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Protective effects of *Lactobacillus paracasei* F19 in a rat model of oxidative and metabolic hepatic injury

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Nardone G, Compare D, Liguori E, Di Mauro V, Rocco A, Barone M, Napoli A, Lapi D, Iovene MR, Colantuoni A. Protective effects of *Lactobacillus paracasei* F19 in a rat model of oxidative and metabolic hepatic injury. *Am J Physiol Gastrointest Liver Physiol* 299: G669–G676, 2010. First published June 24, 2010; doi:10.1152/ajpgi.00188.2010.—The liver is susceptible to such oxidative and metabolic stresses as ischemia-reperfusion (I/R) and fatty acid accumulation. Probiotics are viable microorganisms that restore the gut microbiota and exert a beneficial effect on the liver by inhibiting bacterial enzymes, stimulating immunity, and protecting intestinal permeability. We evaluated *Lactobacillus paracasei* F19 (LP-F19), for its potential protective effect, in an experimental model of I/R (30 min ischemia and 60 min reperfusion) in rats fed a standard diet or a steatogen [methionine/choline-deficient (MCD)] diet. Both groups consisted of 7 sham-operated rats, 10 rats that underwent I/R, and 10 that underwent I/R plus 8 wk of probiotic dietary supplementation. In rats fed a standard diet, I/R induced a decrease in sinusoid perfusion ($P < 0.001$), severe liver inflammation, and necrosis besides an increase of tissue levels of malondialdehyde ($P < 0.001$), tumor necrosis factor-α ($P < 0.001$), interleukin (IL)-1β ($P < 0.001$), and IL-6 ($P < 0.001$) and of serum levels of transaminase ($P < 0.001$) and lipopolysaccharides ($P < 0.001$) vs. sham-operated rats. I/R also induced a decrease in *Bacterioides*, *Bifidobacterium*, and *Lactobacillus* s*pps* ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively) and an increase in *Enterococcus* and *Enterobacteriaceae* ($P < 0.01$ and $P < 0.001$, respectively) on intestinal mucosa. The severity of liver and gut microbiota alterations induced by I/R was even greater in rats with liver inflammation and steatosis, i.e., MCD-fed animals. LP-F19 supplementation significantly reduced the harmful effects of I/R on the liver and on gut microbiota in both groups of rats, although the effect was slightly less in MCD-fed animals. In conclusion, LP-F19 supplementation, by restoring gut microbiota, attenuated I/R-related liver injury, particularly in the absence of steatosis.

ischemia-reperfusion; *Lactobacillus paracasei* F19; nonalcoholic fatty liver disease; probiotics

ISCHEMIA-REPERFUSION (I/R) is an inevitable complication of liver surgery (23, 45, 52). It consists of an early phase characterized by the induction of a cascade of proinflammatory mediators, followed by a subacute phase characterized by a massive infiltration of neutrophils with further production of inflammatory mediators that leads to severe hepatic injury, multiorgan failure, and death in many cases (5, 22, 33, 42). However, despite this large body of data, I/R of the liver remains a complicated and unclear process.

The animal model of liver I/R is a well-tested tool with which to examine the pathogenetic mechanisms underlying I/R. Indeed, animal model studies demonstrated that I/R injury is associated with hepatic neutrophil sequestration and Kupffer cell activation that in turn trigger the release of inflammatory mediators, namely, tumor necrosis factor-α (TNF-α), implicated in several pathological changes (30, 39). However, I/R causes severe inflammation not only of the liver but also of the extrahepatic organs. Portal venous congestion results in extensive mesenteric venous congestion, which considerably slows down blood flow in the intestinal wall and causes stagnant tissue anoxia, abnormalities in small bowel transit, mucosal barrier failure, and intestinal overgrowth of *Enterobacter spp* (1, 6). The mucosal barrier failure and modified gut microbiota induce endotoxin translocation to extraintestinal sites, such as the liver, where they trigger proinflammatory cytokine expression via Kupffer cell activation (27, 46, 47). Given this close interplay between the gut and the liver, attempts to restore the gut microbiota may have a beneficial effect on liver tissue.

Probiotics are viable microorganisms that, when administered in adequate amounts, confer a health benefit to the host. They may positively affect the gut microbiota and be effective in the prevention and treatment of specific pathological conditions (8, 40). Probiotics may positively affect the gut microbiota and liver health via various mechanisms, i.e., by inhibiting intestinal bacterial enzymes, stimulating host immunity, competing for limited nutrients, inhibiting bacteria mucosal adherence and epithelial invasion, protecting intestinal permeability, and controlling bacterial translocation from the gut to the bloodstream (12, 32, 44). The biological activity of probiotics depends prevalently on delivering anti-inflammatory mediators that downregulate proinflammatory cytokines, including interferon-γ and TNF-α, via the nuclear factor-κB pathway (15, 26, 38). Therefore, probiotics provide a means with which to control hepatic cellular stress and promote host health.

The aim of our study was to evaluate whether *Lactobacillus paracasei* F19 (LP-F19) protects against liver injury in an experimental model of I/R of the liver. In addition, because the shortage of organs for transplantation has led to the use of steatosic grafts, we also examined the effect of I/R and LP-F19 dietary supplementation in rats fed a steatogen diet.

