Probiotics Reduce the Inflammatory Response Induced by a High-Fat Diet in the Liver of Young Rats

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the pediatric population. Preliminary evidence suggests a potential therapeutic utility of probiotics for this condition. Here, we tested the potential effect of the probiotic VSL#3 (a multistrain preparation composed of Streptococcus thermophilus and several species of Lactobacillus and Bifidobacterium) on oxidative and inflammatory damage induced by a high-fat diet in the liver of young rats.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the pediatric population, and with the rapid rise in childhood obesity, there has been an increase in the prevalence, recognition, and severity of this condition (1,2).

The disease is related to excessive accumulation of hepatic fat and represents a spectrum of conditions ranging from fat accumulation alone (steatosis without inflammation) to steatohepatitis (NASH) with macrovesicular steatosis in hepatocytes, associated with inflammation and fibrosis. Therefore, NASH is thought to be an intermediate stage in the progression from steatosis to cirrhosis. The fatty acid excess is converted to triglycerides and stored in the cytoplasm, predisposing the hepatocytes to oxidative stress and to activation of inflammatory pathways (3). In the last decade, the “2-hit” model has been proposed for the pathogenesis of NASH (4). Liver fat accumulation and insulin resistance characterize the first hit and are responsible for the development of steatosis. The main factors initiating the second hit are oxidative stress and subsequent lipid peroxidation, together with the production of proinflammatory cytokines, principally tumor necrosis factor (TNF)α (5,6), and hormones derived from adipose tissue (7,8).

The PPAR family plays a key role in modulating hepatic triglyceride accumulation and inflammatory processes. In particular, PPARα regulates fatty acid β-oxidation (9) and catalyzes, preventing and decreasing hepatic fat storage (10). Loss of its gene expression in mice results in hepatic steatosis under conditions of increased fatty acid metabolism in liver, such as fasting or consuming a high-fat diet (11,12).
Probiotics have been proposed to prevent and treat different inflammatory conditions of the gastrointestinal tract (13,14). These therapeutic effects might be related to a variety of direct and indirect mechanisms, including modulation of local microbiota, epithelial barrier function, and the immune system (15). Because the probiotic modulatory effect on the intestinal microflora could influence the gut-liver axis, these microorganisms have also been proposed as possible adjunctive therapy in some types of chronic liver disease (16,17). A recent meta-analysis in adult patients suggests that probiotics could be useful in NAFLD treatment and that further research elucidating the mechanisms of such effects is needed (18). Preliminary data obtained in rat models of alcoholic and nonalcoholic steatohepatitis showed that the treatment with probiotics could be effective in limiting liver damage (19–21), but the exact mechanism of these effects is still largely undefined.

The aim of the present study was to test the effect of probiotics on inflammatory and oxidative mechanisms involved in the pathogenesis of liver damage in an experimental animal model of NAFLD. We focused the study on the main pathological mechanisms involved in the pathogenesis of NAFLD. We chose VSL#3, a multistrain cocktail composed of Streptococcus thermophilus and several species of Lactobacillus and Bifidobacteria, that was previously used in vitro and in vivo studies (17,21). We used a previously established animal model that reproduces the key features of NASH by feeding young male rats a high-fat liquid diet (HFD) (22,23). Compared with genetically modified mice (e.g. ob/ob), this animal model better resembles the pathological events implicated in the pathogenesis of NAFLD induced by a fatty diet. Here, 4 wk after treatment with probiotics in rats receiving HFD, we investigated the modifications of several hepatic oxidative and inflammatory parameters. We also evaluated the involvement of nuclear factor-κB (NF-κB) pathway in the probiotic effect.

Materials and Methods

Diets and drugs. The HFD was purchased from Research Diets (product no. L01004). This liquid diet had 71% of energy derived from fat, 11% from carbohydrates, and 18% from protein and its composition was been previously described by Lieber-DeCarli (22,23). The fat composition of this diet was 48.5 g/L corn oil, 28.4 g/L olive oil, and 2.7 g/L safflower oil. A standard nonpurified pelleted diet (Std; Global diet 2018), described by Guglielmetti et al. (24), was purchased from Harlan Italy, and had 15% of energy derived from fat, 22% from protein, and 63% from carbohydrate (Table 1). The Std and HFD diets contained 13.7 and 41.8 MJ/kg, respectively. Because the 2 diets differed in several aspects, the nonpurified pelleted diet may have introduced other variables not present in the HFD; therefore, the Std diet was included for reference. The probiotic mixture, named VSL#3, is a commercial probiotic product provided by Professor Claudio De Simone, Department of Experimental Medicine, University of L’Aquila, L’Aquila, Italy. VSL#3 contains 4 species of Lactobacillus, 3 species of Bifidobacteria: B. infantis, B. breve, and B. longum, representing 17.7% of the mixture; and 47.5% of Streptococcus salivarius subsp. Thermophilus.

