Probiotics modulate gut microbiota and improve insulin sensitivity in DIO mice

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Abstract

Obesity and type 2 diabetes are characterized by subclinical inflammatory process. Changes in composition or modulation of the gut microbiota may play an important role in the obesity-associated inflammatory process. In the current study, we evaluated the effects of probiotics (*Lactobacillus rhamnosus*, *L. acidophilus* and *Bifidobacterium bifidumi*) on gut microbiota, changes in permeability, and insulin sensitivity and signaling in high-fat diet and control animals. More importantly, we investigated the effects of these gut modulations on hypothalamic control of food intake, and insulin and leptin signaling. *Swiss* mice were submitted to a high-fat diet (HFD) with probiotics or pair-feeding for 5 weeks. Metagenome analyses were performed on DNA samples from mouse feces. Blood was drawn to determine levels of glucose, insulin, LPS, cytokines and GLP-1. Liver, muscle, ileum and hypothalamus tissue proteins were analyzed by Western blotting and real-time polymerase chain reaction. In addition, liver and adipose tissues were analyzed using histology and immunohistochemistry. The HFD induced huge alterations in gut microbiota accompanied by increased intestinal permeability, LPS translocation and systemic low-grade inflammation, resulting in decreased glucose tolerance and hyperphagic behavior. All these obesity-related features were reversed by changes in the gut microbiota profile induced by probiotics. Probiotics also induced an improvement in hypothalamic insulin and leptin resistance. Our data demonstrate that the intestinal microbiome is a key modulator of inflammatory and metabolic pathways in both peripheral and central tissues. These findings shed light on probiotics as an important tool to prevent and treat patients with obesity and insulin resistance.

1. Introduction

Obesity, insulin resistance and type 2 diabetes (T2D) are metabolic disorders resulting from a combination of genetic and environmental factors including caloric intake and sedentary lifestyle [1,2]. In the past 10 years, data coming from different sources showed an important role of gut microbiota in the pathogenesis of obesity and type 2 diabetes [3–10].

Approximately 10^{13} and 10^{14} microorganisms live in a symbiotic manner in the human gastrointestinal tract. This relationship is essential for host physiology and metabolic health [11–13]. In this regard, the intestinal bacteria help in the host nutrients absorption and in the integrity of intestinal immune barrier [13,14] and regulate host fat storage genes, thus regulating host energy homeostasis [15]. It is known that high-fat diet (HFD) can cause imbalances in the gut microbiota composition and in the intestinal epithelial layer integrity and that germ-free animals are protected from this deleterious effect of HFD [16].

Numerous strategies have been proposed to regulate these imbalances in gut microbiota composition (also known as intestinal dysbiosis) in obesity, such as the use of antibiotics, prebiotics and probiotics [7,17–19]. The latter, whose definition is live microbial food supplements which help maintain the balance of intestinal microflora [18,19], have shown in some studies to be effective in the treatment of insulin resistance in both rodents and humans; however, the deep mechanisms that lead to such benefits are poorly understood [4,18–20]. Indeed, multistrain probiotics appear to be efficient against a wide range of intestinal issues [21,22]. Despite the absence of strong evidence for metabolic disease, we hypothesized that probiotic mixture would be more suitable to test in an experimental model of obesity. Furthermore, it is known that some strains have important immune modulatory actions besides their effects on gut epithelial barrier function [23–25]. Actually, *Lactobacillus rhamnosus* is a special strain to treat a wide range of experimental liver diseases, with interesting metabolic modulations in this organ [26–28]. Thus, in the current study, we have used a mixture of probiotic content *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium bifidum* to treat obese mice.

Emerging evidence has shown not only that gut microbiota is important for intestinal physiology but also that microbiota–gut–brain axis strongly influences the central nervous function and

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behavior [29–35]. Accordingly, it was previously hypothesized that energy balance regulation by central nervous system signaling is probably subject to an influence of gut microbiota [13]. Based on this hypothesis, we can also speculate that gut microbiota may also exert an influence in the control of food intake by hypothalamus, thus contributing to evolution of obesity. In the current study, we evaluated the effects of probiotics on gut microbiota, changes in permeability, and insulin sensitivity and signaling in HFD and control animals. More importantly, we investigated the effects of these gut modulations on hypothalamic control of food intake, and insulin and leptin signaling.

2. Research design and methods

2.1. Materials

Antibodies anti-JNK (SC1648), anti-phosphorylated JNK (SC6254), anti-IRβ (SC711), anti-phosphorylated IRβ (SC25103), anti-IRS1 (SC559), anti-phosphorylated IRS-1 (SC33956), anti-JAK2 (SC278), anti-MyD88 (SC11356), anti-NOD1 (SC 398696), anti- STAT3 (SC483), anti-phosphorylated STAT3 (SC8001), anti-occludin (SC5562) and anti-ZO-1 (SC10804) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except as follows: anti-Akt (#9272), anti-phosphorylated Akt (#9271), anti-α-tubulin (#2144), anti-phophorylated JAK2 (#3776) and anti-TLR4 (#2219), obtained from Cell Signaling Technology (Beverly, MA, USA). Routine reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless otherwise specified.

2.2. Animals

UNICAMP Central Animal Breeding Center (Campinas, São Paulo, Brazil) provided 8-week-old male Swiss mice. Animals ($n=6$ per group) were housed in individual cages with free access to water and rodent chow under a 12-h light/dark cycle. The mice composed randomly two groups: one group on chow diet (8% fat, 26% protein, 54% carbohydrate, as a percentage of total kcal) (C group) and the second group on an HFD (55% of energy derived from fat, 29% from carbohydrates and 16% from protein) (DIO group) for 12 consecutive weeks. After that, some mice in the DIO and C groups received daily a pool of probiotics for 5 weeks, and they composed the DIOPB and CPB groups, respectively. The Ethics Committee of the State University of Campinas approved all procedures.

2.3. Probiotic administration protocol

A pool of probiotics that included L. rhamnosus, L. acidophilus and B. bifidum (Futureceuticals, Momence, IL, USA) was given for 5 weeks daily (6×10^8 CFU of each strain; final concentration of 1.8×10^9 CFU of bacteria). Prior of gavage, the probiotics were diluted in 300 µl of sterile water. DIO and C groups received only the vehicle (sterile water).

2.4. Hormones and LPS determination

Enzyme-linked immunosorbent assay kits were used to measure insulin (Millipore, St. Charles, MO, USA), TNF-α, IL-6 and glucagon like peptide 1 (GLP-1) (Thermo Fisher Scientific, Rockford, IL, USA). LPS was quantified using a commercially available Limulus Amebocyte Assay (Cambrex, Walkersville, MD, USA), according to the manufacturer's protocol.

