Nutrition 30 (2014) 1423–1432

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Evaluation of immune response, microbiota, and blood markers after probiotic bacteria administration in obese mice induced by a high-fat diet

Ivanna Novotny Núñez Ph.D. [a], Carolina Maldonado Galdeano Ph.D. [a,b], Alejandra de Moreno de LeBlanc Ph.D. [a], Gabriela Perdigón Ph.D. [a,b,*]

[a] Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Tucumán, Argentina
[b] Cátedra de Inmunología. Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

ARTICLE INFO

Article history:
Received 6 December 2013
Accepted 30 March 2014

Keywords:
Obesity
Probiotic
Fermented milk
Humoral immune response
Gut immunity
Gut microbiota

ABSTRACT

Objective: Obesity is associated with alterations in intestinal microbiota and immunity. The aim of this study was to determine the effect of probiotic *Lactobacillus casei* CRL 431 administration on intestinal and humoral immune response, clinical parameters, and gut microbiota was evaluated using a high-fat diet to induce obesity in a mouse model.
Methods: Adult mice received a conventional balanced diet or a high-fat diet supplemented with milk, milk fermented by *Lactobacillus casei* (FM), *L. casei* as suspension, or water over 60 d. Histology of liver and small intestine (SI), immunoglobulin A-positive cells and macrophages in SI, phagocytic activity of spleen and peritoneal macrophages, and humoral immune response to ovalbumin were studied. Clinical parameters in serum and gut microbiota were also analyzed.
Results: FM was the most effective supplement for decreasing body weight and clinical parameters in serum. The histology of liver and SI was also improved in obese mice given FM. These animals had increased numbers of immunoglobulin A-positive cells and macrophages in SI. The gut microbiota showed that obese mice given probiotics had increased Bacteroides and bifidobacteria. Administration of FM or *L. casei* as suspension enhanced the phagocytic activity of macrophages. The anti-ovalbumin specific immune response was not increased by any supplement assayed.
Conclusion: Administration of probiotics to obese hosts improved the gut microbiota and the mucosal immunity altered by obesity, down-regulated some biochemical parameters in blood associated with metabolic syndrome, and decreased liver steatosis. These results demonstrate the potential use of probiotics in obese individuals to decrease the body weight and to improve the biochemical and immunologic parameters altered by obesity.

Introduction

Obesity has become a serious public health problem and has reached epidemic proportions worldwide, not only in industrialized countries but also in developing countries. Each year, as a result of obesity or overweight, at least 2.6 million individuals are more likely to suffer a heart attack or to die from pathologies related to obesity [1].

Obesity has a multifactorial origin and is strongly associated with: (1) an inflammatory process in which adipose tissue plays an important role [2], (2) liver steatosis, and (3) insulin resistance [3].

This work was financially supported by (Consejo Nacional de Investigaciones Científicas y Técnicas) grant, CONICET PIP 0652 and CIUNT 26/D 442 (Universidad Nacional de Tucumán), 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 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 declared no competing interests.

* Corresponding author: Tel.:+54 381 431 0465 × 132; fax: +54 381 431 0465 × 124.
E-mail address: perdigon@cerela.org.ar (G. Perdigón).

0899-9007/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nut.2014.03.025

Intestinal microbiota is also modified in the obese host [4]. Studies in animal models and humans showed that the composition of the gut microbiota differs in lean versus obese hosts [5]. These studies suggested the potential role of gut microbiota in the development of obesity and the possible beneficial effect of modifying the gut microbiota as a tool for future treatments. In light of this, probiotic supplementation of the diet appears to be beneficial in combating obesity and its related disorders, due especially to the anti-inflammatory effects of these microorganisms [6].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, provide a health benefit to the host [7]. The beneficial effect of probiotic microorganisms on the obese host has been reported [8,9], and different mechanisms of action were suggested. Probiotics can modulate the lipid profile associated with obesity [10]. A recent study showed that *Bifidobacterium pseudocatenulatum* CECT 7765 modulated metabolic parameters in mice fed a high-fat diet by modifying the expression of key regulators of fatty acid and cholesterol metabolism and transport, lipid levels, and glucose levels in the liver [11]. It has been demonstrated that the combination of two probiotic lactobacillus altered hepatic lipid metabolism in a mouse diet-induced obesity model [12]. It was also described that probiotics can beneficially affect immunologic disorders associated with obesity. In light of these findings, the gene expression of different cytokines and transcription factors in immune cells was analyzed using obese models or obese individuals. Probiotic *Lactobacillus gasseri* SBT2055 prevented body weight gain, and proinflammatory gene expression in the adipose tissue of mice fed a high-fat diet [13]. Another recent study showed that the administration of probiotic yogurt modulated gene expression in the peripheral blood mononuclear cells of overweight and obese individuals [14]. The immunomodulatory effect of different probiotic strains was also demonstrated [15–19].

L. casei CRL 431 is a probiotic bacterium. The mechanisms involved in the immune stimulation mediated by *L. casei* CRL 431 have been extensively studied using experimental models [20–23].

This probiotic strain was also evaluated in clinical studies performed in humans, documenting its effects in different conditions [15–19]. *L. casei* CRL 431 is contained in probiotic products that are consumed in Argentina, and also in other countries such as Chile and Costa Rica. Considering these previous results, specifically the capacity of *L. casei* CRL 431 to activate the gut immune response [20–23], our hypothesis was that supplementation with this probiotic strain would exert a beneficial effect in the intestinal ecosystem on the alterations of the microbiota and the immune response observed in obese hosts, and thereby decreasing other alterations associated with obesity (such as metabolic syndrome, gut immunity, and liver damage). The aim of this study was to evaluate the effect of continuous administration of probiotic bacterium *L. casei* CRL 431 or milk fermented by *L casei* (FM) on different parameters in the obese host by analyzing some parameters of the gut and systemic immune response, microbiota, and blood markers in an obesity mouse model induced by a high-fat diet.

Methods and materials

Bacterial strain and fermented milk

L. casei CRL 431 was obtained from the CERELA Culture Collection (San Miguel de Tucumán, Argentina). Cultures were grown overnight at 37°C in 5 mL sterile Mann-Rogosa-Sharp (MRS) broth (Britania, Buenos Aires, Argentina). The cells were harvested by centrifugation at 5000g for 10 min, washed three times with fresh phosphate-buffered saline (PBS) and then resuspended in 5 mL of sterile 10% (wt/vol) reconstituted non-fat milk. The *L. casei* CRL 431 suspension was diluted 1:30 in water and administered ad libitum to the mice. The final concentration of probiotic bacteria in the drinking water was $2 \pm 1 \times 10^8$ CFU/mL. This count was periodically controlled at the beginning of the administration and each 24 h to avoid modifications of more than one logarithmic unit. The content of the bottles was replaced daily.

To obtain the FM, non-fat dried milk was rehydrated (10% wt/vol) and autoclaved (115°C for 15 min). Overnight cultures of *L. casei* CRL 431 were grown in MRS broth as previously explained. The milk was inoculated with 2% vol/vol of the *L. casei* culture, and incubated at 37°C for 24 h. The final concentration of the probiotic bacteria in the fermented milk was $8 \pm 2 \times 10^8$ CFU/mL. This fermented product was prepared every 2 d and the microbial concentration was monitored. The bottles with FM were replaced daily.

