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Effects of a *Lactobacillus paracasei* B21060 based synbiotic on steatosis, insulin signaling and toll-like receptor expression in rats fed a high-fat diet☆,☆☆,★

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Abstract

Insulin resistance (IR) has been identified as crucial pathophysiological factor in the development and progression of non-alcoholic fatty liver disease (NAFLD). Although mounting evidence suggests that perturbation of gut microflora exacerbates the severity of chronic liver diseases, therapeutic approaches using synbiotic has remained overlooked. Here, we show that a synbiotic composed by *Lactobacillus paracasei* B21060 plus arabinogalactan and fructo-oligosaccharides lessens NAFLD progression in a rat model of high fat feeding. IR and steatosis were induced by administration of high fat diet (HFD) for 6 weeks. Steatosis and hepatic inflammation, Toll-like receptor (TLR) pattern, glucose tolerance, insulin signaling and gut permeability were studied.

Liver inflammatory markers were down-regulated in rats receiving the synbiotic, along with an increased expression of nuclear peroxisome proliferator-activated receptors and expression of downstream target genes. The synbiotic improved many aspects of IR, such as fasting response, hormonal homeostasis and glycemic control. Indeed it prevented the impairment of hepatic insulin signaling, reducing the phosphorylation of insulin receptor substrate-1 in Ser 307 and down-regulating suppressor of cytokine signaling 3. Gene expression analysis revealed that in the liver the synbiotic reduced cytokines synthesis and restored the HFD-dysregulated TLR 2, 4 and 9 mRNAs toward a physiological level of expression. The synbiotic preserved gut barrier integrity and reduced the relative amount of Gram–negative Enterobacteriales and *Escherichia coli* in colonic mucosa.

Overall, our data indicate that the *L. paracasei* B21060 based synbiotic is effective in reducing the severity of liver injury and IR associated with high fat intake, suggesting its possible therapeutic/preventive clinical utilization.

Keywords: Non-alcoholic fatty liver disease; Insulin resistance; Glucose tolerance; Inflammation; Gut permeability; Toll-like receptor

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly recognized clinical practice condition characterized by insulin resistance (IR), hepatic steatosis and frequently type 2 diabetes (T2DM). The pathophysiology of NAFLD is still not completely defined. Tilg and Moschen have proposed a "parallel hits" hypothesis on the evolution of inflammation in NAFLD [1], as opposed to the so-called "two hits" previously suggested for the development of non-alcoholic steatohepatitis (NASH) [2]. This new model suggests that different hits may act in parallel, and that gut- or adipose tissue-derived factors may have a key role in the onset of liver inflammation. The cytokines tumor necrosis factor α (TNF-α) and interleukin (IL)-6 represent a link between IR and liver inflammatory process, activating several mechanisms involved in hepatocyte apoptosis and inhibition of insulin signaling [3,4].

Evidences suggest the modulation of gut microflora as potential target for the prevention and treatment of NAFLD [5–9]. Probiotics are live microbial that have beneficial effects on human health and disease modulating intestinal microbiota composition and function, improving epithelial barrier function, and reducing inflammation [10]. Immune and epithelial cells can discriminate among different microbial species through the activation of Toll-like receptors (TLR) [11].

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We have recently obtained experimental and clinical evidences that selected probiotics, could be effective against NAFLD [12,13]. The effects of probiotics are clearly related to specific strains and dosage [14]. It has been reported that some lactic acid bacteria affect the progression of diabetes mellitus [15–18]. These studies show that ingestion of determined lactic acid bacteria prevents or delays the disease onset in various experimental models of diabetes, induced by a chemical or by diet, or genetically modified animals (db/db) [19]. A variety of in vitro experiments and in vivo studies provided experimental evidence to support the probiotic roles in lowering serum cholesterol and ameliorating lipid profiles [20]. It has been demonstrated that *L. paracasei* B21026, alone or in combination with prebiotics, is effective to limit infectious diseases and to regulate immune system [21,22]. A recent study has highlighted the striking difference among species and strains of lactobacilli such as *L. plantarum NCIMB8826, L. rhamnosus GG* and *L. paracasei* B21060 in modulating immune and inflammatory response [23]. This latter strain of lactobacillus, the most active, was isolated from the feces of breast-fed babies and its non-occasional presence in the physiological intestinal microflora was established by genetic identification methods [24].

Based on these findings, it seemed of great interest to assess the influence of a synbiotic preparation containing *Lactobacillus paracasei* B21060 on glucose homeostasis using an animal model of NAFLD. This synbiotic is commercially available in Europe as a formulation containing prebiotics (arabinogalactan and fructo-oligosaccharide) that are able to improve probiotic strain survival [7]. Here, we hypothesize that this synbiotic could limit inflammatory liver damage and insulin signalling impairment by restoring intestinal permeability and, thus, preventing the imbalance of TLR pattern in a model of IR and steatosis in young rats.

2. Materials and methods

2.1. Diets and synbiotic

High fat diet (HFD) provided in pellet with 58% of energy derived from fats, 18% from proteins, and 24% from carbohydrates (5.56 kcal/g) was purchased from Laboratorio Dottori Piccioni (Gessate, Milan, Italy). The composition of this diet has been previously described [25]. The control standard (STD) pellet diet had 15% of energy from fats, 22% from proteins, and 63% from carbohydrates (3.30 kcal/g). The synbiotic formulation containing viable lyophilized *L. paracasei* B21060 mixed with prebiotics fructo-oligosaccharides and arabinogalactan (Flortec, Bracco, Milan, Italy) was available as powder and dispensed in 6 g bag containing about 2.5×10^9 CFU of the bacteria.

2.2. Animal model and experimental design

After weaning, young male Sprague–Dawley rats (113.5±1.1 g; Harlan, Corezzano, Italy) were randomly allocated in 3 groups (at least $n=8$) as follows: (1) control group, receiving STD and vehicle (tap water); (2) HFD-fed group, receiving vehicle; and (3) HFD-fed group, receiving the synbiotic by gavage once daily [HFD+SYN; *L. paracasei* B21060 2.5×10^7 bacteria/100 g body weight (bw); fructo-oligosaccharides 7 mg/100 g bw, and arabinogalactan 5 mg/100 g bw]. The synbiotic treatment started together with the HFD and continued for 6 weeks.

The HFD, administered for a long period of time (up to 6 months), creates a nutritional model of IR and NASH in non-genetically modified animals [26]. In our experiments, we administered HFD in young rats for a shorter period of time (6 weeks) to induce the early events of NAFLD due to fat overnutrition in young animals, excluding age and gender influences.

All procedures involving animals 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 of the European Communities Council Directive of November 24, 1986 (86/609/ECC). Prior to sample collection, animals, kept overnight fasted, were euthanized by an intraperitoneal injection of a cocktail of ketamine/xylazine, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering. Blood samples from animals were collected by cardiac puncture and serum obtained. Liver and white adipose tissue were excised and immediately frozen.

