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Changes in Intestinal Bifidobacteria Levels Are Associated with the Inflammatory Response in Magnesium-Deficient Mice[1–3]

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Abstract

Magnesium (Mg) deficiency is a common nutritional disorder that is linked to an inflammatory state characterized by increased plasma acute phase protein and proinflammatory cytokine concentrations. Recent studies have shown that changes in the composition of gut microbiota composition participate in systemic inflammation. In this study, therefore, we assessed the potential role of gut microbiota in intestinal and systemic inflammation associated with Mg deficiency in mice. For this purpose, mice were fed a control or Mg-deficient diet (500 mg vs. 70 mg Mg/kg) for 4 or 21 d. Compared with the mice fed the control diet, mice fed the Mg-deficient diet for 4 d had a lower gut bifidobacteria content (minus 1.5 log), a 36–50% lower mRNA content of factors controlling gut barrier function in the ileum (zonula occludens-1, occludin, proglucagon), and a higher mRNA content (by ~2-fold) in the liver and/or intestine of tumor necrosis factor-α, interleukin-6, CCAAT/enhancer binding protein homologous protein, and activating transcription factor 4, reflecting inflammatory and cellular stress. In contrast, mice fed the Mg-deficient diet for 21 d had a higher cecal bifidobacteria content compared with the control group, a phenomenon accompanied by restoration of the intestinal barrier and the absence of inflammation. In conclusion, we show that Mg deficiency, independently of any other changes in nutrient intake, modulates the concentration of bifidobacteria in the gut, a phenomenon that may time-dependently affect inflammation and metabolic disorders in mice. J. Nutr. doi: 10.3945/jn.109.117374.

Introduction

Magnesium (Mg) is the second most abundant intracellular cation after potassium and plays an essential role in numerous fundamental cellular reactions (1). Mg deficiency is associated with several metabolic disorders, including type 2 diabetes (2,3), metabolic syndrome (4–6), dyslipidemia (7–9), and hypertension (10–13). In addition, systemic inflammation has been observed in subjects with hypomagnesemia (14,15). Gut and systemic inflammation in rodent models of Mg deficiency are characterized by leukocyte and macrophage activation with increased production of inflammatory cytokines and acute phase proteins (14,16,17). The mechanism underlying the immunomodulation occurring during Mg deficiency remains poorly described.

In our laboratory, we have demonstrated a link between gut microbiota and systemic inflammation that drives the metabolic disturbances in obesity (18–21). Lipopolysaccharide (LPS)[7] is a major component of the outer membrane of Gram-negative bacteria, which, once in the circulation, can trigger the secretion of proinflammatory cytokines (21,22). In several models of obese mice, a decrease in cecal content of lactic acid-producing bacteria, such as bifidobacteria, correlated with higher intestinal permeability. Increased permeability promotes LPS translocation that drives endotoxemia and inflammation (19,20).

Therefore, in this study, we analyzed the intestinal and systemic inflammatory response occurring in Mg-deficient mice and related these data to changes in gut microbiota composition.

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[3] Supplemental Table 1 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.
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[7] Abbreviations used: ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer binding protein homologous protein; CRP, C-reactive protein; IL, interleukin; LPS, lipopolysaccharide; Occ, occludin; PG, proglucagon; TNFα, tumor necrosis factor-α; ZO-1, zonula occludens-1.

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Materials and Methods

Animals and diets. Male C57Bl/6J mice (9 wk old; Charles River) were housed in groups of 4 mice per cage at 22°C in a 12-h-light/-dark cycle and given free access to diet and water.

After an acclimatization period of 1 wk, the mice (n = 8/group) were fed a control (Mg+) (D10001, Research Diets) or a Mg-deficient diet (Mg−) (D16601, Research Diets) for 4 or 21 d (**Table 1**). Distilled water was provided for drinking. The Mg concentration was 500 mg/kg in the control diet and 70 mg/kg in the deficient diet.

Food intake, taking into account spillage, was recorded daily during the treatment as previously described (23,24).

All experiments were approved by the local animal ethics committee and the housing conditions were as specified by the Belgian Law of November 14, 1993 on the protection of laboratory animals (agreement no. LA 1230314).