MATERIALS AND METHODS

Animals and experimental procedures. The study design is shown in Fig. 1. Fifty-four male Wistar rats (Charles River, Calco, Italy), weighing 200–250 g, were randomized in the following two groups: 27 rats fed a standard diet (SD) and 27 rats fed a methionine/choline-

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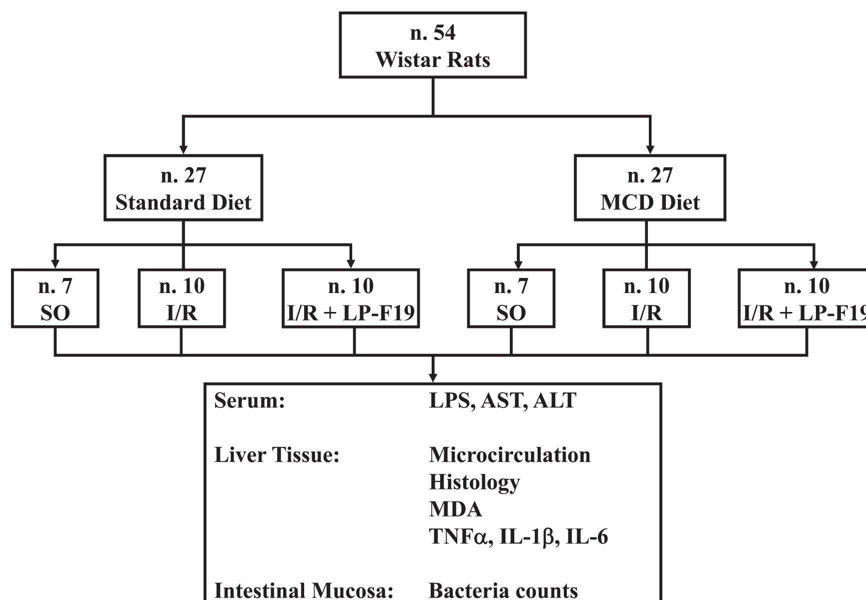

Fig. 1. Study design. SO, Sham-operated; I/R, ischemia-reperfusion; MCD, methionine/choline-deficient diet; LP-F19, *Lactobacillus paracasei* F19; LPS, lipopolysaccharide; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MDA, malondialdehyde; TNF-α, tumor necrosis factor-α; IL, interleukin; *n*, no. of rats.

deficient (MCD) diet for 8 wk before the surgical procedure. In each group, seven rats were sham-operated (SO) to determine baseline conditions, 10 rats underwent I/R of the liver (30 min ischemia and 60 min reperfusion), and 10 rats underwent I/R after dietary supplementation with LP-F19 for 8 wk.

An MCD diet is an animal model of nonalcoholic fatty liver disease (NAFLD) that reproduces several aspects of human diseases, namely, liver steatosis, inflammation, and fibrosis (41). Probiotic supplementation consisted of 3×10^7 colony-forming units (CFU) live LP-F19 (donated by SIFFRA, Rome, Italy, as lyophilized product and stored at 4°C until used) (20), suspended in physiological saline by daily oral gavage. Preliminary experiments showed that this dose of LP-F19 is the most suitable for animals of the weight and size examined.

Liver I/R was performed in animals anesthetized with intraperitoneal pentobarbital (5 mg/100 g body wt) placed on a heating pad to maintain body temperature (36 ± 0.5°C). The carotid artery and jugular vein were catheterized (PE-10 catheters; Clay-Adams, New York, NY) to enable continuous macrohemodynamic monitoring and administration of fluorescent dyes. Hepatic ischemia was induced by clamping the hepatoduodenal ligament, including the artery and portal vein for 30 min followed by reperfusion for 60 min. After reperfusion, all animals were killed. The liver was exteriorized and placed on a special platform for intravital fluorescence microscopy and covered with Saran wrap to avoid dehydration. Liver and small bowel tissue and blood samples were collected under sterile conditions and stored at −80°C (liver and small bowel tissues) and −20°C (blood samples) and fixed in 10% formalin (liver and small bowel tissue). The SO groups, fed a SD or MCD diet, were treated in the same fashion but spared hepatoduodenal ligament clamping.

The animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, Washington, D.C., USA, 1996), and the study was approved by the Ethics Committee of the University of Naples Federico II.

Intravital fluorescence microscopy. A Leica DM FL microscope equipped with long-distance objectives [×5, numerical aperture (NA): 0.25; ×10, NA: 0.30; ×40, NA: 0.40] and ×10 eyepieces was used. Epi-illumination of the liver surface was provided by a 50-W mercury lamp using appropriate filters for fluorescent dyes (Leitz N2 and Leitz I2) and a heat filter. The hepatic microcirculation was televised with a DAGE MTI 1000 low-light level camera connected to a Panasonic monitor and recorded by a computer-based frame grabber (Pinnacle PC 10 Plus; Avid Technology, Tewksbury, MA). Sodium fluorescein caused hepatocyte labeling, whereas Rhodamine 6G caused leukocyte labeling. The number of leukocytes adhered to the sinusoids was expressed as cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment. During a 30-s observation period, leukocytes not moving or detached from the endothelial lining were counted (n = 10 sinusoids/animal). The number of leukocytes extravasated was expressed as cells per square millimeter of tissue.

Microvascular measurements, such as vessel diameter and sinusoid perfusion, were performed with a computerized program (MIP; IFC, Pisa, Italy), by scanning a region of interest that comprised a total of 100 lobules (×280 magnification). Sinusoid perfusion was investigated, at higher magnification (×700), on 10–15 liver lobules for 30 and 60 s. The number of perfused sinusoids was expressed as a percentage of all visible sinusoids. Necrosis points were identified by scanning a region of interest that comprised a total of 100 lobules (×280 magnification). We report the percent changes of perfused lobes in each experimental group.