Experimental groups

Protocol to induce obesity by a high-fat diet and sampling procedure

Five-wk-old female BALB/c mice (25 ± 2 g), were obtained from the closed random bred colony maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Mice were divided into two groups: non-obese (N) and obese (O). The non-obese group was fed ad libitum with conventional balanced diet (45% carbohydrates, 32% fat, 23% proteins, 6% raw fiber, 10% total minerals, 1.3% calcium, 0.8% phosphorus, 12% moisture, and vitamins) provided by ACA Nutrition Animal (San Nicolas, Buenos Aires, Argentina). Considering that the daily intake of a mouse is 3 g of food, the caloric contribution of the conventional balanced diet would be 16.8 kcal/d. The obese group was fed ad libitum with a high-fat diet that was made in the laboratory using the same conventional balanced diet, with bovine lard and sugar added (both for human consumption, purchased in the supermarket), over 60 d. The high-fat diet contained 43.4% of the conventional balanced diet, 43.4% of bovine lard, and 13.2% of sugar; its caloric contribution was 18.6 kcal/d. These caloric values do not include calories provided by the different dietary supplements. No great variation in calorie intake was observed between the two groups; the main difference was in the percentage of fat between the two diets (conventional and high-fat diet).

Each group was divided into four subgroups (one control and three test) according to the addition of the dietary supplement.

Mice in the N group received a conventional balanced diet and water ad libitum during entirety of the experiment.

We performed three test groups to evaluate the effect of different dietary supplements: milk (N + milk), FM (N + FM), or a suspension of *L. casei* CRL 431 (N + Lc), ad libitum for the duration of the experiment (Fig. 1).

Mice in group O received a high-fat diet and water ad libitum for the duration of the experiment.

We performed the following test obese groups: O + milk, O + FM, and O + Lc. Each test group received a high-fat diet supplemented with milk, FM, or suspension of *L. casei*, respectively for the duration of the experiment (Fig. 1).

Each mouse consumed between 3 and 4 mL of water, milk, FM, or Lc daily.

Mice (n = 6/group) were maintained in collective cages (three animals/cage) in an environmentally controlled room (20°C ± 2°C and 70% ± 5% of humidity), with a 12-h light–dark cycle and unlimited free access to water and the respective food. They were weighed every 5 d during the 60-d experimental period. Three mice from each control and test group were sacrificed by cervical dislocation at day 60. The rest of the animals from each group (three mice) were maintained in the same conditions and used for evaluation of the humoral immune response. Liver was removed for histologic studies. Blood was recovered by cardiac puncture to analyze clinical parameters and the small and large intestine for histologic and microbiology studies, respectively. Both spleen and peritoneal macrophages were extracted for phagocytic activity evaluation.

The experiments were repeated three times. All animal protocols were preapproved by the Animal Protection Committee of CERELA (CRL-BIOT-LI-2010/1 A) and all experiments comply with the current laws of Argentina.

Evaluation of the humoral immune response to ovalbumin antigen

To evaluate systemic immunity, the assay was performed at day 60 of the experiment (Fig. 1). Three mice from each group were injected subcutaneously, three times every 48 h, with 15 μg of chicken egg albumin (OVA; Sigma, St. Louis, MO, USA) in PBS solution. The mice from N or O test groups continued receiving the corresponding diet supplemented with milk, FM, or *L. casei* during and after immunization until the end of the experiment. The mice were sacrificed 10 d after the last OVA injection (day 75 of the experiment), and blood was collected to determine specific anti-OVA immunoglobulin (Ig)G in the serum by enzyme-linked immunosorbent assay (ELISA) test, using plates coated with ovalbumin and a goat anti-mouse IgG antibody conjugated with biotin-SP (Jackson Immuno Research Labs Inc, West Grove, PA) to detect the specific anti-OVA IgG, as described previously [24]. Peritoneal and spleen macrophages were also isolated on day 75 to analyze the phagocytic activity according to a previously described technique [24].

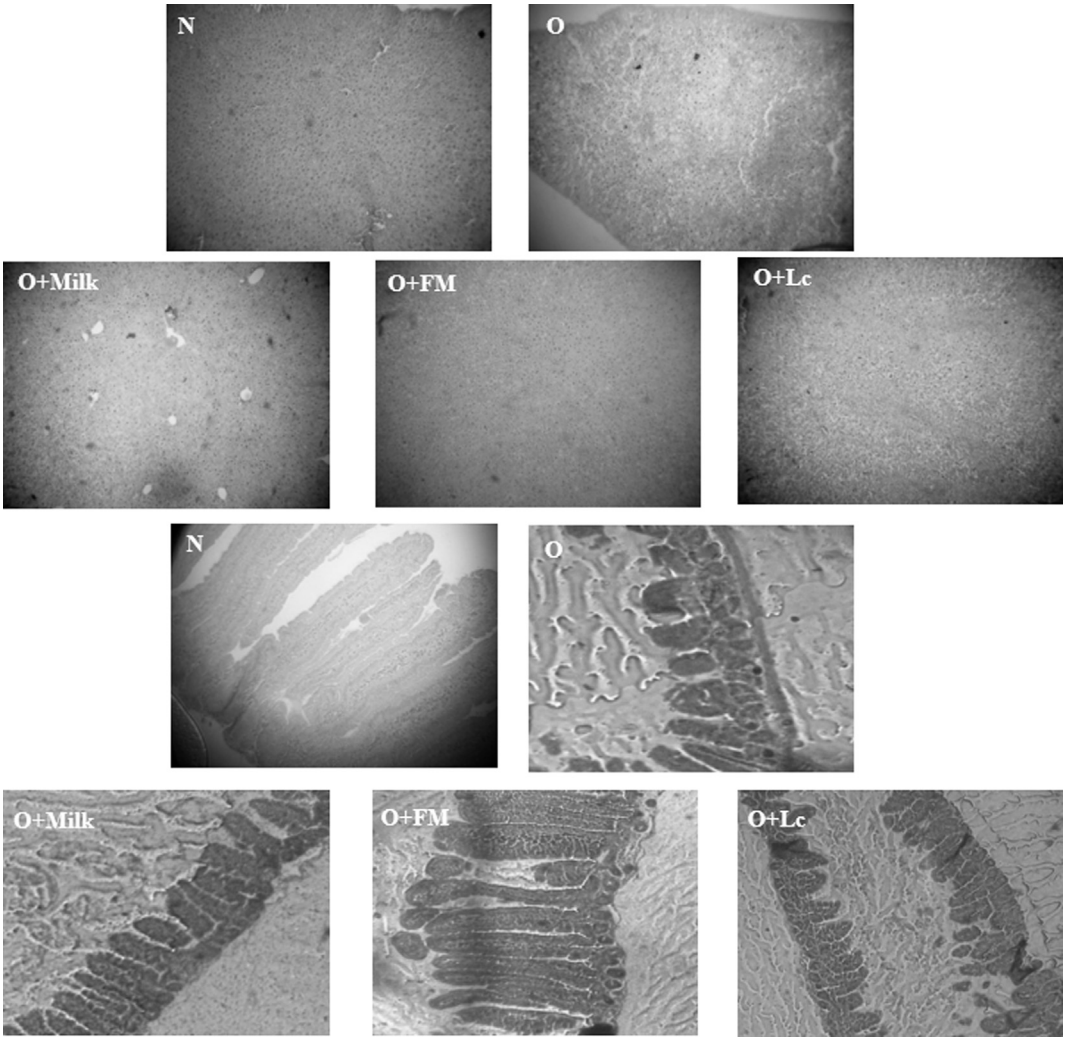

Fig. 1. Design of the experimental protocol. *Control groups received conventional balanced diet (non-obese or N group) or high-fat diet (obese or O group) without special supplements. **Test groups received conventional balanced diet with special supplements (N + milk, N + fermented milk [FM], or N + *L. casei* CRL 431 [Lc]) or high-fat diet with the different dietary supplements (O + milk, O + FM, or O + Lc). Systemic immunization protocol is also schematized and shows the days of OVA injections (first, second, and third).

