Research Article

Received: 3 March 2021 Revised: 24 June 2021 Accepted article published: 30 July 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11448

Fermented goat's milk modulates immune response during iron deficiency anemia recovery

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Abstract

BACKGROUND: Iron deficiency and iron overload can affect the normal functioning of the innate and adaptive immune responses. Fermented milk products may enhance immune functions, but little is known about the effect of fermented milks on modulation of the immune response during iron deficiency anemia and recovery with normal or high dietary iron intake. Eighty male Wistar rats were randomly assigned to a control group fed a standard diet or to an anemic group fed a diet deficit in iron. Control and anemic groups were fed for 30 days with diets based on a fermented goat's or cow's milk product, with normal iron content or iron overload.

RESULTS: In general, during anemia recovery lectin and alternative complement pathway activity and lactoferrin decreased, because it improves iron homeostasis, which is critically important in immune system functions. Fermented goat's milk diet enhanced immune function during iron deficiency recovery, suppressed oxidant-induced eotaxin and fractalkine expression due to the concurrent reduction of free radical production and pro-inflammatory cytokines, and decreased monocyte chemoattractant protein-1 and monocyte migration and adhesion. The increase in interferon-γ can confer immunological colonization of gut microbiota and downregulate inflammation.

CONCLUSION: Fermented goat's milk consumption enhances immune function, modifying complement pathway activity and decreasing pro-inflammatory cytokines as well as lactoferrin concentration, due to the improvement of iron homeostasis, which is critically important in the normal function of the immune system.

Supporting information may be found in the online version of this article.

Keywords: fermented goat's milk; iron deficiency anemia; iron overload; cellular immunity; immune function biomarkers; complement pathways

INTRODUCTION

The World Health Organization (WHO) has established as a priority in public health the need to maintain adequate information on nutrition and immune response.[1] Recent studies suggest that there is a close relationship between infection/immunity and nutritional status.[2] It is generally agreed that moderate to severe malnutrition is associated with impaired immune response.[3]

Iron is an essential element for normal development of the immune system[4] because it is necessary for immune cell proliferation, particularly lymphocytes, associated with the generation of specific defense responses to prevent infection.[5]

The WHO states that the most prevalent micronutrient deficiency worldwide is iron deficiency,[6] showing a reduction in lymphocyte DNA synthesis, number and cytotoxic activity T cells.[7,8]

Similarly, iron overload can affect the immune response by decreasing antibody-mediated and mitogen-stimulated phagocytosis of monocyte–macrophage system cells, inducing T lymphocyte subset alterations and modifying the distribution of lymphocytes.[9]

Fermented and probiotic milk products contain beneficial live microorganisms that interact with the microbiota and the cells of the intestinal wall, and improve gut microbiota.[10] Consumption

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of fermented milk products may have a positive impact on immune function.[11]

Fermented milk products contain lactic acid bacteria, which produce activation of the mucosal and systemic immunity via coordination interactions between the epithelial cells, the microbiota and intestinal immune cells.[12]

Milk products contain bioactive peptides that are involved in lymphocyte proliferation, natural killer cell function, antibody synthesis and cytokine production.[13,14]

Fermented goat's milk reduces serum cortisol levels, decreasing the detrimental effect on immune function, and increases melatonin levels, with multiple immunomodulatory and anti-inflammatory functions[15] – findings that together with the benefits induced in iron metabolism positively influence the immune status.[16]

Despite the scientific evidence indicating that iron deficiency alters the normal function of the immune system, and that fermented milk products may enhance immune function, little is known about the effect of fermented milk consumption on modulation of the immune response during iron deficiency and its recovery. This study aims at assessing immune system homeostasis in a severe iron deficiency anemia model after recovery with a fermented goat's milk-based diet, evaluating its effects on cellular immunity, immune function biomarkers, lactoferrin and complement pathways.

MATERIAL AND METHODS

Animals, housing conditions and ethical declaration

In total, 80 male Wistar albino rats (*Rattus norvegicus*), newly weaned, weighing 36–42 g, were provided by the Animal Experimental Unit at the University of Granada (Spain). Animals were kept in a thermoregulatory room (20–22 °C), with relative humidity between 55% and 60% and a light–dark cycle of 12/12 h. Protocols were approved by the Animal Welfare Committee of the University of Granada (ref. 11022011), and were carried out in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament and the Council, 2010) and care of animals in the Declaration of Helsinki (National Research Council, 2011).

Experimental design and diets

Experimental design included a pre-experimental period for nutritional ferropenic anemia induction, followed by an experimental period of feeding with semi-synthetic diets to be tested based on a fermented product of goat's or cow's milk (Fig. 1).

Pre-experimental period (PEP)

Eighty rats were randomly assigned to two experimental groups fed *ad libitum* for 40 days with AIN-93G diet, with normal iron content (control group, 45 mg kg^{-1} diet), or low iron content (anemic group, 5 mg kg^{-1} diet), for experimental induction of anemia.[17] At the end of PEP, peripheral blood samples were collected from the tail vein for hematological and biochemical control of anemia.

Experimental period (EP)

Both pre-experimental groups were fed *ad libitum* for 30 days with diets based on a fermented goat's or cow's milk product, with normal iron content (45 mg kg^{-1} diet) (FGMD or FCMD group, respectively) or iron overload (450 mg kg^{-1} diet)[18] (FGMD + Fe or FCMD + Fe group, respectively). At the end of EP, rats were anesthetized and sacrificed by aortic abdominal cannulation and total bleeding.

The blood obtained was applied in two fractions. A fraction of blood was collected in ethylenediaminetetraacetic acid K3 tubes, and another fraction withdrawn into a tube without anticoagulant was left to clot and centrifuged for 5 min at 3000 rpm. Serum was separated and stored at −80 °C.

The ingredients and chemical composition of experimental diets were described in Moreno-Fernández *et al.*,[19] and are detailed in Supporting Information Table S1. Diets' fermentation and dehydration protocol were prepared according to Moreno-Fernández *et al.*,[20] and the detailed protocol can be found elsewhere (Supporting Information Appendix S1).