2.5. Tissue extraction, immunoprecipitation and protein analysis by immunoblotting

Overnight-fasted mice were anesthetized; the abdominal cavity was opened, the vena cava was exposed, and NaCl (0.9% wt./vol.) solution or insulin (10^{-6} mol/l) was injected. Liver, ileum, muscle and hypothalamus were removed, minced coarsely and homogenized in lysis buffer. Lysates were process as previously described [36].

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was obtained from different tissues for all four groups of mice according to the methods published previously [37]. Quantitative PCR was performed in an ABI 5700 Sequence Detector System (Applied Biosystems) using SybrGreen PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) (primers described in Supplementary Table 1).

2.7. Metagenome profile

Fecal samples were frozen in liquid nitrogen and stored at −80°C until use. At this time, DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA from each sample was used to amplify the V2–V3 regions of 16S rRNA genes. PCR amplification, pyrosequencing of the PCR amplicons and quality control of raw data were performed as described previously [38]. Samples were then sequenced in GS FLX Titanium (Roche Applied Science, Mannheim, Germany). The readouts obtained from the sequencing were analyzed by bioinformatics using basic local alignment search tool X (BLASTX), observed with METAREP software and compared according to phylum prevalence among groups.

2.8. Liver and adipose tissue histology

Liver and epididymal white adipose tissues were excised and processed as described previously [7]. Sections (5 µm) were obtained and stained by hematoxylin and eosin to assess morphology. Tissue analyses were performed blinded by an expert pathologist (A.A.S.). Adipose tissue was evaluated by crown-like structure (CLS) density (average CLS number within 10 high-power fields per animal) and mean adipocyte surface area, using Imagelab Analysis software, as previously described [7].

2.9. Adipose tissue immunohistochemistry

Tissue sections, 5 µm, were mounted on silanized glass slides, processed and incubated with an epidermal-growth-factor-like compound containing mucin-like hormone receptor-like 1 (F4/80) primary antibody, as previously described [39]. Antibody staining was performed using an IHC-peroxidase kit (Advance HRP, Dako CytoMation, Carpinteria, CA, USA) according to the manufacturer's instructions. Three different high-power fields from three different sections were evaluated blinded by an expert pathologist (A.A.S.). The total number of nuclei of F4/80-expressing cells was counted for each field, and the area occupied by these cells was measured.

2.10. In vivo experiments

Hyperinsulinemic–euglycemic clamp was performed in anesthetized overnight-fasted mice immediately after catheterization. Prime insulin (Human recombinant insulin, Eli Lilly, Indianapolis, IN, USA) was continuously infused (30 mU kg^{-1} min^{-1}) up to 120 min. Blood glucose was measured at 5-min intervals, and glucose (5% wt./vol.) was infused at a variable rate to maintain blood glucose at fasting levels. Glucose tolerance test was performed in 8-h fasted mice as described before [36]. To determine insulin sensitivity in the hypothalamus, stereotaxic surgery was employed with 26-gauge stainless steel indwelling guide cannula aseptically placed into the third ventricle (coordinates of −1.8 mm posterior to the bregma and 5.0 mm below the surface of the skull) in Swiss mice under anesthesia as previously described [8,40]. Fasted mice for 12 h received intracerebroventricular (ICV) infusion of vehicle or insulin (200 mU in 2 µl; Eli Lilly and Company, Indianapolis, IN, USA) at 8:00 a.m. Food

intake was recorded over 4 and 12 h. Next day, same mice were fasted for 12 h and received ICV infusion of vehicle or insulin 15 min before the hypothalamic tissue dissection. To determine leptin sensitivity in the hypothalamus, after 5 weeks of probiotics treatment, five mice from each group received an intraperitoneal (IP) injection of recombinant leptin (3 mg/kg body weight; Calbiochem, San Diego, CA, USA) or saline at 6:00 p.m. on 5 consecutive days, as previously described [41]. Food intake were measured daily after the leptin injection. For hypothalamic analysis of the leptin signaling, immuno-blotting was performed, and the leptin was IP injected 45 min before of the hypothalamic tissue dissection.

2.11. Statistical analysis

Data are expressed as means±S.E.M., and the number of independent experiments is indicated. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (Scion Image; Scion Corporation, Frederick, MD). For statistical analysis, the groups were compared using a two-way analysis of variance with the Bonferroni test for *post hoc* comparisons and *t* test for comparison of only two groups. The level of significance adopted was $P<.05$.

3. Results

3.1. Probiotic reduces weight gain and fat pad improves glucose tolerance and insulin resistance in DIO mice

Probiotic-treated mice receiving an HFD gained significantly less weight (DIOPB 11.4±1.2 g *vs.* DIO 16.9±1.4 g; $P<.05$) during the 5 weeks of the experiment and had reduced food intake compared with animals that did not receive probiotics (DIOPB 2.9±0.2 g/day *vs.* DIO 3.4±0.3 g/da; $P<.01$). In order to avoid this bias, we submitted the nontreated animals to pair feeding on an HFD, and this group was called diet-induced obesity mice per fed (DIOPF).

Fig. 1 shows comparative data regarding the controls (C), controls treated with probiotics for 5 weeks (CPB), DIOPF and DIO mice treated with probiotics for 5 weeks (DIOPB). As expected, the DIOPF and DIOPB mice showed a significant increase in both body and epididymal fat weight, along with higher levels of fasting glucose and insulin, when compared with animals fed on chow diet (Fig. 1A–D). However, the treatment with probiotics provided a marked decrease in weight gain (DIOPB 12.8±1.1 g *vs.* DIOPF 14.7±1.2 g; $P<.05$) and fat pad weight in 5 weeks and also in fasting blood glucose and serum insulin (Fig. 1A–D). Throughout the intraperitoneal glucose tolerance test (IPGTT), DIOPB mice showed improved glucose and insulin profiles compared to DIOPF animals (Fig. 1E and F). Likewise, the DIOPF group showed a striking reduction in the glucose infusion rate measured by the hyperinsulinemic–euglycemic clamp compared with control animals, and the probiotic treatment partially restored insulin sensitivity in DIOPF (Fig. 1G). It is important to mention that the probiotic treatment did not induce significant difference in any physiologic and metabolic parameters studied in animals fed on chow diet (Fig. 1A–G).

