg by analysis), induced experimentally during
126 40 d by a method developed previously by our research group [11]. On day 40 of
127 the study, two blood samples per rat were collected from the caudal vein (with
128 EDTA to measure the hematological parameters) and the rest of the blood was
129 centrifuged (1500 x g, 4ºC, 15 min) without anticoagulant to separate the serum
130 and subsequent analysis of serum Fe, total Fe binding capacity (TIBC), ferritin
131 and serum hepcidin.
132 After inducing experimentally the Fe-deficiency anemia (day 40 of the
133 study), the rats were placed on an experimental period in which both groups
134 (control and anemic) were fed for 30 days with either fermented cow milk or
135 fermented goat milk-based diet, with normal-Fe content (45 mg/kg) or Fe-
136 overloaded (450 mg/kg) to induce chronic Fe-overload [12]. Diets were prepared
137 with fermented cow or fermented goat milk powder to provide 20% of protein and
138 10% of fat (Table 1). During the experimental period, diet intake was also
139 controlled (pair feeding of all the animals with 80% of the average intake) and
140 deionized water was also available *ad libitum*. The Fe content (mg/kg) in the diets
141 by analysis was: normal-Fe diet: 42.7 (fermented cow milk-based diet), 43.5
142 (fermented goat milk-based diet) and Fe-overload diets: 472.2 (fermented cow
143 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 -80°C 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 450nm 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 260/280nm. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis kit (Bio-Rad) in a 20µl reaction, with 1µg 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 20µl using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and SYBR Green detection using Sso Avdvanced 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 [(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 [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

269 The relative expression pattern of genes relevant to Fe metabolism is
270 shown in Figure 2A-F. Fermented goat milk up-regulated enterocyte Dcytb gene
271 expression in both groups of animals either with normal-Fe or Fe-overload ($P < 0.001$),
272 previously induced anemia decreased Dcytb expression in the animals fed
273 fermented cow milk with normal-Fe content ($P < 0.01$) and Fe-overload down-
274 regulated Dcytb expression in both groups (control and anemic rats) fed fermented
275 cow milk with normal-Fe content ($P < 0.001$) and in the anemic group fed
276 fermented goat milk ($P < 0.001$) (Figure 2A).

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

285 Similarly, DMT1 relative protein expression was higher in the animals fed
286 fermented goat milk either with normal-Fe ($P < 0.001$) or Fe-overload ($P < 0.01$).
287 Anemia increased DMT1 protein expression in animals fed fermented cow milk
288 with normal Fe content ($P < 0.05$) and decreased this protein expression in animals
289 fed fermented goat milk with normal-Fe content ($P < 0.001$). Fe-overload
290 decreased DMT1 protein expression in all animals fed fermented goat milk in
291 comparison with normal-Fe content groups ($P < 0.001$ for control animals and $P <$
292 0.05 for anemic animals) (Figures 3A and 3C).

293 Fermented goat milk induced an up-regulation of Ftl1 expression in
294 control and anemic animals, especially in the anemic groups either with normal-
295 Fe or Fe-overload ($P < 0.05$). Anemia up-regulated enterocyte Ftl1 expression in
296 the animals fed both fermented milks with Fe-overload ($P < 0.01$). Fe-overload up-
297 regulated Ftl1 expression in the anemic groups fed fermented cow milk ($P < 0.05$)
298 and fermented goat milk ($P < 0.01$) (Figure 2C).

299 Fermented goat milk consumption induced a marked up-regulation of
300 FPN1 mRNA in control and anemic rats fed with normal-Fe or Fe-overload ($P <$
301 0.001). Anemia increased FPN1 expression in all the groups of rats fed fermented
302 milks ($P < 0.001$), except in those fed fermented goat milk diet with Fe-overload,
303 which recorded a down-regulation of this gene ($P < 0.001$). Fe-overload in animals
304 fed fermented cow milk down-regulated FPN1 gene expression in control rats ($P <$
305 0.05) and up-regulated this gene expression in anemic animals ($P < 0.05$), however
306 in the animals fed fermented goat milk, Fe-overload up-regulated FPN1 in control
307 animals ($P < 0.001$) and down-regulated this gene in anemic rats ($P < 0.01$) (Figure
308 2D).