Hematology and serum biochemical evaluation

Hematological parameters of control and anemic rats in the PEP and after feeding for 30 days with fermented cow's or goat's milk-based diets with normal Fe content or Fe overload in the EP are detailed in Supporting Information Appendix S1.Using an automated coagulation analyzer (Mythic 22CT, C2 Diagnostics, Grabels, France), complete blood counts, including total and differential leukocyte count, were estimated. Neutrophil-to- lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were calculated as the ratio of neutrophil cell and platelet count to lymphocyte cell count, respectively.

Serum levels of total protein, albumin, uric acid, urea, total cholesterol and triglycerides were determined in individual samples in an automatic BS-200 chemistry analyzer (Shenzhen Mindray Bio-Medical Electronics Co. Ltd, Shenzhen, China). Globulin concentrations were calculated by the difference between the total serum protein and the albumin concentrations. Albumin-to-globulin ratio (A/G) was also calculated.

Immune function biomarkers

Eoxatin, fractalkine, granulocyte colony-stimulating factor (G-CSF), interferon (INF)-γ, tumor necrosis factor (TNF)-α, Lipopolysaccharide-inducible CXC chemokine (LIX), monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-4, IL-6 and IL-10 were determined using the RCYTOMAG-80K Milliplex MAP kit rat cytokine/chemokine magnetic bead panel (Millipore, Darmstadt, Germany). The plate was read on a LABScan 100 analyzer (Luminex Corporation, Austin, TX, USA) with xPONENT software for data acquisition. The average values for each set of duplicate samples or standards were within 15% of the mean. Immune function parameter concentrations were determined by comparing the mean of duplicate serum samples with the standard curve for each assay.

Lactoferrin

Lactoferrin concentration was determined in serum samples using a rat LTF/LF (lactoferrin) enzyme-linked immunosorption assay (ELISA) kit (MyBioSource, San Diego, CA, USA). The absorbance of the reaction was measured at 450 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA), and the color intensity was directly related to concentration present in the sample.

Functional assessment of complement pathway activity

The classical, lectin and alternative complement pathway activity in rat serum samples was assessed with ELISA kits, (HIT 410, HIT 411 and HIT 412; Hycult Biotech, Uden, Netherlands). Wells for classical pathway evaluation were precoated with immunoglobulin M, those for lectin pathway were coated with mannan and, for alternative pathway, with lipopolysaccharides

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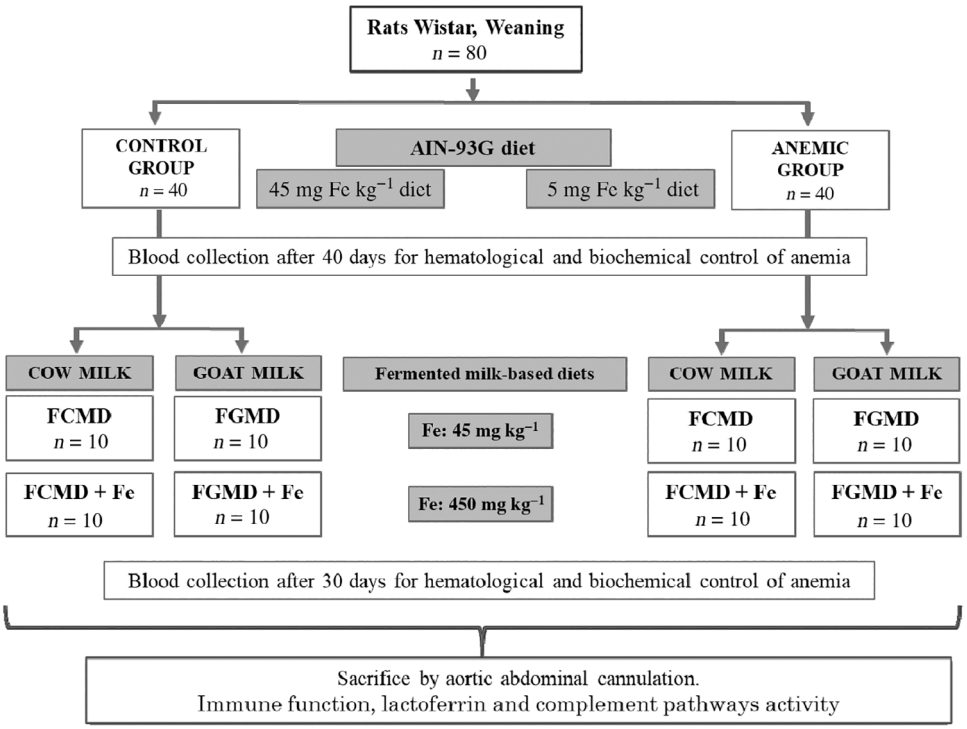

Figure 1. Experimental design of study.

(LPS). Samples as well as standard serum and negative control serum were tested in duplicate at a fixed dilution. Samples were incubated for 60 min at 37 °C. Biotinylated tracer antibody was added to bind to the bound C5b-9 in samples and control, streptavidin peroxidase and substrate. Absorbance values were read at 450 nm with a multifunctional microplate reader (BioTek Instruments, Winooski, VT, USA).

Statistical analysis

The data were presented as mean ± standard error of the mean (SEM). SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Significance in difference between control group and anemic group during the (PEP) was tested by Student's *t*-test. Differences due to diet, anemia and iron content in the diet during the EP were tested using two-way ANOVA followed by Tukey's multiple comparison test when the main effects and interactions were significant. For all statistical analyses, a *P*-value of <0.05 was regarded as statistically significant.

RESULTS

Serum biochemical evaluation

Prior to serum biochemical evaluation, all the hematological parameters studied[19] were assessed to check anemia induction after 40 days of iron deprivation and recovery after 30 days with fermented cow's or goat's milk-based diets with normal Fe content or Fe overload (Supporting Information Tables S2 and S3).

Serum biochemical parameters after iron deprivation are shown in Table 1. Urea and cholesterol concentration were lower in the control group ($P < 0.05$). In the experimental period (Table 2), total protein concentration was higher in control and anemic groups fed FGMD + Fe ($P < 0.01$) compared to animals fed FCMD + Fe.

