Nutrition 31 (2015) 1000–1007

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Lactobacillus casei CRL 431 administration decreases inflammatory cytokines in a diet-induced obese mouse model

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ARTICLE INFO

Article history:
Received 3 December 2014
Accepted 17 February 2015

Keywords:
Cytokines
Probiotic
Diet-induced obesity
Adipocytes
Small intestine
Liver

ABSTRACT

Objectives: Obesity is a chronic disease associated with an inflammatory process in which cytokines play an important role. Probiotic microorganisms have been associated with modulation of the host immune system. The aim of this study was to evaluate the influence of the probiotic bacterium *Lactobacillus casei* CRL 431 on the cytokine response in a model of mice under high-fat diet (HFD) conditions.

Methods: BALB/c mice received a conventional balanced diet or an HFD. The test groups received milk, milk fermented by *L. casei* (FM), or *L. casei* as suspension in the drinking water. Proinflammatory and regulatory cytokine producer cells were evaluated in the small intestine and liver; the cytokine levels in the intestinal fluids were also evaluated. The percentages of immune cells as macrophages (F4/80), NKT, CD4+, CD8+ populations were determined in the liver. Adipocytes were also isolated and cultured to evaluate cytokines and the chemokine monocyte chemoattractant protein (MCP)-1 produced by them.

Results: The administration of probiotic *L. casei* CRL 431 exerted an anti-inflammatory response in mice fed an HFD, evidenced mainly by decreasing proinflammatory cytokines, such as interleukin (IL)-6, IL-17, and tumor necrosis factor-α. Probiotic administration also was associated with fewer immune-infiltrating cells in the liver of mice that received the HFD and decreased secretion of MCP-1 by the adipocytes. This last observation could be associated with less macrophage accumulation in the adipose tissues, which is characteristic in the obese host and contributes to maintaining the inflammatory response in this organ. The results obtained show an anti-inflammatory effect of *L. casei* CRL 431 when it is administered as a supplement of the HFD in a mouse model.

Introduction

Obesity is defined as a chronic disease characterized by an excessive accumulation of fat or adipose tissue hypertrophy. Obesity has become a serious public health problem that is increasing and has reached epidemic proportions worldwide [1]. It has a multifactorial origin and is strongly associated with metabolic syndrome (MetS), causing different diseases such as cardiovascular diseases, type 2 diabetes mellitus, and sleep apnea [2,3]. Obesity is considered an inflammatory process in which the adipose tissue plays an important role [4]. Poor eating habits and sedentary lifestyles are associated with obesity-related diseases. People tend to choose some dietary supplements that, in addition to their nutritional properties, can exert some effect on their health. In this context, products containing probiotic microorganisms often are included in the daily diet.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [5]. Previous studies have described the capacity of certain probiotic microorganisms or fermented products to exert

This work was financially supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0652 and 1071) and CIUNT (Universidad Nacional de Tucumán, CIUNT 26/D442), Argentina. INN, CMG, and AdMdL carried out the microbiologic work, the animal studies, and the immunologic determinations; performed the statistical analyses; and prepared the figures. GP conceived of the study. CMG, AdMdL, and GP designed the experiments. INN, CMG, AdMdL, and GP wrote the draft of the manuscript. All authors read and approved the final version of the manuscript. The authors have no conflicts of interest to declare.

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http://dx.doi.org/10.1016/j.nut.2015.02.006
0899-9007/

a beneficial balance in the gut microbiota, which improves the immunologic status of the host and modulates the cytokine release in the lamina propria of the small intestine [6–8]. The beneficial effects of certain probiotics on body weight and on some immunologic and metabolic parameters were evaluated in animal models of obesity, diabetes, and hyperlipidemia [9–11]. It also has been reported that probiotic administration to obese hosts improves gut microbiota [12,13], modulates genes associated with metabolism and inflammation in the liver and adipose tissue [14], and improves symptoms associated with MetS [15].

Supplementing the diet with probiotics could be a possible alternative to combating obesity and other disorders associated to it, especially those caused by the anti-inflammatory effects exerted by these microorganisms [16,17].

Lactobacillus casei CRL 431, a probiotic bacterium that affects the intestinal immune system, has been extensively studied using murine models [18–21].

Recently, we evaluated the effect of the administration of *L. casei* CRL 431 as a supplementation for a high-fat diet (HFD) in a mouse model [22]. It was demonstrated that *L. casei* CRL 431 administration as suspension or as fermented milk (FM) decreased the body weight and biochemical parameters in blood that are associated with MetS. This beneficial effect was associated with the improvement of gut microbiota by increasing bifidobacteria and by avoiding a decrease of bacteroides. Both bacterial populations were found diminished in mice receiving HFDs [23]. The histology of liver and small intestine, affected by the HFD intake, were also improved in mice that received *L. casei* CRL431 [22].

The aim of this study was to evaluate the influence of the probiotic bacterium *L. casei* CRL 431 and its FM on the cytokine response (in small intestine, liver, and adipocytes) when they are administered as diet supplements for mice fed an HFD. We hypothesized that the proinflammatory cytokines, which are increased during the diet-induced obesity, can be modulated by the probiotic administration.

Material and methods

Bacterial strain and fermented milk

L. casei CRL 431 was obtained from the CERELA Culture Collection (San Miguel de Tucumán, Argentina). Overnight cultures were grown at 37°C in 5 mL sterile Mann-Rogosa-Sharp (MRS) broth (Britania, Buenos Aires, Argentina). The cells were harvested by centrifugation at 5000*g* for 10 min, washed three times with fresh phosphate-buffered saline (PBS) solution and then resuspended in 5 mL of sterile 10% (wt/vol) nonfat milk. This bacterial suspension was diluted 1:30 in water and administered ad libitum to the mice. The final concentration of probiotic bacteria was $2 \pm 1 \times 10^8$ CFU/mL, a concentration used previously [18,24]. This count was periodically controlled at the beginning of the administration and every 24 h to avoid modifications of >1 logarithmic unit.

To obtain the FM, nonfat dried milk (Svelty, Nestlé Argentina S.A.) was rehydrated and autoclaved (115°C for 15 min). Overnight cultures of *L. casei* CRL 431 grown in MRS broth were used to inoculate (2% vol/vol) sterile milk, and then incubated at 37°C for 24 h. Final concentration of the probiotic bacteria in the FM was $4 \pm 2 \times 10^8$ CFU/mL. This fermented product was prepared every 2 d and the microbial concentration was monitored.