Liver histology. Sections, 4 μm thick, were stained with hematoxylin-eosin, Periodic acid Schiff, and Gomori's reticulin. Ten light microscopy fields (×200) were assessed on each section and evaluated for the degree of inflammatory cell infiltration, necrosis, steatosis, and fibrosis (17). Inflammatory cell infiltration was scored as follows: *grade 0*, absent; *grade 1*, focal isolated periportal lymphocytes (<5 foci/field); *grade 2*, periportal aggregate lymphocytes (>5 foci/field); and *grade 3*, intralobular lymphocytes. Necrosis was scored as follows: *grade 0*, absent; *grade 1*, sporadic (isolated hepatocytes); *grade 2*, parcellar (3–5 hepatocytes); and *grade 3*, extensive (>5 hepatocytes). The score for steatosis was as follows: *grade 0*, no fat; *grade 1*, fatty hepatocytes occupying <33% of the hepatic parenchyma; *grade 2*, microvacuolar fatty hepatocytes occupying 34–66% of the hepatic parenchyma; and *grade 3*, macrovacuolar fatty hepatocytes occupying >66% of the hepatic parenchyma. Last, fibrosis was scored as follows: *grade 0*, absent; *grade 1*, thin isolated septa; *grade 2*, periportal fibrosis; and *grade 3*, periportal and intralobular fibrotic septa.

Liver malondialdehyde assay. Hepatic tissue malondialdehyde (MDA) was measured by the thiobarbituric acid colorimetric assay according to the manufacturer's instructions. MDA levels were measured with a spectrofluorimeter (absorbance 530 and 550 nm; Perkin Elmer) and expressed as nanomoles per milligram protein.

Table 1. *In vivo microscopic parameters of the hepatic microcirculation in rats fed a standard or MCD diet that underwent I/R with and without LP-F19 diet supplementation*

	Standard Diet					MCD Diet				
	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†
Sinusoid perfusion rate, %	99 ± 1	85 ± 4	<0.001	95 ± 5	<0.001	90 ± 5	75 ± 3	<0.001	85 ± 5	<0.001
Sinusoid diameter, μm	10.2 ± 0.7	8.0 ± 1.0	<0.001	11.2 ± 0.9	<0.001	8.4 ± 0.7	7.9 ± 0.4	<0.5	10.1 ± 0.5	<0.001
Necrosis points/100 lobules, n	0	8 ± 2		3 ± 2	<0.001	1 ± 0.5	10 ± 2	<0.001	5 ± 2	<0.001
Adhered leukocytes, cells/mm^2	0	28 ± 5		14 ± 6	<0.001	5 ± 2	30 ± 4	<0.001	18 ± 6	<0.001
Extravasated leukocytes, cells/mm^2	0	7 ± 2		3 ± 1	<0.001	0	11 ± 3		4 ± 1	<0.001

Values are means ± SD; n, no. of rats. SO, sham operated; I/R, ischemia-reperfusion; I/R-L, ischemia-reperfusion plus *Lactobacillus paracasei* F19 (LP-F19) dietary supplementation; MCD, methionine/choline-deficient diet. *I/R vs. SO; †I/R-L vs. I/R.

Western blot analysis. Equivalent amounts of 20 μg of liver proteins were loaded and separated by electrophoresis on 10% SDS-polyacrylamide gels at 120 V for 2 h and electrotransferred to a nitrocellulose membrane at 100 V for 1 h on an electromagnetic broiler. Membranes were blocked with 1× Tris-buffered saline containing 20% of inactivated FBS and 0.5% of Triton X-100 for 1 h and then incubated with rat polyclonal anti-TNF-α, interleukin (IL)-1β, and IL-6 (1:2,000 dilution; Pierce Endogen Biotechnology, Rockford, IL) antibodies at 4°C overnight. The membranes were washed in 1× PBS, pH 7.6, containing 0.3% Tween 20. Membranes were then incubated with peroxidase-conjugated rabbit anti-rat IgG horseradish (1:4,000 dilution; Stressgen Bioreagents, Victoria, BC, Canada) for 2 h at 23°C and detected by chemiluminescence reaction ECL (ECL-plus; Amersham Biosciences, Cologno Monzese, MI, Italy).

Serum transaminases. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected in blood samples collected from the abdominal aorta by automated biochemistry (Eurokit, Gorizia, Italy), according to the manufacturer's instructions, and expressed as International units per liter (IU/l) of serum (normal values ALT <35 IU/l, AST <40 IU/l).

Endotoxin assay. Lipopolysaccharide (LPS) levels were assessed in sera collected from the portal veins using the BioWhittaker QCL-1000 chromogenic limulus amoebocyte lysate test kit according to the manufacturer's instructions (BioWhittaker, Walkersville, MD). Opti-

