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Basic nutritional investigation

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

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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

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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