Experimental groups: protocol to induce obesity with HFD and sampling procedure

Mice were provided for CERELA from a closed randomly bred colony. The experimental protocol using mice was performed in three independent experiments. Twenty-four female BALB/c mice (5 wk old, weighing 25 ± 2 g) were used for each trial, and considering the three repetitions, the total number of mice used was 72. Mice were divided into two groups: group 1 included nonobese mice (N) fed ad libitum with a conventional balanced diet (45% carbohydrates, 32% fat, 23% proteins, 6% raw fiber, 10% total minerals, 1.3% calcium, 0.8% protein, 12% moisture and vitamins) provided by ACA Nutrition Animal [San Nicolas, Buenos Aires, Argentina], and group 2 included mice fed the HFD (diet-induced obese group [O]). The HFD was made in the laboratory using the same conventional balanced diet but with bovine lard and sugar (both for human consumption, purchased in the supermarket) added. The HFD contained 43.4% conventional balanced diet, 43.4% bovine lard, and 13.2% sugar; its caloric contribution was 18.6 kcal/d [22]. Each group was divided into three subgroups according to the addition of the dietary supplements. The total number of mice evaluated for each group was nine (three in each repetition of the assays).

The nonobese group had a control group (NC) in which animals received conventional balanced diet and water ad libitum over a 2-mo period, and three test subgroups received conventional balanced diet supplemented daily with milk (N + milk), FM (N + FM), or a suspension of *L. casei* CRL 431 in the drinking water (N + Lc), ad libitum. The diet-induced obese group had a control (OC) group of animals that received HFD and water ad libitum over a 2-mo period, and three test subgroups that received HFD supplemented with milk (O + milk), FM (O + FM), or the suspension of *L. casei* (O + Lc) during the experiment. The administration of the supplements was ad libitum. The volume consumed was measured daily in each cage, and considering the number of mice, each mouse consumed an average of 3 to 4 mL/d of liquid. The bottles containing diet supplements were replaced daily to maintain their quality.

Mice were maintained in a room with a 12-h light/dark cycle at 20°C ± 2°C. They were weighed every 2 d. Mice in each control and test group were sacrificed by cervical dislocation at day 60 and small intestine, liver, and adipose tissue from each mouse were removed for further studies. All animal protocols were preapproved by the Animal Protection Committee of CERELA (CRL-BIOT-LI-2010/1 A) and all experiments complied with the current laws of Argentina.

Detection of cytokine-positive cells and TLR-5–expressing cells in small intestine and liver by immunohistochemistry

Cells positive for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10, IL-6, IL-17, and toll-like receptor (TLR)-5 were studied in the lamina propria of the small intestine and in the liver by indirect immunofluorescence assay following a previously described technique [19]. For the small intestine, representative lengths (3–5 cm) were taken from the different sections (*duodenum*, jejunum, and ileum). Rabbit antimouse TNF-α, IFN-γ, IL-10, IL-6 (ProSci Inc., Poway, CA, USA), goat antimouse IL-17 (BD Bioscience, San Diego, CA, USA), or rabbit antimouse TLR-5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) polyclonal antibodies were used as primary antibodies and goat antirabbit or rabbit antigoat antibodies conjugated with fluorescein isothiocyanate (FITC; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) were used as secondary antibodies.

The number of fluorescent cells was counted by two researchers (two individual blinded counts per sample) and the results were expressed as the number of positive cells for each cytokine in 10 fields of vision as seen at 1000× using a fluorescence light microscope (Carl Zeiss, Germany).

Determination of the cytokines levels in the small intestinal fluids

Small intestine (duodenum, jejunum, and ileum) was removed and the intestinal content was collected by washing with 1 mL of PBS and immediately centrifuged at 5000*g* for 15 min at 4°C. The supernatant was separated and stored at −20°C (or −80°C if they would be not analyzed within the next 7 d) until determination of cytokine (IFN-γ, IL-10, IL-6) concentrations using BD OptEIA cytokine enzyme-linked immunosorbent assay (ELISA) set (BD Bioscience). The results were expressed as the concentration of each cytokine in the intestinal fluids (pg/mL).

Isolation of liver mononuclear cells

The liver of each mouse was removed aseptically and placed in sterile tubes containing 1 mL of Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Gibco, Invitrogen, NY, USA) following a previously described technique [25]. The samples were homogenized under sterile conditions using a metal mesh. They were centrifuged at 2500*g* for 10 min and resuspended in Percoll (Sigma, St. Louis, MO, USA) 35% in DMEM, and centrifuged at 2500 to 2800*g* for 20 min. The resulting pellets were lightly mixed with sterile red blood cell lysing buffer (Sigma) for 2 to 4 min. Hemolysis was stopped with the addition of PBS, the samples were centrifuged again at 1800*g* for 10 min, and the pellets resuspended in 2 mL of sterile PBS. The cell concentration was adjusted to 3×10^6 cells/mL, counting the viable cells by trypan blue exclusion method in Neubauer chamber.

Determination of macrophages (F4/80+), and T populations (CD4+, CD8+, and NKT cells) in liver

Mononuclear cells obtained from the liver of each mouse (3×10^6 cells/mL) were incubated in darkness with the appropriate monoclonal antibodies: Peridinin Chlorophyll Protein Complex (PerCP-Cy5.5)-conjugatedBM8 (antimouse F4/80 antigen. Pan Macrophage Marker, e-Bioscience, San Diego, CA, USA) for macrophages, FITC-labeled rat antimouse CD8 α-phycoerythrin (PE)–labeled rat antimouse CD4, and allophycocyanin (APC)-conjugated antimouse NK1.1 (BD

Biosciences Pharmingen, San Diego, CA USA) for CD8+, CD4+, and natural killer (NK) T cells. Cells were then analyzed with a FACSCalibur flow cytometer (BD Bioscience) equipped with excitation laser source at 488 and 635 nm. Samples were run through the flow cytometer, and 500 000 events were analyzed for each sample with FCS Express 4 Flow Cytometer (De Novo Software, Glendale, CA, USA).

Using the forward- and side-scatter properties of macrophages and lymphocytes in laser light, two gates were drawn. The percentages of F4/80+ were determined in both gates and the percentages of CD4+, CD8+, and NK1.1 + cells in the gate corresponded to lymphocytes.