Determination of clinical parameters

Mice were sacrificed on day 60, and blood was collected by cardiac puncture, centrifuged at 1000g for 10 min and the serum was separated. Serum glucose, triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined using enzymatic methods, provided in commercial kits (Wiener Lab., Rosario, Argentina). Results were expressed as concentration of each parameter in the serum (mg/dL).

Analysis of the intestinal microbiota

Large intestines were aseptically removed at 60 d, weighed and placed into sterile tubes containing 5 mL of peptone water (0.1%). The samples were immediately homogenized under sterile conditions using a micro homogenizer (MSE, England), to disintegrate the tissue without damaging the microorganisms present in the lumen, and the total populations of some microorganisms were determined. Serial dilutions of the homogenized samples were obtained and aliquots (0.1 mL) of the appropriate dilution were spread onto the surface of the different agarized media (obtained from Britania, Buenos Aires, Argentina): MacConkey agar for *Enterobacter*; MRS for *Lactobacilli*; Reinforced Clostridial Agar (RCA) for total anaerobes; RCA containing 0.2% LiCl, 4 mg/L colistin, 1% aniline blue and adjusted to pH 5.0 with acetic acid, for bifidobacteria; and Brucella Agar Base for Bacteroides. MacConkey and MRS were aerobically incubated at 37°C for 24 and 48 h, respectively, and the other culture media were anaerobically incubated at 37°C for 72 to 96 h.

Histologic samples of liver and small intestine

The liver and the small intestine were removed, fixed in formaldehyde 10% solution in PBS pH 7.0. After fixation, the tissues were dehydrated and embedded in paraffin using conventional methods [25]. Livers were cut into small pieces (five per organ) to facilitate the dehydration and embedding processes, and all the pieces were placed in the same paraffin block. For small intestine, five small pieces (~1.5 cm) were cut, covering different parts of the intestine along its entire length, and these five pieces were placed in the same paraffin block.

Serial tissue sections of 4 μm from each paraffin block (corresponding to one mouse), were made using a rotation microtome. Nine slices from each subgroup (obtained from three mice per experiment) were stained with hematoxylin & eosin to be analyzed in the optical microscopy.

Immunofluorescence assay for IgA-secreting cells and macrophages in the small intestine tissues

IgA-positive cells in the lamina propria of the small intestine tissues were determined by direct immunofluorescence assays. Slides were incubated with α-chain monospecific antibody conjugated with fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO, USA). Macrophages were determined by indirect immunofluorescence as described previously [26]. The BM8 monoclonal antibody (Affinity Purified antimouse F4/80 Antigen-Pan Macrophage Marker, e-Bioscience, San Diego, CA, USA) was used to evaluate these cells. The results were expressed as the number of positive cells per 10 fields of vision (magnification 1000 ×) using a fluorescent light microscope.

Ex vivo phagocytosis assay of peritoneal and spleen macrophages

Peritoneal and spleen macrophages were isolated from mice of all control and test groups before OVA immunization on day 60 and after OVA immunization on day 75. Phagocytosis assay was performed using *Saccharomyces boulardii* suspension (Hansen CBS 5926 from Floratil, MERCK Quimica, Argentina) at a concentration of 10^7 cell/mL, as described previously [24]. Equal volumes of opsonized *S. boulardii* were mixed with 10^6 cells/mL of macrophage's suspensions. The mixture was incubated for 30 min at 37°C. Phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells counted using an optical microscope.

Statistical analysis

Three mice from each group were sacrificed in each sample on day 60 and on day 75 of the 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 ANOVA general linear model followed by a Tukey's post hoc test and $P < 0.05$ was considered significant.

No significant differences were observed between the three independent replicates; results from three replicates were combined and, with exception of the body weight evaluation, the comparisons were obtained from nine animals. For the body weight, six mice from each group (three sacrificed at day 60, and three after immunization protocol) were evaluated in each repetition of the experiment and the comparisons were obtained from 18 animals.

Results

Effect of milk, FM, or Lc on body weight

Body weight increased significantly in the mice from O group compared with N group. Each mouse consumed between 3 and 4 mL of water, milk, FM or Lc suspension per day, meaning there were no major differences in the quantity of consumption for the different products that could influence animal weight. N + FM or N + Lc mice showed a significant decrease ($P < 0.05$) in body weight beginning on day 35 compared with N + milk mice. Only the animals with induced obesity given FM showed a significant decrease in body weight compared with O group, from day 40 until the conclusion of the experiment (Fig. 2). A significant increase in body weight gain in O + Lc mice was seen at days 50 and 55; however, these differences were related to a decrease in body weight in the mice from group O that was reverted at day 60, without significant differences with the O + Lc group.

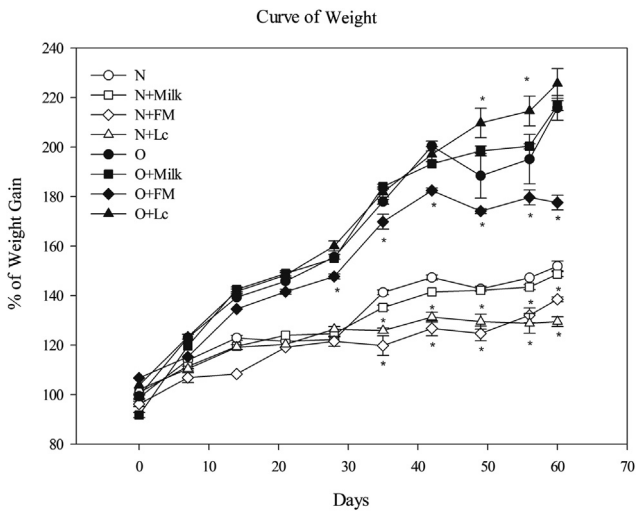

Fig. 2. Changes in body weight. Mice from non-obese groups (N) (open symbols) and obese groups (O) (filled symbols) without special supplement or receiving different dietary supplement (milk, fermented milk, or *L. casei* CRL 431 diluted in the drinking water [Lc]) were weighed weekly. For each trial, each group consisted of six mice. Data are expressed as percentage of weight gain above of the initial weight (100%). Each point represents the mean ± SEM of the percentage of weight gain obtained from the three independent trials (N = 18). *Significant decrease ($P < 0.05$) compared with the respective control at the same time point.

Effect of milk, FM, or Lc on clinical parameters in blood serum

The analysis of the glucose in blood serum showed a significant increase ($P < 0.05$) in group O compared with all N groups. The administration of milk and *L. casei* to obese mice did not modify glucose concentration compared with group O. O + FM mice were the only animals that showed a significant decrease ($P < 0.05$) of glucose concentration compared with the O and O + milk groups; however, they did not reach the values of non-obese mice (Table 1).