2.3. Histological analysis of liver tissue and transaminase levels

Liver sections were stained with hematoxylin and eosin or Oil Red O. Steatosis was graded on a scale of 0 (absence of steatosis), 1 (mild), 2 (moderate) and 3 (extensive). AST and ALT were measured in serum samples by standard automated procedures, according to manufacturer's protocols (AST Flex reagent cartridge, ALT Flex reagent cartridge; Dade Behring, Newark, DE, USA). Blood nonesterified fatty acids (NEFA) were determined as previously described [27].

2.4. Oral glucose tolerance test and insulin resistance assessment

At fifth week of treatment, fasted rats received glucose (2g/kg; per os) and glycaemia was measured at 0, 30, 60, 90 and 120 min after glucose administration. The area under the curve (AUC) was calculated from time zero, as the integrated and cumulative measure of glycemia up to 120 min for all animals. Glucose and insulin levels were measured by the glucometer One Touch UltraSmart (Lifescan, Milpitas, CA, USA) and by rat insulin radioimmunoassay kit (Millipore Corporation, Billerica, MA, USA), respectively. As index of insulin resistance, homeostasis model assessment (HOMA) was calculated, using the formula [HOMA=fasting glucose (mmol/L)×fasting insulin (μU/ml)/22.5].

2.5. Western blotting

Liver and visceral white adipose tissues were homogenized and total protein lysates were subjected to SDS-PAGE. Blots were probed with anti-suppressor of cytokine signaling 3 (SOCS3, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-peroxisome proliferator-activated receptor α (PPARα; Santa Cruz Biotechnology), or anti-TLR4 (Imgenex, San Diego, CA, USA), or anti-PPARγ (Novus Biologicals, Littleton, CO, USA), or anti-glucose transporter4 (GLUT4, Santa Cruz Biotechnology). To evaluate nuclear factor-κB (NF-κB) activation, IκB-α (Santa Cruz Biotechnology) and NF-κB p50 (Santa Cruz Biotechnology) were measured in liver cytosolic or nuclear extracts, respectively. Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich; Milan Italy) or lamin A (Chemicon, Temecula, CA, USA) was performed to ensure equal sample loading.

2.6. Immunoprecipitation

Immunoprecipitation of insulin receptor substrate (IRS)-1 was performed incubating 1.5 mg of liver lysate with 2 μg of an antibody against total IRS-1 (Santa Cruz Biotechnology). The immunoprecipitates were subjected to SDS-PAGE, and immunoblotted with an antibody against total IRS-1 or phospho-IRS-1^{Ser307} (1:1000, Cell Signaling Technology, Danvers, MA, USA).

2.7. Real-time semi-quantitative polymerase chain reaction (PCR)

Total RNA, isolated from liver, colon and visceral adipose tissue, was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with an ABIPrism HT7900 fast Real-time PCR System instrument and software (Applied Biosystem). The primer sequences are reported in Table 1. The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1–100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/L of each primer (EUROGENTEC Explera s.r.l, Ancona, Italy) in a final volume of 25 μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

2.8. Measurement of gut permeability in vivo

In another experiment, after 6 weeks on HFD, rats were fasted for 6 h and then gavaged with 4,000 kDa FITC-labeled dextran diluted in water (TdB Consultancy AB, Uppsala, Sweden) (500 mg/kg, 125 mg/ml). After 2 h, blood (500 μl) was collected from intracardiac puncture and centrifuged (3000 rpm for 15 min at RT), and FITC-dextran concentration in plasma was determined by spectrophotometry (excitation wavelength 485 nm; emission wavelength 535 nm; HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, MA, USA), as previously described [28].

2.9. Immunofluorescence analysis of occludin and zonula occludens (ZO)-1

Colon segments were immediately removed, washed with phosphate-buffered saline (PBS), mounted in embedding medium (Pelco Cryo-Z-T, Ted Pella inc, Redding, California), and stored at −80°C until use. Cryosections (7 μm) were fixed in formaldehyde 2%+PBS at RT for 10 min for occludin or in methanol for 10 min at RT for ZO-1. Non-specific background was blocked by incubation with normal goat serum in PBS and 0.1% Triton X-100. Sections were incubated for 2h with rabbit anti-occludin (1:50 for occludin, Santa Cruz Biotechnology) or rabbit anti-ZO-1 (1:100 for ZO-1; Invitrogen, Camarillo, CA, USA). Sections were probed with goat anti-rabbit Alexa Fluor 488 antibodies (1:200, Invitrogen). Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), and visualized on a fluorescence microscope using a 640 objective, and images were stored digitally with Leica software. Two negative controls were used: slides incubated with or without

Table 1
Real-time PCR primer sequences

Target gene	Forward primer (5′→3′)	Revere primer (3′→5′)	Accession number
Adiponectin	AATCCTGCCCAGTCATGAAG	TCTCCAGGAGTGCCATCTCT	NM_144744
CD14	GTGCTCCTGCCCAGTGAAAGAT	GATCTGTCTGACAACCCTGAGT	AF_087943
CPT-1a	CGCTCATGGTCAACAGCAACTACT	CTCACGGTCTAATGTGCGACGA	NM_031559
FGF21	AGATCAGGGAGGATGGAACA	ATCAAAGTGAGGCGATCCATA	NM_130752.1
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008 XM_216453
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC	NM_012589
MyD88	TGGCCTTGTTAGACCGTGA	AAGTATTTCTGGCAGTCCTCCTC	NM_198130.1
Occludin	TTGGGAGCCTTGACATCTTGTTC	TCCGCCATACATGTCATTGCTTGGTG	NM_031329.2
RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT	NM_009078.2
TLR2	GTACGCAGTGAGTGGTGCAAGT	TGGCCGCGTCATTGTTCTC	NM_198769 XM_227315
TLR4	CTACCTCGAGTGGGAGGACA	ATGGGTTTTAGGCGCAGAGTT	NM_019178
TLR9	ATGGCCTGGTAGACTGCAACT	TTGGCGATCAAGGAAAGGCT	NM_198131
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675
ZO-1	CCATCTTTGGACCGATTGCTG	TAATGCCCGAGCTCCGATG	NM_001106266.1

primary antibody. All the staining were performed in duplicate in non-serial distant sections, and analyzed in a double-blind manner by two different investigators.

2.10. Semi-quantitative and qualitative assessment of Enterobacteriales order and Escherichia coli species by sequence analysis of the microbial 16S rRNA gene

Semi-quantitative PCR was performed to investigate modifications in Gram-negative bacteria relative amount in animals receiving HFD alone or in combination with the synbiotic. For microbial content, DNA was extracted from colon tissue by the NucleoSpin Tissue (Macherey-Nagel, Düren, Germany).

Group-specific primers based on 16S rRNA gene sequences PCR assay were forward Enterobacteriales order, CCTTGGTGATTGACGTTACTCGCA; reverse Enterobacteriales order, CCACGCTTTCGCACCTGAGC; forward Escherichia coli CATGCAGTCGAACGGTAA-CAGGA; reverse Escherichia coli, CTGGCACGGAGTTAGCCGGTG (Eurofins MWG Operon; Huntsville, AL, USA).

The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 50 ng DNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer) in a final volume of 25 μl. PCR was performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories) The relative amount of 16S rRNA was normalized to RPL19 rRNA levels as housekeeping gene, and the data were analyzed according to the 2-ΔΔCT method.