Tissue samples. At the end of the study period, mice were anesthetized (ketamine/xylazine intraperitoneal, 100 and 10 mg/kg of body weight, respectively). The liver was immediately clamped in liquid N_2 and kept at −80°C. Ileum tissue, proximal colon tissue, and cecal content were collected and stored at −80°C. A fraction of ileum was washed with PBS, mounted in embedding medium (Tissue-tek, Sakura), and stored at −80°C until use for immunofluorescence analysis. The jejunal mucosa was scraped off and stored at −80°C. Liver, spleen, fatty tissues (epididymal, subcutaneous, and visceral), cecal content, and cecal tissue were weighed.

Blood samples. Portal vein blood was centrifuged 3 min at 13,000 × g and stored at –80 °C. Serum LPS concentrations were determined using a kit based on a Limulus amebocyte extract (LAL kit; Cambrex Bioscience) as previously described (20); samples were diluted 1:100 in LPS-free water. Cava vein blood was centrifuged 3 min at 13,000 × g and stored at –80 °C. The serum Mg concentration was determined using a Quantichrom magnesium assay kit (DIMG-250 Bioassay Systems).

Disaccharidase activity. The jejunal sucrase, lactase, and maltase activities were assessed in the jejunal mucosa following the method of Dahlqvist (25).

TABLE 1 Composition of Mg+ and Mg− diets

Ingredient	Mg+		Mg−	
	composition, %	*kJ/kg diet*	*composition, %*	*kJ/kg diet*
Casein	20	3349	20	3349
DL-Methionine	0.3	50	0.3	50
Corn starch	15	2512	15	2512
Sucrose	50	8374	50	8374
Cellulose, BW200	5	0	5	0
Corn oil	5	1884	5	1884
Mineral mix S10001[1]	3.5	69	0	0
Mineral mix S16601[2]	0	0	3.5	83
Vitamin mix V10001[3]	1	167	1	167
Choline bitartrate	0.2	0	0.2	0
Total	100	16405	100	16419

[1] Containing the following (g/kg mineral mix): 500 calcium phosphate, 24 magnesium oxide, 220 potassium citrate.1H_2O, 52 potassium sulphate, 74 sodium chloride, 0.55 chromium potassium sulphate.12H_2O, 0.3 cupric carbonate, 0.01 potassium iodate, 6 ferric citrate, 3.5 manganous carbonate, 0.01 sodium selenite, 1.6 zinc carbonate, and 118 sucrose.

[2] Containing the following (g/kg mineral mix): 500 calcium phosphate, 220 potassium citrate.1H_2O, 52 potassium sulphate, 74 sodium chloride, 0.55 chromium potassium sulphate.12H_2O, 0.3 cupric carbonate, 0.01 potassium iodate, 6 ferric citrate, 3.5 manganous carbonate, 0.01 sodium selenite, 1.6 zinc carbonate, and 142 sucrose.

[3] Containing the following (g/kg vitamin mix): 0.8 retinol palmitate, 1 cholecalciferol, 10 *dl*-α tocopherol acetate, 0.05 mg menadione, 0.02 biotin, 0.001 cyanocobalamin, 0.2 folic acid, 3 nicotinic acid, 1.7 calcium pantothenate, 0.7 pyridoxine, 0.6 riboflavin, 0.6 thiamin, and 978.42 sucrose.

Immunofluorescence analysis of occludin and ZO-1. The immunofluorescence analysis of occludin (Occ) and zonula occludens-1 (ZO-1) in ileum was performed as previously described (20).

Real-time quantitative PCR. Total RNA was isolated from liver tissue, ileum, and proximal colon using the TriPure reagent (Roche). cDNA was prepared by RT of 1 g total RNA using the Kit Reverse transcription System (Promega). Real-time PCR were performed with a StepOnePlus instrument and software (Applied Biosystems) as described (19). RPL19 RNA was chosen as an invariant standard. Gene transcripts for tumor necrosis factor-α (TNFα), interleukin (IL)-6, Occ, ZO-1, CCAAT/enhancer binding protein homologous protein (CHOP), activating transcription factor 4 (ATF4), CD68, F4/80, C-reactive protein (CRP), IL-1β, and proglucagon (PG) were amplified using gene-specific primers (**Supplemental Table 1**).