309 With regard to FPN1 protein expression, a higher expression of this
310 protein was recorded in control animals fed fermented goat milk with normal-Fe
311 content in comparison with fermented cow milk ($P < 0.001$). Anemia increased
312 FPN1 protein expression in the animals fed fermented cow milk with normal-Fe
313 content ($P < 0.001$) and reduced this protein expression in the animals fed
314 fermented goat milk with normal-Fe content ($P < 0.001$). Fe-overload increased
315 FPN1 protein expression in control rats fed fermented cow milk ($P < 0.001$) and
316 reduced this protein expression in control ($P < 0.001$) and anemic ($P < 0.05$) rats
317 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

343 signaling. In a study conducted by Katz et al.,[14] vitamin A attenuated
344 inflammatory signaling, and induced a recovery of the intracellular Fe-related
345 proteins abnormalities, as well as alleviated intracellular Fe sequestration in
346 duodenal cells. These results were serum hepcidin independent, thus they reflects
347 the local intestinal compartmental events (at enterocyte level) and suggest that by
348 applying anti-inflammatory compounds, less Fe is locked in inflamed intestinal
349 epithelial cells, leading to its increased bioavailability by up-regulating the genes
350 involved in Fe homeostasis. Taking into account that goat milk contains more
351 vitamin A than cow milk,[7, 15] this vitamin might be contributing to the increased
352 expression of Fe-status related genes.

353 In addition, goat milk fat is richer in medium chain triglycerides (MCT),
354 which are able to pass through the mitochondrial membrane independently of
355 carnitine, do not need re-esterification and are oxidized in the mitochondria,
356 therefore providing fast energy discharge available for several metabolic
357 pathways, conferring metabolic and energetic advantages to the enterocytes.[7]
358 Through goat milk consumption, the higher amount of MCT would provide more
359 energy, inducing a trophic effect[16] in the enterocyte and contribute to the up-
360 regulation of the genes studied and hence improving duodenal Fe absorption.

361 Regulation of Fe uptake in the organism occurs in the enterocyte, which
362 determines how much Fe is acquired in the intestinal lumen. Subsequent to
363 reduction of dietary ferric iron (Fe^{3+}) by Dcytb, ferrous Fe (Fe^{2+}) is transported
364 across the brush-border membrane of enterocytes via DMT1.[17] In our case an up-
365 regulation of Dcytb was recorded in all the groups fed fermented goat milk,
366 revealing a higher capacity of dietary Fe reduction and thus conferring more
367 substrate to DMT1, improving therefore the bioavailability by the enterocyte.

368 Anemia induced an over-expression of Dcytb mRNA in the groups fed fermented
369 goat milk with normal-Fe content, however this trend was inverse in the case of
370 rats fed fermented cow milk with normal-Fe content, revealing that Fe
371 bioavailability by DMT1 would be lower in the case of cow milk. An up-
372 regulation of Dcytb and DMT1, has been previously reported in situation of Fe-
373 deficiency, as a compensatory mechanism to promote Fe absorption,[18] findings in
374 agreement with those obtained in the current study.

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

391 Fe^{2+} leaves enterocytes via FPN1-mediated transport, being the only
392 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.[22] FPN1 gene mutations have been reported, and, although they are not common, they represent an important group of Fe-loading disorders.[23] 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,[24] 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.[25] 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

418 correlates inversely with the activity of FPN1 and Fe absorption.[26] Once more
419 these data indicate the better Fe status recovery and absorption with fermented
420 goat milk.

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

434 It is known that duodenal Fe absorption correlates with Fe status of the
435 body. In the present study, most of the genes showed significantly higher mRNA
436 abundance during Fe depletion with fermented goat milk compared with cow
437 milk. This finding suggests that up-regulation of genes coding for proteins
438 involved in Fe absorption, such as Dcytb, DMT1 and FPN1, play a key role in
439 enhanced absorption of Fe during IDA recovery. In addition, enhanced expression
440 of TfR1 has been reported in IDA[30, 31] therefore the down-regulation of this gene
441 in the animals consuming fermented goat milk reveals, once again, the adapted
442 genetic regulation of Fe metabolism during Fe repletion.

