Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 25 (2014) 81–90

Journal of Nutritional Biochemistry

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

Giuseppina Mattace Raso[a], Raffaele Simeoli[a], Anna Iacono[a,d], Anna Santoro[a], Paola Amero[a], Orlando Paciello[b], Roberto Russo[a], Giuseppe D'Agostino[a], Margherita Di Costanzo[c], Roberto Berni Canani[c], Antonio Calignano[a], Rosaria Meli[a,*]

[a]*Department of Pharmacy, University of Naples "Federico II", 80131 Naples, Italy*
[b]*Department of Pathology and Animal Health, University of Naples "Federico II", 80131 Naples, Italy*
[c]*Department of Pediatrics and European Laboratory for the Investigation of Food Induced Diseases, University of Naples "Federico II", 80131 Naples, Italy*
[d]*Hospital Pharmacy Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy*

Received 18 April 2013; received in revised form 24 July 2013; accepted 3 September 2013

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].

☆ Specific author contributions: R.S., A.I., O.P., A.S., P.A. and M.D.C. performed research. G.M.R. analyzed data and wrote the manuscript. G.D. and R.R. reviewed the manuscript. A.C. and R.B.C. contributed to the study design, data analysis and reviewed manuscript. R.M. designed research, analyzed data and wrote the paper.

☆☆ Fundings: Preliminary data of this study were supported by a grant from Italian Ministry of University and Research (MIUR) PRIN 2005.

★ Potential competing interests: The authors have no financial and commercial conflicts of interest.

* Corresponding author. Tel.: +39 081 678413; fax: +39 081 678403.
E-mail address: meli@unina.it (R. Meli).

0955-2863/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2013.09.006

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