Real-time quantitative PCR for microbial cecal content. The cecal contents of Gram-positive *Bifidobacterium* spp. and *Lactobacillus* spp. were quantified as described previously (19). Results were expressed as [Log_{10} (bacterial cells/g of cecal content)].

Statistical analysis. Results are presented as mean ± SEM. Mg+ and Mg− mice were compared at each time using Student's t-test with GraphPad Prism version 4.00 for Windows. Associations between variables were assessed by Pearson correlation test. $P < 0.05$ was considered significant.

Results

Mg− mice had a lower body weight than Mg+ mice after 21 d. We assessed Mg deficiency from the serum Mg concentration. The serum Mg levels in the Mg− mice were 43 and 60% lower than the corresponding control values after 4 and 21 d, respectively (**Table 2**).

Mice fed a Mg-deficient diet for 4 d did not differ in food intake or body weight compared with Mg+ mice, although epididymal adipose tissue weight was ~22% lower (Table 2). After 21 d, Mg− mice had lower body weight and white adipose tissue mass than Mg+ mice. The progressive loss of fat mass and body weight in the Mg− mice may in part be explained by the lower total energy intake observed in these mice during the 21 d of the study period compared with Mg+ mice (Table 2).

Mg deficiency modulated gut microbiota. After 4 d, Mg− mice had ~43% less cecal content of bifidobacteria compared with Mg+ mice (**Fig. 1***A*). The lactobacilli content did not differ between the groups at d 4 (Fig. 1*B*). Cecal tissue and content weight did not differ between Mg+ and Mg− mice at d 4 (Table 2). Surprisingly, after 21 d, Mg− mice had higher cecal tissue and content weights (Table 2) and a higher intestinal content of bifidobacteria and lactobacilli (Fig. 1*C*,*D*). The cecal content of bacteroides did not differ between Mg+ mice and Mg- mice after either 4 or 21 d (data not shown).

We measured the jejunal disaccharidase activity to assess carbohydrate digestibility in the upper part of the intestine. Jejunal lactase, maltase, and sucrase activities were lower in Mg− mice compared with Mg+ mice after 4 d (**Supplemental Fig. 1**).

4 d of Mg deficiency induced colonic inflammation and decreased factors controlling ileal permeability. After 4 d of Mg deficiency, the expression of Occ and ZO-1, 2 tight junction proteins, was ~37% less in the ileum of Mg− compared with Mg+ mice (**Table 3**). The immunofluorescence assay performed on ileum sections of 4 d Mg+ mice revealed an intact network of Occ and ZO-1 proteins that were predominantly localized along

TABLE 2 Body and relative organs weight, energy intake, and serum Mg in mice fed a Mg+ or Mg− diet for 4 or 21 d[1]

	4 d of treatment		21 d of treatment	
	Mg+	Mg−	Mg+	Mg−
Body weight, *g*	22.89 ± 0.35	22.79 ± 0.41	23.79 ± 0.53	21.92 ± 0.40*
Spleen, *g/100 g* body weight	0.28 ± 0.01	0.32 ± 0.03	0.31 ± 0.01	0.30 ± 0.02
Liver, *g/100 g* body weight	3.64 ± 0.12	3.87 ± 0.08	3.91 ± 0.10	3.59 ± 0.08*
EAT,[2] *g/100 g* body weight	1.74 ± 0.10	1.36 ± 0.11*	1.23 ± 0.05	1.10 ± 0.05
SAT,[3] *g/100 g* body weight	0.95 ± 0.05	0.95 ± 0.12	1.44 ± 0.06	1.33 ± 0.06
VAT,[4] *g/100 g* body weight	0.68 ± 0.05	0.77 ± 0.11	0.64 ± 0.05	0.47 ± 0.03*
Cecal tissue, *g/100 g* body weight	0.26 ± 0.01	0.28 ± 0.02	0.24 ± 0.02	0.31 ± 0.01*
Cecal content, *g*	0.11 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.17 ± 0.01*
Total energy intake, *kJ/mouse*	160.02 ± 4.06	143.27 ± 9.38	953.84 ± 27.80	793.82 ± 6.32*
Serum Mg, *mmol/L*	0.79 ± 0.03	0.45 ± 0.06*	0.80 ± 0.02	0.32 ± 0.02*