Isolation and culture of adipocytes to determine cytokines and the MCP-1

Adipose tissues were obtained from mesenteric and visceral fat of each mouse, removed aseptically, weighed to standardize the amount extracted and placed in sterile tubes containing 1 mL of DMEM medium, as previously described [26,27]. The samples were mechanically disaggregated, and then were subjected to enzymatic digestion in 1 mL of DMEM medium supplemented with collagenase type I (Sigma Aldrich) at a concentration of 2 mg/mL, and were stirred with magnetic bars for 1 h at 37°C. All these procedures were conducted under sterile conditions. Subsequently, the products of enzyme digestion (adipocytes) were centrifuged at 1000*g* for 10 min. The adipocytes were placed in sterile culture dishes and incubated at 37°C and 5% carbon dioxide for 24 h. After incubation, the culture supernatants were collected and stored at −20°C (or −80°C if they would be not analyzed within the next 7 d) until cytokine determinations.

The concentration of different cytokines (IL-6, IL-10, IFN-γ and TNF-α) and the chemokine MCP-1 were measured by ELISA using commercial sets (BD Biosciences Pharmingen). The results were expressed as the concentration of each cytokine and chemokine in the culture supernatants (pg/mL).

Statistical analysis

For each trial, test and control groups contained three animals that were sacrificed after 2 mo of experiment. Each experiment was repeated three times. Statistical analyses were performed using MINITAB 15 software (Minitab Inc., State College, PA, USA).

Comparisons were accomplished by an analysis of variance general linear model followed by a Tukey's post hoc test, and $P < 0.05$ was considered significant. No significant differences were observed among the three independent replicates; results from three replicates were combined and the comparisons were obtained from nine animals.

Results

Fermented milk or L. casei administration as suspension modulate the number of cytokine and TLR-5–positive cells in the lamina propria of the small intestine

No significant differences in the number of IFN-γ–positive cells in the small intestine were observed between the different study groups (Fig. 1A).

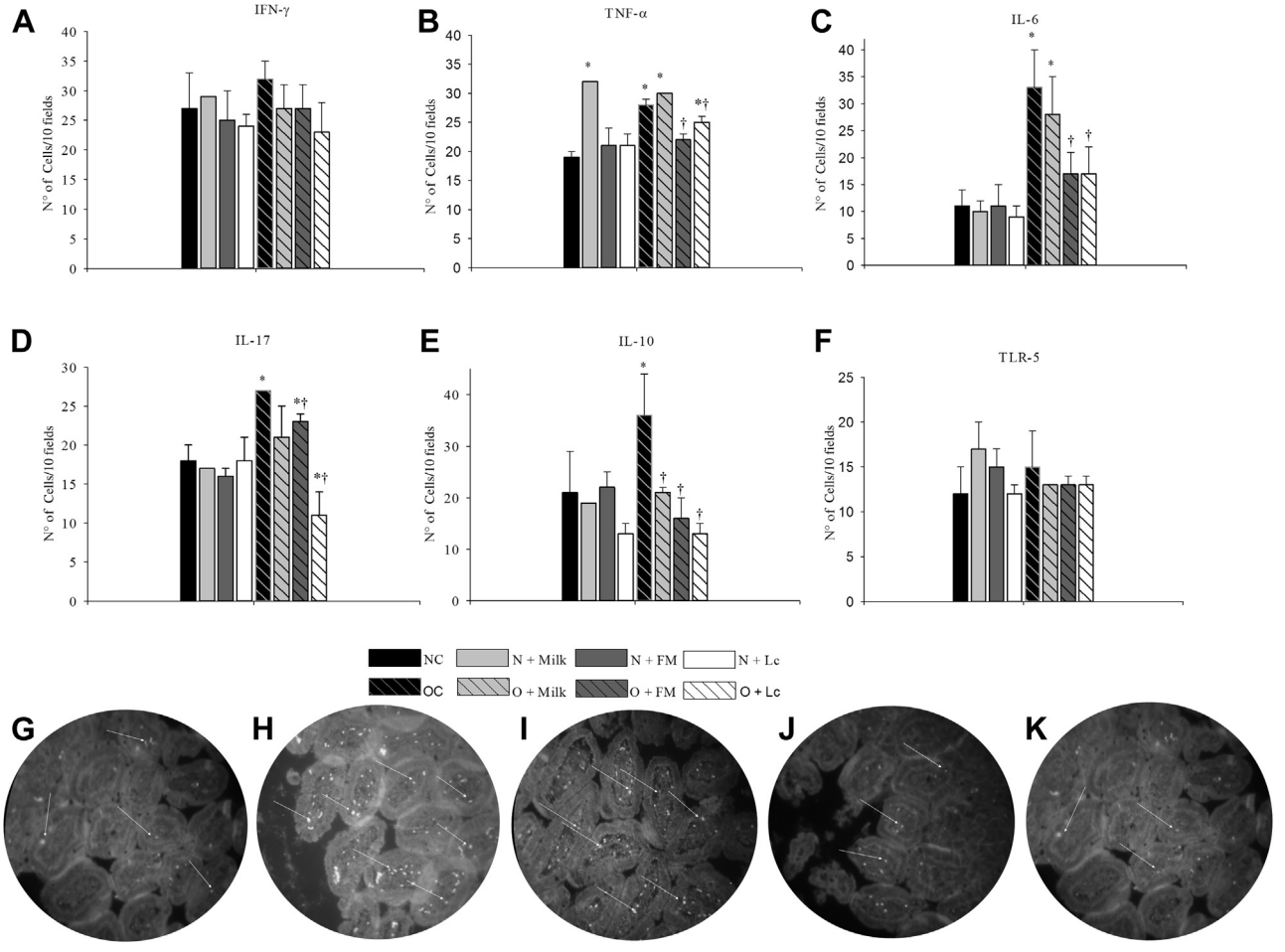

Fig. 1. Effect of high-fat diet (HFD) and probiotic administrations on cytokine-positive cells in the small intestine. The numbers of cytokine-positive cells (A–E) and TLR-5–expressing cells (F) were determined by indirect immunofluorescence on the small intestine tissue slides of mice from different groups: nonobese control (NC), nonobese mice given milk, fermented milk (FM) or *Lactobacillus casei* (Lc; N + milk, N + FM and N + Lc), control mice fed the HFD (diet-induced obese control, OC), and diet-induced obese mice that received milk, FM, or *L. casei* (O + milk, O + FM, and O + Lc). The results were expressed as the number of positive cells per 10 fields of vision (1000×). Data correspond to the means ± SD of nine animals from three separated experiments. Representative figures (100×) of interleukin (IL)-6–positive cells from mouse of NC (G), OC (H), O + milk (I), O + FM (J), and O + Lc (K) are shown in the bottom. Arrows show the positive cells (fluorescent cells). *Significant difference ($P < 0.05$) with NC. †Significant difference ($P < 0.05$) with OC.