TGs increased significantly ($P < 0.05$) in N + FM and N + Lc mice compared with the N group. All the obese groups also showed significant increases in TG concentrations compared with the N group. Supplementation with FM or *L. casei* suspension to diet-induced obese mice (O + FM and O + Lc, respectively) significantly decreased concentrations of TG ($P < 0.05$) compared with the O group, without reaching the values obtained in the N group (Table 1).

Total cholesterol, LDL-cholesterol and HDL-cholesterol concentrations showed significant increases in the serum of the O group compared with the N group. Probiotic administration to non-obese mice did not modify cholesterol levels in blood serum; however, the obese groups given probiotic supplements showed significant ($P < 0.05$) decreases of cholesterol concentrations (total, LDL and HDL) compared with the O group. Diet induced obese mice that received milk (O + milk) also decreased total and HDL-cholesterol levels compared with the O group, but showed an important variability in the LDL-cholesterol concentration (Table 1).

Effect of milk, FM or L. casei supplementation on intestinal microbiota

The study of the intestinal microbiota did not show differences for total anaerobes or lactobacilli counts between control and test subgroups for both non-obese and obese groups. For enterobacteria, a significant increase was observed in the non-obese mice that received any special diet supplement (N + milk, N + FM and N + Lc) compared with the N group. Enterobacteria counts were not affected by the high fat diet. The control and test groups with induced obesity maintained the mean $\log_{10}$ CFU/g similar to the mean values obtained from the N group.

No significant modifications for bifidoacteria counts were observed between the O and N groups. This population showed a significant increase ($P < 0.05$) in the non-obese or obese subgroups that received FM or *L. casei* supplementation, compared with other control and test groups. Bacteroides counts increased significantly ($P < 0.05$) in non-obese mice that receive probiotic (N + FM and N + Lc) compared with the N group. This population decreased significantly ($P < 0.05$) in the large intestine of the O group compared with the N group, while the three supplementations to diet-induced obese mice increased bacteriodes counts compared with the O group. The O + FM which showed the highest counts (Table 2).

Table 1
Biochemical parameters in blood serum

Group	Glucose	TG	TC	LDL-C	HDL-C
N	0.50 ± 0.09[a]	1.30 ± 0.06[a]	0.60 ± 0.09[a]	0.45 ± 0.08[a]	0.73 ± 0.11[a]
N + milk	0.47 ± 0.13[a]	1.35 ± 0.14[a,b]	0.85 ± 0.02[b]	0.61 ± 0.03[b]	0.77 ± 0.08[a]
N + FM	0.29 ± 0.20[a]	1.83 ± 0.10[c]	0.50 ± 0.06[a]	0.40 ± 0.38[a,b]	0.78 ± 0.03[a]
N + Lc	0.29 ± 0.25[a]	1.56 ± 0.09[b]	0.75 ± 0.13[a,b]	0.56 ± 0.04[a,b]	0.75 ± 0.07[a]
O	1.27 ± 0.07[b]	3.45 ± 0.65[e]	1.96 ± 0.19[c]	1.22 ± 0.20[c]	1.12 ± 0.01[b]
O + milk	1.23 ± 0.01[b]	3.38 ± 0.18[d]	1.32 ± 0.33[d,e]	0.71 ± 0.34[a,b,c]	0.60 ± 0.31[a,c]
O + FM	1.14 ± 0.04[c]	2.23 ± 0.50[e]	1.03 ± 0.15[d]	0.51 ± 0.22[a,b]	0.78 ± 0.14[a,c]
O + Lc	1.35 ± 0.35[b,c]	2.37 ± 0.41[e]	1.63 ± 0.03[e]	0.67 ± 0.12[a,b]	0.98 ± 0.07[c]

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride
Biochemical parameters were measured in the serum of mice from different experimental groups: non-obese control (N), obese control (O), and the test subgroups that received the special dietary supplement: milk, fermented milk (FM), or *L. casei* CRL 431 (Lc). Results were obtained using enzymatic methods and were expressed as mg/dL. Values are the means for N = 9 mice ± SD obtained in each group (from three independent experiments). Means for each clinical parameter without a common letter (a,b,c,d,e) differ significantly ($P < 0.05$)

Effect of milk, FM or L. casei administration on the liver and small intestine histology

The histology of the liver was affected by the high fat diet in the O group, where a steatosis was observed compared with the N group (Fig. 3 A and 3 B). Mice from the O + milk group did not show significant differences in the liver histology compared with the O group (Fig. 3 C). These histologic alterations were improved in mice that received the high fat diet supplemented with FM or *L. casei* (Fig. 3 D and 3 E). The addition of any assayed dietary supplements to N mice did not induce modification in the liver histology (data not shown).

The histology of the small intestine showed that the villi were shorter in the O group than in the N group (Fig. 3 F and 3 G). The administration of FM to obese animals improved these histologic alterations (Fig. 3 I) as well as the administration of milk or *L. casei* (Fig. 3 H and 3 J, respectively); however, this improvement was lower in these mice than in the animals receiving FM (O + FM).

Effect of the diet and diet supplementations to obese mice on the number of intestinal IgA secreting cells and macrophages

The number of IgA + cells did not show differences between control and test groups in the non-obese mice. IgA + cells decreased significantly in the O group compared with the N group. Only mice from the O + FM group had significantly increased ($P < 0.05$) numbers of IgA + cells in the small intestine compared with other obese groups, reaching values similar to the N group (Fig. 4 A). For the number of macrophages, no significant differences were observed between non-obese mice with different diet supplementations. The O group showed a significant ($P < 0.05$) decrease with respect to the N group. Mice from O + milk, O + FM or O + Lc maintained the number of macrophages without significant differences compared with the N group (Fig. 4 B).

Effect on systemic antibody response

Specific anti-OVA IgG did not show significant differences in the serum of mice from the N control group and the three non-obese test groups (N + milk, N + FM or N + Lc). Specific anti-OVA IgG decreased significantly in the serum of mice that received the high fat diet (O group) compared with the N group, being O + milk and O + FM, the groups that showed the lowest values, only *L. casei* supplementation maintained the values of the O control group (Fig. 5 A).

Determination of the phagocytic activity of macrophages isolated from spleen and peritoneum before and after OVA immunization

The percentage of phagocytic activity of spleen macrophages increased significantly in mice from N + FM compared with the N group, previous and after OVA immunization. The O group had significantly decreased phagocytic activity of spleen macrophages compared with the N group before the immunization. This activity prior to immunization was increased in the O + FM and O + Lc groups. The obese group (O) showed a significant ($P < 0.05$) increase in the phagocytic activity in the samples taken after immunization, compared with the N group (Fig. 5 B). Diet-induced obese mice that received *L. casei* (O + Lc) had significantly increased capacity in the macrophages isolated from spleen compared with the O group, reaching values higher than the N group before the immunization (Fig. 5 B).