Rat model. After weaning, young male Sprague-Dawley rats (113.0 ± 2.5 g; Charles River Laboratories) were randomly divided into 3 groups (n = 6 rats each group) as following: 1) a control group, receiving a Std diet; 2) a HFD diet-fed group; and 3) HFD rats treated by gavage with VSL#3 (HFD + VSL#3; 13 × 109 bacteria·kg–1·d–1). HFD and Std diet-fed rats were gavaged with water as vehicle.

It has been previously shown that the rats receiving HFD developed the pathologic changes of NASH (23). To explore the preventive effects of probiotics, VSL#3 was administered by gavage to 1 group of rats fed the HFD for 4 wk. All animal procedures were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/EEC).

All rats were weighed at the beginning of the study and before killing to calculate the body weight gain during the experimental period (4 wk). Throughout the treatment, food intake was monitored twice a week. Bioelectrical impedance analysis was applied to body composition before killing using a BIA 101 analyzer, modified for the rat (Akern). Fat-free mass was calculated using the bioelectrical impedance analysis (50 kHz) prediction equation of Ibagan et al. (25) and fat mass was determined as the difference between body weight and fat-free mass.

After 4 wk of treatment, all rats were anesthetized by an overdose of enflurane and killed.

Blood biochemistry. After killing, livers were excised, weighed, and immediately frozen in liquid nitrogen and used for later experimental procedures. Blood was collected by cardiac puncture and centrifuged at 1300 × g; 15 min at 4°C and sera were stored at −70°C for later measurements of aspartate amino transferase (AST), alanine amino transferase (ALT), triglycerides, and total cholesterol, by standard automated procedures, according to the manufacturer’s protocols (AST Flex reagent cartridge, catalog no. DF41A; ALT Flex reagent cartridge, catalog no. DF43A; CHOL Flex reagent cartridge, catalog no. DF27; TGL Flex reagent cartridge, catalog no. DF69A, Dade Behring). Insulin concentrations were measured by using a rat insulinRIA kit (Linco Research).

Liver malondialdehyde and triglycerides. The malondialdehyde (MDA) concentration was measured as an indicator of lipid peroxidation. Briefly, livers were homogenized in 1.15% KCl solution and processed as previously described (26). The sample absorbance was measured by spectrophotometry and MDA values were calculated by comparison with the OD532 of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dimethyl acetel) 99% (Sigma).

We measured the hepatic triglyceride concentration using a commercially available kit (Triglyceride G-Test kit, Wako Pure Chemical Industries).

Liver TNFα. Portions of liver tissues were homogenized and TNFα levels were evaluated using a colorimetric commercial kit (Quantikine Rat TNFα-Immunoassay, catalog no. RTA00, R&D System) according to the manufacturer’s instructions. All TNFα determinations were performed in duplicate serial dilutions.

Western blotting. Liver tissue (0.1 g) was disrupted by homogenization on ice in a lysis buffer. Protein tissue lysates, mitochondrial extracts, or nuclear and cytosolic fractions were subjected to SDS-PAGE (8% polyacrylamide). Western blot analysis was performed as previously described (27). In particular, the blot was probed with specific antibodies
against inducible nitric oxide synthase (iNOS) (BD Biosciences Transduction Laboratories; 1:1000), cyclooxygenase (COX)-2 (Cayman Chemical; 1:1500), PPARα (Santa Cruz Biotechnology; 1:500), liver carnosine palmitoyltransferase (LCPT) I (Santa Cruz Biotechnology; 1:2000), 3-nitrotyrosine (Upstate Biotechnology; 1:5000), IκBα (Santa Cruz Biotechnology; 1:1000), and NF-κB p65 (Santa Cruz Biotechnology; 1:500), Western blot for β-actin (in cell lysates) or lamin protein (in nuclei lysates) (Sigma; 1:10,000) was performed to ensure equal sample loading.