Albumin level was higher in control and anemic groups fed FGMD + Fe ($P < 0.05$) and in anemic group fed FGMD ($P < 0.05$) *versus* animals fed FCMD + Fe. Globulin level was higher in the anemic group fed FGMD ($P < 0.05$) and in control and anemic groups fed FGMD + Fe ($P < 0.05$) compared to FCMD + Fe. Anemia decreased globulin level in animals fed FCMD ($P < 0.05$). A/G ratio was lower in the anemic group fed FGMD or FGMD + Fe ($P < 0.05$) compared to FCMD or FCMD + Fe, respectively.

Uric acid concentration was higher in control and anemic groups fed FCMD ($P < 0.01$) *versus* those fed FGMD and in the anemic group fed FCMD + Fe ($P < 0.05$) compared to FGMD + Fe. Anemia decreased uric acid concentration in animals fed FCMD ($P < 0.05$).

Urea concentration was higher in control and anemic groups FCMD ($P < 0.001$) compared to animals fed FGMD. Iron overload increased urea concentration in control and anemic groups fed FGMD ($P < 0.05$).

Table 1. Serum biochemical parameters of control and anemic rats in the pre-experimental period ($n = 40$ animals per group)

	Pre-experimental period	
	Control group	Anemic group
Total protein (g dL^{-1})	5.35 ± 0.18	5.81 ± 0.15
Albumin (g dL^{-1})	2.98 ± 0.06	3.31 ± 0.08
Globulin (g dL^{-1})	2.38 ± 0.12	2.50 ± 0.14
A/G ratio	1.28 ± 0.04	1.44 ± 0.17
Uric acid (mg dL^{-1})	1.17 ± 0.15	1.54 ± 0.19
Urea (mg dL^{-1})	34.79 ± 1.99	41.82 ± 1.77*
Cholesterol (mg dL^{-1})	71.83 ± 5.20	91.40 ± 9.64*

Data are shown as mean values ± SEM. Asterisk indicates significant difference from the control group (*$P < 0.05$; Student's *t*-test).

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Table 2. Serum biochemical parameters of control and anemic rats in the pre-experimental period ($n = 40$ animals per group)

		Fermented cow's milk-based diet		Fermented goat's milk-based diet		Two-way ANOVA		
	Iron content	Control group	Anemic group	Control group	Anemic group	Diet	anemia	iron content
Total protein (g dL^{-1})	Normal	5.28 ± 0.14	5.07 ± 0.09	5.86 ± 0.11	5.96 ± 0.12	NS	NS	NS
	Overload	5.35 ± 0.22a	5.06 ± 0.17A	6.17 ± 0.18b	6.05 ± 0.12B	<0.01	NS	
Albumin (g dL^{-1})	Normal	3.14 ± 0.04	3.03 ± 0.03A	3.18 ± 0.02	3.22 ± 0.04B	NS	NS	NS
	Overload	2.99 ± 0.08a	2.93 ± 0.08A	3.37 ± 0.06b	3.27 ± 0.05B	<0.05	NS	
Globulin (g dL^{-1})	Normal	2.44 ± 0.10	2.05 ± 0.09 AC	2.68 ± 0.10	2.74 ± 0.09B	<0.05	<0.05	NS
	Overload	2.36 ± 0.17a	2.13 ± 0.10A	2.80 ± 0.12b	2.78 ± 0.12B	<0.05	NS	
A/G ratio	Normal	1.30 ± 0.04	1.50 ± 0.07A	1.20 ± 0.04	1.18 ± 0.03B	<0.05	NS	NS
	Overload	1.34 ± 0.14	1.39 ± 0.04 A	1.21 ± 0.03	1.20 ± 0.06B	<0.05	NS	
Uric acid (mg dL^{-1})	Normal	1.48 ± 0.06a	1.07 ± 0.04 AC	0.79 ± 0.09b	0.88 ± 0.18B	<0.01	<0.05	NS
	Overload	1.24 ± 0.07	1.43 ± 0.11A	1.01 ± 0.07	1.08 ± 0.09B	<0.05	NS	
Urea (mg dL^{-1})	Normal	46.0.1 ± 1.41a	44.40 ± 1.11A	34.82 ± 0.84b	36.49 ± 1.13 B	<0.001	NS	<0.05
	Overload	42.89 ± 2.61	44.37 ± 1.21	43.15 ± 0.38D	41.23 ± 0.70D	NS	NS	
Cholesterol (mg dL^{-1})	Normal	45.69 ± 2.15a	45.60 ± 1.75A	40.10 ± 1.98b	36.81 ± 1.29 BC	<0.01	<0.05	<0.01
	Overload	39.63 ± 3.16D	40.20 ± 1.67D	36.70 ± 2.85D	37.48 ± 1.79	NS	NS	

NS, not significant. Mean values among groups of controls rats with different lower-case letters (a,b) in the same row are significantly different ($P < 0.05$; Tukey's test). A, B Mean values among groups of anemic rats with different upper-case letters (A,B) in the same row are significantly different ($P < 0.05$, Tukey's test). Mean values from the corresponding group of control rats (C) are significantly different ($P < 0.05$; Student's *t*-test). Mean values from the corresponding group of rats fed with normal Fe content (D) are significantly different ($P < 0.05$, Student's *t*-test).

Cholesterol concentration was higher in control and anemic groups fed FCMD ($P < 0.01$) compared to animals fed FGMD. Anemia decreased cholesterol concentration in animals fed FGMD ($P < 0.05$). Iron overload decreased cholesterol concentration in control and anemic groups fed FCMD and control group fed FGMD ($P < 0.01$).

Cellular immunity

Results of white blood cell lines after anemia induction are shown in Table 3. Monocyte percentage and PLR was higher in anemic group ($P < 0.05$).

At the end of the experimental period, white blood cell count (WBC) was higher in anemic groups fed FGMD ($P < 0.01$) or FGMD + Fe ($P < 0.001$), and in the control group fed FGMD + Fe ($P < 0.05$), compared to FCMD. Anemia increased WBC in animals fed FGMD or FGMD + Fe ($P < 0.05$) and reduced in animals fed FCMD ($P < 0.05$). With regard to iron overload, WBC was lower in control and anemic groups fed FCMD ($P < 0.01$).