Table 2
Changes in the intestinal microbiota of the large intestine

Groups	*Enterobacteriaciae*	*Lactobacilli*	Total anaerobes	*Bifidobacteria*	*Bacteroides*
N	4.1 ± 0.1[a]	8.1 ± 0.6[a]	8.9 ± 0.7[a,b]	1.9 ± 0.1[a,c]	2.6 ± 0.5[a,d]
N + milk	7.1 ± 1.1[b]	8.3 ± 0.7[a,c]	9.3 ± 0.3[a,b]	1.8 ± 0.02[a]	2.9 ± 0.2[a]
N + FM	5.6 ± 0.6[c,d]	7.9 ± 0.3[b,a]	9.0 ± 0.1[a]	7.1 ± 0.5[b]	4.9 ± 0.04[b,e]
N + Lc	5.6 ± 0.1[d]	8.9 ± 0.2[a,c]	9.6 ± 0.2[b]	7.7 ± 0.4[b]	3.8 ± 0.07[c]
O	3.9 ± 1.4[a,c]	9.0 ± 0.4[a,c]	9.4 ± 0.5[a,b]	2.0 ± 0.06[c]	2.1 ± 0.03[d]
O + milk	4.0 ± 1.9[a,d]	8.8 ± 1.0[a,b,c]	9.1 ± 0.5[a,b]	2.1 ± 0.03[d]	3.7 ± 1.1[a,c,e]
O + FM	4.9 ± 1.2[a,b,d]	8.9 ± 0.6[a,b,c]	9.4 ± 0.3[a,b]	7.6 ± 0.1[b]	4.2 ± 0.7[e]
O + Lc	4.1 ± 0.9[a,c]	9.2 ± 0.2[c]	9.5 ± 0.4[a,b]	7.6 ± 0.1[b]	3.9 ± 0.2[c]

FM fermented milk; Lc, *L. casei* CRL 431; N, non-obese control; O, obese control
Results are expressed as means ± SD of the $\log_{10}$ CFU/g of large intestine. Each mean represents data from nine animals (from three independent experiments). Means for each medium without a common letter (a,b,c,d,e) differ significantly ($P < 0.05$)

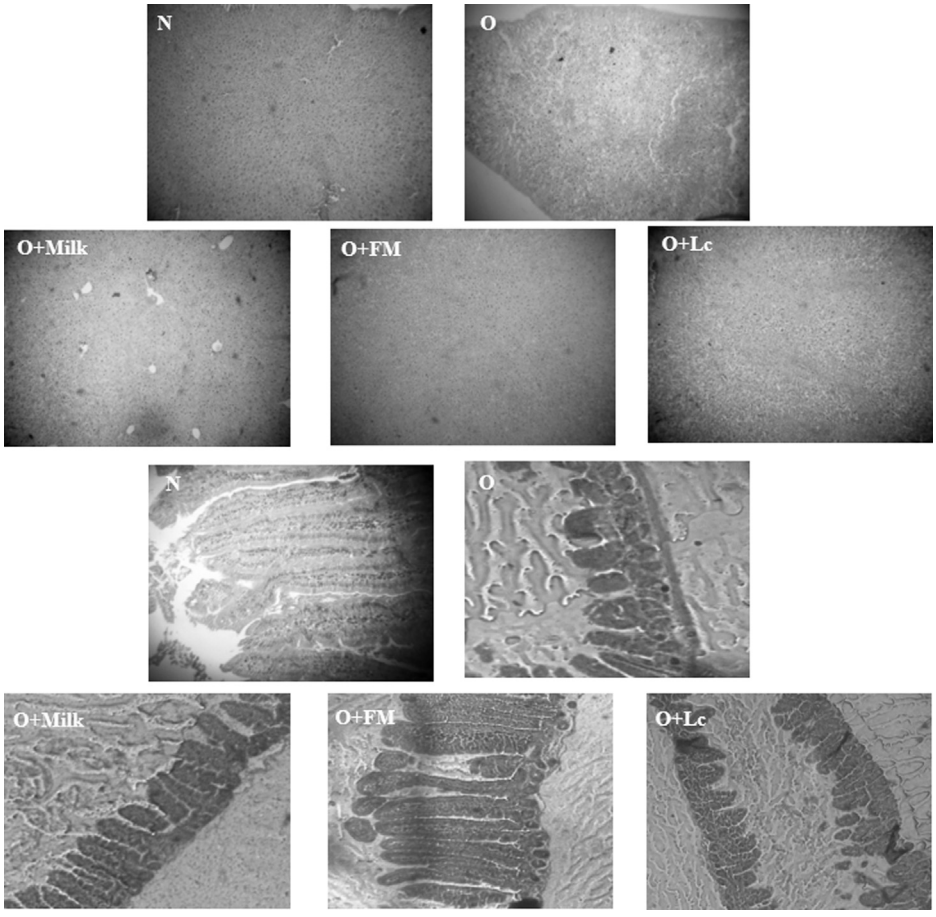

Fig. 3. Histologic study comparing the different experimental groups on day 60. Slices from liver or small intestine of mice were analyzed after staining with hematoxilin & eosin. Figure shows representative microphotographs for each group (100 ×). N group shows the normal histology of the liver and small intestine. Mice from O group had liver steatosis and the villi of the intestine were shorter than in the N group. Milk supplementation of obese mice did not induce beneficial changes in the histology of liver and small intestine; however, fermented milk and *L. casei* CRL 431 administration to the obese mice improved the histologic alterations caused by obesity, being the mice that received fermented milk, the group that showed histologic characteristics similar to the N group animals.

The phagocytic activity of peritoneal macrophages before immunization showed that mice from the O and O + milk groups decreased significantly this activity with respect to the N group; however, mice from the O + FM and O + Lc groups increased significantly ($P < 0.05$) the phagocytic activity of peritoneal macrophages, reaching values even higher than the N group (Fig. 5 C). After OVA immunization, the O group had significantly decreased the phagocytic activity compared with the N group. The supplementation with milk, FM or *L. casei* to diet-induced obese mice increased significantly ($P < 0.05$) the phagocytic activity compared with the O group, reaching values close to N mice (Fig. 5 C).

Discussion

Obesity and related health problems are increasing rapidly worldwide. Dietary habits and lifestyles are associated with obesity-related diseases [27]. Actually, people tend to choose dietary supplements that can exert a beneficial health effect, in addition to their nutritional values. In this context, there are many studies which have demonstrated the effects of probiotics on obesity and metabolic diseases, considering body weight, lipid levels and hepatic metabolism [14,28,29]. The present study was carried out in order to analyse the effect of probiotic supplementation in relation to eating habits in high fat diet-induced obese mice, using BALB/c animal model and receiving the probiotic bacterium *L. casei* CRL 431 in milk as a bacterial suspension, or contained in FM. The final concentration of probiotic bacteria in the drinking water was $2 \pm 1 \times 10^8$ CFU/mL, the same used previously in other animal models. For the FM, we decided to incubate the milk with *L. casei* CRL 431 until it reached a probiotic concentration similar to those present in the probiotic products on the market ($8 \pm 2 \times 10^9$ CFU/mL).

In this study the effect of this probiotic bacterium in the improvement of the some metabolic parameters altered by obesity was analysed. How this probiotic bacterium was able to improve the intestinal ecosystem, and whether it can influence the liver or other immune cells distant from the gut, such as peritoneal macrophages, were also evaluated. Macrophages were evaluated because they are key cells which link innate and systemic immunity.