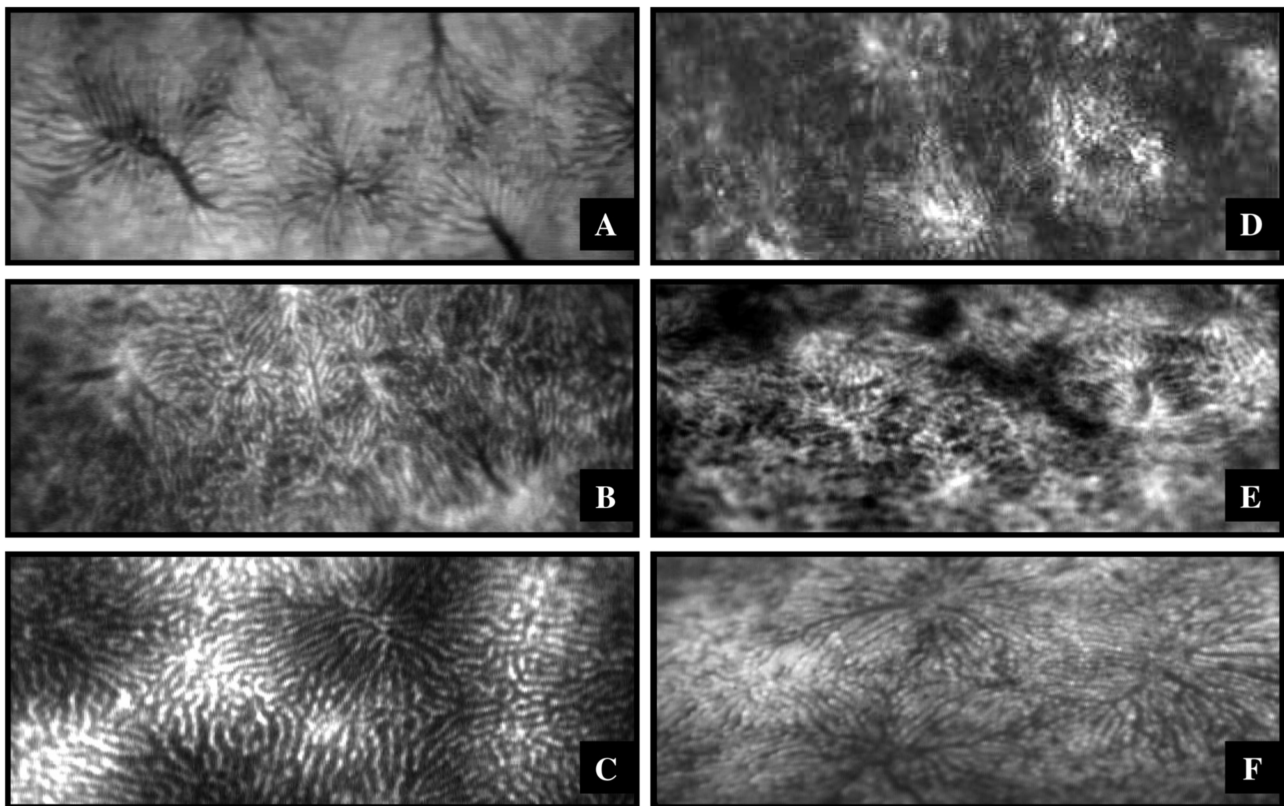

Fig. 2. In vivo microscopic images of the liver microcirculation. *A*: rat fed a standard diet. Note normal hepatic lobular microvasculature with black colored sinusoid between two layers of hepatocytes filled with fluorescine (white color). *B*: rat that underwent I/R and received a standard diet. Note the change in hepatic microvasculature characterized by structural remodeling of lobular sinusoids. A point of necrosis associated with leukocyte infiltration can be seen on *left*. *C*: rat that underwent I/R and received a standard diet plus LP-F19 supplementation. Note the recovery of the hepatic lobular microvasculature characterized by normal perfusion of sinusoids. *D*: rat fed a MCD diet. Note the change in hepatic structure characterized by decreased sinusoidal diameter and diffuse vascular derangement. *E*: rat that underwent I/R and received a MCD diet. Note the dramatic changes in hepatic structure, i.e., marked derangement of microvasculature, points of necrosis, and diffuse leukocyte infiltration. *F*: rat that underwent I/R and received a MCD diet plus LP-F19 supplementation. Note the recovery of hepatic lobular structure characterized by improved sinusoidal perfusion and decreased points of necrosis and leukocyte infiltration.

Table 2. *Liver histologic finding in rats fed a standard or MCD diet that underwent I/R with and without LP-F19 dietary supplementation*

	Standard Diet					MCD Diet				
	SO (*n* = 7)	I/R (*n* = 10)	*P**	I/R-L (*n* = 10)	*P*†	SO (*n* = 7)	I/R (*n* = 10)	*P**	I/R-L (*n* = 10)	*P*†
Inflammation	0	2.3 ± 0.4		1.2 ± 0.4	<0.001	1.3 ± 0.4	2.9 ± 0.3	<0.001	1.7 ± 0.4	<0.001
Necrosis	0	1.7 ± 0.3		0.7 ± 0.4	<0.001	1.6 ± 0.4	2.6 ± 0.6	<0.001	1.8 ± 0.4	<0.01
Steatosis	0	0		0		1.8 ± 0.4	2.7 ± 0.4	<0.01	2.1 ± 0.5	<0.05
Fibrosis	0	0		0		1.2 ± 0.6	2.3 ± 0.5	<0.001	17 ± 0.4	<0.05

Values are means ± SD; *n*, no. of rats. *I/R vs. SO; †I/R-L vs. I/R.

cal densities were measured using an ELISA plate reader (Spectra I; Tecan, Gratz, Austria) at 405 nm. The sensitivity of the assays was 3 pg/ml.

Bacteriological analysis. The small intestine specimens were washed in sterile saline solution, dried with sterile paper, and weighed. Each sample was placed in a sterile tube with 2 ml of sterile saline and homogenized. The homogenates were diluted 1:1 in sterile saline solution, and 100 μl of the sample solution were inoculated in MacConkey plates and CNA agar plates and incubated for 24 h at 37°C under aerobic conditions to isolate *Enterobacteriaceae* and *Enterococcus*, respectively. All isolates were identified using biochemical methods (RAPID ID 32 E System-API; BIO Merieux). Furthermore, 100 μl of the sample solution were inoculated in MRS agar plates and incubated for 48 h at 37°C under anaerobic conditions to isolate *Lactobacillus spp*. All isolates were identified using biochemical methods (API 50 CH System; BIO Merieux). Finally, 100 μl of the sample solution were inoculated in Schaedler plates and incubated for 48 h at 37°C under anaerobic conditions to isolate anaerobic Gram positive and Gram negative bacteria (*Bacteroides* and *Bifidobacterium spps*). All the isolates were identified using biochemical methods (API 20 A System; BIO Merieux). The bacteria adhering to mucosa was quantified as colony-forming units ($\log_{10}$ CFU/g, means ± SD).