2.11. Statistical analysis

Data are presented as mean±S.E.M. Statistical analysis was performed by analysis of variance test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software, San Diego, CA, USA). Statistical significance was set at $P<0.05$.

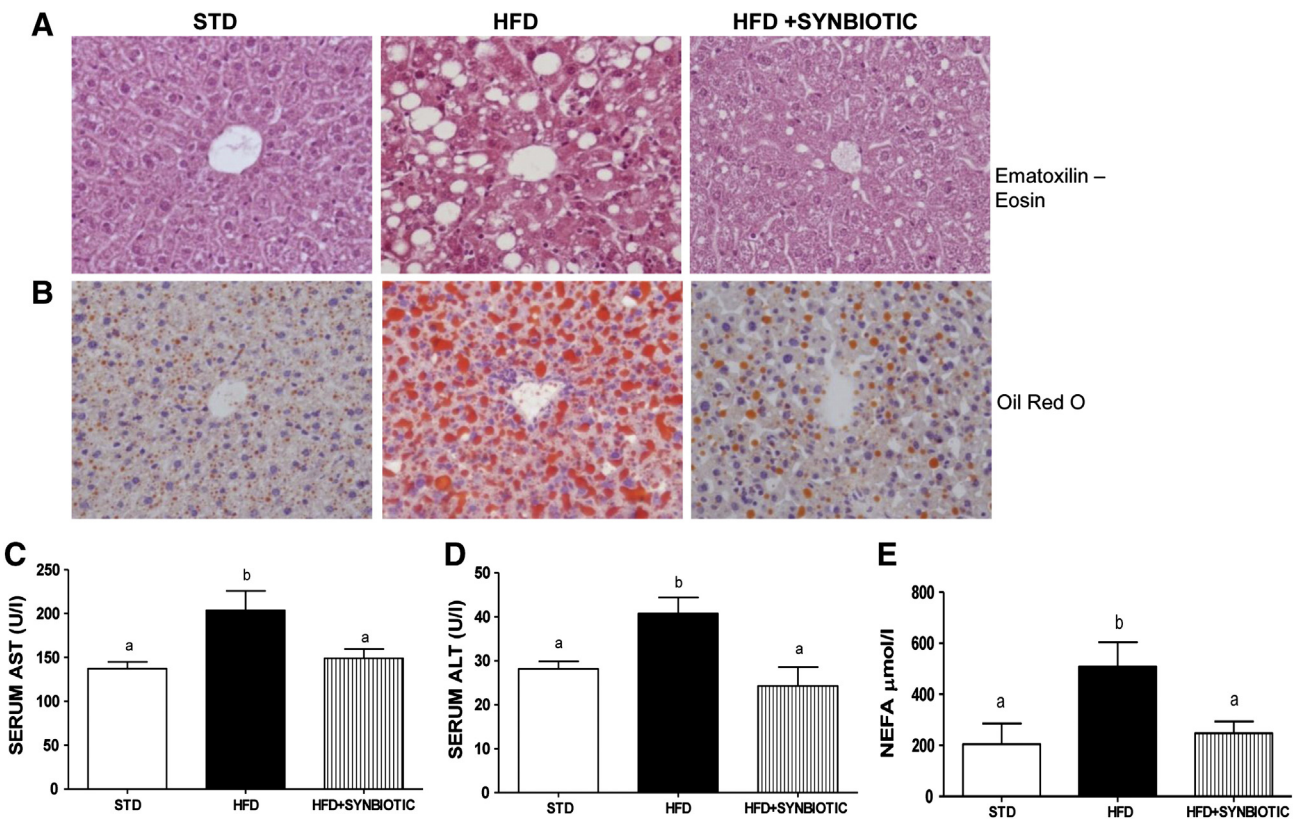

Fig. 1. The synbiotic effects on liver damage in HFD-fed rats. Paraffin-embedded sections of the liver ($n=4$ each group) were stained with hematoxylin-eosin (A) or oil red O (B). Micrographs in both panels are representative pictures with magnification 400×. Circulating AST (C), ALT (D), and NEFA (E) were measured (n=8, each group). Means without a common superscript letter are significantly different ($P<0.05$).

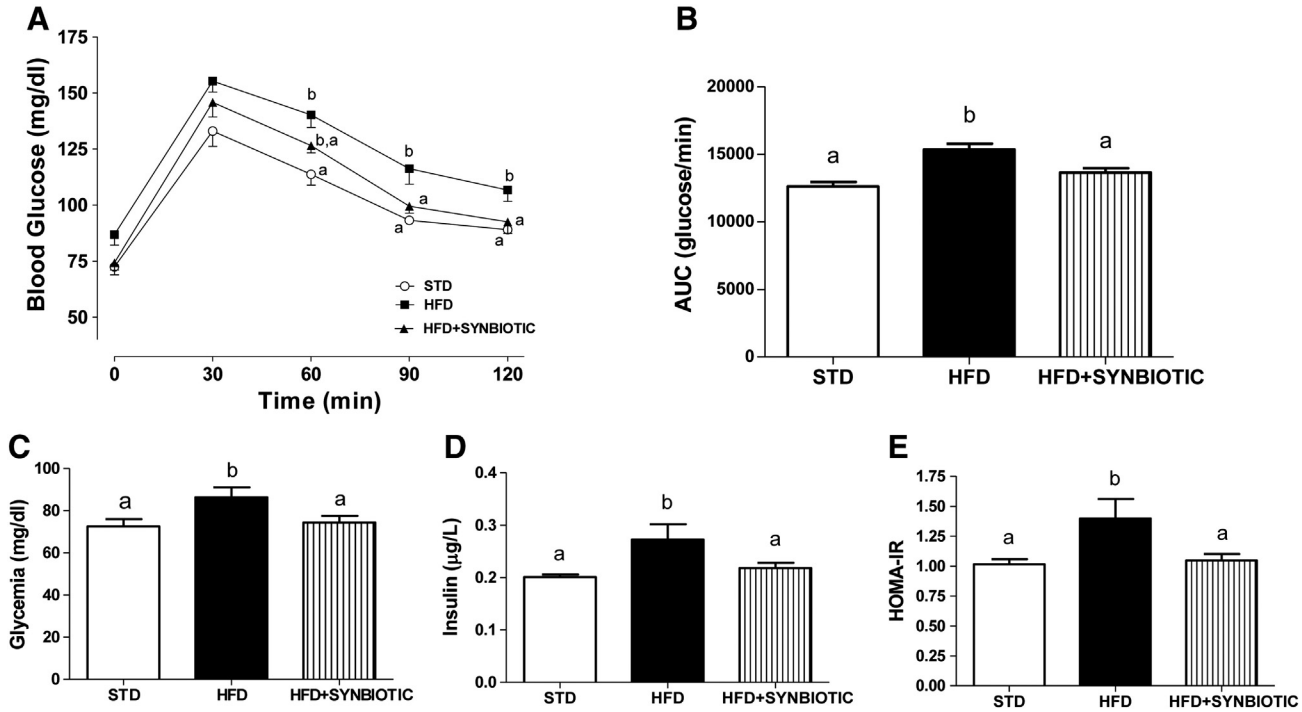

Fig. 2. The synbiotic effects on glucose homeostasis. Glucose tolerance test (A) in STD and HFD-fed rats (n=6, each group) was performed and AUC evaluated (B). Fasting glucose (C), insulin levels (D), and HOMA-IR (E) were also reported (n=8, each group). Means without a common superscript letter are significantly different (P<0.05).