The first beneficial effect obtained with the FM administration was a significant decrease in body weight of mice given high fat diet (O + FM) in concordance with other authors [30–32]. It is important to note that oral administration of *L. casei* suspension or FM for a long period of time (60 d) maintained the body weight of non-obese animals (receiving a conventional balanced diet) below the weights observed in non-obese mice (N) or in non-obese mice given milk (N + milk). These observations led us to look more deeply into the study as associated with this loss of

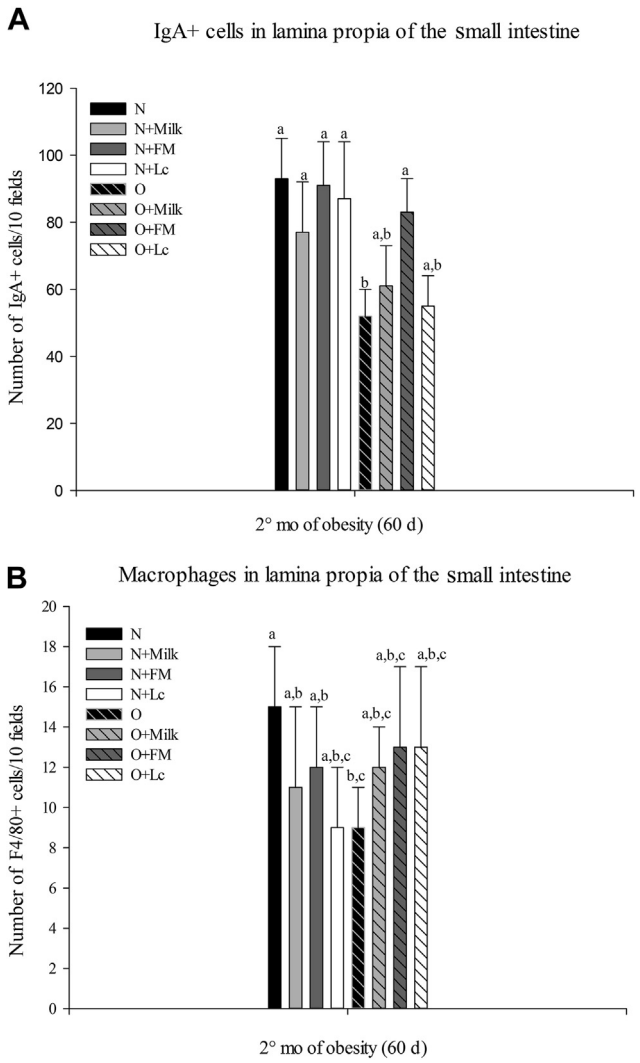

Fig. 4. IgA-secreting cells and macrophages in the lamina propria of the small intestine on day 60. The number of IgA-positive cells (A) and macrophages (B) were determined by direct and indirect immunofluorescence, respectively on the small intestine tissue slides of mice from different groups: non-obese control (N) and non-obese mice given milk, fermented milk or *L. casei* CRL 431 (N + milk, N + FM, and N + Lc), obese control (O) and diet-induced obese mice that received milk, fermented milk, or *L. casei* CRL 431 (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 ± SEM of results of nine animals from three separated experiments. [a,b,c] Means for each cell population without a common letter differ significantly ($P < 0.05$).

body weight, comparing the effects on the biochemical parameters, microbiota and immune system, between obese and non-obese mice.

In our model of obesity, as in other models described by other authors, the biochemical parameters determined in blood serum are altered in obese hosts [33,34] and they represent a risk factor for other diseases [35]. The glucose concentration increased in the blood serum of obese mice and decreased in the mice given high fat diet supplemented with FM, without reaching blood glucose levels of N group; perhaps the time of probiotic administration was not enough to restore the normal levels in our obesity model. It is also important to note that in the group of mice that received the conventional diet, the administration of FM decreased the glucose concentration compared with the N group, showing its potential to improve glucose levels.

The lipid profile was evaluated as it can be an indicator of coronary heart disease, and also a sign of metabolic syndrome when in combination with high blood sugar. The increased TG, TC, HDL-C and LDL-C concentrations observed in O mice were significantly decreased in mice that received probiotics (O + FM and O + Lc), and the FM group (O + FM) had values similar to the N group. These results are in concordance with those obtained by other authors with administration of probiotics and prebiotics [12,33,36,37].

The important role played by the intestinal microbiota, which is able to regulate gut immunity, metabolism, and the histologic structure of the gut, has been well demonstrated [38,39]. In this sense, obesity was also associated with differences in gut microbioma [40,41]. One study reported on mice that received a high-fat diet, resulting in reduced gram-negative bacteria, especially *Bacteroides*, and some gram-positive bacteria, such as *Bifidobacterium* species [42]. Similar observations were reported in a human trial in which obese participants had fewer *Bacteroidetes*. After weight loss, the relative proportion of *Bacteroides* increased [43]. In this sense, beneficial properties of certain probiotics are related to desirable changes in the gut microbiota, such as increased *Lactobacilli* or *Bifidobacteria* populations. In the present work, the probiotic supplementation of obese mice (O + FM and O + Lc) prevented the diminution of *Bacteroides* in the large intestine, as this population was diminished in obese hosts [42,43]. *Bifidobacteria* showed significant increases in the large intestine of mice that received FM or *L. casei* suspension, independent of the administration of conventional or high-fat diet. This effect was observed previously for our group with another *L. casei* probiotic strain (*L. casei* DN-114001) administered to mice [26]. In a pilot study, individuals who consumed *L. casei* strain Shirota showed an increase in *Lactobacillus* and *Bifidobacteria* levels in the gut [44]. This last finding shows another beneficial effect associated with probiotic administration in our obesity model. One study reviewed the relationship between microbiota and obesity and found that the alteration observed in the gut microbiota after high-fat diet ingestion affected intestinal barrier function and favored endotoxemia, thus increasing oxidative and proinflammatory processes in plasma and peripheral tissues, and the risk for insulin resistance [45]. The study also showed that these parameters could be reverted by the administration of prebiotics, which stimulate the growth of *Bifidobacterium* and *Lactobacillus* species in the colon, thus re-establishing gut homeostasis. These results agree with our findings on gut bacteria changes after probiotic administration.

The improvements in the microbiota and in the biochemical parameters induced by probiotic administration in obese mice were also evidenced by a decrease in liver steatosis, as described for other probiotics [46,47], and by the restoration of the villi length in the small intestine, which were altered by the high-fat diet given to the mice. It should be noted that our observations were only qualitative because our main point was to evaluate whether the improvement in the gut ecosystem in obese mice was correlated with liver histology improvement after probiotic supplementation. We wanted to demonstrate the influence of gut recovery after probiotic feeding on tissues or immune cells distant from the gut, such as the liver or peritoneal macrophages.