3.2. Effects of probiotic treatment on insulin signaling in liver and muscle of DIO mice

As expected, in liver and muscle of mice fed on HFD, insulin-stimulated Akt[Ser 473] phosphorylation levels were significantly reduced compared to the control animals (Fig. 2A–B). On the other hand, the probiotic treatment completely recovered the insulin-induced Akt[Ser 473] phosphorylation levels in both tissues when compared to the obese DIOPF (Fig. 2A–B). There was no difference in the Akt protein expression among C, CPB, DIOPF and DIOPB groups.

3.3. Effects of probiotic treatment on the TLR4 signaling pathway in DIO mice

The TLR4 protein levels in liver and muscle tissues were higher in the DIOPF animals compared to corresponding controls, though the
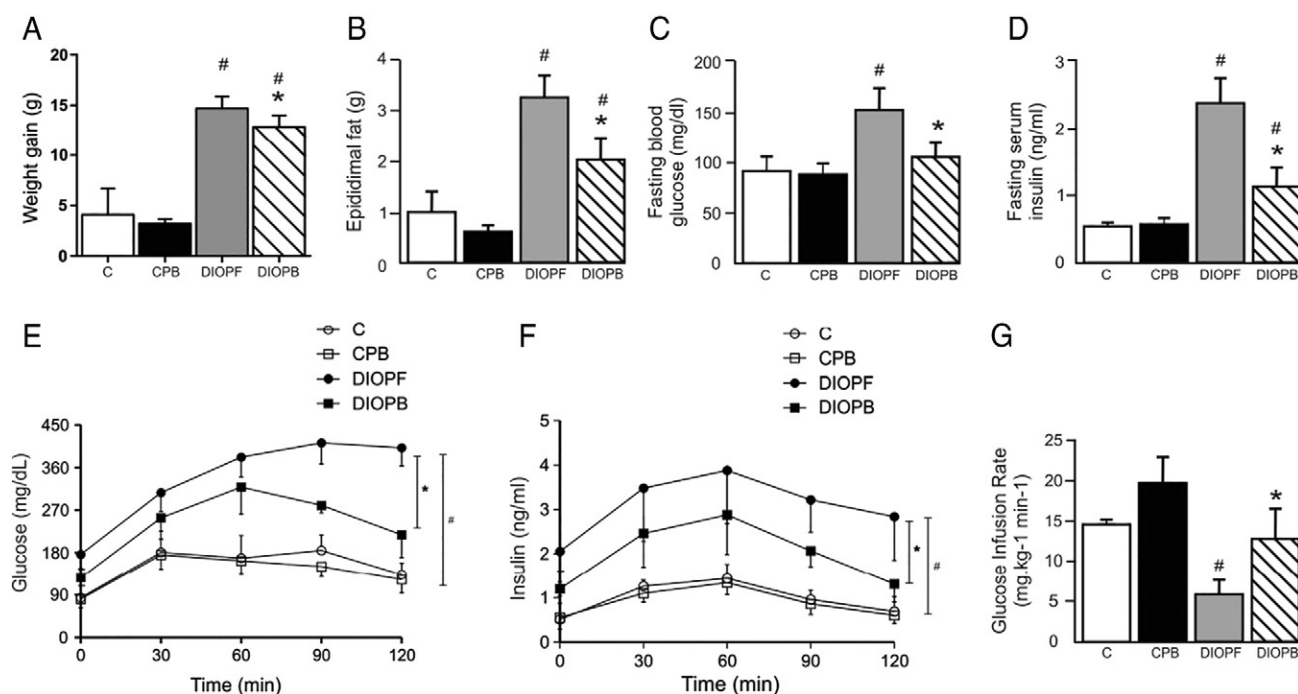

Fig. 1. Physiologic and metabolic parameters in control mice, control mice submitted to the administration of probiotics, obese mice (pair-fed DIOPF) and obese mice submitted to the administration of probiotics (DIOPB). (A) Weight gain. (B) Epididymal fat pad weight. (C) Fasting blood glucose. (D) Fasting serum insulin. (E) Glucose tolerance test (GTT). (F) Insulin response curve during the GTT. (G) Hyperinsulinemic–euglycemic clamp. Data are presented as means±S.E.M. of 6 to 10 mice per group. #$P<.05$ *vs.* control, *$P<.05$ *vs.* DIOPF.
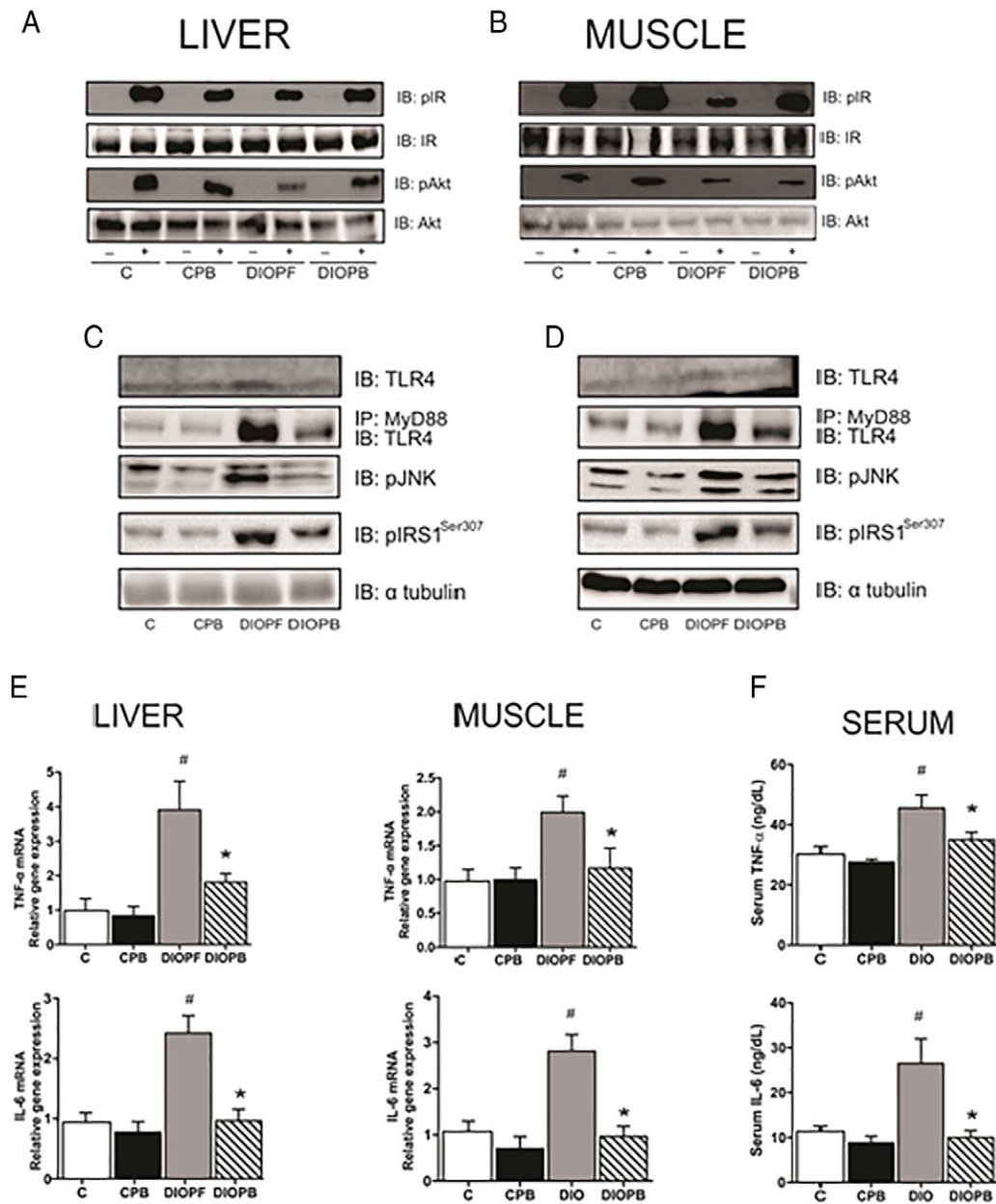
Fig. 2. Effects of probiotic administration on insulin signaling, TLR4 signaling pathway and cytokine mRNA level in DIO mice. Representative blots of the IRb tyrosine phosphorylation of (upper panel) and Akt serine phosphorylation (lower panels) of control, CPB, DIOPF and DIOPB mice in extracts from liver (A) and muscle (B). TLR4 protein, TLR4/MyD88 interaction, pJNK and pIRS1^{Ser307} in liver (C), muscle (D) of control, CPB, DIOPF and DIOPB mice. Total protein expression of α-tubulin (C and D lower panels). Determination of TNF-α and IL-6 mRNA expression by real-time PCR in the liver and muscles (E). Circulating levels of TNF- α and IL-6 in the same groups (F). Data are presented as means±S.E.M. from six mice per group. #P<.05 $vs.$ control, *P<.05 $vs.$ DIOPF. IB, immunoblotting; p, phosphorylated.