Ftl1 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 biomlecules. 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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615 **Table 1**. Composition of the experimental diets
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Component	g/Kg diet
Pre-experimental period, Standard (non-milk) diet[a]	
Casein	200
Lactose	0
Fat (virgin olive oil)	100
Wheat starch	500
Constant ingredients[b]	200
Experimental period	
Fermented cow milk-based diet[c]	
Protein	205
Lactose	295
Fat	100
Wheat starch	200
Constant ingredients[b]	200
Fermented goat milk-based diet[c]	
Protein	206
Lactose	291
Fat	100
Wheat starch	203
Constant ingredients[b]	200

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620 [a] The diets were prepared according to the recommendations of the AIN-93G for
621 control rats (45 mg Fe/Kg diet) (Reeves et al., 1993), or with low Fe content (5 mg
622 Fe/Kg diet) (Pallarés et al., 1993), for anaemic groups.
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624 [b] The constant ingredients consisted of (g/Kg diet): fibre (micronized cellulose)
625 50, sucrose 100, choline chloride 2.5, L-cystine 2.5, mineral premix 35, vitamin
626 premix 10.
627
628 [c] Specific vitamin and mineral premixes supplements for fermented goat and cow
629 milk-based diets were formulated taking into account the mineral and vitamin
630 contents of the fermented milk powder supplied in order to meet the
631 recommendations of the AIN-93G for normal-Fe diets (45 mg Fe/Kg diet) (Reeves
632 et al., 1993) or Fe-overload (450 mg Fe/Kg diet) (Raja et al., 1994).
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645 **Table 2**. PCR primer sequences and annealing temperatures
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Gene	Forward Sequence	Reverse Sequence	Annealing temperature (°C)
β-Actin	GGGGTGTTGAAGGTCTCAAA	TGTCACCAACTGGGACGATA	57
Dcytb	AGATTGCCATGGACCTGGAA	GAGCCCATGGAAGCAGAAAG	57
DMT1	GGCATGTGGCACTGTATGTG	CCGCTGGTATCTTCGCTCAG	59
Ftl1	GCCCTGGAGAAGAACCTGAA	AGTCGTGCTTCAGAGTGAGG	59
FPN1	GAACAAGAACCCACCTGTGC	AGGATGGAACCACTCAGTCC	57
TfR1	CAAATGGTTCGTACAGCAGGC	CTCCACGAGCAGAATACAGC	59
Hamp	CCTATCTCCGGCAACAGACG	GGGAAGTTGGTGTCTCGCTT	59

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685 **Table 3**. Hematological parameters of control and anemic rats
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	Normal-Fe Control group (n = 40)	Low-Fe Anemic group (n = 40)
Total blood		
Hb concentration (g/L)	133.88 ± 2.98	60.22 ± 2.87 *
RBCs (10^{12}/L)	7.14 ± 0.20	3.08 ± 0.24 *
Hematocrit (%)	40.01 ± 1.13	12.76 ± 1.33 *
MCV (fL)	55.76 ± 0.53	36.91 ± 0.37 *
MCH (pg)	19.47 ± 0.15	14.10 ± 0.63 *
MCHC (g/dl)	35.54 ± 0.36	30.21 ± 0.82*
RDW (%)	16.23 ± 0.35	19.16 ± 0.39 *
Platelets (10^9/L)	733 ± 72.15	2123 ± 119 *
WBCs (10^9/L)	8.91 ± 0.39	8.53 ± 0.88
Lymphocytes (10^6/ml)	7.98 ± 0.58	5.76 ± 0.84*
Serum		
Fe (μg/L)	1336 ± 99.12	601 ± 55.98 *
TIBC (μg/L)	2675 ± 189	17935 ± 598 *
Transferrin saturation (%)	49.02 ± 5.87	3.93 ± 0.41 *
Ferritin (μg/L)	79.74 ± 2.12	49.65 ± 1.64 *
Hepcidin, ng/mL	16.87 ± 0.46	13.48 ± 0.62*