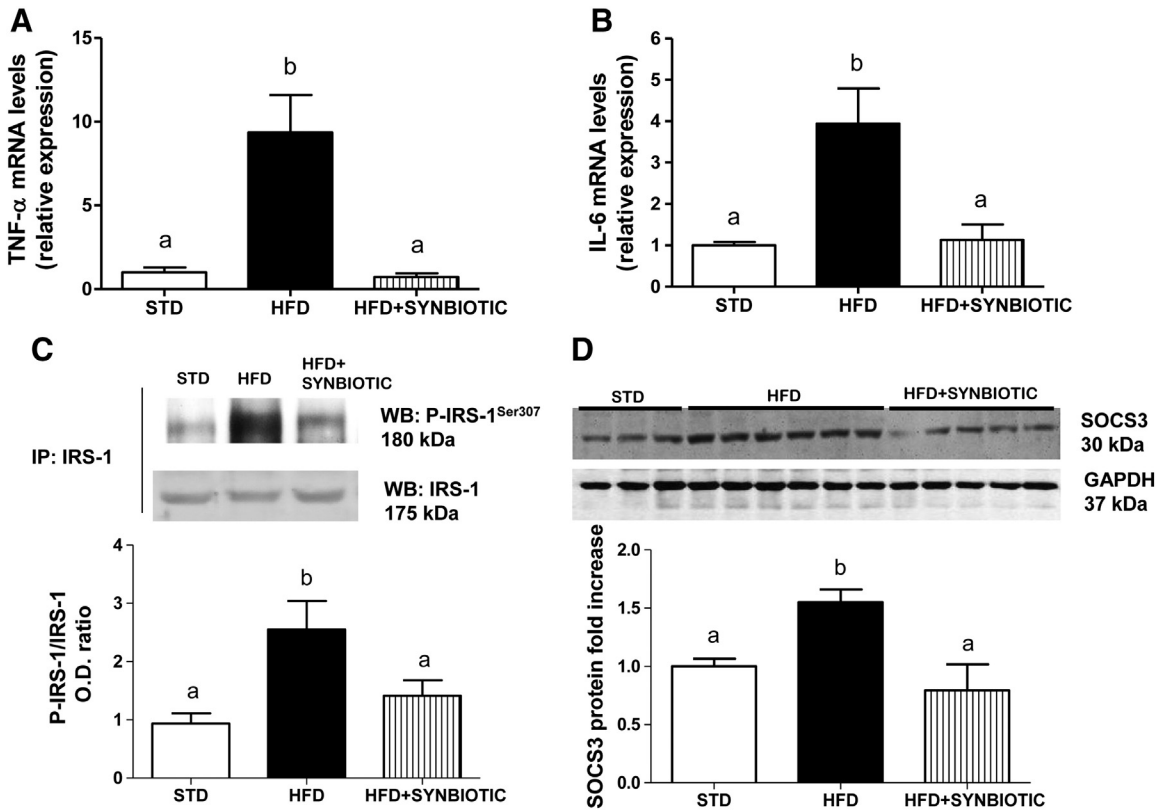

Fig. 3. The synbiotic effects on TNF-α and IL-6 gene expression in liver and insulin signaling. TNF-α (A) and IL-6 (B) mRNAs expression (relative expression to STD) are reported (n= 8 each group). Panels C and D show representative western blot analysis of P-IRS-1$^{Ser\ 307}$ of IRS-1 immunoprecipitate from liver tissues and SOCS3 expression, respectively. Means without a common superscript letter are significantly different (P<0.05).

3. Results

3.1. Effects of the synbiotic on liver steatosis and damage

Liver sections from HFD-fed rats demonstrated hepatic damage compared to control animals. As shown in Fig. 1A, foci of inflammatory cell infiltration and hepatocyte necrosis or apoptosis appeared throughout the lobule. HFD-fed rats showed microvesicular steatosis of grade 2 (Fig 1B). In the animals treated with synbiotic the severity of steatosis was reduced at grade 1, with a microvescicular pattern of lipids accumulation mainly in perivenular and periportal region. Scattered inflammation and occasionally apoptotic nuclei were observed, showing that treatment prevents the inflammation induced by HFD. Accordingly, the increase in AST, ALT (Fig. 1C and D) and NEFA (Fig. 1E) were reduced by the synbiotic.

Weight gain of HFD fed animals did not significantly change among groups after 5weeks (STD 209.2±7.2, HFD 225.0±6.6, and HFD+synbiotic 213.3±9.3 g), in accordance, at this experimental time, also fat mass did not vary among groups (STD 29.05±2.50, HFD 31.78±1.26, and HFD+synbiotic 30.30±2.88 g). Moreover, food intake, expressed as grams of food taken daily, did not differ between rats fed the HFD treated with vehicle (15.45±0.58 g/day/rat) or treated with synbiotic (15.45±0.82 g/day/rat).

3.2. Effect of the synbiotic on glucose homeostatasis

The synbiotic administration caused a significant reduction of glycemia 90 min after glucose load (Fig. 2A). A marked and significant increase of AUC values was shown in HFD group (Fig. 2B), and this effect resulted significantly inhibited by synbiotic.

As shown in Fig 2C and D, the increase in serum glucose and insulin levels induced by HFD was prevented in rats receiving the synbiotic. Accordingly, IR assessed by the HOMA index was reduced (Fig. 2E). No significant difference in body weight was observed among all groups.

3.3. Effects of the synbiotic on TNF-α and IL-6 gene liver expression

The raise of pro-inflammatory cytokines is one of the early events in many types of liver injury. In particular, TNF-α and IL-6 are two prototypic inflammatory cytokines involved in metabolic impairment, initiating the pathogenesis of hepatic IR. As shown in Fig. 3A and B, HFD induced a significant increase in hepatic TNF-α and IL-6 mRNAs, and the synbiotic significantly prevented the transcription of both genes. As known, TNF-α and IL-6 are involved in IR due to their ability to impair insulin signaling through the phosphorylation of IRS-1 in Ser 307 and up-regulation of SOCS3, respectively. As depicted in Fig. 3C, Western blot analysis of P-IRS-1$^{Ser\ 307}$ of immune-precipitated