Table 3. Total and differential leukocyte count of control and anemic rats in the pre-experimental period ($n = 40$ animals per group)

	Pre-experimental period	
	Control group	Anemic group
WBC ($10^3/\mu L$)	8.88 ± 0.38	8.04 ± 0.99
Neutrophils (%)	8.92 ± 1.01	7.77 ± 1.53
Lymphocytes (%)	81.56 ± 1.58	78.00 ± 2.73
Monocytes (%)	2.92 ± 0.26	6.53 ± 1.29*
Eosinophils (%)	2.02 ± 0.69	2.10 ± 1.21
Basophils (%)	4.58 ± 0.29	5.60 ± 0.62
NLR	0.12 ± 0.02	0.10 ± 0.01
PLR	2.49 ± 0.70	4.92 ± 0.54*

Data are shown as mean values ± SEM. WBC, white blood cells; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio. Asterisk indicates significant difference from the control group (*$P < 0.05$; Student's *t*-test).

Neutrophil level was higher in the control group fed FGMD or FGMD + Fe ($P < 0.05$). Anemia increased neutrophil level in animals fed FCMD ($P < 0.05$) and FCMD + Fe ($P < 0.01$), and reduced it in animals fed FGMD ($P < 0.01$). With regard to iron overload, neutrophil level was reduced in the control group fed both fermented milk-based diets ($P < 0.01$).

Anemia reduced lymphocyte level in animals fed FCMD + Fe ($P < 0.05$). Iron overload increased lymphocyte level in the control group fed both fermented milk-based diets ($P < 0.05$).

Monocyte levels were lower in control and anemic groups fed FGMD + Fe ($P < 0.05$) and in the anemic group fed FGMD ($P < 0.01$), compared to FCMD. Anemia increased monocyte level in animals fed FCMD and FCMD + Fe ($P < 0.05$). Iron overload increased monocyte level in the anemic group fed FCMD ($P < 0.05$).

Eosinophil percentage was lower in the control group fed FGMD ($P < 0.05$) and increased in the anemic group fed FGMD + Fe ($P < 0.05$) compared to animals fed FCMD or FCMD + Fe, respectively. Anemia increased eosinophils in animals fed FCMD + Fe ($P < 0.05$). Iron overload reduced eosinophil level in the control group fed FCMD ($P < 0.05$).

With regard to basophil level, FGMD reduced it in control ($P < 0.05$) and anemic animals with high iron content ($P < 0.01$). Anemia increased basophils in animals fed FCMD + Fe ($P < 0.01$). Iron overload increased basophil level in the anemic group fed FCMD ($P < 0.01$) and reduced the levels in the control group fed FGMD ($P < 0.05$).

NLR and PLR were lower in the anemic group fed FGMD ($P < 0.05$) or FGMD + Fe ($P < 0.05$; $P < 0.01$) and PLR was lower in the control group fed FGMD + Fe ($P < 0.01$) compared to animals fed FCMD.

Anemia increased NLR and PLR in animals fed FCMD ($P < 0.05$) and NLR in animals fed FCMD + Fe ($P < 0.05$). Iron overload increased PLR in control and anemic groups fed FCMD ($P < 0.01$) (Table 4).

Table 4. Total and differential leukocyte count of control and anemic rats fed for 30 days with fermented cow's or goat's milk-based diets with normal iron content of iron overload in the experimental period ($n = 10$ animals per group)

		Fermented cow's milk-based diet		Fermented goat's milk-based diet		Two way ANOVA		
%	Iron content	Control group	Anemic group	Control group	Anemic group	Diet	Anemia	Iron content
WBC ($10^3/\mu L$)	Normal	1.66 ± 0.12a	1.12 ± 0.12A	1.79 ± 0.16a	2.17 ± 0.39BC	<0.05	<0.05	<0.01
	Overload	0.99 ± 0.15aD	0.79 ± 0.10AD	1.80 ± 0.13b	2.22 ± 0.18B	<0.01	NS	
Neutrophils (%)	Normal	14.37 ± 1.42a	18.40 ± 1.44C	17.77 ± 1.29b	14.46 ± 2.58	<0.05	<0.05	<0.01
	Overload	11.78 ± 1.12aD	17.87 ± 1.53C	14.04 ± 1.17bD	16.00 ± 1.23	<0.05	<0.05	
Lymphocytes (%)	Normal	73.14 ± 3.82	73.50 ± 1.91	75.23 ± 2.14	78.42 ± 2.36	NS	NS	<0.05
	Overload	81.01 ± 1.94D	70.62 ± 3.59C	80.24 ± 1.58D	76.71 ± 2.52	NS	<0.05	
Monocytes (%)	Normal	1.41 ± 0.27	2.33 ± 0.21AC	1.81 ± 0.20	1.45 ± 0.45B	<0.05	<0.05	<0.05
	Overload	1.86 ± 0.25a	3.03 ± 0.35ACD	1.24 ± 0.12b	1.59 ± 0.16B	<0.01	<0.05	
Eosinophils (%)	Normal	2.07 ± 0.78a	1.58 ± 0.42	1.00 ± 0.21b	1.15 ± 0.19	<0.05	<0.05	<0.05
	Overload	0.85 ± 0.14aD	1.81 ± 0.49C	1.32 ± 0.18b	1.72 ± 0.33	<0.05	<0.05	
Basophils (%)	Normal	4.65 ± 0.69	4.28 ± 0.51	3.98 ± 0.22	3.11 ± 0.52	NS	NS	<0.01
	Overload	3.61 ± 0.32a	7.29 ± 0.74ACD	2.40 ± 0.21bD	2.98 ± 0.22B	<0.01	<0.05	
NLR	Normal	0.22 ± 0.04	0.31 ± 0.05AC	0.26 ± 0.05	0.20 ± 0.04B	<0.05	<0.05	<0.05
	Overload	0.15 ± 0.03	0.34 ± 0.09AC	0.18 ± 0.03	0.22 ± 0.03B	<0.05	<0.05	
PLR	Normal	6.16 ± 0.65	9.28 ± 0.24AC	5.08 ± 0.83	6.99 ± 0.70B	<0.05	<0.05	<0.01
	Overload	13.44 ± 2.61aD	17.84 ± 3.99AD	5.47 ± 0.90b	6.55 ± 1.17B	<0.01	NS	

NS, not significant. Mean values among groups of controls rats with different lower-case letters (a,b) in the same row are significantly different ($P < 0.05$; Tukey's test). Mean values among groups of anemic rats with different upper-case letters (A,B) in the same row are significantly different ($P < 0.05$; Tukey's test). Mean values from the corresponding group of control rats (C) are significantly different ($P < 0.05$; Student's *t*-test). Mean values from the corresponding group of rats fed with normal Fe content (D) are significantly different ($P < 0.05$; Student's *t*-test).