use of PB was able to return TLR4 protein levels to baseline (Fig. 2C–D). As expected, the HFD activated the signaling of TLR4 in liver and muscle tissues of DIOPF animals as evidenced by the greater interaction of TLR4 with its adaptor protein MyD-88 along with increased activity of its downstream JNK (Fig. 2C–D). Such activation resulted in increased IRS-1^{Ser307} phosphorylation (Fig. 2C–D). By contrast, the administration of probiotics to obese animals was able to reduce TLR4 activation, downstream JNK phosphorylation and the subsequent IRS1^{Ser307} phosphorylation (Fig. 2C–D). With regard to the control groups, the probiotic treatment did not provide changes on the TLR4 signaling (Fig. 2C–D).

3.4. Probiotics reduce tissue cytokines mRNA level in DIO mice

To verify whether alterations in TLR4 signaling were reflected in increased proinflammatory cytokines, we examined TNF-α and IL-6 mRNA expression in the tissues of the studied groups, and these mRNA levels were higher in liver and muscle of the DIOPF as compared to the control group (Fig. 2E). In accordance with TLR4 signaling, the relative amounts of TNF-α and IL-6 transcripts were markedly reduced in liver and muscle from DIOPB animals compared with DIOPF group (Fig. 2E). These benefits were also observed in circulating levels of TNF-α and IL-6 (Fig. 2F).

3.5. Effects of probiotic treatment on gut microbiota of DIO mice

The metagenomic sequencing analysis from feces of all groups showed profile changes among all studied groups. Among the control mice, treatment with probiotics increased the prevalence of *Firmicutes* and *Actinobacteria*, as well as decreased the occurrence of *Bacteroidetes* compared to untreated animals. In addition, by comparing control and DIO mice, regardless probiotic treatment, there were a greater increase in the prevalence of *Bacteroidetes* and a decrease in the *Firmicutes* and *Actinobacteria* phylum in the obese animals. Although this result disaccords with most studies in animals, we would like to emphasize that we previously observed similar result

with *Swiss* mice [7]. Finally, the administration of probiotics in obese animals provided a continued presence of *Bacteroidetes*, an increase in the prevalence of *Actinobacteria*, along with a reduction in the phylum *Firmicutes* compared to DIOPF. The probiotics also promoted an increased variety of phyla presented in these animal feces (data not shown).