IgA-positive cells and macrophages in the lamina propria of the small intestine were evaluated considering that mucosal IgA is an important component of the gut barrier [48] and macrophages are mediators of intestinal immune homeostasis, inflammation

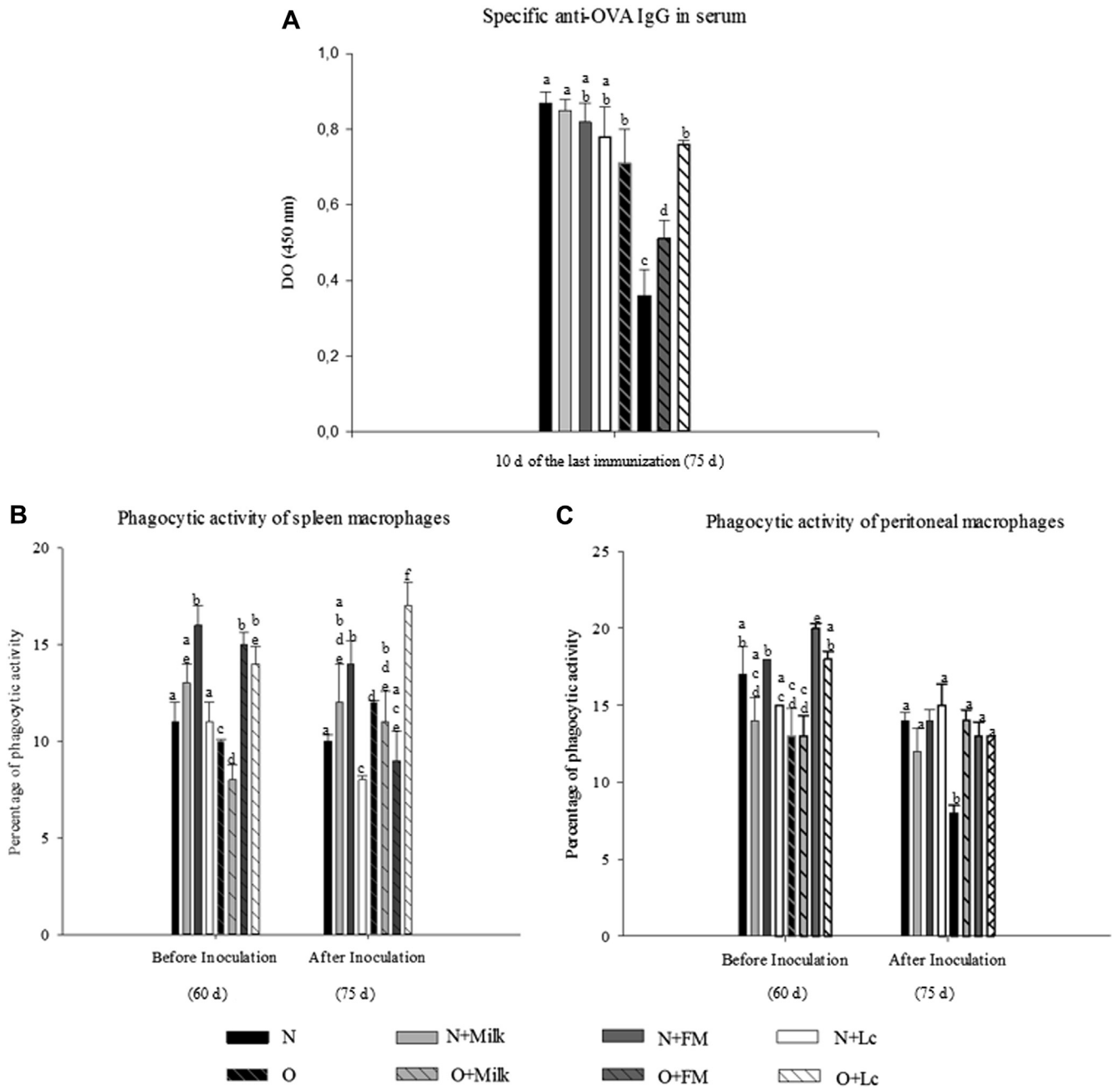

Fig. 5. Specific anti-OVA antibodies and phagocytic activity of macrophages. Specific anti-OVA IgG (A) was determined in blood serum by enzyme-linked immunosorbent assay in the samples obtained 10 d after the third OVA injection (day 75 of the experience). Results are expressed as OD (450 nm). Each bar point represents the mean of N = ± SD mice from each group. Phagocytic activity of macrophages isolated from spleen (B) and peritoneum (C) were analyzed. Mice were sacrificed at day 60 (before OVA inoculation) and 10 d after the third OVA injection to isolate the macrophages from peritoneum and spleen. The values for phagocytic activity were expressed as mean for n = 9 ± SD of percentage of phagocyting macrophages in 200 cells counted. Means values without a common letter (a,b,c,d,e,f) differ significantly ($P < 0.05$).

and key cells to link innate with systemic immunity [49]. Improvements in both cell populations in the diet-induced obese mice given FM were observed, thus showing the importance of FM administration in reinforcing the first line of defense in gut mucosa that can be impaired by intake of a high-fat diet.

The effect on systemic immunity was analyzed with the knowledge that obesity can be associated with altered immune responses, as well as cytokine release in response to systemic challenges [50]. The phagocytic activity of the spleen and peritoneal macrophages was increased in the obese mice that received probiotics before and after OVA immunization, in concordance with previous work performed in malnourished mice given probiotic fermented milk containing *L. casei* DN-114001 or its bacterial-free supernatant [24]. However, this effect was not reflected on systemic immunity, where the anti-OVA response was not increased with any of the probiotic supplementation assayed in the diet-induced obese mice.

Conclusion

The results obtained in the present work showed that *L. casei* CRL 431 administration can have a beneficial effect in

the obese host when it is introduced as a dietary supplement. *L. casei* CRL 431 was able to influence the intestinal ecosystem. The effect on the gut improved the biochemical parameters, the architecture of the small intestine, liver histology, and gut immunity, and the activity of the peritoneal macrophages; however the specific immune response against the OVA antigen was not increased. The positive effect found could be related to the positive balance observed in the intestinal microbiota of the obese mice that received probiotic supplementation.

The importance of a probiotic strain included in fermented milk was also demonstrated. The obese mice that received FM were the ones that showed the best improvement in the most of the parameters analyzed, most significantly in liver steatosis.

Studies of other possible immunologic beneficial effects associated with probiotic administration to obese mice, such as the cytokines in gut, liver, and adipose tissues, to modulate the inflammatory response induced by obesity, are currently under way.

References

[1] World Health Organization. Obesity. World Health Organization; 2013. Available at: http://www.who.int/gho/ncd/risk_factors/obesity_text/en/. Accessed June 23, 2014.

[2] Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 2004;92:347–55.

[3] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–72.

[4] Bajzer M, Seeley RJ. Physiology: obesity and gut flora. Nature 2006;444:1009–10.

[5] DiBaise JK, Zhang H, Crowell MD, Krajmalnik-Brown R, Decker GA, Rittmann BE. Gut microbiota and its possible relationship with obesity. Mayo Clin Proc 2008;83:460–9.

[6] Wan YM, Zhu YQ, Xia B, Luo J. Treating TNBS-induced colitis in rats with probiotics. Turk J Gastroenterol 2011;22:486–93.

[7] Food and Agriculture Organization of the United Nations and World Health Organization. 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. Accessed June 23, 2014.

[8] Mekkes MC, Weenen TC, Brummer RJ, Claassen E. The development of probiotic treatment in obesity: a review. Benef Microbes; 2013:1–10.

[9] Aronsson L, Huang Y, Parini P, Korach-Andre M, Hakansson J, Gustafsson JA, et al. Decreased fat storage by Lactobacillus paracasei is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). PLoS One 2010;5.