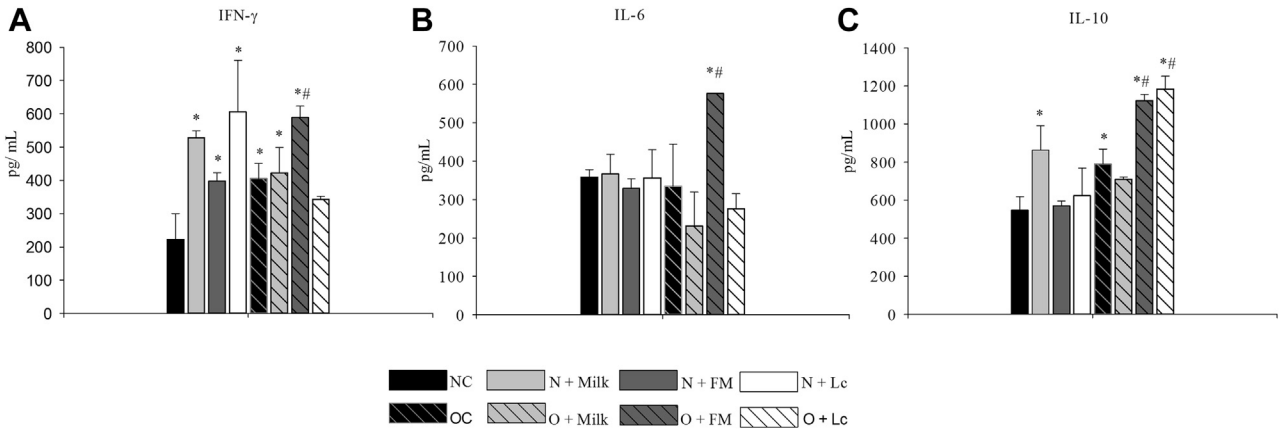

Fig. 2. Effect of high-fat diet and probiotic administration on the release of cytokine to the intestinal fluid. IFN-γ (A), IL-6 (B) and IL-10 (C) concentrations were determined in the intestinal fluid of mice from the different control and test groups. Results were expressed as pg/mL of each cytokine. Data correspond to the mean ± SD of nine animals from three separated experiments. *Significant difference ($P < 0.05$) with nonobese control (NC). #Significant difference ($P < 0.05$) with diet-induced obese control (OC).

The number of TNF-α–positive cells increased significantly ($P < 0.05$) in the nonobese mice that consumed milk compared with the NC group. The other diet supplements did not modify the number of TNF-α–positive cells. In mice fed the HFD, the number of TNF-α–positive cells was significantly increased in control mice (OC) and in the mice from the O + milk and O + Lc groups, compared with the NC group. However, the number of cytokine-positive cells in the small intestine of mice from the O + FM and O + Lc groups was significantly lower ($P < 0.05$) than in the mice from OC group (Fig. 1B).

No significant changes were observed in the number of IL-6–, IL-10–, and IL-17–positive cells in the small intestine of nonobese mice that received probiotic compared with the NC group. The numbers of IL-6–, IL-10–, and IL-17–positive cells were significantly increased ($P < 0.05$) in the OC group compared with the NC group. Probiotic administration to mice fed a HFD (O + FM and O + Lc) significantly decreased ($P < 0.05$) the number of cells positive for these cytokines compared with OC group (Fig. 1). Figure 1G–K show examples of this diminution obtained for IL-6–positive cells in one representative mouse from each group. IL-6–positive cells were increased in the O + milk group, without significant differences with the OC mice (Fig. 1C and H). Regarding the production of IL-17, counts of these cytokine-producing cells in mice from the O + Lc group decreased significantly ($P < 0.05$), reaching values even lower than in the NC group (Fig. 1D).

The number of TLR-5–positive cells did not show any significant differences in the mice fed the HFD, whether or not they received dietary supplements (OC, O + milk, O + FM, or O + Lc), and the NC group (Fig. 1E).

Effect of milk, FM, or L. casei administration on the release of cytokines to the small intestine fluid

Figure 2 shows the concentration of the cytokines IFN-γ, IL-10, and IL-6 evaluated in the small intestine fluid. IFN-γ increased significantly ($P < 0.05$) in nonobese mice given any of the dietary supplement (Fig. 2A), and IL-10 concentrations increased significantly in mice from the N + milk group, both compared with NC group. IFN-γ and IL-10 increased significantly ($P < 0.05$) in OC mice compared with the NC group (Fig. 2A and C). No significant differences were observed in the IL-6 concentrations in the intestinal fluid in mice from these groups (Fig. 2B). FM administration to mice fed the HFD (O + FM) increased the levels of IFN-γ significantly ($P < 0.05$) compared with their respective controls. IL-6 increased significantly ($P < 0.05$) in mice fed the HFD given FM compared with both control groups as well as the other test groups (Fig. 2B). IL-10 concentrations increased significantly ($P < 0.05$) in the fluid of HFD mice that received probiotic (O + FM and O + Lc) compared with the OC group; these levels also were significantly increased ($P < 0.05$) compared with the NC group (Fig. 2C).

Effects of probiotic and HFD administration on liver immune cells

Mononuclear cells were isolated from the liver of the mice and analyzed by flow cytometry. The addition of any supplement to the diet of nonobese mice did not modify the number of evaluated immune cells in the liver, maintaining values similar to NC (data not shown). The percentage of NKT cells (recognized by the antibody anti-NK1.1) decreased in the livers of mice in the HFD groups compared with the NC group. The O + FM group was the group that had the most significant increase ($P < 0.05$) in the percentage of these cells in the liver compared with the OC, O + milk, and O + Lc, reaching values of the NC group.

The percentage of CD8+ cells did not vary significantly between HFD mice or mice from the NC group.

The study of CD4+ cells showed a significantly increased percentage ($P < 0.05$) in the liver of HFD mice compared with the NC group. HFD mice given probiotic supplementation demonstrated a significant decrease ($P < 0.05$) in the percentage of these cells compared with the OC group, but did not reach the NC values (Table 1).

The percentage of macrophages (F4/80 + cells) decreased significantly ($P < 0.05$) in the OC group than in the NC group, and the three dietary supplementations (O + milk, O + FM, and O + Lc) were able to increase these values, reaching levels similar to those of the NC group (Table 1).

Probiotic administration influences the number of cytokine-positive cells in the liver of diet-induced obese mice

Significant differences were not observed in the number of IFN-γ and IL-10 positive cells in the liver of nonobese mice that received probiotic administration compared with the NC group.

