Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Bifidobacterium CECT 7765 Improves Metabolic and Immunological Alterations Associated with Obesity in High-Fat Diet-Fed Mice

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Objectives: To evaluate the effects of administration of *Bifidobacterium pseudocatenulatum* CECT 7765 on metabolic and immune alterations in obese mice.

Design and Methods: Adult male wild-type C57BL-6 mice were fed a standard diet or high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT 7765 for 7 weeks. The assessments included biochemical and immunological parameters, insulin resistance, glucose tolerance, histology of liver, white-adipose and intestinal tissues, immunocompetent cell functions, and microbiota-related features.

Results: *B. pseudocatenulatum* CECT 7765 reduced serum cholesterol, triglyceride, and glucose levels and decreased insulin resistance and improved glucose tolerance in obese mice. This strain reduced serum levels of leptin, interleukin (IL)-6 and monocyte chemotactic protein-1, while increased those of IL-4 in HFD-fed mice. *B. pseudocatenulatum* CECT7765 reduced liver steatosis and the number of larger adipocytes and number of fat micelles in enterocytes of obese mice. The strain also improved the function of macrophages and dendritic cells in relation to phagocytosis, cytokine production, and induction of T-lymphocyte proliferation. The strain administration increased bifidobacteria and reduced enterobacteria and the inflammatory properties of the gut content in HFD-fed mice.

Conclusion: *B. pseudocatenulatum* CECT 7765 was shown to ameliorate both metabolic and immunological dysfunctions related to obesity in HFD-fed mice.

Obesity (2013) **21**, 2310-2321. doi:10.1002/oby.20330

Introduction

Obesity constitutes a global epidemic nowadays, although its adverse consequences on health have only been seriously considered for the past 10 years (1). Obesity is the result of a metabolic energy imbalance and is also characterized by immunological dysfunction and chronic, low-grade inflammation (2).

Recent scientific evidence supports the notion that the gut microbiota is involved in body weight regulation by influencing metabolic and immunologic functions (3). Obesity has also been related to phylum and group-specific changes in the microbiota. Although not all results are consistent (4), it has been shown that obesity correlates with a decrease in the relative proportion of *Bacteroidetes* to *Firmicutes* while weight loss in obese human subjects subjected to dietary intervention correlated to an increase in Bacteroidetes (5) or the *Bacteroides-Prevotella* group (6) proportions. In addition, reduced numbers of *Bifidobacterium* spp. were associated with overweight in pregnant woman by comparisons with normal weight woman (7). In genetically obese *fa/fa* rats lacking the leptin receptor, associations between decreased numbers of *Bifidobacterium* spp. and obesity have also been reported, suggesting that *Bifidobacterium* spp. may play a role in defining an obese or lean phenotype (8). Therefore, dietary modulation of the intestinal microbiota has been proposed as a way of improving the efficacy of weight-loss treatments (3,5,9). Nevertheless, there is limited knowledge of the obesity-related benefits of bacteria commercialized as probiotics (*Lactobacillus* spp. and *Bifidobacterium* spp.), particularly, in relation to their potential effects on associated immunological alterations.

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Disclosure: The authors declare no conflict of interest.

Funding agencies: This work was supported by grants AGL2011-25169 and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Science and Innovation (MICINN, Spain).

Additional Supporting Information may be found in the online version of this article.

Received: 9 December 2011 **Accepted:** 4 December 2012 **Published online 18 February 2013. doi:10.1002/oby.20330**

The objective of this study was to evaluate the effects of oral administration of *Bifidobacterium pseudocatenulatum* CECT 7765 on both metabolic and immunological cell functions in mice with high-fat diet (HFD)-induced obesity.

Methods

Bacterial strains and culture conditions

The following *Lactobacillus* and *Bifidobacterium* strains were studied: *L. casei* ATCC 393, *L. casei* IATA-2E11, *L. plantarum* CECT 4185, *L. plantarum* 299v (Probi, Lund, Sweden), *L. plantarum* IATA-L1, *L. reuteri* IATA-LACA8, *B. catenulatum* LMG 110437, *B. pseudocatenulatum* CECT 5776, *B. pseudocatenulatum* CECT 7765, *B. longum* IATA-F1, and *B. longum* BB536 (Morinaga Milk Industry, Zama, Japan). These strains were obtained from the American Type Culture Collection [ATCC], Spanish Type Culture Collection (CECT), Belgian Co-ordinate Collections of Microorganisms (BCCM/LMG), Comercial Química Massó SA, Barcelona, Spain (BB536), or isolated in our laboratory from commercial products (299v) or from stools of breast-fed infants and identified by sequencing of amplified 16S rDNA regions with the universal primers 27f, 530f, U-968f as previously described (10,11).