Statistical analysis. Data are reported as means ± SD and analyzed using the SPSS package for Windows. The Kruskall-Wallis test (nonparametric ANOVA) and Dunn's multiple-comparison post test

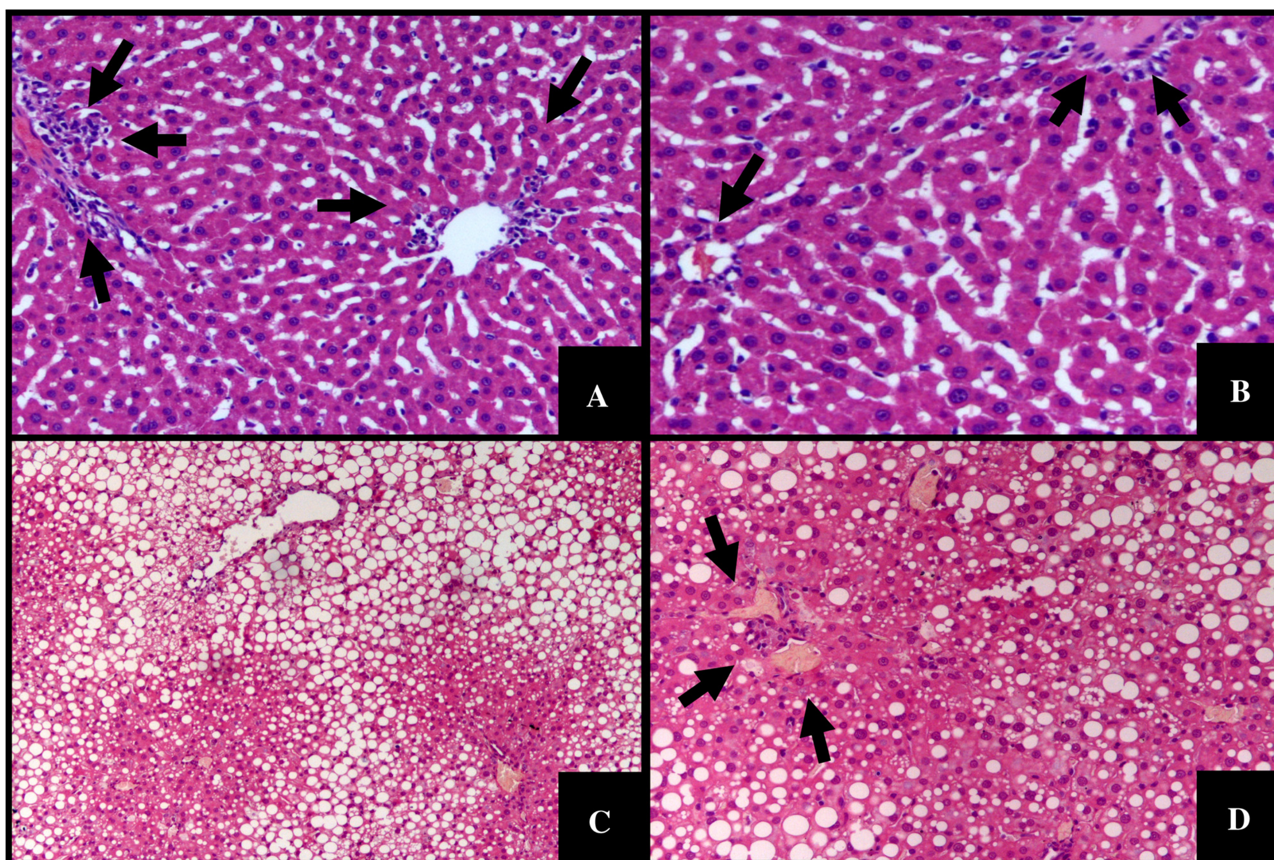

Fig. 3. Liver histology. *A*: rat that underwent I/R and received a standard diet. Note inflammatory infiltrate characterized by an abundance of lymphocytes and monocytes, which is more evident in the portal space and around centrolobular veins (arrows) [hematoxylin & eosin (H&E) staining, orig. magnification ×20]. *B*: rat that underwent I/R and received a standard diet plus LP-F19 supplementation. Note that the inflammatory process is almost absent in the portal space and the few mononuclear cells at centrolobular vein level (arrows) (H&E, original magnification: ×40). *C*: rat that underwent I/R and received a MCD diet. Note diffuse steatotic damage characterized by macrovacuoles diffusely distributed (Periodic acid Schiff, original magnification ×10). *D*: rat that underwent I/R and received a MCD diet plus LP-F19 supplementation. Note sporadic macrovacuolar deposits around the vascular structures (arrows) (Periodic acid Schiff, original magnification ×20).

were used to compare data between groups. A P value <0.05 was taken as the level of significance.

RESULTS

Intravital fluorescence microscopy. Data related to the hepatic microcirculation are reported in Table 1 and Fig. 2. In the SO group fed a SD (baseline), all hepatic sinusoids were perfused, and there were no points of necrosis or leukocytes adhering to the vessel wall. In SD-fed animals, I/R induced a significant decrease in sinusoid perfusion and sinusoid diameter associated with points of necrosis, leukocyte adherence to the vessel wall, and leukocytes extravasated. LP-F19 dietary supplementation in I/R-treated rats significantly increased sinusoid perfusion and sinusoid diameter vs. SO rats. The number of points of necrosis, of leukocytes adhering to the vessel wall, and of extravasated leukocytes decreased.