Table 1
Immune populations in liver*

Groups†	NKT	CD8+	CD4+	F4/80+
NC	80 ± 8[a]	3 ± 1[a]	21 ± 2[a]	58 ± 8[a]
OC	63 ± 5[b]	5 ± 1[a]	63 ± 4[b]	34 ± 11[b]
O + milk	61 ± 6[b]	6 ± 2[a]	48 ± 11[b,c]	63 ± 8[a]
O + FM	88 ± 6[a]	3 ± 2[a]	40 ± 13[b]	72 ± 10[a]
O + Lc	64 ± 6[b]	5 ± 1[a]	36 ± 2[c]	58 ± 6[a]

FM, fermented milk; Lc, *Lactobacillus casei*; NC, nonobese control; NKT, natural killer T cell; OC, obese control

[a,b,c] Mean for each population without a common letter differs significantly ($P < 0.05$)

* Mononuclear cells isolated from liver of mice were labeled with the specific antibodies and the percentages of cells positive were analyzed by flow cytometry. Results are expressed as average of the percentage obtained for N = 9 mice (from three independent experiments) ± SD.

† O + milk, O + FM and O + Lc: correspond to groups of mice fed high-fat diet and received milk, FM, or a suspension of *L. casei*, respectively.

Milk supplementation increased the number of IL-6–positive and TNF-α–positive cells in non-obese mice, whereas the administration of *L. casei* suspension increased IL-17–positive cells and also IL-6–positive cells (Fig. 3). Cytokine-positive cells increased in the OC group compared with the NC group. None of probiotic dietary supplements were able to restore the normal values (NC group) of IFN-γ–positive and IL-6–positive cells (Fig. 3A); however, in mice that received milk or FM ($P < 0.05$) the number of IL-6–positive cells were significantly decreased compared with the OC group The administration of *L. casei* suspension to HFD mice (O + Lc) increased ($P < 0.05$) the number of IL-6–positive cells in the liver to levels higher than in OC mice (Fig. 3B). HFD mice that received probiotic demonstrated a significant decrease ($P < 0.05$) in the counts of both TNF-α–positive and IL-17–positive cells, reaching the values observed in the NC group (Fig. 3C and D). The number of IL-10–positive cells decreased significantly ($P < 0.05$) in HFD mice given milk or FM compared with the OC group; however, this cytokine remained at an increased level the O + Lc mice (Fig. 3E).

The analysis of TLR-5 expression in liver did not show any significant differences between groups of mice that received conventional balanced diet or HFD or between the test groups given special supplements and the respective controls (data not shown).

Probiotic administration modifies amounts of certain cytokine produced by adipocytes

Figure 4 shows results from the study of different cytokines and the chemokine MCP-1 in the supernatant obtained from cultures of adipocytes. The most remarkable differences between the diet supplementations were obtained for IL-6, TNF-α, and MCP-1, which increased significantly ($P < 0.05$) in the culture supernatant of adipocytes isolated from OC mice compared with those from the NC group (Fig. 4A, B, and D). Adipocytes obtained from HFD mice receiving probiotics (O + FM or O + Lc) showed