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689 Data are shown as the mean values ± SEM.
690 Hb, hemoglobin; RBCs, red blood cells; MCV, mean corpuscular volume; MCH,
691 mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; RDW, red
692 cell distribution width; WBCs, white blood cells; TIBC, total Fe-binding capacity.
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694 *Significantly different from the control group ($P < 0.001$, Student's t test).
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703 **Table 4.** Haematological parameters from control and anemic rats fed for 30 days with fermented cow or goat milk-based diets with normal-
704 Fe content or Fe-overload (experimental period)
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		Fermented cow milk		Fermented goat milk		2-WAY ANOVA		
	Fe content	Control group	Anemic group	Control group	Anemic group	Diet	Anemia	Fe content
Hb concentration (g/l)	Normal	129.55 ± 2.89	129.33 ± 2.61	131.95 ± 2.76	129.15 ± 2.48	NS[1]	NS	< 0.001
	Overload	142.63 ± 2.63C	141.10± 2.92AC	141.30 ± 2.98C	147.35 ± 3.01BC	< 0.05	NS	
RBCs (10^{12}/l)	Normal	7.06± 0.18	7.08± 0.22	7.39 ± 0.21	7.21± 0.20	< 0.05	NS	< 0.05
	Overload	6.94 ± 0.17a	7.19 ± 0.24	8.01 ± 0.30 bC	7.11 ± 0.21	< 0.01	NS	
Haematocrit %	Normal	40.02± 1.19a	39.01± 0.97A	41.93± 1.23b	42.95 ± 0.98B	< 0.01	NS	< 0.01
	Overload	39.37 ± 1.33a	44.89 ± 2.76C	44.86 ± 1.26 bC	45.42 ± 1.35C	< 0.05	NS	
MCV (fL)	Normal	57.68 ± 0.54	55.34 ± 0.59	57.25 ± 0.56	55.04 ± 0.53	NS	NS	NS
	Overload	56.79 ± 0.58	53.18± 0.55A	56.44 ± 0.53	56.15± 0.53B	< 0.05	NS	
Platelets (10^9/l)	Normal	933.00 ± 70.32	963.00 ± 66.45	926.00 ± 79.65	935.33 ± 66.89	NS	NS	NS
	Overload	939.67 ± 71.37	965.50 ± 72.22	933.59 ± 81.32	945.86 ± 70.26	NS	NS	
Serum Fe (µg/l)	Normal	1346 ± 86.18	1355 ± 86.35	1352 ± 88.96	1326 ± 94.35	NS	NS	< 0.01
	Overload	1591± 100C	1587 ± 102C	1556 ± 99C	1576 ± 97C	NS	NS	
TIBC (µg/l)	Normal	2787 ± 158	2798 ± 137	2785 ± 144	2789 ± 166	NS	NS	< 0.01
	Overload	3145 ± 177C	3254 ± 175C	3251 ± 169C	3195± 166C	NS	NS	
Transferrin saturation (%)	Normal	45.98 ± 0.91	45.32 ± 0.89	46.65± 0.76	46.37± 0.94	NS	NS	< 0.01
	Overload	47.76± 1.32C	47.88± 1.02C	49.59± 0.98C	48.96± 1.05C	NS	NS	
Serum ferritin (µg/l)	Normal	83.25 ± 1.75	82.97 ± 1.68	84.33 ± 1.77	82.34± 1.82	NS	NS	< 0.01
	Overload	87.73 ± 1.87C	86.87± 1.91C	87.91± 1.88C	86.65± 1.96C	NS	NS	
Serum hepcidin (ng/mL)	Normal	14.25 ± 0.59a	14.42± 0.49A	16.85± 0.55b	16.66 ± 0.59B	< 0.01	NS	NS
	Overload	15.75 ± 0.62aC	14.98± 0.58A	17.01 ± 0.61b	16.73± 0.63B	< 0.01	NS	