**Metalloproteinase activity.** We performed gelatin-zymography to determine the metalloproteinase (MMP) activity of the proenzyme forms (pro-MMP-9 and pro-MMP-2) and MMP-2 active form, as previously described (28). The low availability of active MMP-9 in the zymographies may have been due to its high level of instability and the removal of active enzyme during the washing of specimens, as has been previously suggested (29). To measure the activities of the detected enzymes, zymograms were read using a ScanJet 3c scanner (Hewlett-Packard). The intensities of the separate bands were analyzed using Sigma Gel measurement software (Jandel).

**Statistical analysis.** All data are presented as means ± SEM. The statistical analysis was performed using Graph-Pad Prism (Graph-Pad Software). The data were tested using Bartlett’s test for equality of variance. We evaluated group differences with 1-way ANOVA followed by Newman-Keuls post hoc comparison unless the data were not normally distributed, in which case evaluation was performed, using Kruskal-Wallis test followed by Dunn’s Multiple Comparison test. A P-value of ≤0.05 was considered significant.

### Results

**Body and organ weights, liver lipids, and serum biochemistry.** The HFD rats had greater body weight gain, fat mass, relative liver weights, and triglyceride concentrations than rats fed the Std diet (Table 2). Serum cholesterol and triglyceride concentrations and AST and ALT levels, markers of liver cell damage, also were greater in the HFD group compared with rats receiving the Std diet.

HFD rats receiving VSL#3 had significantly lower body weight, triglyceride concentration, and AST serum level compared with HFD-fed rats (Table 2). Food intake did not differ between rats fed the HFD treated with vehicle (183.8 ± 7.2 mL) and VSL#3 (187.3 ± 1.4 mL).

The rats receiving the HFD had also higher plasma insulin concentrations (653.3 ± 63.1 pmol/L) compared with rats receiving the Std diet (241.7 ± 83.4; P < 0.05), suggesting the onset of insulin resistance. VSL#3-treated rats had significantly lower triglyceride concentrations (Table 2) compared with rats receiving the HFD; conversely, insulinemia was not significantly changed (491.7 ± 115.9 pmol/L).

**Effect of VSL#3 on lipid peroxidation, iNOS, and S-nitrosylation induced by HFD in liver.** Peroxidation of phospholipids generates MDA and other MDA-like aldehydes and ketones; however, MDA is the major product that reacts with thiobarbituric acid. HFD induced an increase in the amount of MDA (Fig. 1A; P < 0.01 vs. Std) and VSL#3 treatment blunted this effect (P < 0.01 vs. HFD).

Because prolonged production of NO by iNOS may result in liver damage and inflammation and considering that peroxynitrite is a specific marker of iNOS activity, we have determined both the levels of nitrotyrosine (downstream reaction products of peroxynitrite) of hepatic proteins and iNOS expression using Western blot analysis. Protein nitrosylation and iNOS expression were increased in liver from the HFD rats compared with rats receiving the Std diet. VSL#3 treatment completely inhibited both alterations induced by the HFD (Fig. 1B,C).

**TNFα and COX-2 modulation by VSL#3 treatment in liver of HFD-fed rats.** To evaluate the specific inflammatory response induced by the HFD, we examined local TNFα production and COX-2 expression in the liver. Tissue TNFα levels increased in the liver of HFD-fed rats (P < 0.01) but were completely inhibited by VSL#3 treatment (P < 0.01; Fig. 2A). Moreover, the 4-wk feeding of the HFD also induced increased COX-2 expression (P < 0.01 vs. Std) that was decreased by VSL#3 treatment (Fig. 2B; P < 0.05).

**Effect of VSL#3 on IκBα degradation and NF-κB p65 nuclear translocation.** To investigate the mechanism by which probiotic may attenuate the development of liver inflammation, we evaluated Western blot analysis the IκBα degradation and NF-κB p65 activation in the cytosolic or nuclear extracts from liver tissue, respectively. The basal level of IκBα was detected in the liver cytosolic fraction from rats fed the Std diet, whereas the HFD induced decreased IκBα (P < 0.05 vs. Std; Fig. 3A). VSL#3 prevented IκBα degradation (P < 0.01 vs. HFD). Moreover, the translocation of the p65 subunit of NF-κB into the nucleus was dramatically increased by HFD (P < 0.01 vs. Std; Fig. 3B). VSL#3 reduced the level of NF-κB p65 in the nuclear fraction (P < 0.01 vs. HFD).