Bacteria were grown in MRS broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (MRS-C Sigma, St. Louis, MO, USA), and incubated at 37°C for 22 h (at stationary growth phase) under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). Cells were harvested by centrifugation (6.000*g* for 15 min), washed twice in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), and resuspended in PBS for *in vitro* stimulation of immunocompetent cells or in 10% skimmed milk for oral administration to mice. Aliquots of these suspensions were frozen in liquid nitrogen and stored at −80°C until used. The number of live cells after freezing and thawing was determined by colony-forming unit (CFU) counting on MRS-C agar after 48 h incubation. For each strain tested, more than 90% cells were alive upon thawing and no significant differences were found during storage time (2 months). One fresh aliquot was thawed for every new experiment to avoid variability in the viability of cultures.

Ability of different bacterial strains to induce cytokine production by macrophages

For evaluating the immunological properties of different *Lactobacillus* and *Bifidobacterium* strains, the Raw 264.7 Mouse leukaemic monocyte macrophage cell line, obtained from the American Type Culture Collection (Rockville, MD, USA), was cultured overnight into 24-well flat-bottom polystyrene microtiter plates (Corning, Cultek, Madrid, Spain) at a concentration of 1×10^5 cells/mL in Dulbeco's Modified Eagles Medium (DMEM) (Sigma^{TM}, St. Louis, MO, USA). Media were changed before stimulation and, then, cells were incubated in the presence of 100 μL of a cell suspension (1×10^7 CFU/mL) of each strain for 24 h. Purified LPS from *Salmonella enterica* serotype Typhimurium (Sigma Chemical Co, Madrid, Spain) was used at a concentration of 1 μg/mL as a positive control. Nonstimulated Raw 264.7 cells were also evaluated as controls of basal cytokine production. The cell culture supernatants were collected and stored at −20°C until used for cytokine determination. TNF-α and MCP-1 were quantified using ELISA Ready SET Go! Kit (BD Bioscience, San Diego, CA, USA). Every parameter was assayed by triplicate in two independent experiments.

Animals, diets, and experimental design

Adult (age 6–8 weeks) male wild-type C57BL-6 mice were purchased from Harlan Laboratories. In the adaptation period (7 days), all animals were housed in each stainless-steel cage in a temperature-controlled (23°C) room with a 12-h light/dark cycle and 40–50% relative humidity and they were fed a standard diet (SD). Then, mice were randomly divided into four groups ($n \geq 6$ mice per group) as follows: (a) a control group that continued receiving the SD and a daily dose of placebo (skimmed milk) by gavage; (b) an obese group that was switched to a HFD and a daily dose of placebo (skimmed milk) by gavage; (c) a group that continued receiving the SD and a daily dose of 5×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage; and (d) an obese group that was switched to the HFD and a daily dose of 5×10^8 CFU *B. pseudocatenulatum* CECT 7765 by oral gavage. This regime was maintained for 7 weeks. The SD (CA.170481—AIN-76A Purified Diet-Rats/Mice) and the HFD (TD.06414—Adjusted Calories Diet—60/Fat, Harlan Laboratories, Madison, WI, USA) were provided by Harlan Laboratories. The HFD provided 18.4% kcal as protein, 21.3% kcal as carbohydrate, and 60.3% kcal as fat (5.1 kcal/g), whereas the SD provided 18.8% kcal as protein, 68.8% kcal as carbohydrate, and 12.4% kcal as fat (3.8 kcal/g). Mice had free access to the diets and to sterile water. Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of University of Valencia (Central Service of Support to Research [SCSIE], University of Valencia, Spain) and the protocol was approved by its Ethic Committee. Body weight was measured weekly and caloric intake was accounted from daily food consumption. At the end of study, animals were fasted for 16 h, anaesthetized, bled by aortic puncture, and killed by cervical dislocation. For analysis of metabolic parameters, blood samples were collected in tubes containing EDTA and centrifuged to obtain plasma, which was kept at −20°C. Feces were collected at the end of the experimental period (7 weeks) for microbiological analyses. The liver, white adipose (perirenal and epididymal), and small intestinal tissues were excised and rinsed with saline solution, then fixed in 10% neutral formalin buffered solution for histological analysis.

Histology of liver, white adipose, and small intestine tissues

Paraffin-embedded tissues were sectioned to a thickness of 4-5 μm and fixed to glass slides. Slides were deparaffinized and stained with haematoxylin–eosin. The severity of steatosis was determined in 100 hepatocytes of two liver tissue sections per mouse and scored as follows: grade 0 when fat was not detected in hepatocytes; grade 1 when fat occupied less than 30% of hepatocytes; grade 2 when fat occupied between 30 and 60% of hepatocytes; grade 3, when fat occupied more than 60% of hepatocytes.

Adipocyte cell sizes were measured in 100 cells of two sections of epididymal adipose tissue per mouse. Adipocyte cell sizes were expressed as area ranges using the following ranges: <2,000, 2,000-4,000; 4,000-6,000; and 6,000-7,000 μm^2.