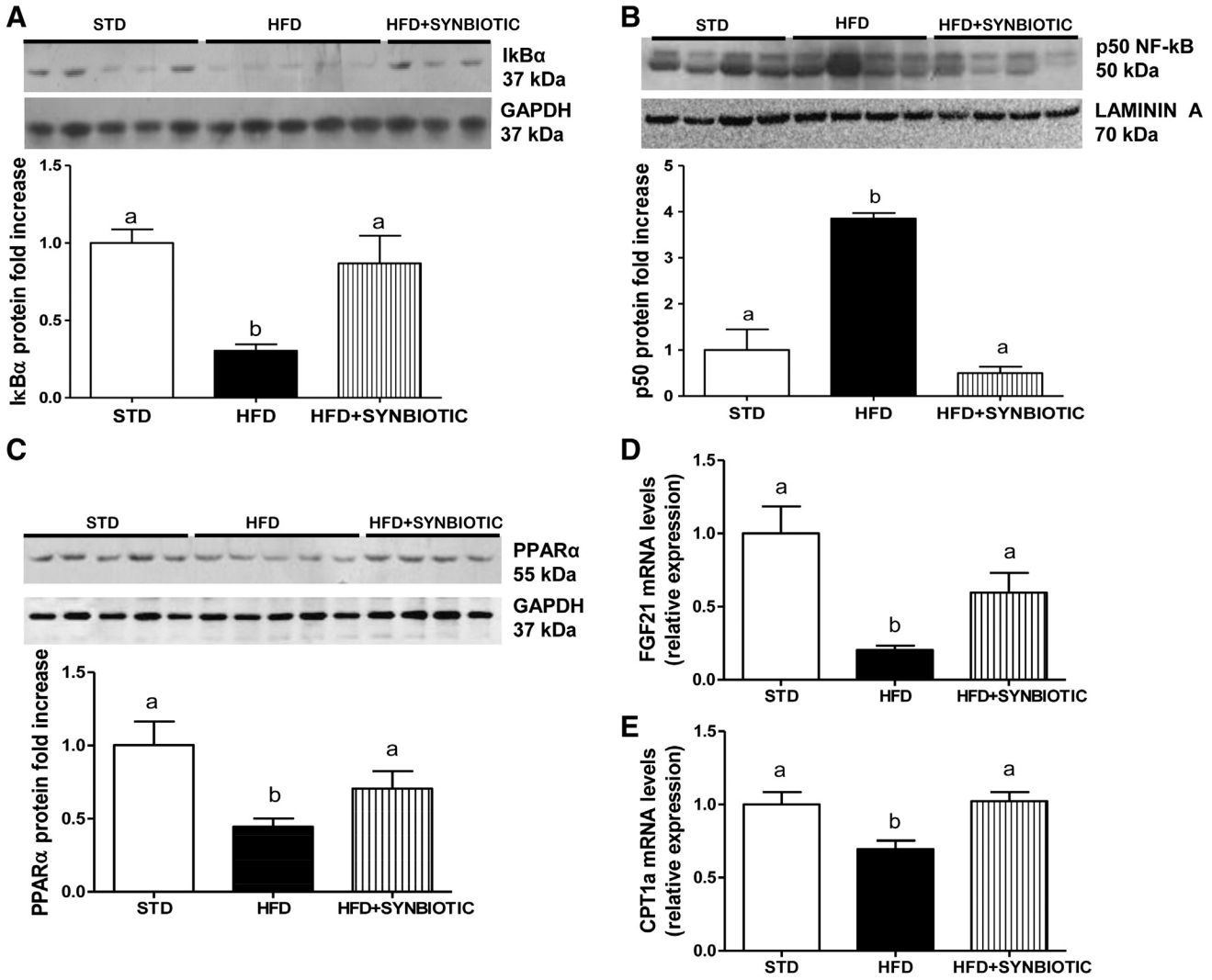

Fig. 4. The synbiotic effect on hepatic activation of NF-κB, PPARα and FGF21 expression. Immunoblot of cytosolic inhibitory protein IκB-α (A), nuclear p50 NF-κB (B) and PPARα (C) protein expression are shown (n=8 each group). FGF21 (D) and CPT1 (E) mRNAs expression (relative expression to STD) are also reported. Means without a common superscript letter are significantly different (P<0.05).

IRS-1 from hepatic tissues showed an increase in serine phosphorylation in HFD group, partially reverted by the synbiotic. Moreover, the increase in SOCS3 in hepatic tissues from HFD rats was significantly inhibited by the synbiotic (Fig. 3D).

3.4. Modulation of hepatic inflammatory transcription factors by the synbiotic

The activation of nuclear factor-κB (NF-κB) was evaluated through the measurement of cytosolic amount of IκBα and nuclear content of p50 NF-κB. In our model, nuclear p50 NF-κB resulted increased in HFD group, related to a decrease of the inhibitory protein IκBα, the synbiotic significantly prevented both effects (Fig. 4A and B). Accordingly, with the metabolic and inflammatory alterations, PPARα expression resulted significantly reduced by HFD and partially restored by the synbiotic (Fig. 4C). The evaluation of fibroblast growth factor (FGF)21 transcription, as a downstream target gene of PPARα, revealed a similar profile of expression of its transcription factor (Fig. 4D). These findings were consistent with carnitine palmitoyltransferase (CPT) 1 expression level, whose transcription was up-regulated by synbiotic, suggesting an increase in the oxidation of fatty acids (Fig. 4E).

3.5. Effect of the synbiotic on hepatic Toll-like receptors pattern

The activation of TLRs family, especially TLR4, by inflammatory cytokines or increased NEFA could modulate insulin sensitivity [29]. As shown in Fig. 5A and B, HFD induced an increase in liver TLR4 mRNA and protein expression. Interestingly, a similar expression profile was also observed for TLR4 co-receptor CD14 (Fig. 5C). The synbiotic significantly inhibited these effects. Notably, TLR2 and TLR9, which are able to detect lipoproteins and unmetilated CpG-containing DNA, respectively, were also up-regulated by HFD and both TLRs were reduced by synbiotic (Fig. 5D and E).

3.6. Modulation of PPARγ, GLUT4 and adiponectin expression in adipose tissue elicited by the synbiotic

To address whether the change in glucose metabolism was related to a modulation of genes expression involved in glucose and fat metabolism in metabolically active tissues, we evaluated the expression of PPARγ and GLUT4 in visceral white adipose tissue. In animals receiving HFD, a significant reduction of PPARγ and GLUT4 was observed after 6 weeks. The synbiotic limited these effects preventing PPARγ decrease (Fig. 6A) and partially limiting the effect