Compared with SO rats fed a SD, SO rats fed a MCD diet showed a decrease in sinusoid perfusion associated with a few isolated points of necrosis. Leukocytes adhering to the vessel wall but not extravasated leukocytes were also detected. In the MCD group, I/R decreased sinusoid perfusion and sinusoid diameter, and there was an increase in the number of points of necrosis, of leukocytes adhering to the vessel wall and of extravasated leukocytes. LP-F19 dietary supplementation in rats treated with I/R significantly increased sinusoid perfusion and sinusoid diameter, decreased the number of points of necrosis, of leukocytes adhering to the vessel wall, and of extravasated leukocytes.

Liver histology. A semiquantitative evaluation of histological findings is reported in Table 2. In the SO group fed a SD, no inflammation, necrosis, steatosis, and fibrosis were observed. In this group, I/R induced a substantial inflammatory infiltrate characterized by lymphomonocytes, particularly in the portal spaces and around the centrolobular veins, associated with mild necrotic phenomena (Fig. 3*A*). LP-F19 dietary supplementation led to a decrease in inflammation and necrosis (Fig. 3*B*).

In the SO group fed a MCD diet, there was a mild to moderate inflammatory infiltrate, fibrosis, and steatosis (*grade 2*, affecting ~40% of the hepatic parenchyma). I/R significantly increased the inflammatory infiltrate, necrosis, fibrosis, and steatosis (*grade 3*, affecting ~70% of the hepatic parenchyma) (Fig. 3*C*). LP-F19 dietary supplementation significantly decreased the I/R-induced inflammatory infiltrate, necrosis, periportal fibrosis, and steatosis (*grade 2*, affecting ~50% of the hepatic parenchyma), without however restoring normal values (Fig. 3*D*).

Liver MDA assay. I/R significantly ($P < 0.001$) increased MDA tissue levels in SD-fed rats from 3.4 ± 1.5 to 49.7 ± 7.2 nmol/mg protein; an even greater increase occurred in MCD-fed rats, i.e., from 16.9 ± 5.1 to 309.7 ± 30.8 nmol/mg protein. LP-F19 dietary supplementation in I/R-treated rats significantly ($P < 0.001$) decreased MDA levels in SD-fed rats from 49.7 ± 7.2 to 22.5 ± 4.6 nmol/mg protein and in MCD-fed rats from 309.7 ± 30.8 to 261.3 ± 22.4 nmol/mg protein.

Western blot analysis. Figure 4 shows the protein expression of TNF-α, IL-1β, and IL-6 together with the results of densitometric analysis obtained in all groups of rats. In rats fed a SD and in rats fed a MCD diet, I/R significantly upregulated TNF-α, IL-1β, and IL-6 expression vs. baseline conditions (SO