[1] Data are presented as mean ± SEM, $n = 8$. *Different from Mg+, $P < 0.05$.
[2] EAT, Epididymal adipose tissue.
[3] SAT, Subcutaneous adipose tissue.
[4] VAT, Visceral adipose tissue.

the apical cellular border (**Fig. 2***A*,*C*). On the contrary, 4 d Mg− mice exhibited discontinuous ZO-1 and Occ staining in the ileum (Fig. 2*B*,*D*). Similarly, Mg− mice had ~ 50% lower ileal content of PG mRNA, a precursor of glucagon-like peptide-2 that participates in the control of gut permeability (20,26–28). These alterations were not associated with any sign of inflammation or stress in the ileum. In fact, at d 4, Mg+ and Mg− mice did not differ in the ileal expression of TNFα and IL-6, 2 cytokines, or of ATF4 and CHOP, 2 markers of cellular stress associated with the inflammatory process (Table 3). In contrast, in the proximal colon of mice fed for 4 d with a Mg-deficient diet, there were no differences in mRNA content of the genes involved in intestinal permeability (PG, Occ, and ZO-1) compared with Mg+ mice; however, there was a higher expression of factors involved in cellular stress and inflammation (Table 3). CD68 and F4/80 expression, 2 markers of monocyte infiltration, was similar in Mg+ and Mg− mice in the ileum and was 25–50% higher in the proximal colon of Mg− mice compared with Mg+ mice (Table 3).

After 21 d of treatment, Mg+ and Mg− mice did not differ in inflammation-related variables, monocyte infiltration, gut barrier function, or metabolic stress in the ileum or in the proximal colon (Table 3). Moreover, the immunofluorescence staining of ZO-1 and Occ in the ileum sections was similar in Mg+ and Mg− mice after 21 d (Fig. 2*E*–*H*).

4 d of Mg deficiency induced systemic inflammation. Because the 4-d Mg deficiency induced systemic inflammation, we assessed systemic inflammation. Mg− mice tended to have higher ($P = 0.11$) plasma LPS concentrations than did Mg+ mice after 4 d (LPS: Mg+, 8.0 ± 1.0 EU/L; Mg−, 12.2 ± 2.1 EU/L). The plasma LPS concentration did not differ between Mg+ and Mg− mice after 21 d (LPS: Mg+, 8.9 ± 3.5 EU/L; Mg−, 10.1 ± 1.6 EU/L).