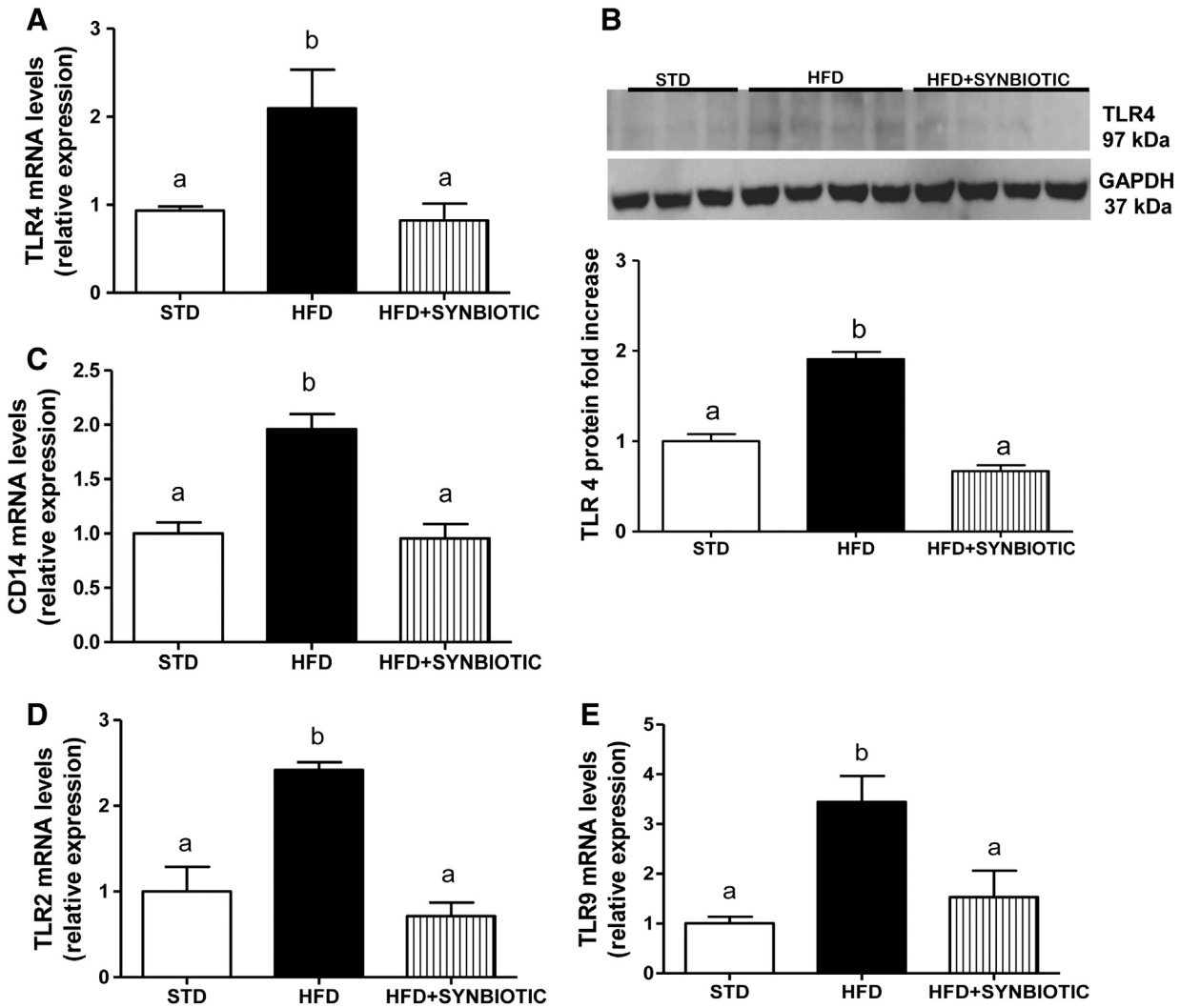

Fig. 5. Effect of synbiotic on hepatic Toll-like receptor pattern. Panels A and B are the results from the PCR and Western blot for TLR4 in livers from 8 rats on STD or HFD or HFD +SYNBIOTIC. Panel C shows mRNA expression of coreceptor CD14. mRNA expression of TLR2 (D) and TLR9 (E) are also shown. All mRNA levels are expressed as relative expression to STD. Means without a common superscript letter are significantly different ($P<0.05$).

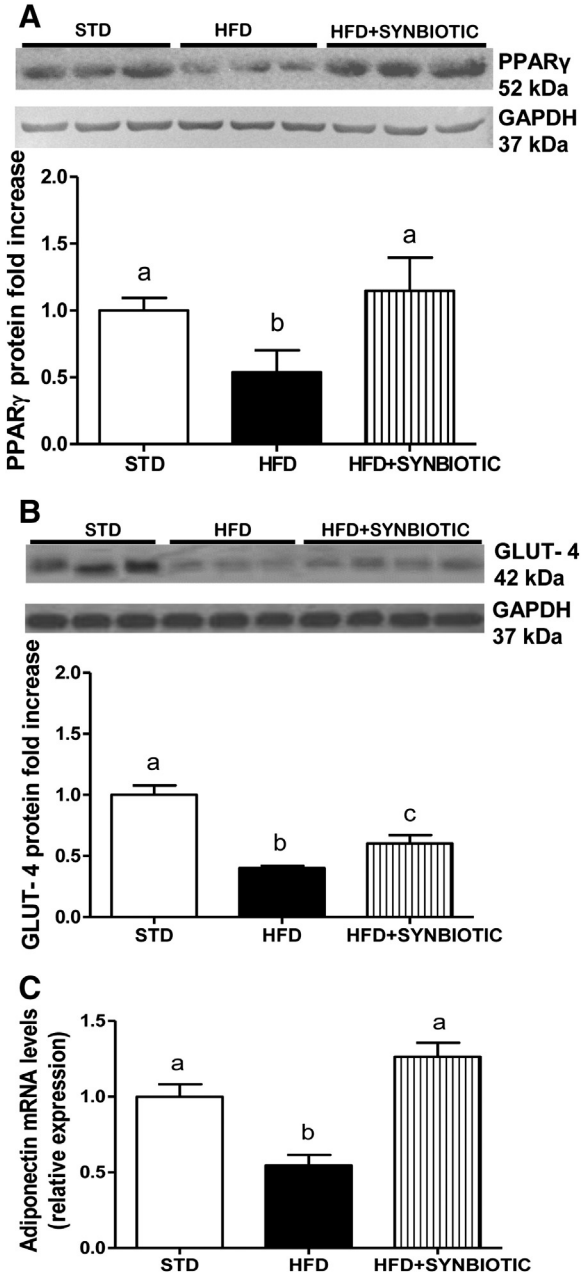

Fig. 6. The synbiotic modulation of metabolic and inflammatory proteins in adipose tissue. Representative Western blot of PPARγ (A) and GLUT4 (B) are shown (n=8 each group). Panel C shows PCR results from adiponectin mRNA expression (relative expression to STD) in adipose tissue (n=8 each group). Means without a common superscript letter are significantly different (P<0.05).

of HFD on GLUT4 expression (Fig. 6B). Moreover, the reduction of adiponectin mRNA in mesenteric adipose tissue from HFD group was abolished by synbiotic (Fig. 6C).

3.7. Effect of the synbiotic on intestinal permeability and tight junction-associated proteins in gut mucosa

As a consequence of HFD feeding, epithelial barrier integrity was altered. There was a significant increase in gut permeability measured in vivo by appearance in plasma of FITC-labeled dextran (Fig. 7A), by a mechanism associated with a reduced expression of the epithelial tight junction proteins ZO-1 and occludin (Fig.7 B-E). These effects were prevented by the synbiotic (Fig. 7 A-E).

3.8. Modulation of Gram-negative bacteria and TLR4 in colonic mucosa

HFD strongly increased 16S rRNA levels of Enterobacteriales order and related specie (*Escherichia coli spp*) at colonic level, while the synbiotic significantly reduced Gram-negative bacteria (Fig. 8A-B). The modulation of Gram-negative bacteria was associated with a significant increase in TLR4 and myeloid differentiation primary response gene 88 (MyD88) in HFD rat intestinal mucosa. Also this effect was significantly blunted by the synbiotic (Fig. 8C and D).