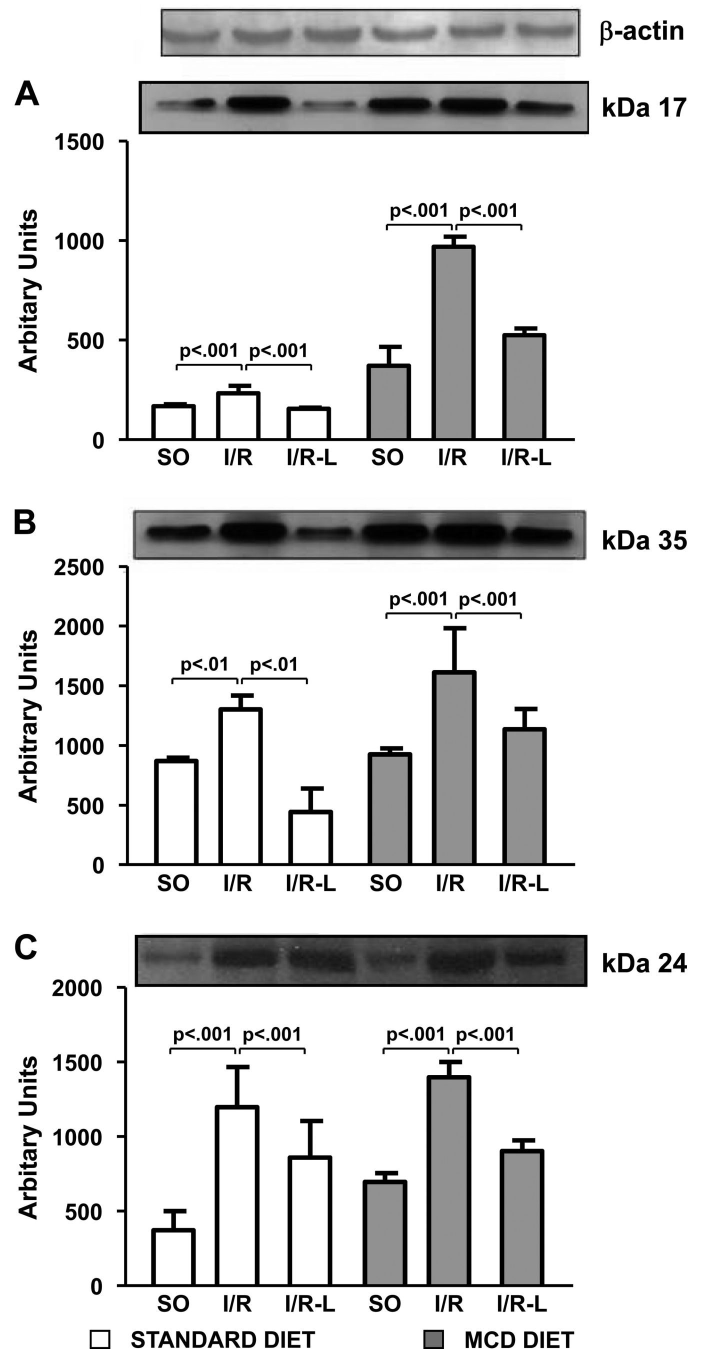

Fig. 4. Expression of cytokines by Western blot analysis of proteins (20 μg) from hepatic tissue homogenates of rats fed a standard or MCD diet that underwent I/R with or without LP-F19 dietary supplementation. *A*: expression of TNF-α. *Top*, representative autoradiograph of experiments with band corresponding to TNF-α migrating with an apparent molecular mass of 17 kDa. Histogram below shows mean ± SD densitometric values of TNF-α band. *B*: expression of IL-1. *Top*, representative autoradiograph of experiments with band corresponding to IL-1 migrating with an apparent molecular mass of 35 kDa. Histogram below shows mean ± SD densitometric values of IL-1 band. *C*: expression of IL-6. *Top*, representative autoradiograph of experiments with band corresponding to IL-6 migrating with an apparent molecular mass of 24 kDa. Histogram below shows mean ± SD densitometric values of IL-6. Kruskall-Wallis test with Dunn's multiple-comparison posttest.

Table 3. *Serum transaminase (IU/l) levels in rats fed a standard or MCD diet that underwent I/R with and without LP-F19 dietary supplementation*

	Standard Diet					MCD Diet				
	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†
AST	95 ± 9.0	1,880 ± 210	<0.001	820 ± 150	<0.001	1050 ± 120	2,930 ± 210	<0.001	1,950 ± 300	<0.001
ALT	90 ± 7.0	1,540 ± 150	<0.001	740 ± 100	<0.001	960 ± 150	2,370 ± 280	<0.001	1,740 ± 270	<0.001

Values are means ± SD; *n*, no. of rats. AST, aspartate aminotransferase; ALT, alanine aminotransferase. *I/R vs. SO; †I/R-L vs. I/R.

animals). The percent increase was greater for IL-6 than for TNF-α and IL-1β. LP-F19 dietary supplementation significantly downregulated TNF-α, IL-1β, and IL-6 expression, particularly in animals not affected by steatosis.

Serum transaminases. As shown in Table 3, in the SO group fed a SD, serum levels of ALT and AST were in the normal range, whereas, in the SO group fed a MCD diet, serum levels of ALT and AST were significantly higher vs. SO fed a SD ($P < 0.001$). In both rats fed a SD and rats fed a MCD diet, I/R led to a significant increase in AST and ALT levels ($P < 0.001$), which was attenuated in the groups given LP-F19 dietary supplementation although values did not return to normal.

Endotoxin assay. Baseline, serum LPS levels were significantly ($P < 0.001$) higher in rats fed a MCD diet than in those fed a SD (120 ± 16 and 43 ± 9 pg/ml, respectively). I/R significantly ($P < 0.001$) increased serum LPS levels in both groups (180 ± 16 and 380 ± 22 pg/ml, rats fed a SD and MCD diet, respectively). LP-F19 dietary supplementation in rats treated with I/R significantly ($P < 0.001$) decreased LPS levels in both groups (95 ± 11 and 220 ± 16 pg/ml, rats fed a standard and MCD diet, respectively).

Bacteriological analysis. The counts of bacterial species ($\log_{10}$ CFU/g ± SD) on intestinal mucosa samples are reported in Table 4. At baseline, the counts of *Enterococcus spp* and *Enterobacteriaceae* were higher, and the counts of *Lactobacillus spp, Bifidobacter spp*, and *Bacterioides spp* were lower in MCD-fed rats than in SD-fed rats. In both groups, I/R induced a significant increase in *Enterococcus spp* and *Enterobacteriaceae* and a significant decrease in *Lactobacillus spp, Bifidobacter spp*, and *Bacterioides spp*. LP-F19 diet supplementation significantly decreased *Enterococcus spp* and *Enterobacteriaceae* and increased *Lactobacillus spp, Bifidobacter spp*, and *Bacterioides spp* in both groups.

DISCUSSION

In this study, LP-F19 dietary supplementation, by restoring the gut microflora and intestinal barrier, protected the liver from I/R-induced injury in both SD-fed animals and in MCD-fed animals, although the effect was less pronounced in MCD-fed animals.

Liver I/R is a well-known model of hepatic injury in which various times of ischemia and reperfusion can be used. Oxygen deprivation during liver ischemia induces severe damage, but more important lesions occur during the first hours of reperfusion, when the blood supply to the organ is restored (18a, 19, 25). Therefore, the degree of hepatic injury depends on the tissue ischemia and reperfusion timeframe. In this study, we carried out hepatic ischemia by clamping the hepatoduodenal ligament to involve both the hepatic artery and portal vein. We chose a short period of ischemia (30 min) and reperfusion (60 min) to avoid massive organ damage and to better evaluate the potential protective role of probiotics. Prolonged ischemia, lasting 60–180 min, causes intense hepatocellular necrosis and inflammation and is thus not appropriate for studies aimed at identifying the protective effect of a given substance (18, 49). Furthermore, a short period of reperfusion avoids the downregulation of multidrug resistance proteins and bile duct injury (11, 51, 59).