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707 [1]NS, not significant.
708 [a,b] 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).
709 [A,B] 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).
710 [C] 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.
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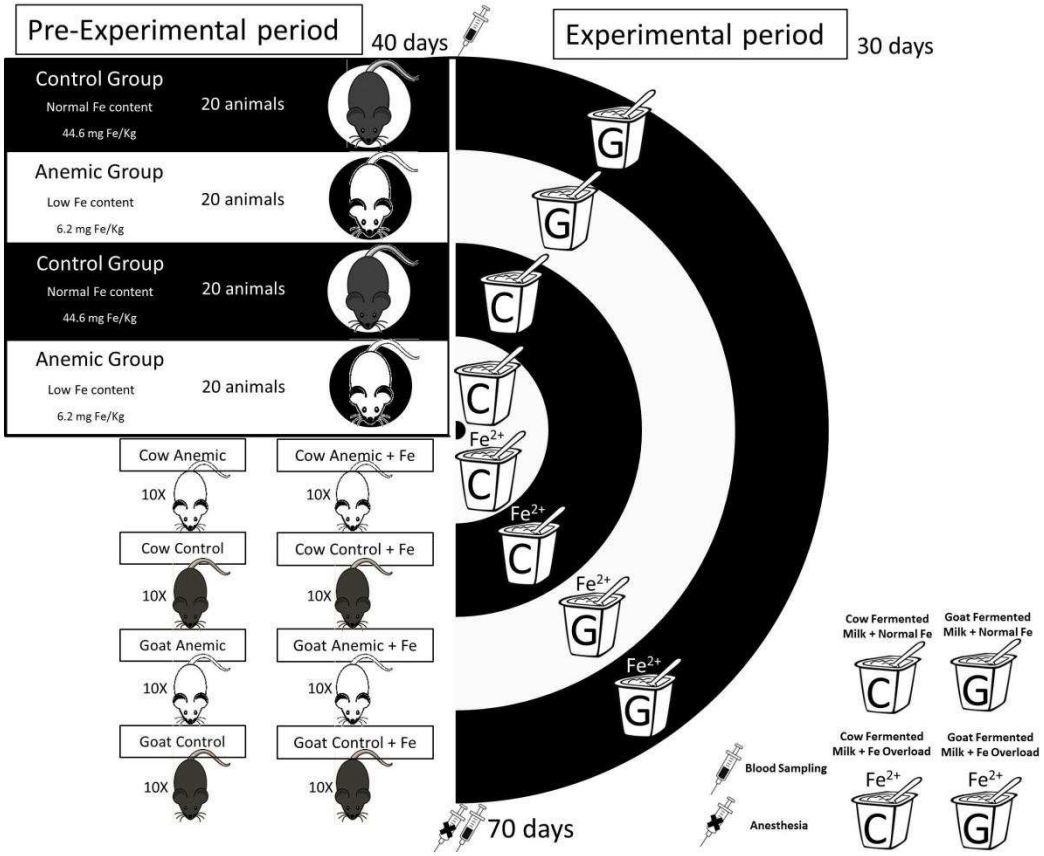
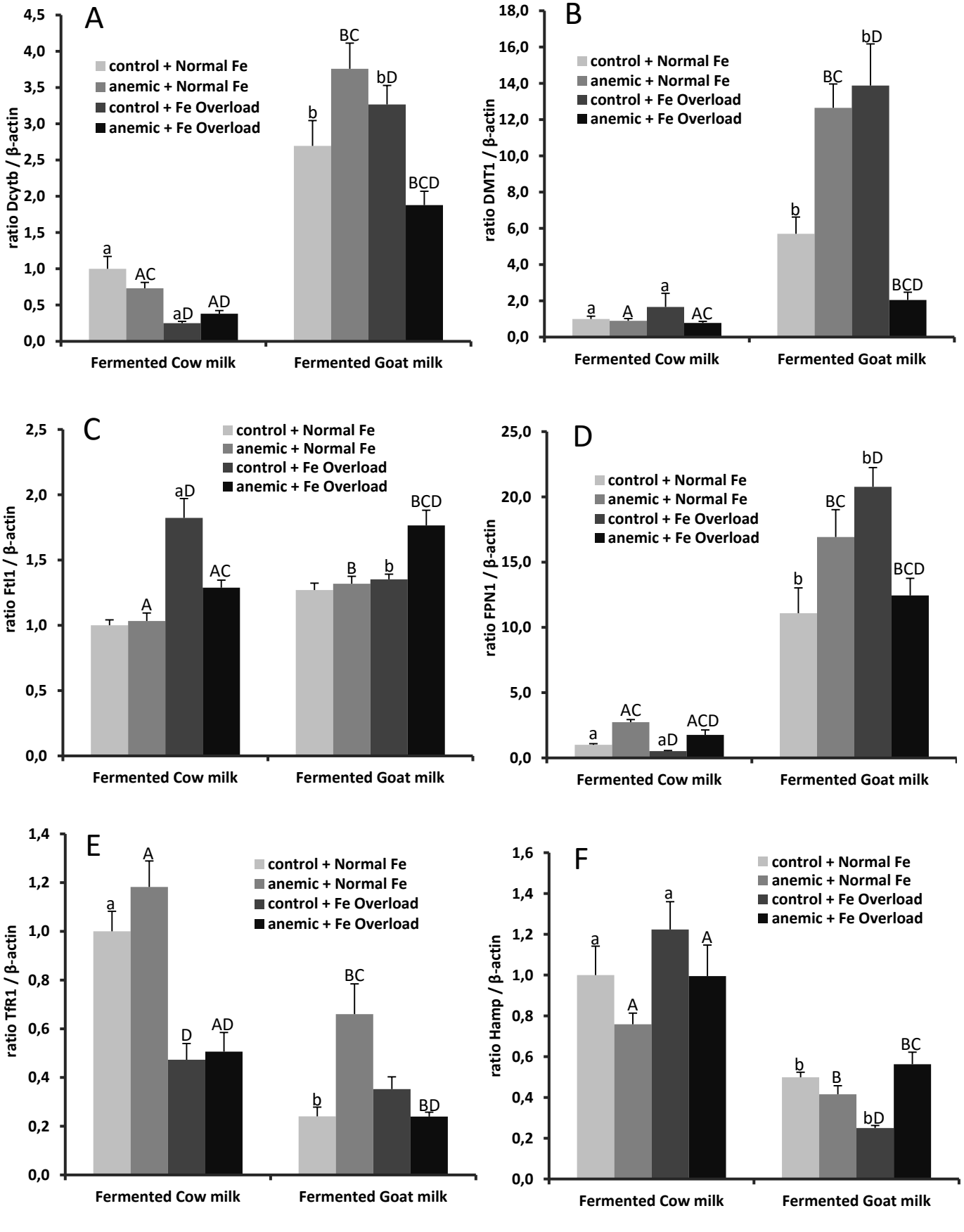