4. Discussion

We show that the synbiotic containing *L. paracasei* B21060 plus arabinogalactan and fructo-oligosaccharide, is able to prevent liver damage and inflammation, steatosis, IR, and imbalance of TLRs pattern in the early stage of NAFLD. The synbiotic not only prevents the increase of hepatic markers of steatosis and NEFA, but also preserves glucose tolerance, reduces fasting glucose and insulinemia modulating HOMA-IR and adiponectin levels. It is now clear that TNF-α and IL-6 represent crucial effectors of IR, that link liver inflammatory process to hormonal and metabolic alterations [4,30]. In our experimental model, the synbiotic reduces TNF-α levels in parallel with a lower Ser307-phosphorylation of IRS-1, demonstrating the recovery of insulin signaling transduction. IL-6, activating the JAK-STAT pathway, stimulates SOCS1 and SOCS3 transcripts that in turn led to ubiquitin-induced degradation of IRS-1 [4]. Here, we show that the synbiotic reduces the transcription of both cytokines, TNF-α and IL-6, and inhibits markedly their above reported pathways, limiting inflammation and IR.

In our experimental conditions, it is plausible to argue that the synbiotic, reducing NEFA and cytokines (i.e., TNFα, IL-6 and IL-1β), modulates the activation of NF-κB pathway induced by HFD [31], reducing IκBα degradation and inhibiting p50. Moreover, the increased expression of TNF-α by HFD is associated with the reduction of PPARα expression in liver and adiponectin synthesis in mesenteric adipose tissue. Adiponectin, an insulin-sensitizing anti-inflammatory adipokine, limits fat accumulation in the liver by a number of mechanisms including induction of PPARα expression [32], reduces liver TNFα expression [33], and inhibits expression of several cytokines in hepatic stellate cells, with a concomitant increase in the release of the regulatory cytokines IL-10 and IL-1RA [34].

The molecular mechanisms by which the synbiotic exerts its beneficial effects on NAFLD are linked to the marked increase of adiponectin and to the partial recovery of PPARα. The level of PPARα which regulates fatty acid β-oxidation and catabolism, was restored by the synbiotic. We previously demonstrated that HFD feeding is associated with the reduction of PPARα expression in liver [12] and according to our findings, the administration of a PPAR agonist or probiotics restores PPARα and improves hepatic steatosis [35,12].

Recently, it has been demonstrated that FGF21, a cytokine/hormone predominantly produced by the liver, was regulated by PPARα [36]. FGF21 regulates glucose and lipid metabolism through pleiotropic actions in pancreas and adipose tissue [37]. In particular, FGF21 is required for the normal activation of hepatic lipid oxidation and triglyceride clearance [38]. In our model FGF21 was significantly reduced by HFD and its decreased expression was partially prevented by synbiotic. Accordingly, the synbiotic not only increased PPARα and FGF21, but also normalized CPT1 transcription, suggesting a role for this pathway in synbiotic-induced decrease in fatty acid accumulation in the liver.

As known, adiponectin up-regulation by PPARγ, provides a connection between the two PPAR isotypes [39]. PPARγ promotes fatty acid uptake and increases insulin sensitivity by up-regulating GLUT4, an insulin dependent glucose transporter in adipose tissue and striated muscle [40] and attenuating the induction of SOCS3 [41].

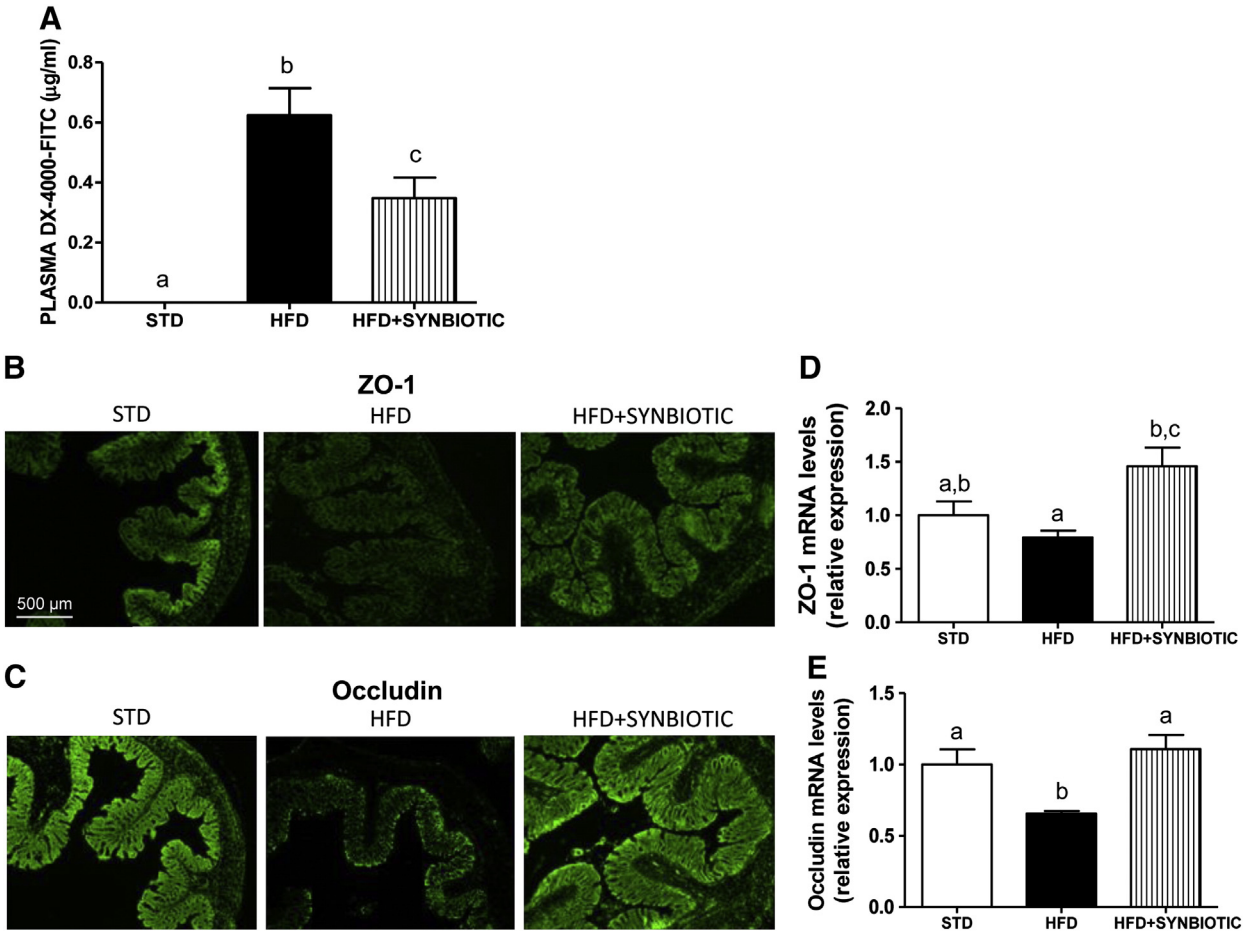

Fig. 7. Effect of the synbiotic on intestinal permeability and tight junction-associated proteins. Panel A shows the measurement of gut permeability by appearance of FITC-labeled dextran in plasma of STD, HFD and HFD+SYNBIOTIC rats (n=8 each group). Immunofluorescent images (5× magnification) and mRNA expression for ZO-1 (B, D) and occludin (C, E) in the colon tissue are shown (n=4 each group). Means without a common superscript letter are significantly different (P<0.05).