Table 5. Immune function biomarkers in serum from control and anemic rats fed for 30 days with fermented cow's or goat's milk-based diets with normal Fe content or Fe overload ($n = 10$ animals per group)

		Fermented cow's milk-based diet		Fermented goat's milk-based diet		Two-way ANOVA		
pg mL^{-1}	Iron content	Control group	Anemic group	Control group	Anemic group	Diet	anemia	iron content
Eotaxin	Normal	13.33 ± 0.55a	16.55 ± 1.12AC	10.37 ± 0.99b	8.99 ± 0.81 BC	<0.001	<0.001	<0.05
	Overload	8.79 ± 0.76D	15.23 ± 1.29AC	9.63 ± 1.01	7.98 ± 0.90B	<0.01	<0.05	
Fractalkine	Normal	44.89 ± 3.22a	66.325 ± 3.39AC	32.303 ± 3.13b	25.68 ± 2.35 BC	<0.001	<0.01	NS
	Overload	47.48 ± 2.83	68.512 ± 3.97AC	35.518 ± 1.21b	28.32 ± 2.43 BC	<0.001	<0.001	
G-CSF	Normal	8.223 ± 0.331	10.123 ± 0.412A	6.926 ± 0.28	24.21 ± 1.09 BC	<0.01	<0.001	<0.01
	Overload	9.382 ± 0.074a	3.618 ± 0.391ACD	13.89 ± 1.31bD	6.43 ± 0.55BCD	<0.001	<0.001	
INF-γ	Normal	702.14 ± 43.89a	685.379 ± 41.13A	801.22 ± 42.2b	795.13 ± 41.41B	<0.01	NS	NS
	Overload	705.54 ± 43.23a	682.225 ± 43.22A	805.76 ± 43.67b	788.12 ± 40.34B	<0.01	NS	
TNF-α	Normal	8.51 ± 0.47a	9.01 ± 0.48A	6.19 ± 0.38b	6.30 ± 0.60B	< 0.01	NS	NS
	Overload	8.43 ± 0.34a	8.65 ± 0.32A	6.31 ± 0.32b	6.20 ± 0.37B	< 0.01	NS	
LIX	Normal	105.230 ± 8.74a	374.401 ± 166.11AC	302.228 ± 20.976b	302.692 ± 16.49B	<0.001	<0.05	<0.001
	Overload	4770.63 ± 437.30aD	4467.58 ± 245.62AD	1039.39 ± 40.83bD	1139.42 ± 45.43BD	<0.001	NS	
MCP-1	Normal	575.22 ± 31.14a	767.12 ± 52.11AC	458.23 ± 28.32b	507.34 ± 44.22B	< 0.001	< 0.01	NS
	Overload	580.351 ± 33.55a	696.896 ± 47.99AC	466.599 ± 39.43b	504.229 ± 52.11B	< 0.001	< 0.05	
IL-4	Normal	28.75 ± 2.18a	27.05 ± 1.19A	36.19 ± 1.05b	35.82 ± 1.03B	< 0.01	NS	< 0.01
	Overload	32.73 ± 1.01a	34.62 ± 2.77AD	43.01 ± 1.08bD	55.74 ± 2.09 BCD	< 0.05	< 0.05	
IL-6	Normal	483.32 ± 48.19a	409.43 ± 44.11A	309.12 ± 51.32b	316.18 ± 42.21B	< 0.01	NS	NS
	Overload	439.25 ± 21.12a	423.35 ± 35.15A	337.19 ± 18.21b	327.43 ± 31.45B	< 0.01	NS	
IL-10	Normal	33.48 ± 0.77a	16.09 ± 0.66 AC	38.07 ± 1.94b	23.48 ± 1.19 BC	< 0.01	< 0.01	< 0.01
	Overload	35.65 ± 2.03a	41.19 ± 1.83ACD	44.79 ± 1.04bD	74.59 ± 3.05BCD	< 0.01	< 0.01	

NS, not significant. Mean values among groups of control rats with different letters (a,b) in the same row are significantly different ($P < 0.05$; Tukey's test). Mean values among groups of anemic rats with different upper-case letters (A,B) in the same row are significantly different ($P < 0.05$; Tukey's test). Mean values from the corresponding group of control rats (C) are significantly different ($P < 0.05$; Student's *t*-test). Mean values from the corresponding group of rats fed with normal Fe content are significantly different ($P < 0.05$; Student's *t*-test).

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Immune function biomarkers

The immune function biomarkers are shown in Table 5. FGMD reduced eotaxin concentration in the control group with normal iron content ($P < 0.001$) and in anemic groups either with normal iron ($P < 0.001$) or high iron content ($P < 0.01$), compared to FCMD. Previously induced anemia increased eotaxin concentration in animals fed FCMD ($P < 0.05$) and FCMD + Fe ($P < 0.001$). Contrarily, anemia decreased it in animals fed FGMD, especially with normal iron content ($P < 0.001$). Iron overload content decreased eotaxin concentration in the control group fed FCMD ($P < 0.05$).

Fractalkine concentration was lower in control ($P < 0.05$) and anemic groups ($P < 0.001$) fed FGMD or FGMD + Fe compared to those fed FCBD or FGMD + Fe, respectively. Anemia reduced fractalkine concentration in animals fed FGMD and increased it in animals fed FCMD ($P < 0.01$) or FCMD + Fe ($P < 0.001$).

Concentration of G-CSF was higher in animals fed FGMD or FGMD + Fe ($P < 0.001$) except in the control group fed FGMD, where no differences where observed. Anemia reduced G-CSF concentration in animals fed FGMD or FCMD + Fe ($P < 0.001$) and increased it in animals fed FGMD ($P < 0.001$). Iron overload decreased the concentration in the anemic group fed FGMD or FCMD ($P < 0.01$) and increased it in control animals fed FGMD ($P < 0.01$).

FGMD increased INF-γ concentration in control and anemic animals, either with normal ($P < 0.01$) or high iron content ($P < 0.01$) compared to animals fed FCBD. Contrarily, TNF-α concentration was lower in animals fed FGMD or FGMD + Fe ($P < 0.01$), compared to animals fed FCMD or FCMD + Fe.