Fig. 3B and C shows the distribution of some genera of *Bacteroidetes* and *Firmicutes* phyla in the DIOPF and DIOPB groups. Although probiotic treatment did not change the prevalence of *Bacteroidetes* in animals on a HFD, there was a major change in the concentration of certain genera, such as *Bacteroides* and *Alistipes,* populations of which increased in a significant way compared to levels in DIOPF (Fig. 3B).
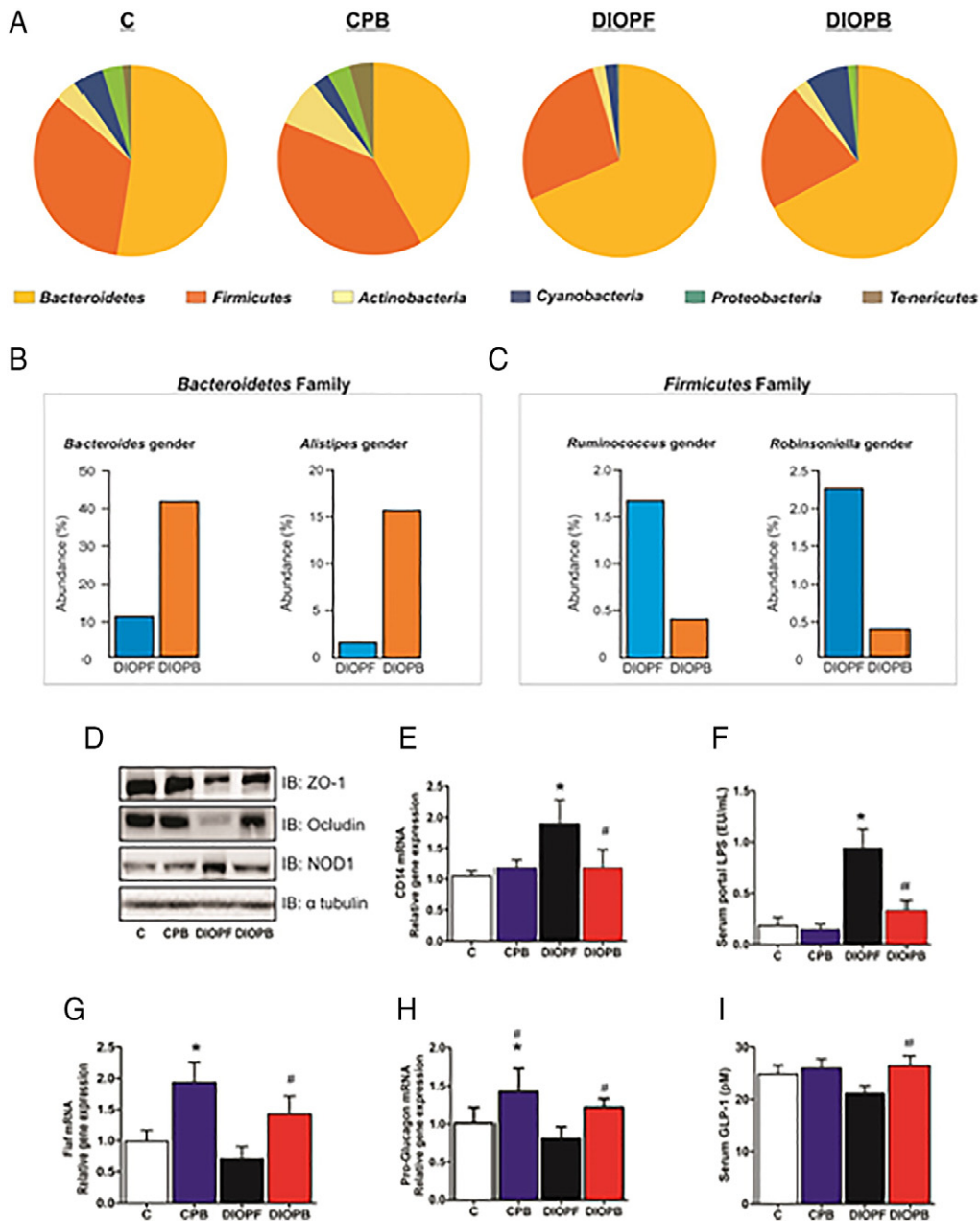

Fig. 3. Effects of probiotic administration on bacterial phylum prevalence in mouse feces and on ileal tight-junction proteins, on gut metabolic and immunologic modulations, and on LPS portal levels. (A) Metagenomic analysis of gut microbiota prevalence in control mice, control mice submitted to the administration of probiotics, DIOPF mice and DIOPB mice. (B–C) Prevalence of some genera inside the Bacteroidetes and Firmicutes families in DIOPF and DIOPB mice. Phylum charts were extracted using METAREP software based on BLASTX analysis of DNA pyrosequencing from mouse feces. Protein expression of ZO1, Occludin and NOD1 protein (D) and CD14 mRNA (E) in ileum. (F) Portal levels of LPS. (G) Ileal levels of Fiaf mRNA. (H) Proglucagon (I) serum concentrations of GLP-1 control, CPB, DIOPF and DIOPB mice. Data are presented as means±S.E.M. from six mice per group. *$P<.05$ *vs.* control group and #$P<.05$ *vs.* DIOPF. IB, immunoblotting.

Previous data have shown that an increased taxonomic family *Lachnospiraceae bacterium* contributes to development of diabetes in obese mice [42–44]. Our data showed that *Robinsoniella* and *Riminococcus* genera from the Lachnospiraceae family were increased in DIOPF mice and showed reduction after PB treatment (Fig. 3C).

3.6. Effects of probiotic treatment on gut metabolic and immunologic homeostasis

We then examined the consequences that changes in gut microbiota provoked on tight-junction proteins and on important immune proteins situated in the ileum. ZO-1 and Occludin protein and mRNA were reduced in DIOPF animals compared with controls, whereas the administration of probiotics restored partially these expressions (Fig. 3D). Furthermore, the main molecules involved in gut microbiota inflammation and bacterial translocation, NOD-1 and CD-14, had their expression increased in ileum of DIO animals, and the treatment with probiotics reversed this negative modulation (Fig. 3D and E). As consequence of these changes, obese mice also showed increased levels of LPS in their portal circulation compared with the control mice, and this was prevented by probiotic treatment (Fig. 3F). Previous data showed that modulation of fasting-induced adipose factor (FIAF, a factor that increases triglycerides accumulation in

adipose tissue) and GLP-1 might have a role in microbiota-induced insulin resistance/obesity [45]. Probiotic treatment in high-fat fed animals increased the expression of FIAF and proglucagon mRNAs in the ileum, as well as serum levels of GLP-1, compared with DIOPF (Fig. 3G–I). Probiotics had no such effect in control group (Fig. 3D–I).