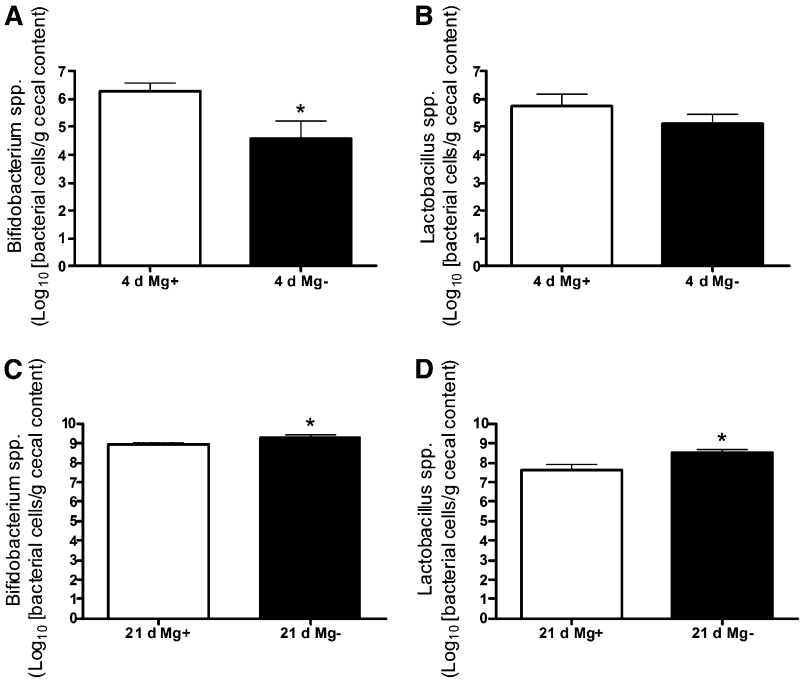

FIGURE 1 Cecal content of *Bifidobacterium spp.*, and *Lactobacillus spp.* in mice fed a Mg+ or Mg− diet for 4 d (*A*,*B*) or 21 d (*C*,*D*). Values are means ± SEM, $n = 8$. *Different from Mg+, $P < 0.05$.

TABLE 3 mRNA levels of factors involved in gut barrier function, inflammation, and cellular stress in mice fed a Mg+ or Mg− diet for 4 or 21 d[1]

	4 d of treatment		21 d of treatment	
	Mg+	Mg−	Mg+	Mg−
Ileum	*relative expression*			
TNFα	1.09 ± 0.18	1.05 ± 0.31	1.06 ± 0.17	0.67 ± 0.13
IL-6	1.08 ± 0.19	1.27 ± 0.20	1.12 ± 0.22	1.41 ± 0.11
ATF4	1.01 ± 0.06	0.98 ± 0.05	1.02 ± 0.09	1.21 ± 0.13
CHOP	1.02 ± 0.07	1.21 ± 0.10	1.04 ± 0.12	1.34 ± 0.15
F4/80	1.03 ± 0.09	0.84 ± 0.11	1.00 ± 0.20	1.24 ± 0.15
CD68	1.02 ± 0.09	0.99 ± 0.18	1.05 ± 0.13	1.11 ± 0.10
Occ	1.03 ± 0.10	0.64 ± 0.06*	1.18 ± 0.24	1.13 ± 0.25
ZO-1	1.03 ± 0.09	0.66 ± 0.07*	1.18 ± 0.24	1.27 ± 0.22
PG	1.09 ± 0.14	0.55 ± 0.13*	1.24 ± 0.34	1.39 ± 0.46
Proximal colon				
TNFα	1.25 ± 0.31	3.00 ± 0.39*	1.25 ± 0.33	0.76 ± 0.25
IL-6	1.03 ± 0.11	3.03 ± 0.68*	1.12 ± 0.20	1.18 ± 0.12
ATF4	1.02 ± 0.07	1.34 ± 0.07*	1.03 ± 0.10	1.39 ± 0.09
CHOP	1.05 ± 0.12	1.64 ± 0.13*	1.01 ± 0.05	1.17 ± 0.08
F4/80	1.01 ± 0.06	1.51 ± 0.18*	1.03 ± 0.09	1.10 ± 0.09
CD68	1.02 ± 0.08	1.28 ± 0.09*	1.00 ± 0.03	1.11 ± 0.09
Occ	1.06 ± 0.13	1.15 ± 0.18	1.09 ± 0.18	0.98 ± 0.10
ZO-1	1.05 ± 0.12	1.25 ± 0.13	1.06 ± 0.14	0.97 ± 0.07
PG	1.06 ± 0.16	0.81 ± 0.26	1.12 ± 0.21	0.36 ± 0.05*

[1] Data are presented as mean ± SEM, $n = 8$. *Different from Mg+, $P < 0.05$.

After 4 d, Mg− mice had a higher hepatic mRNA content of IL-6 and TNFα than Mg+ mice (**Fig. 3***A*). After 21 d, the hepatic mRNA content of TNFα, IL-1β, CD68, and CRP did not differ between Mg+ and Mg− mice, except for significantly lower IL-6 mRNA content in Mg− mice (Fig. 3*B*).

Bifidobacteria content correlated negatively with liver and proximal colon markers of inflammation. Cecal bifidobacteria content correlated negatively with inflammatory markers in the liver (IL-6: $r = -0.65$, $P = 0.0003$; TNFα: $r = -0.44$, $P = 0.02$) and in the proximal colon (IL-6: $r = -0.42$, $P = 0.02$; TNFα: $r = -0.64$, $P = 0.0002$) of Mg+ and Mg− mice after 4 and 21 d.