In general, concentration of LIX was lower in animals fed FGMD or FGMD + Fe ($P < 0.001$), except in the control group with normal iron content, where the concentration increased ($P < 0.001$). Only in animals fed FCBD with normal iron content did anemia increase ($P < 0.001$) LIX concentration. Iron overload increased the concentration in control and anemic groups fed FGMD or FCMD ($P < 0.001$).

With regard to MCP-1, FGMD reduced the concentration in control and anemic groups either with normal iron or high iron content ($P < 0.001$). Anemia increased the concentration in animals fed FCMD ($P < 0.01$) or FCMD + Fe ($P < 0.05$).

FGMD increased IL-4 concentration in control and anemic groups, either with normal iron ($P < 0.01$) or high iron content ($P < 0.05$). Anemia only increased the concentration in animals fed FGMD + Fe ($P < 0.05$). In general, high iron content increased

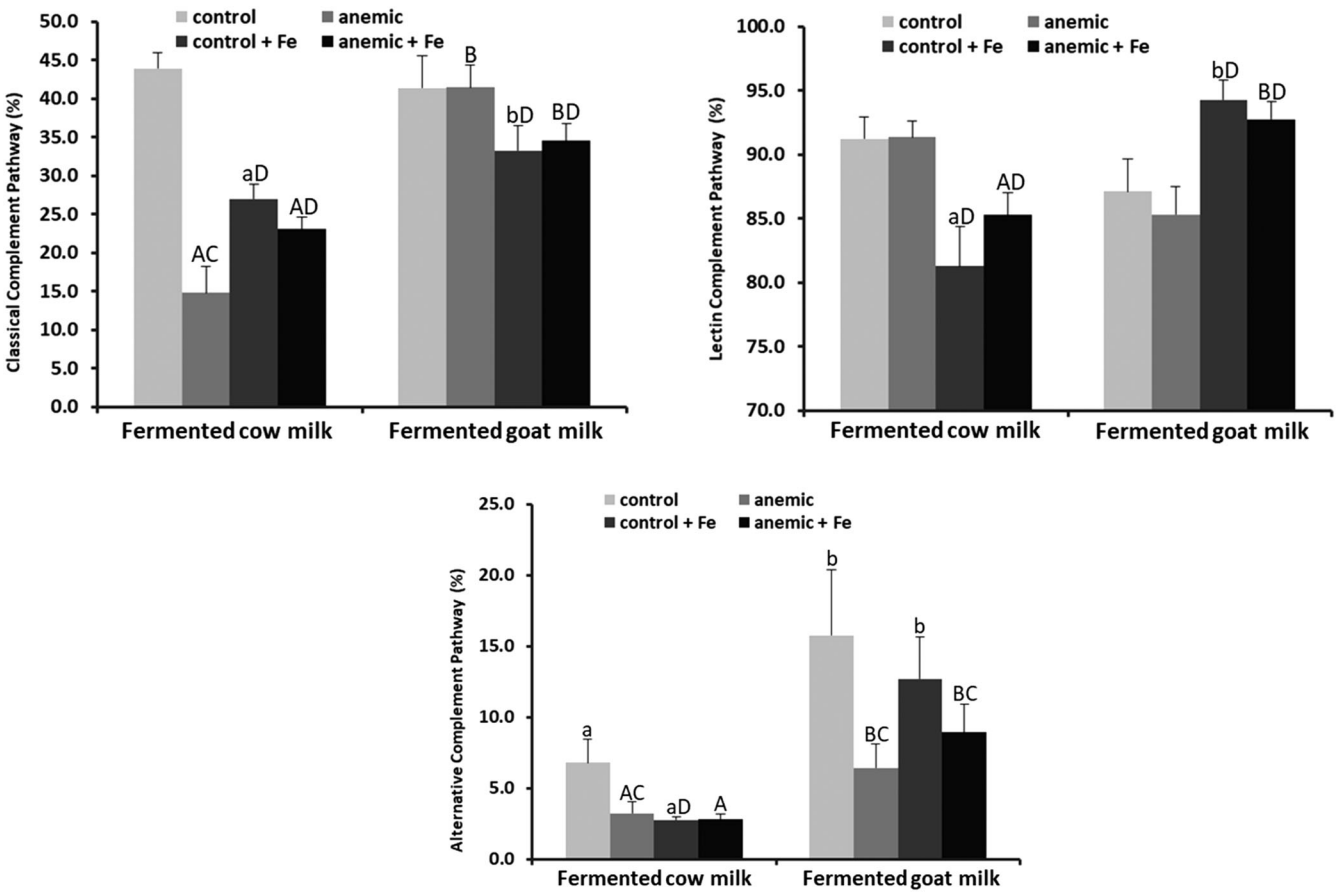

Figure 2. Functional assessment of complement pathway activity in serum from control and anemic rats fed for 30 days with fermented cow's or goat's milk-based diets with normal Fe content or Fe overload ($n = 10$ animals per group). Mean values among groups of controls rats fed with different diets and having different lower-case letters (a,b) are significantly different ($P < 0.05$, Tukey's test). Mean values among groups of anaemic rats fed with different diets and having different upper-case letters (A, B) are significantly different ($P < 0.05$,Tukey's test). Mean values from the corresponding group of control rats (C) are significantly different ($P < 0.05$, Student's *t*-test). Mean values from the corresponding group of rats fed with normal Fe content (D) are significantly different ($P < 0.05$, Student's *t*-test).