**Effect of VSL#3 treatment on PPARα and LCPT I expression.** The basal level of PPARα was detected in the liver homogenates from Std diet-fed rats. PPARα decreased in liver from HFD rats (P < 0.05) and VSL#3 treatment prevented the HFD-induced PPARα decrease (P < 0.01; Fig. 4A). The expression of LCPT I, the rate-limiting enzyme in mitochondrial fatty acid oxidation, was also measured (Fig. 4B). Its expression in the liver of HFD-fed rats significantly increased, whereas the treatment with VSL#3 restored its expression (Fig. 4A).

**Evaluation of hepatic pro- and active MMP-2 and proMMP-9 activity.** Activation of MMP plays an important role in the evolution of the inflammatory processes, as well as in the pathogenesis of inflammatory disease. In particular, MMP-2 and MMP-9 are involved in liver damage. Here, we investigated the levels of gelatinase A and B in livers obtained from rats fed a

### Table 2

<table>
<thead>
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<th>Std</th>
<th>HFD</th>
<th>HFD + VSL#3</th>
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<tr>
<td>Body weight gain, g/4 wk</td>
<td>102.5 ± 1.12b</td>
<td>141.7 ± 3.33b</td>
<td>132.5 ± 6.65b</td>
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<td>Fat mass, g/100 g body weight</td>
<td>24.92 ± 1.09b</td>
<td>31.65 ± 1.70b</td>
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<td>Relative liver weight, g/100 g body weight</td>
<td>3.78 ± 0.12b</td>
<td>4.45 ± 0.11b</td>
<td>3.71 ± 0.20b</td>
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<td>Liver triglycerides, mmol/g</td>
<td>1.13 ± 0.04b</td>
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<td>2.35 ± 0.11b</td>
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<tr>
<td>Serum AST, U/L</td>
<td>164.30 ± 5.05</td>
<td>253.00 ± 1.08b</td>
<td>168.30 ± 24.25</td>
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<td>Serum ALT, U/L</td>
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<td>59.75 ± 2.87b</td>
<td>53.50 ± 4.65b</td>
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<td>Serum cholesterol, mmol/L</td>
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<td>2.79 ± 0.05b</td>
<td>2.64 ± 0.02b</td>
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<tr>
<td>Serum triglycerides, mmol/L</td>
<td>0.70 ± 0.10b</td>
<td>1.1 ± 0.06b</td>
<td>0.75 ± 0.03b</td>
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1 Values are means SEM, n = 6. Means without a common letter differ, P ≤ 0.05.
Std diet or the HFD diet alone or in combination with VSL#3. Pro- and active MMP-2 and proMMP-9 were significantly up-regulated in liver from HFD-fed rats. VSL#3 treatment significantly reduced their activity (Fig. 5).

**Discussion**

NAFLD is a common cause of chronic liver disease both in adults and children. The aim of the present study was to evaluate the antioxidative and antiinflammatory effect elicited by the probiotic mixture VSL#3 in an experimental model of NASH induced in young rats.

We showed that a 4-wk HFD was able to induce a significant increase of body weight gain and fat mass in young rats compared with rats fed with a Std maintenance diet. In HFD-fed rats, liver weight and AST serum level were elevated and these modifications reproduce typical aspects of NASH (22,23).

In our experimental condition, the HFD evoked the mechanisms involved in the second hit of liver injury, in particular the oxidative stress evidenced through the increased MDA content and liver protein S-nitrosylation, accompanied with the

**FIGURE 1** MDA content in homogenates (A), and protein nitrotyrosilated levels (B) and iNOS expression (C) in the liver from Std, HFD, or HFD+VSL#3 groups. MDA values are means ± SEM, n = 6. The data in panel C represent relative density normalized to β-actin. Means without a common letter differ, \( P < 0.01 \).

**FIGURE 2** Liver TNFα levels (A) were obtained from Std, HFD, or HFD+VSL#3 groups treated with VSL#3 (HFD+ VSL#3). Representative Western blot of COX-2 expression in liver homogenates from all groups is shown (B). COX-2 modulation was revealed by densitometric analysis of all protein bands (n = 6 rats from each group). The data in panel B represent relative density normalized to β-actin. Values are means ± SEM, n = 6. Means without a common letter differ, \( P < 0.05 \).