Vascular liver damage induces Kupffer cell activation that, in turn, triggers the release of inflammatory mediators and cytokines, implicated in several pathological changes (9, 50, 57). In our study, liver TNF-α, IL-1β, and, in particular, IL-6 were upregulated. IL-6 has a high anti-inflammatory and protective potential because it induces IL-1 receptor antagonist and soluble TNF receptor p55 and promotes hepatocyte regeneration (4, 53). Treatment for 10 days with IL-6 prevented the susceptibility of fatty liver to I/R injury, increased hepatic peroxisome proliferator-activated receptor-α, and decreased serum TNF-α levels (21). In addition, IL-6 may enhance intestinal barrier function and protect enterocytes from stress-induced apoptosis (56). Taken together, these data seem to suggest that the increase in IL-6 may be a compensatory mechanism with which to balance the increase of IL-1β and TNF-α. However, in our study, LP-F19 downregulated IL-6 expression to the same degree as the proinflammatory cyto-

Table 4. *Ileal mucosa bacteria counts (log10 CFU/g) in rats fed a standard or MCD diet that underwent I/R with and without LP-F19 diet supplementation*

	Standard Diet					MCD Diet				
	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†	SO (n = 7)	I/R (n = 10)	P*	I/R-L (n = 10)	P†
Enterococcus spp.	2.78 ± 0.7	3.71 ± 0.5	<0.01	3.23 ± 0.4		3.30 ± 0.7	4.71 ± 0.5	<0.001	3.14 ± 0.3	<0.001
Enterobacter spp.	2.89 ± 0.6	4.14 ± 0.5	<0.001	3.71 ± 0.5		3.72 ± 0.4	4.97 ± 0.7	<0.01	3.86 ± 0.9	<0.01
Lactobacillus spp.	4.78 ± 0.4	3.28 ± 0.5	<0.001	4.14 ± 0.7	<0.01	3.96 ± 0.5	3.07 ± 0.5	<0.05	3.90 ± 0.5	<0.01
Bacteroides spp.	4.22 ± 0.7	2.71 ± 0.7	<0.01	3.86 ± 0.9	<0.01	3.50 ± 0.5	2.23 ± 0.5	<0.001	2.97 ± 0.7	<0.05
Bifidobacterium spp.	4.33 ± 0.9	2.86 ± 0.7	<0.001	3.71 ± 0.5	<0.05	3.45 ± 0.5	2.14 ± 0.5	<0.001	2.86 ± 0.7	<0.05

Values are means ± SD; *n*, no. of rats. *I/R vs. SO; †I/R-L vs. I/R.

kines TNF-α and IL-1β. Consequently, further studies are required to clarify the role of IL-6 in hepatic injury.

In our study, we also found a significant increase of the intestinal content of *Enterococcus spp* and *Enterobacteriaceae* (Table 4) as well as of serum LPS levels. The increase of LPS serum level mirrors the overgrowth of Gram negative anaerobic bacteria and a failure of the gut barrier. These events may further contribute to and aggravate liver injury. Indeed, NAFLD, including steatosis and steatohepatitis, is a frequent complication of intestinal bacteria overgrowth (12, 29, 34, 43). Miele et al. (31) very recently reported that NAFLD in humans is associated with increased gut permeability and increased prevalence of small bowel bacterial overgrowth (31). In line with these data, the administration of antibiotics, such as polymyxin B and metroinidazole, as well as anti-TNF-α antibodies reduces the severity of steatosis in an animal model and in humans (13, 36, 37). Improved intestinal epithelial function and decreased bacterial translocation and endotoxemia were observed in experimental animals and humans after probiotic treatment (14). In addition, the administration of VSL#3, a probiotic preparation of eight different live, freeze-dried bacteria, had a beneficial effect on liver steatosis in *ob/ob* mice and in a small cohort of patients with NAFLD (24, 28). In contrast, in a mouse model of steatohepatitis, VSL#3 supplementation had a beneficial effect on liver fibrosis but did not protect against inflammation and steatosis (55).

Lactobacillus spp and *Bifidobacterium spp* are considered to be the most important gut microorganisms for maintenance of colonization resistance and intestinal barrier function (48, 54). Xing et al. (58) found that *Bifidobacterium catenulatum* ZYB0401 combined with *Lactobacillus fermentum* ZYL0401 restored intestinal microflora and prevented liver injury in hepatic I/R of rats. In the present study, we used the LP-F19 lactobacillus strain because of its in vitro activity against several pathogens, tolerance to acid and bile, as well as genetic stability (7, 10). LP-F19 dietary supplementation protected the liver from I/R injury in both SD-fed and MCD-fed animals, although the effect was less pronounced in animals with steatosis. This was not unexpected because the fatty liver can contain unsaturated fatty acids that undergo lipid peroxidation in the presence of reactive oxygen species and is thus more sensitive to I/R injury (16). This coincides with our finding that I/R induced more severe liver injury in MCD-fed rats, as demonstrated by the high tissue levels of MDA, which is a marker of lipid peroxidation.

In conclusion, in our study, I/R induced severe hepatic injury that was greater in rats fed a MCD diet. LP-F19 dietary supplementation, by restoring the gut microflora and intestinal barrier function, attenuated the I/R-related liver damage, particularly in animals without steatosis.

Therefore, manipulation of gut microbiota by means of probiotics could represent an additional tool with which to counteract the impact of oxidative and metabolic stress on the liver and also after transplantation. At present, between 10 and 25% of donor livers are estimated to be affected by steatosis (3). In this context, our data suggest that steatosic graft may not be the ideal choice for liver transplantation because it is more susceptible to oxidative stress.

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DISCLOSURES

No conflict of interest exists for each of the authors.

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