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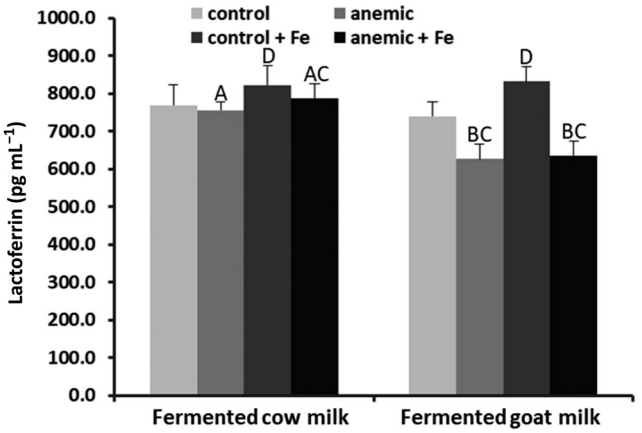

Figure 3. Lactoferrin concentration in serum from control and anemic rats fed for 30 days with fermented cow's or goat's milk-based diets with normal Fe content or Fe overload ($n = 10$ animals per group). Mean values among groups of control rats fed with different diets and having different lower-case letters (a,b) are significantly different ($P < 0.05$, Tukey's test). Mean values among groups of anaemic rats fed with different diets and having different upper-case letters (A,B) are significantly different ($P < 0.05$,Tukey's test). Mean values from the corresponding group of control rats (C) are significantly different ($P < 0.05$, Student's t-test). Mean values from the corresponding group of rats fed with normal Fe content (D) are significantly different ($P < 0.05$, Student's *t*-test).

IL-4 concentration in animals fed both fermented milks ($P < 0.01$) except in the control group fed FCMD.

IL-6 concentration was lower in control and anemic groups fed FGMD or FGMD + Fe ($P < 0.01$).

Concentration of IL-10 was higher in control and anemic groups fed FGMD ($P < 0.01$) or FGMD + Fe ($P < 0.01$). Anemia reduced the concentration in animals fed both fermented milks with normal iron content ($P < 0.01$); however, it increased the IL-10 concentration in animals fed both fermented milks with high Fe content ($P < 0.01$). With regard to iron overload, in general it increased IL-10 in control and anemic groups fed both fermented milk-based diets ($P < 0.01$), except in the control group fed FCMD.

The classical, lectin and alternative complement pathway activity is shown in Fig. 2. FGMD increased classical complement pathway activity in animals either with normal ($P < 0.05$) or high iron content ($P < 0.001$), except in the control group with normal iron content, where no differences were observed. Anemia decreased the activity in animals fed FCMD ($P < 0.001$). Iron overload decreased classical complement pathway activity in the control group fed both fermented milks ($P < 0.001$) and in the anemic group fed FGMD ($P < 0.001$), whereas it increased the activity in the anemic group fed FCMD ($P < 0.001$) (Fig. 2(a)).

With regard to lectin complement pathway activity, FGMD + Fe increased the activity in control and anemic groups ($P < 0.01$). Iron overload increase the activity in control and anemic groups fed FGMD ($P < 0.01$) compared to normal iron content (Fig. 2(b)).

FGMD increased the alternative complement pathway activity in control and anemic animals either with normal or high iron content ($P < 0.001$). Anemia decreased the activity in animals fed both fermented milks either with normal ($P < 0.001$) or high iron content ($P < 0.01$), except in animals fed FCMD, where no differences where observed. Iron overload decreased the activity only in the control group fed FCMD ($P < 0.05$) (Fig. 2(C)).

Lactoferrin concentration is shown in Fig. 3. FGMD decreased lactoferrin concentration in anemic groups either with normal or high iron content ($P < 0.05$). Anemia decreased the concentration in animals fed FGMD ($P < 0.05$) or FGMD + Fe ($P < 0.01$) and in animals fed FCMD + Fe ($P < 0.05$). In the control group fed both fermented milks, iron overload increased lactoferrin concentration ($P < 0.05$).

DISCUSSION

A better understanding of the physiology of the immune system during ferropenic anemia recovery and iron replenishment should facilitate new nutritional strategies to improve the immune response and hematological status. Scientific evidence supports that dairy is an important component of a healthy dietary pattern and associated with positive health outcomes.[21,22]

In the current study, immune system homeostasis in a severe iron deficiency model during has been explored and it was observed that fermented goat's milk consumption positively modulates the immune system response. The pre-experimental period was developed to induce iron deficiency and, subsequently, iron replenishment with dietary iron overload was carried out; therefore the benefits observed with fermented goat's milk do not merely represent an improvement in cell count due to recovery of the iron status and hematological parameters, but also a modulation of the immune system. In the current study, monocytes increased during anemia induction, which is in agreement with the results of Theurl *et al.*,[23] reporting that this was a consequence of ferroportin downregulation by hepcidin and cytokines during anemia.

Platelets increased during iron deficiency; therefore PLR ratio also increased in the current study. Iron deficiency is a cause of thrombocytosis; however, the exact mechanism of this platelet increase has not been completely elucidated. Pro-inflammatory cytokines play a key role in thrombocytosis by increasing the synthesis of acute-phase proteins, decreasing hepatic albumin biosynthesis, and stimulating megakaryocytic proliferation and thrombopoietin production.[24]

Iron plays a key role in innate immunity, and iron overload inhibits macrophage functions because iron storage in macrophages directly affects the binding activity of pro-inflammatory transcription factors.[25] One of the major responses is the withdrawal and sequestration of iron from the systemic circulation, coordinated by two genes of iron homoeostasis – hepcidin and ferroportin – such that there is reduced iron availability for bacterial utilization. Hepcidin regulates iron levels and location, reduces the absorption of dietary iron in the duodenum, decreases the release of iron from macrophages that recycle old erythrocytes and other cells, and stabilizes the stored iron from hepatocytes, because hepcidin binds to ferroportin, leading to degradation of lysosomes.[26] In the current study, an increase in serum hepcidin has been recorded during iron deficiency, which would decrease iron efflux from the duodenal cells, restricting iron availability to pathogens. T-helper cells type 1 (Th-1) produce IFN-γ,[27] and in the current study iron deficiency impaired IFN-γ in the animals consuming FCMD because these cells are extremely sensitive to disturbances of iron homeostasis; however, this impairment is not recorded when consuming FGMD, owing to its positive role in iron homeostasis. In addition, changes in the total iron content of the macrophage will alter NADPH oxidase activity in iron deficiency and iron overload; it is decreased and increased, respectively, which may compromise the efficacy of these macrophages to cope with the pathogens.[28] Deleterious effects induced by iron deficiency include reduced neutrophil function, decreased myeloperoxidase activity and possibly impaired intracellular antibacterial activity, depressed T-lymphocyte counts, defective T-lymphocyte-induced proliferative response and impaired natural killer cell activity.[29]