Discussion

Several studies have described systemic inflammation in Mg-deficient rodents, characterized by infiltration of leukocytes and phagocytic cells in various tissues (such as heart, lung, skeletal muscle, intestine, and thymus) and higher plasma levels of a number of positive acute phase proteins and cytokines, such as IL-6 and TNFα (14,29). However, a higher plasma TNFα concentration has not always been observed during Mg deficiency (17). Moreover, the mechanism underlying the immunomodulation linked to Mg deficiency remains poorly described.

In our laboratory, we have demonstrated a link between gut microbiota and inflammation in several models of obesity (20,30). The concentration of bifidobacteria correlated inversely with endotoxemia, fat mass development, and disturbances in glucose metabolism in high-fat–fed mice (18).

In this study, we show that the cecal content of bifidobacteria, and to a lesser extent of lactobacilli, decreases during short-term (4 d) Mg deficiency. This effect occurred independently of any

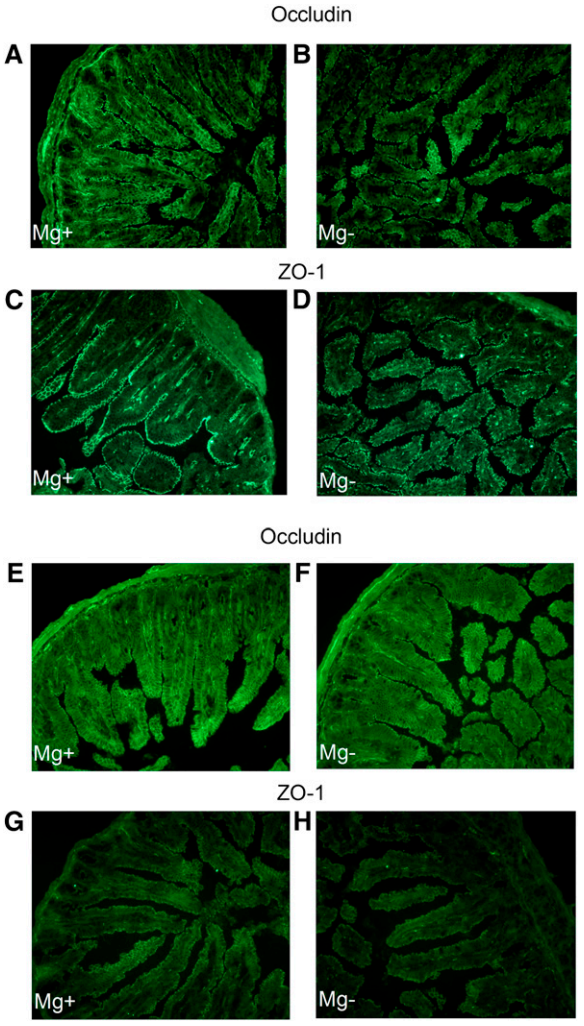

FIGURE 2 Immunofluorescence staining of the ileum epithelial tight junction proteins in mice fed a Mg+ or Mg− diet for 4 d (Occ: *A,B*; ZO-1: *C,D*) or 21 d (Occ: *E,F*; ZO-1: *G,H*).

significant change in food intake and was therefore not attributable to any changes in other nutrients. Some studies have revealed the importance of Mg in bifidobacteria physiology (31,32). Mg requirements are much higher for Gram-positive than for Gram-negative bacteria (33).

Interestingly, the concentrations of bifidobacteria and lactobacilli were higher in Mg− mice than in Mg+ mice after 21 d of treatment. This phenomenon may be interpreted as an adaptive mechanism. Despite a lower nutrient intake, Mg− mice had a greater cecal content and tissue weight than Mg+ mice at 21 d. This observation suggests increased fermentation in Mg− mice, which could be explained by decreased activity of the intestinal enzymes involved in carbohydrate digestion, leading to an increased availability of nutrients in the ceco-colon for fermentation. In fact, we observed reduced activity of the disaccharidases in Mg− mice compared with Mg+ mice.