Consistently with the modulation of adiponectin synthesis, the synbiotic also modulates PPARγ and GLUT4 expression in visceral adipose tissue.

To address the synbiotic mechanisms on HFD-induced hepatic alterations, we evaluated gut permeability. The synbiotic is able to significantly inhibit the modification of gut permeability induced by HFD. In fact it significantly reduces the amount of FITC-dextran at plasmatic level and restores the HFD-induced alteration in tight junction proteins expression and distribution. The synbiotic also prevents the increased transcription of TLR4 in the colonic mucosa of HFD animals, suggesting a reduction of TLR4 inflammatory pathways.

TLRs are involved in bacterial sensing and are crucial for "liver tolerance" in the healthy liver [42]. Here, we demonstrate that the synbiotic is able to limit the increased transcription and expression of TLRs and co-receptor CD14 or MyD88 at intestinal and liver level and restores the imbalance of Gram negative bacteria (Enterobcteriales and in particular *E. coli*) induced by HFD. Recently, it has been demonstrated that during HFD-induced diabetes, commensal intestinal bacteria translocate in pathological manner from intestine towards the tissues where they trigger a local inflammation. This metabolic bacteremia was reversed by a *Bifidobacterium animalis* strain, which reduced the mucosal adherence and bacterial translocation of gram-negative bacteria from the *Enterobacteriaceae* group [43]. Moreover, an increase in Enterobacteriaceae family within Enterobacteriales order has been associated with gut inflammation; induction of experimental colitis in rodents was followed by an increase in this family, suggesting that it may be a consequence of gut inflammation rather than a cause [44]. In our model the increased amount of Enterobacteriales induced by HFD, and probably associated to gut inflammation, was restored by this *L. paracasei* strain, which is well known modulator of the inflammatory process [23].

In this study, the increased expression of hepatic TLRs due to HFD confirms a greater exposure of the liver to ligands for these receptors (i.e. PAMPs and DAMPs) deriving from the intestine. Our data are in agreement with previous studies showing that the administration of probiotics (i.e. *Lactobacillus* and *Bifidobacterium*) or prebiotics (i.e., inulin and oligofructose) can modulate the microbiota and improve gut permeability, thus controlling the occurrence of endotoxemia [17,45–47].

Also NEFA and other non-bacterial substances, may act as ligands for TLR2, TLR4, and TLR9 [29,48,42]. In NAFLD patients, elevated NEFA levels are commonly observed [49]. Very recently, it was demonstrated that free fatty acids could stimulate NF-κB activation in hepatocytes in the early stage of HFD-induced NAFLD through the TLR4 [50].

Here, we demonstrate that the synbiotic reduces inflammation and its mediators, not only through an effect on NEFA and intestinal permeability, but also inhibiting NF-κB activation through the down-regulation of TLR pattern. Accordingly, we evidenced the same profile of activity of the synbiotic on TLR2 expression. Both TLR2 and TLR4 recognizes NEFAs [51], and share the same signaling cascade leading to NF-κB activation. The reduction of TLR2 by the synbiotic may contribute to the inhibition of the effects of HFD, impacting on IR and tissue damage. Consistently with our data, Ehses et al. [52] have

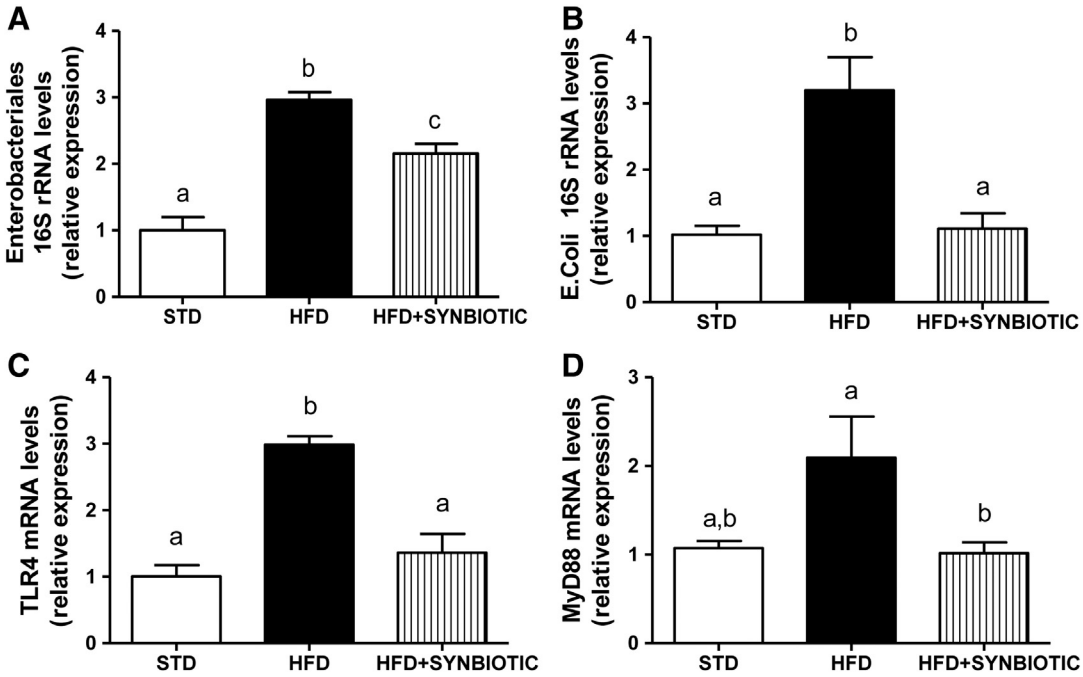

Fig. 8. Enterobacteriales and *Escherichia coli* modification and TLR4 and MyD88 transcription in colonic mucosa. Relative amount of Enterobacteriales order (A) and semi-quantitative analysis of *Escherichia coli* species (B) are shown. mRNA abundance of TLR4 (C) and MyD88 (D) are also shown. Means without a common superscript letter are significantly different ($P<0.05$).

reported that TLR2 deficient mice are protected from IR and β cell dysfunction induced by HFD, linking TLR2 to the increased dietary lipid and the alteration of glucose homeostasis. Finally, the synbiotic significantly inhibits the HFD-related increase in TLR9 synthesis. Intracellular TLR9 activates innate immune defenses against viral and bacterial infection and plays a role in the pathogenesis of NASH [53].

In conclusion, our results support probiotics as innovative, preventive and therapeutic strategy for NAFLD, using synbiotic preparations containing selected strain with clear and demonstrated beneficial immunomodulatory effects. Among probiotics, *L. paracasei* B21060, can be considered a potential approach, limiting the main pathogenetic events involved in the onset of IR and steatosis induced by HFD. This synbiotic, alone or in combination with other therapies, could be useful in the treatment of fatty liver in children who are hardly able to follow a program of hypocaloric diet and regular physical activity.

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