3.7. Effects of probiotic treatment on histology in liver and adipose tissue

Histological sections from livers of DIOPF group showed the presence of innumerous fat vesicles compared to control sections, which characterizes hepatic steatosis. Nevertheless, treatment with probiotics prevented this fat accumulation in the liver parenchyma (Fig. 4A). Morphologic analysis of epididymal fat pad revealed significant differences on adipocyte size from DIO groups compared to control groups. However, between the DIO groups, we observed that the treatment with probiotics reduced the area of the adipocytes significantly but still far from the values of the controls animals. The HFD provided a huge macrophage infiltration in the adipose tissue, determined by the F4/80+ staining, but the administration of probiotics decreased such infiltration as well as the number of crown-like structures, which were abundant in DIOPF animals (Fig. 4B–D).
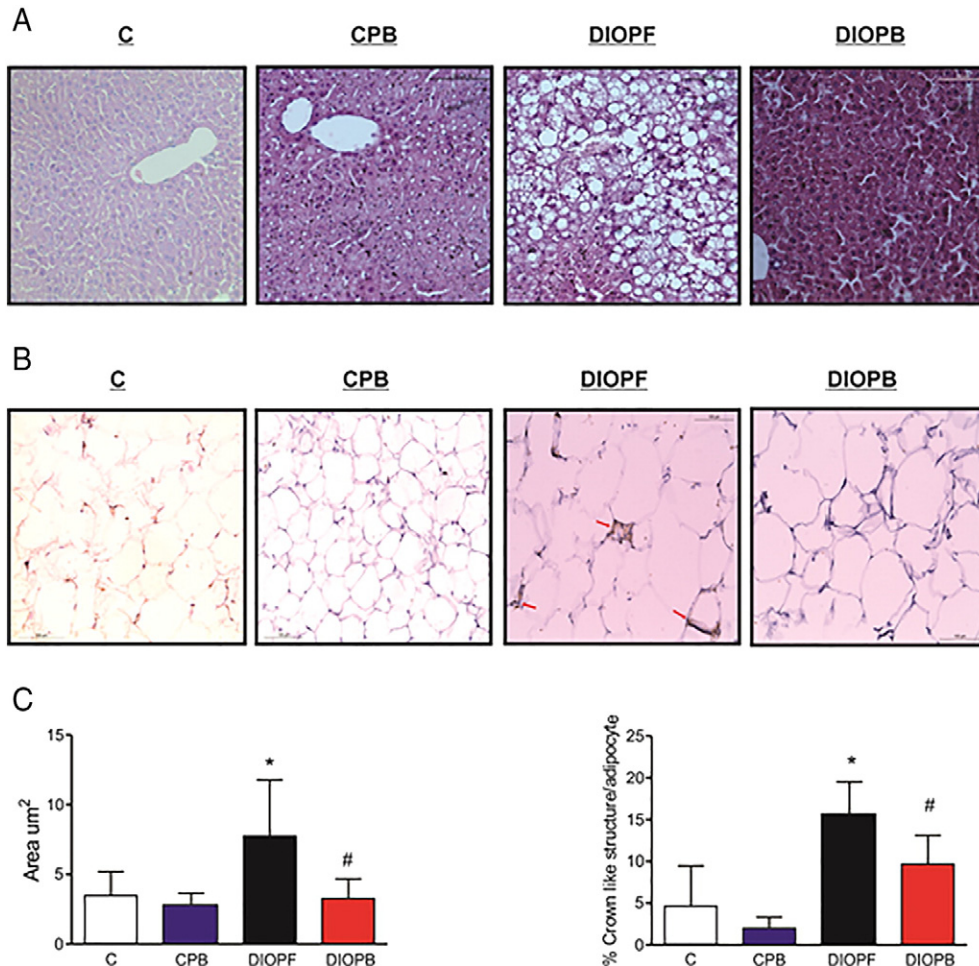

Fig. 4. Morphologic characterization of liver and adipose tissue samples. (A–D) Hematoxylin and eosin staining of 5-μm histological sections of liver parenchyma from control, CPB, DIOPF mice and DIOPB mice (A). F4/80 immunostaining of 5-μm histological sections of epididymal fat pad from control, CPB, DIOPF mice and DIOPB mice (B). *Red arrows* indicate crown-like structures. Scale bar=100 μm of all pictures. Original magnification × 200. Quantification of adipocytes area (square micrometers) (C). Prevalence of crown-like structures (D). Each bar represents the mean±S.E.M. from five different experiments. *$P<.05$ *vs.* control group and #$P<.05$ *vs.* DIOPF.

3.8. Effects of probiotics on hypothalamic insulin and leptin signaling

As mentioned before, probiotic-treated mice receiving an HFD gained less weight than DIO mice (DIOPB 11.4±1.2 g vs. DIO 16.9±1.4 g during treatment; P<.05) and had reduced food intake compared with animals that did not receive probiotics (DIOPB 2.9±0.2 g/day vs. DIO 3.4 ±0.3 g/da; P<.01). We then investigated whether probiotics treatment improved the response to anorexigenic hormones such as leptin and insulin in mice on HFD. The administration of probiotics in mice fed HFD improved leptin sensitivity, as evidenced by reduced food intake induced by leptin in DIOPF (Fig. 5A). In accordance, there was an increase in JAK2 and STAT3 phosphorylation in the hypothalamus of DIOPB compared to DIOPF mice (Fig. 5B). In addition to greater leptin sensitivity, we observed an enhanced anorexigenic effect in response to ICV insulin administration in the DIOPB group after 4 and 12 h (Fig. 5C). In parallel, Akt phosphorylation was increased in response to ICV insulin in the hypothalamus of DIOPB compared to DIOPF mice (Fig. 5D). We further measured mRNA levels of NPY and POMC in the hypothalamus since these neuropeptides have major action regulating food intake. Probiotics treatment reduced NPY mRNA without changes in POMC mRNA levels in the hypothalamus of DIOPB compared to DIOPF mice (Fig. 5E and F). We also evaluated the effect of probiotics on several inflammatory signals in the hypothalamus. Similar to the effects observed in peripheral tissues, HFD-fed animals showed increment in TLR4 and IL-6 expression in the hypothalamus, and probiotic treatment was able to reduce TLR4 and IL-6 mRNA levels in this tissue (Fig. 5G and H). There were no changes in the TNF-α mRNA levels in the hypothalamus of DIOPB compared to DIOPF mice (Fig. 5I).

4. Discussion

Here we show that HFD animals treated with probiotics have changes in gut microbiota and the intestinal gene expression of metabolic and immunological molecules that resulted in reductions in circulating LPS, which attenuated TLR4 activation and improved insulin signaling and inflammatory profiles in liver and muscle. In addition, the probiotic treatment improved hypothalamic insulin and leptin signaling pathways and modulated the control food intake (Fig. 6).