Bifidobacteria are known to reduce intestinal LPS and to improve mucosal barrier function (34–37). In our study, Mg− mice had a lower cecal bifidobacteria content at d 4 than Mg+ mice and this was associated with a lower ileal content of ZO-1 and Occ mRNA, 2 tight junction proteins that control gut barrier function. Analogous to the observation previously made in obese animals (20), we can relate the decrease in ZO-1 and Occ to a lower expression of PG in the ileum of Mg− mice at 4 d.

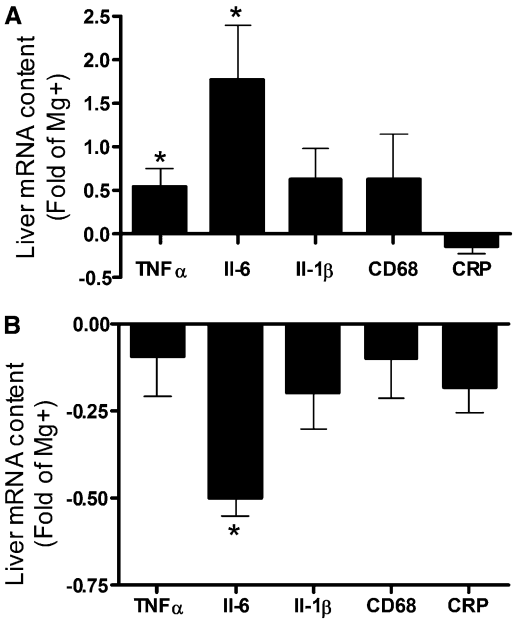

FIGURE 3 Liver content of TNFα, IL-6, IL-1β, CD68, and CRP mRNA in mice fed a Mg+ or a Mg− diet for 4 d (*A*) or 21 d (*B*). Values are mean ± SEM, n = 8. See Supplemental Table 1 for primer sequences and abbreviations. *Different from Mg+, $P < 0.05$.

PG mRNA is translated into glucagon-like peptide-2, an intestinotrophic peptide that has been shown to decrease gut permeability (26–28).

The higher intestinal permeability observed after 4 d of Mg deficiency could explain the higher portal LPS concentration. Systemic exposure to LPS is an important inflammatory trigger (22) and is commonly associated with hepatic inflammation (20). Chmielinska et al. (38) suggested the presence of increased intestinal permeability in Mg-deficient rats. These investigators described increased expression of the CD14 receptor, which specifically binds LPS in the heart, gut, and liver tissue of Mg-deficient rats.

Our results are in agreement with this previous observation, because mice deficient in Mg for 4 d had a higher mRNA content of IL-6 and TNFα, 2 proinflammatory cytokines, in the liver and in the proximal colon than control mice. Moreover, Mg− mice exhibited a higher expression of CD68 and F4/80 in the proximal colon, suggesting monocyte infiltration. Inflammation is often accompanied by cellular alterations. Several reports have demonstrated that inflammatory cytokines can induce endoplasmic reticulum stress and, therefore, activate the unfolded protein response (39). Moreover, a study conducted in mice demonstrated that LPS induced the expression of ATF4 and CHOP in the lung (40). In the present study, the proximal colon of Mg− mice at 4 d, which exhibited a higher IL-6 and TNFα mRNA content, also had a higher expression of stress markers, such as ATF4 and CHOP, compared with Mg+ mice. The impact of Mg deficiency on cellular and inflammatory stress seemed confined to the proximal colon, because in the ileum, these markers were not modified by Mg deficiency.

We can, therefore, suggest that decreased bifidobacteria content as a result of short-term Mg deficiency may participate in increased intestinal permeability, resulting in colonic and systemic inflammation. Despite a progressive loss of body weight, the mice fed a Mg-deficient diet for a longer period had no signs of inflammation, in accordance with restoration of gut microbiota composition (more bifidobacteria and lactobacilli, less bacteroides).

In conclusion, we show that targeted nutritional modulation of dietary mineral content (i.e. Mg level) may drive changes in gut microbiota composition, which has consequences on host metabolism.

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