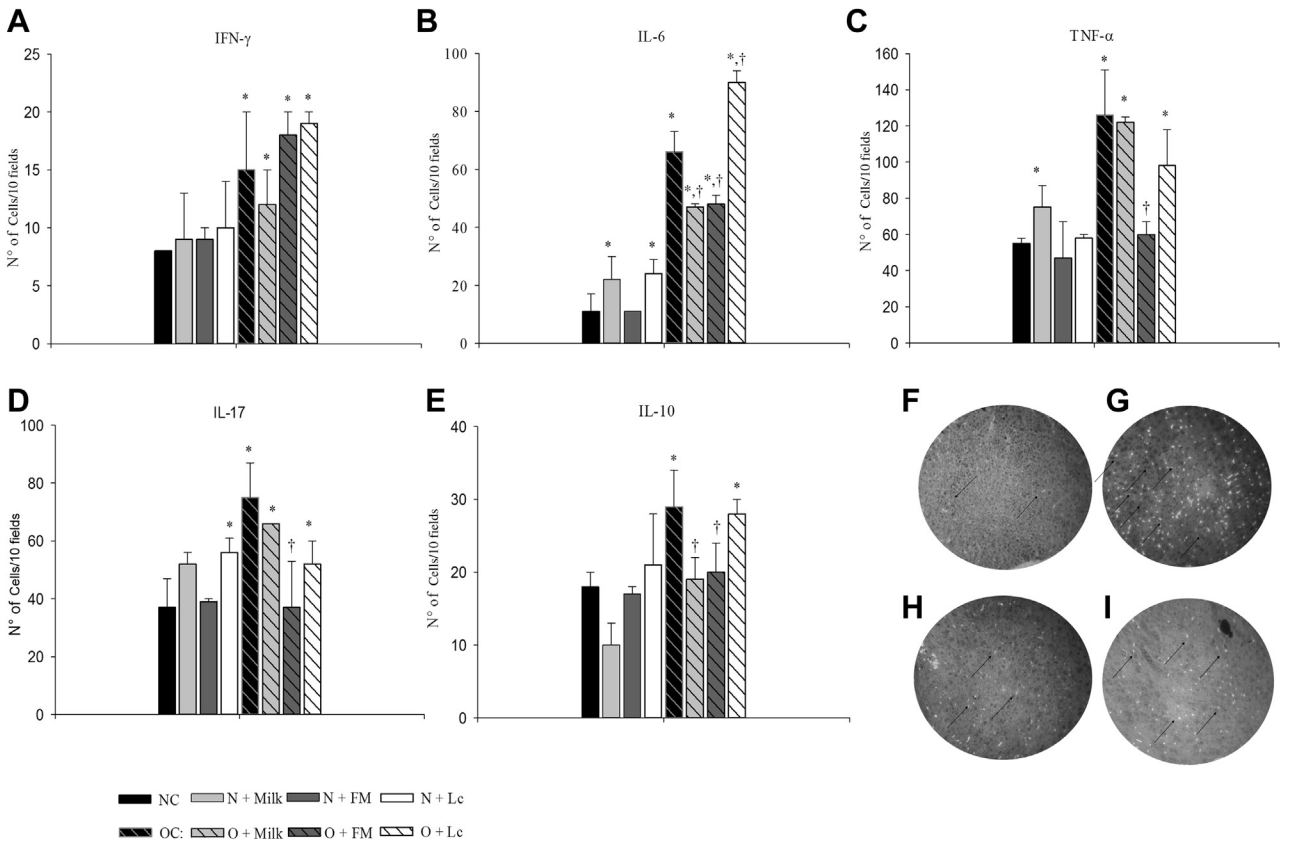

Fig. 3. Effect of high-fat diet (HFD) and probiotics on cytokine-positive cells in the liver. The numbers of cells positive for IFN-γ (A), IL-6 (B), TNF-α (C), IL-17 (D), and IL-10 (E) were determined by indirect immunofluorescence on the liver tissue slides of mice from different groups: nonobese control (NC), nonobese mice given milk, fermented milk (FM) or *Lactobacillus casei* (Lc; N + milk, N + FM, and N + Lc), HFD control mice (OC), and diet-induced obese mice that received milk, FM, or *L. casei* (O + milk, O + FM and O + Lc). The results were expressed as the number of positive cells per 10 fields of vision (1000×). Data correspond to the means ± SD of nine animals from three separated experiments. *Significant difference ($P < 0.05$) with NC. †Significant difference ($P < 0.05$) with OC. Representative figures (100×) of tumor necrosis factor-α–positive cells from a mouse of NC (F), OC (G), O + FM (H), and O + Lc (I) are shown. Arrows show the positive cells (fluorescent cells).

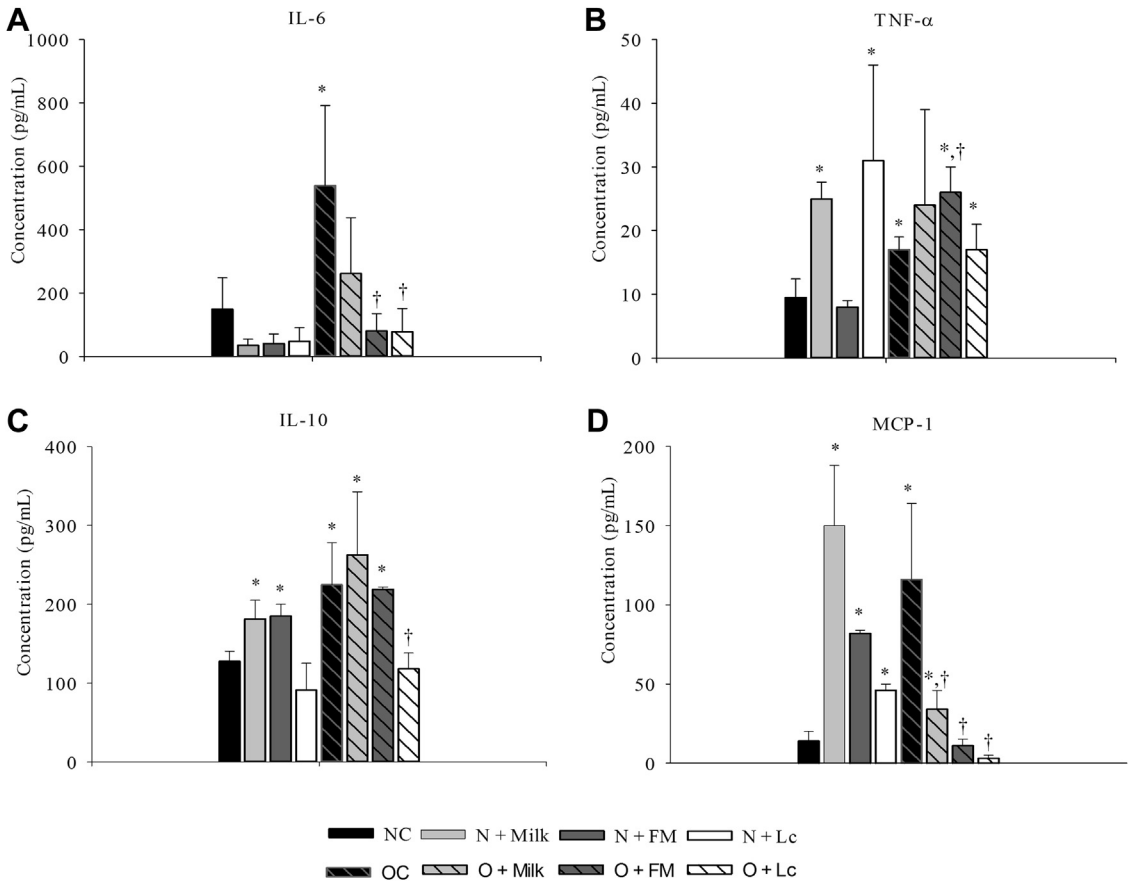

Fig. 4. Effect of probiotic administration to diet-induced obese mice on the cytokines and monocyte chemoattractant protein (MCP)-1 production by adipocytes. Adipocytes were isolated from mesenteric and subcutaneous fat of nonobese control (NC), diet-induced obese control mice (OC), and mice fed the high-fat diet and received milk, FM, or *Lactobacillus casei* (Lc) as suspension (O + milk, O + FM, or O + Lc). Cytokines (A-C) and MCP-1 (D) concentrations were determined in the supernatant of adipocyte cultures. Results were expressed as pg/mL. Data correspond to the mean ± SD of nine animals from three separated experiments. *Significant difference ($P < 0.05$) with NC. †Significant difference ($P < 0.05$) with OC.

lower concentrations of IL-6 and MCP-1 in the culture supernatant compared with adipocytes from the OC group (Fig. 4A and D). TNF-α levels remained high in the culture supernatant of adipocytes isolated from HFD mice (±dietary supplement) compared with the NC group (Fig. 4B). IL-10 levels also were significantly lower ($P < 0.05$) in the adipocyte culture supernatants from the O + Lc group compared with the other HFD groups (Fig. 4C). Non-obese mice receiving either of the dietary supplements demonstrated a significant increase ($P < 0.05$) in the concentration of MCP-1 in the supernatant of adipocyte cultures compared with the NC group (Fig. 4D).