Our metagenomic analysis of gut microbiota in the DIO groups showed an increase in *Bacteroidetes* prevalence and a slight reduction in *Firmicutes*. This result is similar to previous studies [46–48], including a work from our group using also *Swiss* mice [7], but is not in agreement with other classical published data [8,49], suggesting that the gut microbiota profile in obesity depends also on genetic background. Differences in animal species and/or strain may explain these controversial results. Another phylum that is important to intestinal homeostasis and was reduced in DIOPF animals compared to controls was the Actinobacteria. Mice subjected to probiotic treatment and fed HFD showed some differences in gut microbiota profiles compared with DIO nontreated animals. Despite the maintenance of Bacteroidetes phylum, there were a significant reduction in Firmicutes prevalence and an increase in Actinobacteria. Genera analysis of *Bacteroides* showed relevant modifications after PB treatment: the genus *Bacteroides* increased considerably, and it is known that it has beneficial activities to the host, including increased adipose tissue lipolysis, intestinal production of GLP-1, as well as reduction of
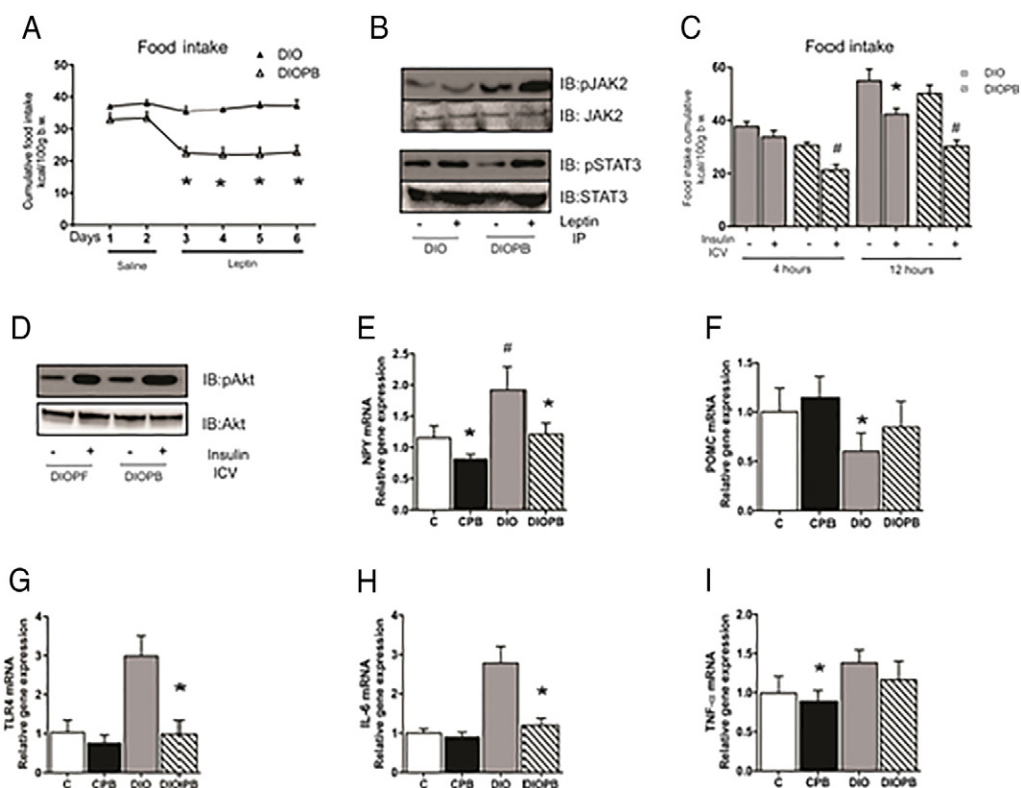

Fig. 5. Effects of probiotic administration on leptin and insulin hypothalamic signaling, on the control of food intake and on proteins involved in the inflammatory signaling. (A) Six days of controlled food intake; the first 2 days with daily IP infusion of saline and the last 4 days with daily IP infusion of recombinant leptin in DIO (not pair-fed) and DIOPB mice. (B) Leptin-induced Jak2 and STAT3 tyrosine phosphorylation in the hypothalamus of DIO and DIOPB mice. (C) Four hours and 12 h of food intake after intrahypothalamic infusion of insulin in DIO and DIOPB mice. (D) Insulin-induced Akt serine phosphorylation in the hypothalamus of DIO and DIOPB mice. NPY (E) and POMC (F) mRNA levels in control, CPB, DIO and DIOPB mice. (G–I) Determination of mRNA expression of TLR-4 (G), IL-6 (H) and TNF-α (I) in control, CPB, DIO and DIOPB mice. Immunoblotting data are representative of four independent experiments. Quantitative PCR and food intake data are presented as means±S.E.M. from six mice per group. #P<.05 vs. control group and *P<.05 vs. DIO, except for panel C. *P<.05 vs. DIO negative, #P<.05 vs. DIOPB negative. IB, immunoblotting; p, phosphorylated.
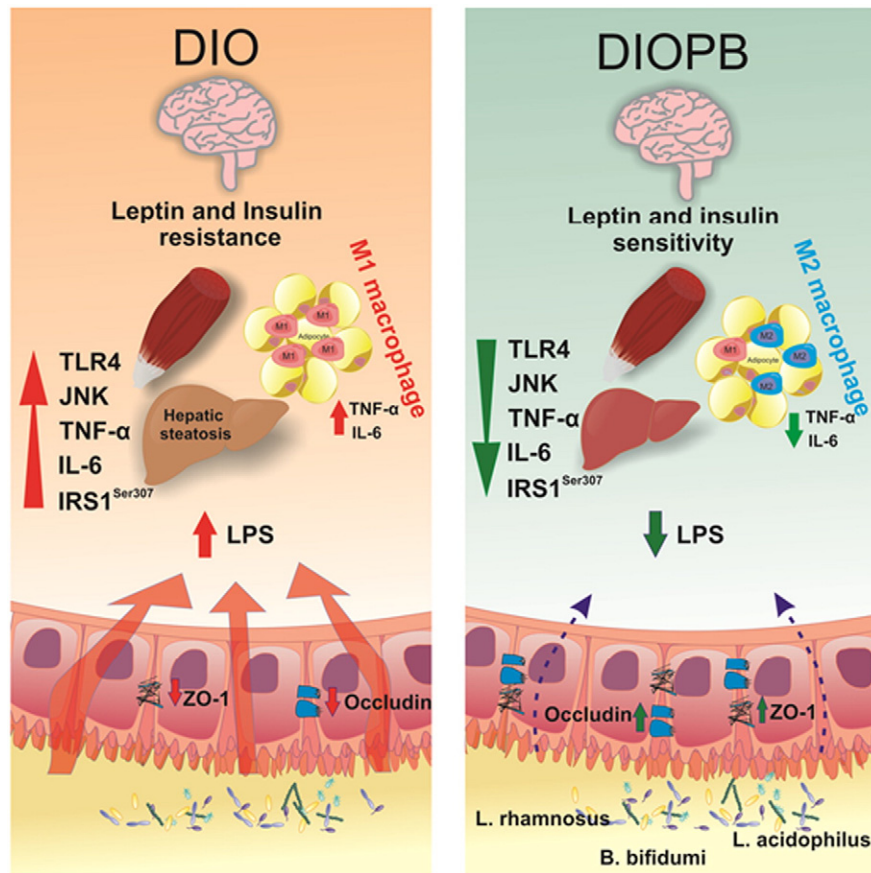
Fig. 6. Effects of probiotic administration on obesity-related features by changes in the gut microbiota profile in mice subjected to an HFD. (A) DIO induced changes in microbiota accompanied by an alteration in intestinal barrier (increased expression of ileal tight-junction proteins ZO-1 and occludin) and an increase in absorption and circulating levels of LPS, accompanied by TLR4 and JNK activation and M1 macrophage infiltration in adipose tissue, all contributing to insulin and leptin resistance. (B) Probiotics (*L. rhamnosus, L. acidophilus and B. bifidum*) treatment by modulating microbiota reversed these inflammatory phenomena and improved insulin and leptin action.