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Macrophages are producers and targets of hepcidin, providing a potential mechanism of auto-regulation and a further link between iron homeostasis and immunity.[30] Iron overload inhibits macrophage functions because the iron content of macrophages directly impacts the binding activity of pro-inflammatory transcription factors.[31]

One of the main immune activators of eosinophil tissue infiltration is eotaxin, which induces release of reactive oxygen species and is a chemoattractant; also, indirectly, it acts as a tissue-damaging agent at sites of inflammation.[32] Fractalkine increases the transfer of inflammatory cells and tissue destruction through increased secretion of TNF-α, matrix metalloproteinases and IFN-γ. Th-1-derived cytokine IFN-γ activate macrophages, thus contributing to the formation of pro-inflammatory cytokines such as TNF-α, IL-1 or IL-6, and the induction of cytotoxic immune effector mechanisms of these cells. By contrast, Th-2 cells produce IL-4, IL-5, IL-9 and IL-13, which in part exert anti-inflammatory actions via inhibition of various macrophage functions and which also activate immune cells.[33] FGMD consumption decreased pro-inflammatory cytokines (IL-1β, IL-2, IL-12p70, IP-10 and TNF-α) and increased anti-inflammatory cytokines (IL-4, IL-13 and IL-10) due to the high content of anti-inflammatory activities of its lipids and the higher content in linoleic acid compared to FCMD. Additionally, fermented goat's milk consumption increases total antioxidant status and decreases 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 from evoked oxidative damage during anemia recovery.[34] In this sense, eotaxin-1 induction is linked to oxidative stress and inflammatory signaling due to reactive oxygen species (ROS) production[35] and FGMD-suppressed oxidant-induced eotaxin and fractalkine expression due to the concurrent reduction of ROS production and pro-inflammatory cytokines. Recently, it has been demonstrated that the probiotic present in fermented milk could activate the gut-associated natural killer cells to secret TNF-α, which is a well-known pro-inflammatory cytokine.[36] In addition, granzyme B (Gzmb) and perforin (Prf) are cytotoxic factors involved in eliminating pathogenic bacteria during infection.[37] In the study of Xiaoxin *et al.*,[38] fermented goat's milk treatment increased TNF-α, while decreasing Gzmb and Prf, which might be the host's normal immune local response to exogenous probiotic bacteria consumption. In contrast, in the current study, TNF-α decreased in the animals fed FGMD; therefore, we can assume that Gzmb and Prf would also increase, boosting the systemic immune response.

Vitamin A has been shown to fulfill important immunomodulatory properties, and its effect on the immune system is also influenced by iron deficiency.[39] In this sense, fermented goat's milk contains more vitamin A than fermented cow's milk,[20] which boosts the immune response even during iron deficiency.

FGMD also induced a decrease in MCP-1, which can also be attributed to the high nutritional value of the fat and decreases the generation of free radicals and monocyte migration and adhesion. Additionally, the ability of fermented goat's milk 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, inhibiting lipid oxidation and reducing hydroperoxides.[40]

In the current study, higher levels of IFN-γ have been recorded in animals fed FGMD. These results are in agreement with those reported by Kao et al.,[41] which can be attributed to their bioactive factors, such as proteins, polyunsaturated fatty acids, oligosaccharides and micronutrients present in goat's milk which confer immunological colonization of gut microbiota, improving intestinal development and downregulating inflammation.

Lactoferrin influences the complex immune machinery, downregulating immune cell activation, migration and growth. Lactoferrin has a high affinity for ferric iron, which deprives microbes of the free iron necessary for their growth and of its propensity to interact with microbial and target host cell surfaces,[42] explaining the low levels of this protein during iron deficiency anemia. On the other hand, because FGMD consumption increases the expression of iron-status-related genes (cytochrome b, ferritin, ferroportin 1 and TfR1) and storage in the target, this explains the lower levels of this immune regulator when consuming FGMD. The downregulation of the pro-inflammatory signaling induced by FGMD[15] can also explain the lower levels of lactoferrin. In addition, the downregulation of lactoferrin when consuming FGMD can be a protective effect against oxidative stress.[34]

It is well known that iron is a central regulator of immune cell proliferation and function.[43] All lymphocyte subsets, which include B and T lymphocytes and natural killer cells, are dependent on transferrin/transferrin receptor (TfR)-mediated iron uptake, and a blockade of this pathway leads to diminished proliferation and differentiation of these cells,[44] explaining the impairment of the immune response recorded during iron deficiency and the improvement of this response induced by FGMD consumption because it enhances iron homeostasis,[16] improving immune cell proliferation and immune function.

The complement system is a crucial element in the activation of innate and adaptive immune response. Activation of complement can occur through three major pathways: classical, alternative and lectin pathways. Iron overload is associated with suppressed functions of the complement system (classic or alternative types). In general, FGMD increased classical, lectin complement and the alternative complement pathway activity in control and anemic animals either with normal or iron overload, while anemia decreased the lectin and alternative activity because, as previously mentioned, FGMD improves iron homeostasis,[16] which is critically important in the normal function of the immune system.[45]

CONCLUSIONS

This study shows that fermented goat's milk consumption during iron deficiency recovery enhances immune function. Fermented goat's milk suppressed oxidant-induced eotaxin and fractalkine expression due to the concurrent reduction of free radical production and pro-inflammatory cytokines. Fermented goat's milk consumption also induced a decrease in MCP-1, which provides a lower substrate for lipid peroxidation and consequently decreases monocyte migration and adhesion. The increase in IFN-γ can be attributed to the bioactive factors of fermented goat's milk, such as proteins, polyunsaturated fatty acids, oligosaccharides and micronutrients, which confer immunological colonization of gut microbiota and downregulate inflammation. Finally, fermented goat's milk increased classical, lectin and alternative complement pathway activity and decreased lactoferrin, while anemia decreased the lectin and alternative complement pathway activity, because it improves iron homeostasis, which is critically important in the normal function of the immune system.

ACKNOWLEDGEMENTS

This work was supported by the Regional Government of Andalusia, Excellence Research Project No. P11-AGR-7648. M García

Burgos was supported by a fellowship from the Spanish Ministry of Education, Culture and Sport (MECD). J Moreno-Fernández was supported by a postdoctoral contract (Contrato Puente) from the University of Granada. Funding for open access charge: University of Granada / CBUA.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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