Discussion

In this study, we analyzed the modifications of cytokine profiles in the small intestine, liver, and adipose tissue exerted by the administration of the probiotic strain *L. casei* CRL 431 as suspension or contained in FM, considering the positive effect observed previously in the body weight and clinical parameters in mice receiving probiotic [22]. In that work, it was demonstrated that mice given a balanced diet had a 50% ± 2% body weight gain in the live body during the 2 mo of the experiment. Administration of *L. casei* decreased the body weight gain by 32% ± 4% whether the diet supplementation was given as suspension or FM. At the end of the 2-mo experiment, HFD mice demonstrated >100% increase in body weight; however, the administration of *L. casei* as a supplement to HFD mice resulted in a significant decrease in body weight, especially when the probiotic was given in FM (75% ± 2% of body weight gain). In this study, the effect of *L. casei* CRL 431 as a diet supplement in HFD animals was evaluated and compared with the effect exerted in mice given a balanced diet. Even when some effects were observed in nonobese mice that received the probiotic under study, the most relevant effects were obtained in the diet-induced obese mice, as the parameters selected for analysis were those reported modified by the HFD. The results at the intestinal level showed that HFD mice demonstrated an increase in the production of different proinflammatory cytokines such as TNF-α, IL-6, IL-17; as well as the regulatory cytokine IL-10. These increases in the OC group could be related to the development of an inflammatory state associated with MetS, as was also demonstrated in other obesity-related pathologies such as diabetes type 2 and arteriosclerosis [28–30]. These results agree with those in a study that demonstrated increased levels of TNF-α and IL-6 in the serum of obese individuals compared with nonobese individuals [31]. Regarding IL-17, it has been suggested that obesity predisposes to the generation of a Th17 response [32]. It was also demonstrated that obesity promotes the selective expansion of Th17 cells [33]. T cells of obese mice are expanded and produce progressively more IL-17 than the cells from lean mice in an IL-6–dependent process. The increases observed for IL-10–positive cells in the small intestine of mice

from the OC group could be due to the body's response to the diminishing inflammatory state caused by obesity. In this sense, it was reported that dendritic cells of obese patients produced a significant increase of IL-10 compared with lean patients [34].

The diminution of the inflammatory state in the small intestine of HFD mice that received FM or *L. casei* suspension was associated with the decrease in the number of the cells positive for proinflammatory cytokines, which was accompanied by counts of IL-10–positive cells similar to those obtained in nonobese animals (Fig. 1), and increased levels of this cytokine in the intestinal fluid (Fig. 2). However, in the present study, we could not distinguish what cell population was implicated in the production of IL-10. It is possible that, in addition to dendritic cells (explained above), T-reg lymphocytes are involved in this antiinflammatory response, as was previously reviewed [35]. These results agree with those demonstrated previously where the increases in the IL-10 levels had been considered an advantage due to their antiinflammatory capabilities [36].

TLR-5 was also evaluated because recent studies showed that mice deficient in TLR-5 presented hyperphagia, dyslipidemia, hypertension, insulin resistance, and increased adiposity, which are conducive to the development of the obesity and MetS [37]. TLR-5 recognizes flagellin, which is the major protein component of flagella in both gram-positive and gram-negative bacteria [38]. TLR-5 is expressed in intestinal epithelial cells and in immunologic cells [37,39]. Our obesity model did not show an association with decreased TLR-5 expression (in small intestine or liver), which may be due to the degree of obesity reached in the 2 mo of experiment.

Considering that probiotic supplementation to HFD mice was related to less influx of mononuclear cells in the liver [22], it was also questioned whether *L. casei* supplementation could modulate the influx of some immune cells such as macrophages (F4/80+), CD4+, CD8+, and NKT cells in this organ. The study of CD4+ and CD8+ cells showed significant increases in diet-induced obese mice compared with the NC group. However, HFD mice that received FM or *L. casei* suspension showed significantly lower T-cell infiltration, especially for CD4+ cells, than the OC group. This fact could be related to a decrease in the inflammation observed (Table 1). NKT cells, whose function is to balance the production of pro- and antiinflammatory cytokines, decreased in the livers of OC mice, and increased significantly only in HFD mice that received FM. These results were similar to those obtained in other studies that showed that a probiotic mixture (VSL #3) significantly improved the depletion of the hepatic NKT cells caused by obesity induced by a HFD in a murine model [11]. The number of macrophages (F4/80+) was also evaluated in the liver and showed a significant decrease in the OC group compared with the NC group; however, the three dietary supplements increased these cells, recovering the values observed in the NC group.

To evaluate whether the modifications in the immune populations observed in the liver could be related to the inflammatory state of this organ, cytokine-positive cells were analyzed in the hepatic tissues. The results obtained were similar to the ones observed in the small intestine samples with increases in the number of proinflammatory cytokines accompanying higher amounts of IL-10-positive cells. The antiinflammatory effect observed in the liver of mice that received the probiotic administration was associated with increases of IL-10-positive cells (Fig. 3). It is also important to note that the inflammatory markers were higher in the liver than in the small intestine when compared with nonobese and HFD mice. This last group duplicated or even increased more than twice the number of cytokine-positive cells in the liver compared with mice that received conventional balanced diet. This antiinflammatory response in the liver from HFD mice that received probiotic also can be associated with the improvement in the liver histology reported previously in these animals [22]. The adipocytes isolated from mice of the different control and test groups were analyzed. Results showed that adipocytes from OC mice produced higher secretion of proinflammatory cytokines compared with adipocytes from NC mice. As was observed in the other organs studied, increases of proinflammatory cytokines were accompanied by increases in the secretion of the regulatory cytokine IL-10 by the adipocytes. The obesity is associated with macrophage accumulation in adipose tissue [40]. The secretion of MCP-1 by the adipocytes was also evaluated, showing that adipocytes from OC mice significantly increased the release of this chemokine compared with nonobese mice. The results obtained from HFD mice that received probiotic showed a reduction in the secretion of IL-6 and MCP-1 by the adipocytes, maintaining a regulated immune response through the secretion of IL-10 (Fig. 4).

The results obtained with the different cytokines analyzed in the small intestine, liver, and adipocytes led us to conclude that the administration of the probiotic strain L. casei CRL 431 to HFD mice induced an anti-inflammatory response, evidenced mainly by the decrease of proinflammatory cytokines, such as IL-6, IL-17, and TNF-α. Most of the parameters analyzed did not show significant differences associated with the how probiotic was administered (i.e., as a suspension or as FM). Probiotic administration was also related to fewer immune-infiltrating cells in the liver and a lower secretion of MCP-1 by the adipocytes was associated with less accumulation of macrophages in the adipose tissue.

Conclusion

The results obtained in this study showed that probiotic supplementation to hosts susceptible to developing obesity can be effective, and that this effect is mainly related to the anti-inflammatory capacity of the selected probiotic strain.