intestinal inflammation [50,51]. Another genus that increased with probiotic administration in DIO animals was *Alistipes*. Serino et al. [52] described that mice that have high prevalence of this genus is protected from type 2 diabetes in an HFD scenario. Thus, despite the unchanged prevalence of the *Bacteroidetes* phylum following probiotic treatment, the modulation of the genera of this phylum might have a role in the improvement of glucose tolerance and insulin sensitivity.

Changes in the gut microbiota of DIO mice promoted by probiotics were accompanied by increased expression of ileal tight-junction proteins (ZO-1 and occludin) and proglucagon mRNA, as well as reduced intestinal expression of pattern recognition receptors (PRRs) CD-14 and NOD1. These modulations might play a role in the reduction in circulating levels of LPS and the increase in GLP-1 serum levels. In agreement with our results, Gomes et al. [18] have recently shown that bacterial translocation needs CD-14 and NOD1 PRRs activation in DIO mice; however, probiotic treatment reversed this situation. It is also known that fermented products of probiotics bacteria can increase gut proglucagon expression, which results in GLP-1 and GLP-2 secretion [53,54]. Another important intestinal protein modulated by probiotics is FIAF. Obesogenic microbiota decreases the expression of this molecule, a factor able to increase lipoprotein-lipase-dependent triglyceride storage in adipose tissue and to increase adipocyte triacylglycerol accumulation [5,55]. Our results showed that probiotic administration increased the ileal expression of FIAF in obese mice, probably contributing to reduce fat storage in these animals.

It is interesting that obese mice treated with probiotics showed a reduction in portal LPS concentrations, which certainly contributed to down-regulate the TLR4–MyD88 interaction in liver and muscle, leading to reduced activation of JNK and IRS-1 serine phosphorylation. As expected, in parallel, there was an increase in activation of insulin signaling in liver and muscle, improving insulin sensitivity and glucose tolerance [56,57].

The positive feedback loop between JNK and proinflammatory cytokines can perpetuate a vicious cycle of low-grade inflammatory signaling, contributing to enhanced insulin resistance [9,58]. Our findings indicate that the probiotic treatment reduced not only the JNK activation but the TNFα, IL-6 and IL-1β mRNA levels in liver and muscle tissue, probably by reducing NFκB translocation into the nucleus.

In obesity, adipose tissue macrophages are an important source of cytokine tissue expression [59]. In addition, a reduction in macrophage infiltration can decrease local inflammation in adipose tissue [60]. In accordance, our findings revealed a reduction in macrophage infiltration in the adipose tissue of DIO mice treated with probiotics, as observed by crown-like structures number. These morphological changes, coupled with reduced expression of inflammatory molecules, are factors that may contribute to improve insulin sensitivity in treated animals. Lipid accumulation in the liver is a hallmark of high-fat-diet-induced insulin resistance [61]. Although in hepatic sections of DIOPF mice there were a large number of fat vesicles amply distributed across the parenchyma, mice treated with probiotics

presented liver parenchyma very similar to that of the controls, with no evidence of hepatic steatosis, in parallel to a marked reduction in inflammatory pathways and cytokine expression in this tissue.

Our data showed that animals fed HFD and treated with probiotics presented decreased weight gain and food intake compared to DIO counterparts. The regulation of food intake by the hypothalamus has been extensively investigated and involves nutrients, hormones and neural signals [62]. Leptin and insulin signaling and action in the hypothalamus have profound anorexigenic effects [62]. Hypothalamic insulin signaling promotes transcriptional and electrical events in neurons and activates PI3K/Akt pathway controlling neuropeptides responsible to maintain energy homeostasis. Leptin in the hypothalamus exerts its effects through LR/JAK2/STAT3 pathway also regulating neuropeptide transcription. Both hormones may increase POMC, which is anorexigenic, and decrease the orexigenic NPY mRNA levels [62,63]. Obesity state is associated with insulin and leptin resistance in the hypothalamus [64]. The mechanism responsible for the resistance is likely due to, at least in part, aberrant hypothalamic activation of proinflammatory molecules, including TLR4, JNK and IKK [55,65]. Here we reported that probiotic administration by gavage in HFD-fed mice induced a striking reduction in hypothalamic TLR4 and IL-6 mRNA levels and a decrease in protein expression of serine kinases JNK and IKK. The reduction of proinflammatory molecules was associated with an improvement of insulin and leptin signaling, action in the hypothalamus and decrease in NPY mRNA levels. These results might contribute to reduce food intake and adiposity in mice treated with probiotics.

Serum GLP1 levels were higher in mice treated with probiotics than their controls. It is well established that activation of GLP1 receptor in multiple sites of the brain alters energy balance [66]. Thus, we cannot rule out a possible contribution of GLP1 lowering body weight and food intake in mice treated with probiotics.

In summary, our data demonstrate that HFD promotes alterations in gut microflora that are reflected in increased intestinal permeability, LPS translocation and systemic low-grade inflammation, resulting in decreased glucose tolerance and hyperphagic behavior. These obesity-related features were reversed by changes in the gut microbiota profile induced by probiotics in mice subjected to an HFD (Fig. 6). In this context, we believe that probiotics can be an important tool to prevent and treat patients with obesity and insulin resistance, but more studies are needed, especially to develop more specific probiotic strains for treating metabolic disorders such as obesity.

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Authors' contributions

R.A.B.: researched data, contributed to discussion, wrote/reviewed/edited manuscript; N.T.: researched data; A.G.O.: researched data, wrote/reviewed/edited manuscript; B.M.C.: researched data; G.Z.R.: researched data; T.G.A.: wrote/reviewed/edited manuscript; J.F.V.: researched data; D.G.: researched data; P.O.P.: reviewed/edited manuscript; A.S.: contributed to discussion, wrote/reviewed/edited manuscript; S.T.O.S., M.J.A.S.: contributed to discussion, wrote/reviewed/edited manuscript.

Conflict of interest

None.

Acknowledgments

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