Figure 1. Experimental design of the study.


A
ratio Dcytb / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
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AC
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BCD
Fermented Cow milk
Fermented Goat milk
B
ratio DMT1 / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
a
A
a
AC
b
BC
bD
BCD
Fermented Cow milk
Fermented Goat milk
C
ratio Ftl1 / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
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BCD
Fermented Cow milk
Fermented Goat milk
D
ratio FPN1 / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
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ACD
b
BC
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BCD
Fermented Cow milk
Fermented Goat milk
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ratio TfR1 / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
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BC
BD
Fermented Cow milk
Fermented Goat milk
F
ratio Hamp / β-actin
control + Normal Fe
anemic + Normal Fe
control + Fe Overload
anemic + Fe Overload
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B
bD
BC
Fermented Cow milk
Fermented Goat milk

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).

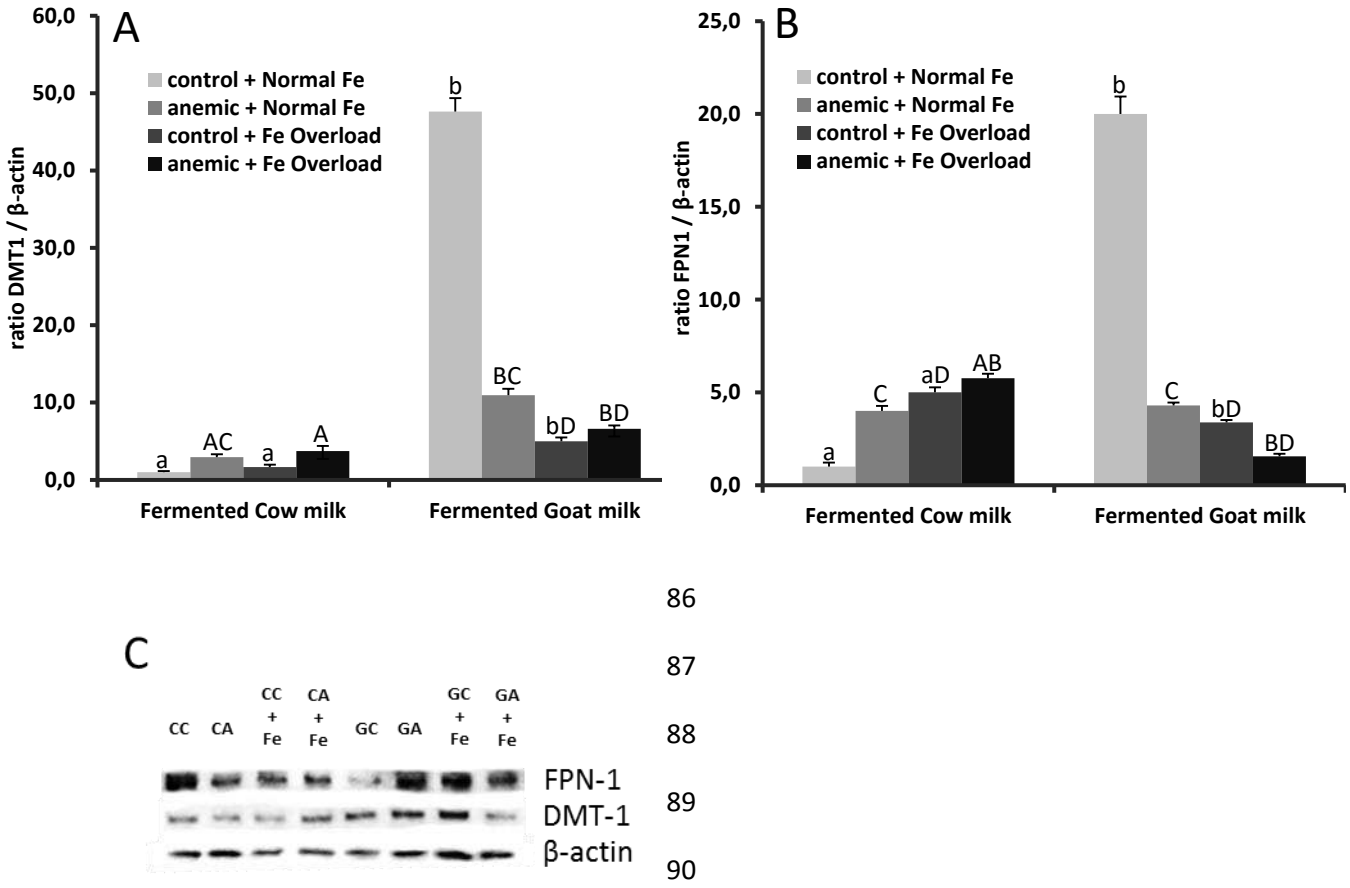


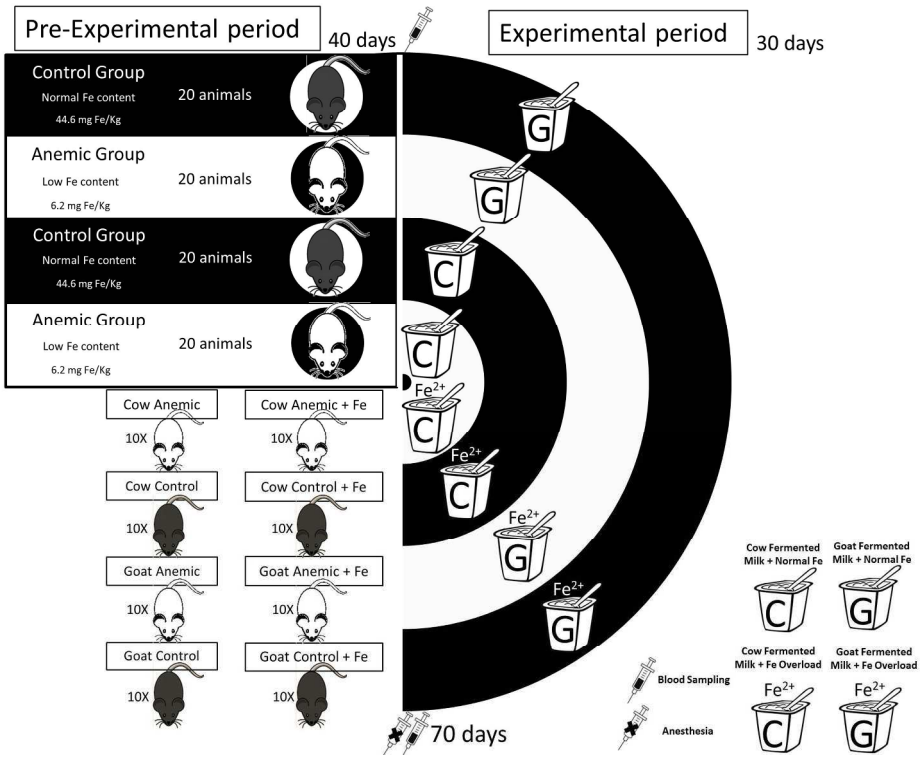
Figure 3. Effect of fermented cow and goat milk in control and anemic rats under normal and Fe overload conditions on expression of DMT1 (A) and FPN1 (B) protein. Data are mean with SEM of 10 animals per group. Representative immunoblots of DMT1, FPN1 and β-actin are shown (C).

[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).



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