**FIGURE 3** (A) Western blot analysis of IκBα levels in the cytosolic fractions of liver lysates Std, HFD, or HFD+VSL#3 groups. The data represent relative density normalized to β-actin. (B) Western blot analysis of NF-κB p65 levels in the nuclear fractions of liver lysates from all groups. A representative blot of lysates obtained is shown. The data represent relative density normalized to lamin. Means without a common letter differ, \( P < 0.05 \).
Values are means accompanied by an increased number in infiltrated lym-
phocytes and macrophages.

In our experimental model, the VSL#3 probiotic mixture was found to markedly reduce the oxidative damage, protein nitro-
ryosilation, and tissue TNFα level, interfering with the key pathogenetic mechanisms responsible of the onset of liver damage.

We demonstrated a direct effect of VSL#3 on liver damage, both reducing inflammatory enzymes such as iNOS and COX-2 and restoring PPARα expression reduced by the HFD.

To determine whether a HFD and probiotic treatment could affect the expression of enzymes linked to fatty acid degrada-
tion, we also evaluated LCPT I. The rate-limiting enzyme of long-chain fatty acid oxidation, LCPT I, ensures the first step of long-chain fatty acid import into the mitochondrial matrix for subsequent metabolism. Surprisingly, we found that the HFD feeding induced a significant increase of this enzyme, despite the downregulation of PPARα that is reported to control its gene transcription. Actually, it has been reported that LCPT I could also be regulated in a PPARα-independent manner (37). In particular, the long-chain fatty acids modulate the transcription of genes responsible of their metabolism, such as LCPT I, increasing its expression (38,39). Therefore, we hypothesize that the strong availability of long-chain fatty acids or change in pancreatic hormone concentrations (glucagon or insulin) could be responsible for the increased LCPT I expression and subsequent fatty acid oxidation in the mitochondria.

In our experimental conditions, VSL#3 treatment significantly reduced serum and liver triglyceride concentrations, associated with a reduction of fat mass, on equal food intake, sustaining the possibility that this mixture could reduce dietary fat absorption with a reduction of long-chain fatty acid availability and a normalization of LCPT I.

The VSL#3 treatment also reduced hepatic gelatinase activity of proMMP-2 and proMMP-9 in HFD-fed rats. Expression and activation of MMP also play an important role in the evolution of liver inflammatory process. Both macrophages and T cells have been shown to produce several MMP, including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12, and their expression appears to be modulated by cytokines (40–42). These MMP, which degrade the basement membrane and extracellular matrix, are important in leukocyte migration as well as in the release of TNFα from its membrane-bound form (43).

Previous data indicated that VSL#3 significantly ameliorates the severity of colitis, decreasing PGE2 generation and MPO and NOS activities (44). In humans, VSL#3 ameliorates a variety of gastrointestinal ailments, reducing the induction and main-
tenance of remission of ulcerative colitis (45,46) and preventing pouchitis and its relapses (14,47).

Recently, experimental and clinical data indicate an increase of intestinal mucosal permeability with a subsequent increase in endotoxin level into the portal circulation, initiating hepatic necroinflammatory cascades and resulting in liver damage (46,47).

Our results strongly support the antiinflammatory and anti-
oxidative activity of VSL#3, which is responsible in the pre-
ventative effect of the early onset of NASH. Another possible mechanism of the protective effect of VSL#3 may include the maintenance of gut integrity. To further characterize the antiin-
flammation mechanism of VSL#3 in HFD-induced liver damage, we focused our attention on IκBα degradation and NF-κB activation. The appearance of IκBα and NF-κB p65 in cytosolic or nuclear fractions of the liver tissue was investigated using Western blot analysis. We found that the antiinflammatory effects of VSL#3 occurred at least in part via prevention of HFD-induced IκBα degradation. Consistent with this result, the appearance of NF-κB p65 in the nuclear fractions was also significantly reduced by this probiotic mix.
Weight loss and regular exercise are considered the first line of treatment for NAFLD in children and adults (48), but they are often difficult to achieve, especially in pediatric patients. Our data demonstrating a protective effect of VSL3# on the major key pathogenetic events of liver damage suggest the potential clinical utility of probiotics in patients affected by this condition.

Literature Cited