References

[1] WHO: Obesity and overweight. Available at: http://www.who.int/mediacentre/factsheets/fs311/en. Accessed February 24, 2015.
[2] Haslam DW, James WP. Obesity. Lancet 2005;366:1197–209.
[3] Romero-Corral A, Montori VM, Somers VK, Korinek J, Thomas RJ, Allison TG, et al. Association of bodyweight with total mortality and with cardiovascular events in coronary artery disease: a systematic review of cohort studies. Lancet 2006;368:666–78.
[4] Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 2004;92:347–55.
[5] FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report 2001. Available at: www.fao.org/es/ESN/probio/probio.htm.
[6] Maldonado Galdeano C, Novotny Nunez I, de Moreno de LeBlanc A, Carmuega E, Weill R, Perdigon G. Impact of a probiotic fermented milk in the gut ecosystem and in the systemic immunity using a non-severe protein-energy-malnutrition model in mice. BMC Gastroenterol 2011;11:64.
[7] Maassen CB, van Holten-Neelen C, Balk F, den Bak-Glashouwer MJ, Leer RJ, Laman JD, et al. Strain-dependent induction of cytokine profiles in the gut by orally administered Lactobacillus strains. Vaccine 2000;18:2613–23.
[8] Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 2002;168:171–8.
[9] Hamad EM, Sato M, Uzu K, Yoshida T, Higashi S, Kawakami H, et al. Milk fermented by Lactobacillus gasseri SBT2055 influences adipocyte size via inhibition of dietary fat absorption in Zucker rats. Br J Nutr 2009;101:716–24.
[10] Yadav H, Jain S, Sinha PR. Antidiabetic effect of probiotic dahi containing Lactobacillus acidophilus and Lactobacillus casei in high fructose fed rats. Nutrition 2007;23:62–8.

[11] Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 2008;49:821–30.
[12] Arora T, Singh S, Sharma RK. Probiotics: interaction with gut microbiome and antiobesity potential. Nutrition 2013;29:591–6.
[13] da Silva ST, Dos Santos CA, Bressan J. Intestinal microbiota, relevance to obesity and modulation by prebiotics and probiotics. Nutr Hosp 2013;28:1039–48.
[14] Park DY, Ahn YT, Park SH, Huh CS, Yoo SR, Yu R, et al. Supplementation of Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032 in diet-induced obese mice is associated with gut microbial changes and reduction in obesity. PLoS One 2013;8:e59470.
[15] Chen J, Wang R, Li XF, Wang RL. Bifidobacterium adolescentis supplementation ameliorates visceral fat accumulation and insulin sensitivity in an experimental model of the metabolic syndrome. Br J Nutr 2012;107:1429–34.
[16] Wan YM, Zhu YQ, Xia B, Luo J. Treating TNBS-induced colitis in rats with probiotics. Turk J Gastroenterol 2011;22:486–93.
[17] Chaves S, Perdigon G, de Moreno de LeBlanc A. Yoghurt consumption regulates the immune cells implicated in acute intestinal inflammation and prevents the recurrence of the inflammatory process in a mouse model. J Food Prot 2011;74:801–11.
[18] Galdeano CM, Perdigon G. Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. J Appl Microbiol 2004;97:673–81.
[19] Perdigon G, Maldonado Galdeano C, Valdez JC, Medici M. Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 2002;56 Suppl 4:S21–26.
[20] Galdeano CM, Perdigon G. The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol 2006;13:219–26.
[21] Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet ME, Perdigon G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin Vaccine Immunol 2007;14:485–92.
[22] Nunez IN, Galdeano CM, de LeBlanc Ade M, Perdigon G. Evaluation of immune response, microbiota, and blood markers after probiotic bacteria administration in obese mice induced by a high-fat diet. Nutrition 2014;30:1423–32.
[23] Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007;50:2374–83.
[24] Castillo NA, Perdigon G, de Moreno de Leblanc A. Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against Salmonella enterica serovar Typhimurium infection in mice. BMC Microbiol 2011;11:177.
[25] Soulard V, Roland J, Sellier C, Gruner AC, Leite-de-Moraes M, Franetich JF, et al. Primary infection of C57 BL/6 mice with Plasmodium yoelii induces a heterogeneous response of NKT cells. Infect Immun 2007;75:2511–22.
[26] Ishii-Yonemoto T, Masuzaki H, Yasue S, Okada S, Kozuka C, Tanaka T, et al. Glucocorticoid reamplification within cells intensifies NF-kappaB and MAPK signaling and reinforces inflammation in activated preadipocytes. Am J Physiol Endocrinol Metab 2010;298:E930–40.
[27] Parra P, Bruni G, Palou A, Serra F. Dietary calcium attenuation of body fat gain during high-fat feeding in mice. J Nutr Biochem 2008;19:109–17.
[28] Yudkin JS, Yajnik CS, Mohamed-Ali V, Bulmer K. High levels of circulating proinflammatory cytokines and leptin in urban, but not rural, Indians. A potential explanation for increased risk of diabetes and coronary heart disease. Diabetes Care 1999;22:363–4.
[29] Pickup JC, Chusney GD, Mattock MB. The innate immune response and type 2 diabetes: evidence that leptin is associated with a stress-related (acute-phase) reaction. Clin Endocrinol 2000;52:107–12.
[30] Pickup JC, Mattock MB, Chusney GD, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia 1997;40:1286–92.
[31] Khaodhiar L, Ling PR, Blackburn GL, Bistrian BR. Serum levels of interleukin-6 and C-reactive protein correlate with body mass index across the broad range of obesity. JPEN J Parenter Enteral Nutr 2004;28:410–5.
[32] Ahmed M, Gaffen SL. IL-17 in obesity and adipogenesis. Cytokine Growth Factor Rev 2010;21:449–53.
[33] Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, et al. Obesity predisposes to Th17 bias. Eur J Immunol 2009;39:2629–35.
[34] O'Shea D, Corrigan M, Dunne MR, Jackson R, Woods C, Gaoatswe G, et al. Changes in human dendritic cell number and function in severe obesity may contribute to increased susceptibility to viral infection. Int J Obes (Lond) 2013;37:1510–3.
[35] Matarese G, Procaccini C, De Rosa V, Horvath TL, La Cava A. Regulatory T cells in obesity: the leptin connection. Trends Mol Med 2010;16:247–56.
[36] Satoh N, Shimatsu A, Kotani K, Himeno A, Majima T, Yamada K, et al. Highly purified eicosapentaenoic acid reduces cardio-ankle vascular index in association with decreased serum amyloid A-LDL in metabolic syndrome. Hypertens Res 2009;32:1004–8.
[37] Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010;328:228–31.
[38] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099–103.
[39] Romagne F. Current and future drugs targeting one class of innate immunity receptors: the toll-like receptors. Drug Discov Today 2007;12:80–7.
[40] Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796–808.