[10] Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, Niafar M, Asghari-Jafarabadi M, Mofid V, et al. Effect of probiotic yogurt containing Lactobacillus acidophilus and Bifidobacterium lactis on lipid profile in individuals with type 2 diabetes mellitus. J Dairy Sci 2011;94:3288–94.

[11] Moya-Perez A, Romo-Vaquero M, Tomas-Barberan F, Sanz Y, Garcia-Conesa MT. Hepatic molecular responses to Bifidobacterium pseudocatenulatum CECT 7765 in a mouse model of diet-induced obesity. Nutr Metab Cardiovasc Dis 2014;24:57–64.

[12] Yoo SR, Kim YJ, Park DY, Jung UJ, Jeon SM, Ahn YT, et al. Probiotics L. plantarum and L. curvatus in combination alter hepatic lipid metabolism and suppress diet-induced obesity. Obesity (Silver Spring) 2013;21:2571–8.

[13] Miyoshi M, Ogawa A, Higurashi S, Kadooka Y. Anti-obesity effect of Lactobacillus gasseri SBT2055 accompanied by inhibition of pro-inflammatory gene expression in the visceral adipose tissue in diet-induced obese mice. Eur J Nutr 2014;53:599–606.

[14] Zarrati M, Shidfar F, Nourijelyani K, Mofid V, Hossein Zadeh-Attar MJ, Bidad K, et al. Lactobacillus acidophilus La5, Bifidobacterium BB12, and Lactobacillus casei DN001 modulate gene expression of subset specific transcription factors and cytokines in peripheral blood mononuclear cells of obese and overweight people. Biofactors 2013;39:633–43.

[15] de Vrese M, Winkler P, Rautenberg P, Harder T, Noah C, Laue C, et al. Effect of Lactobacillus gasseri PA 16/8, Bifidobacterium longum SP 07/3, B. bifidum MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. Clin Nutr 2005;24:481–91.

[16] Larsen CN, Nielsen S, Kaestel P, Brockmann E, Bennedsen M, Christensen HR, et al. Dose-response study of probiotic bacteria Bifidobacterium animalis subsp lactis BB-12 and Lactobacillus paracasei subsp paracasei CRL-341 in healthy young adults. Eur J Clin Nutr 2006;60:1284–93.

[17] Vlieger AM, Robroch A, van Buuren S, Kiers J, Rijkers G, Benninga MA, et al. Tolerance and safety of Lactobacillus paracasei ssp. paracasei in combination with Bifidobacterium animalis ssp. lactis in a prebiotic-containing infant formula. A randomised controlled trial. Br J Nutr 2009;102:869–75.

[18] Rizzardini G, Eskesen D, Calder PC, Capetti A, Jespersen L, Clerici M. Evaluation of the immune benefits of two probiotic strains Bifidobacterium animalis ssp. lactis, BB-12(R) and Lactobacillus paracasei ssp. paracasei, L. casei 431(R) in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. Br J Nutr 2012;107:876–84.

[19] Gaon D, Garcia H, Winter L, Rodriguez N, Quintas R, Gonzalez SN, et al. Effect of Lactobacillus strains and Saccharomyces boulardii on persistent diarrhea in children. Medicina (B Aires) 2003;63:293–8.

[20] 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.

[21] 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):S21–6.

[22] 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.

[23] 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.

[24] 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.

[25] Sainte-Marie. A paraffin embedding technique for studies employing immunofluorescence. J Histochem Cytochem 1962;10:150–6.

[26] de Moreno de LeBlanc A, Dogi CA, Galdeano CM, Carmuega E, Weill R, Perdigon G. Effect of the administration of a fermented milk containing Lactobacillus casei DN-114001 on intestinal microbiota and gut associated immune cells of nursing mice and after weaning until immune maturity. BMC Immunol 2008;9:27.

[27] Craigie AM, Lake AA, Kelly SA, Adamson AJ, Mathers JC. Tracking of obesity-related behaviours from childhood to adulthood: a systematic review. Maturitas 2011;70:266–84.

[28] Aggarwal J, Swami G, Kumar M. Probiotics and their effects on metabolic diseases: an update. J Clin Diagn Res 2013;7:173–7.

[29] Poutahidis T, Kleinewietfeld M, Smillie C, Levkovich T, Perrotta A, Bhela S, et al. Microbial reprogramming inhibits Western diet-associated obesity. PLoS One 2013;8:e68596.

[30] Kang JH, Yun SI, Park HO. Effects of Lactobacillus gasseri BNR17 on body weight and adipose tissue mass in diet-induced overweight rats. J Microbiol 2010;48:712–4.

[31] Karlsson CL, Molin G, Fak F, Johansson Hagslatt ML, Jakesevic M, Hakansson A, et al. Effects on weight gain and gut microbiota in rats given bacterial supplements and a high-energy-dense diet from fetal life through to 6 months of age. Br J Nutr 2011;106:887–95.

[32] Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, Louis P, et al. Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes (Lond) 2008;32:1720–4.

[33] Cano PG, Santacruz A, Trejo FM, Sanz Y. Bifidobacterium CECT 7765 improves metabolic and immunological alterations associated with obesity in high-fat diet-fed mice. Obesity (Silver Spring) 2013;21:2310–21.

[34] Shen J, Obin MS, Zhao L. The gut microbiota, obesity and insulin resistance. Mol Aspects Med 2013;34:39–58.

[35] Sikaris KA. The clinical biochemistry of obesity. Clin Biochem Rev 2004;25:165–81.

[36] Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, et al. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 2011;60:2775–86.

[37] Hu X, Wang T, Li W, Jin F, Wang L. Effects of NS Lactobacillus strains on lipid metabolism of rats fed a high-cholesterol diet. Lipids Health Dis 2013;12:67.

[38] Sommer F, Backhed F. The gut microbiota–masters of host development and physiology. Nat Rev Microbiol 2013;11:227–38.

[39] Joyce SA, Gahan CG. The gut microbiota and the metabolic health of the host. Curr Opin Gastroenterol 2014;30:120–7.

[40] Wolf KJ, Lorenz RG. Gut microbiota and obesity. Curr Obes Rep 2011;1:1–8.

[41] Hullar MA, Lampe JW. The gut microbiome and obesity. Nestle Nutr Inst Workshop Ser 2012;73:67–79.

[42] 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.

[43] Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022–3.
[44] Rao AV, Bested AC, Beaulne TM, Katzman MA, Iorio C, Berardi JM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathol 2009;1:6.
[45] Morales P, Brignardello J, Gotteland M. The association of intestinal microbiota with obesity. Rev Med Chil 2010;138:1020–7.
[46] Solga SF, Buckley G, Clark JM, Horska A, Diehl AM. The effect of a probiotic on hepatic steatosis. J Clin Gastroenterol 2008;42:1117–9.
[47] 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.
[48] Brandtzaeg P. Update on mucosal immunoglobulin A in gastrointestinal disease. Curr Opin Gastroenterol 2010;26:554–63.
[49] Sheikh SZ, Plevy SE. The role of the macrophage in sentinel responses in intestinal immunity. Curr Opin Gastroenterol 2010;26:578–82.
[50] Lawrence CB, Brough D, Knight EM. Obese mice exhibit an altered behavioural and inflammatory response to lipopolysaccharide. Dis Model Mech 2012;5:649–59.