The ratio of fat micelles to enterocyte was determined in 100 cells from two sections of small intestine tissue of each mouse by counting ten 100× light microscope fields. All parameters were measured with a NIKON Eclipse 90i Microscopic, using the NIS Elements BR 2.3 basic research software (Kingston, Surrey, UK).

All histology analyses were conducted by an experienced histologist in a blind fashion.

Analysis of serum cytokines

Inflammatory cytokines, including interleukin (IL)-1β (sensibility of 34.7 pg/mL), IL-6 (sensibility 2.2 pg/mL), TNF-α (sensibility of 2.1 pg/mL), and IFN-γ (sensibility of 6.5 pg/mL) and anti-inflammatory cytokines, including IL-4 (sensibility of 0.7 pg/mL) and IL-10 (sensibility of 5.4 pg/mL), and the chemokines IP-10 (sensibility of 5.4 pg/mL), and MCP-1 (sensibility 42.0 pg/mL) were quantified using FlowCytomix Multiple Analyte Detection kit Bendes Med Systems, GmbH, Vienna, Austria) according to the manufacturer's instructions. Samples were analyzing with a FACS Canto cytometer (Becton, Dickinson and Company, NJ, USA).

Analysis of metabolic parameters

Serum leptin and insulin concentration was determined using enzyme-linked immunosorbent assay (ELISA) (BD Bioscience). Biochemical parameters were also quantified in plasma using enzymatic assay kits for glucose (Glucose Liquid Kit; Química Analítica Aplicada SA, Spain), cholesterol (Cholesterol Liquid kit, Química Analítica Aplicada, SA, Spain) and triglyceride (Triglyceride Liquid kit, Química Analítica Aplicada, SA, Spain).

Intraperitoneal insulin sensitivity test (IPIST) and oral glucose tolerance test (OGTT)

Insulin sensitivity and oral glucose tolerance tests were performed *in vivo* after 6 weeks of treatment. Food was removed 2 h after the onset of the daylight cycle and, after a 3-h fasting period, 5 UI/Kg insulin (Humulina regular, *Lilly*, Giessen, Germany) were injected intraperitoneally and glucose was administered orally at a dose of 2 g/kg. Blood samples were taken with heparinized capillary tubes from the tail vein at 0, 15, 30, 45, 60, 90, 60 and 120 minutes for glucose determination. Plasma glucose levels were analyzed with glucose test strips (Ascensia Esyfill, Bayer, Tarrytown, NY; USA) and a glucometer (Ascensia VIGOR, Bayer Tarrytown, NY; USA), with a detection level ranging from 30 to 550 mg glucose/dL. The area under the glucose curve (AUC) was estimated by plotting the glucose concentration (mg/dL) versus time (min).

Isolation and cytokine production by peritoneal macrophages

Peritoneal cells were collected by washing the peritoneal cavity of different mouse groups, with 5 mL of sterile cold Dulbeco's Modified Eagles Medium (DMEM) (Sigma^{TM}), containing 10% inactivated (56°C for 30 min) fetal bovine serum (Gibco, Barcelona, Spain), 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma^{TM}). Isolated macrophages were plated into flask (Corning, Cultek) at a concentration of 1×10^6 cells/mL in DMEM and incubated for 2 h at 37°C in an atmosphere containing 5% CO_2, and nonadhered cells were washed out with warm PBS. To evaluate the effects of different stimuli, we cultured adhered macrophages overnight into 24-well flat-bottom polystyrene microtiter plates (Corning, Cultek, Madrid, Spain) at a concentration of 1×10^5 cells/mL in DMEN and media were changed before stimulation. To evaluate the different response to a common stimulus, we incubated macrophages from different mouse groups in the presence of purified LPS from *S. enterica* serotype Typhimurium (Sigma Chemical Co, Madrid, Spain) at a concentration of 1 μg/mL. Nonstimulated peritoneal macrophages were also evaluated as controls of basal cytokine production. To evaluate the immune properties of fecal samples related to the microbiota, we incubated macrophages from control mice in the presence of fecal samples (30 μL of 10-fold dilution) from the different mouse groups for 24 h. Fecal samples used as stimuli were collected from six mice in each experimental group at the end of the study, diluted 10-fold in PBS and homogenized for 3 min. The cell culture supernatants were collected and stored at −20°C until used for cytokine determination. TNF-α and IL-10 were quantified using ELISA Ready SET Go! Kit (BD Bioscience, San Diego, CA, USA). Every parameter was assayed in triplicate in two independent experiments.

Bactericidal activity of peritoneal macrophages

The bactericidal activity of peritoneal macrophages was analyzed according to Vieira et al. (13). Cells were washed with serum free DMEM and nitroblue tetrazolium (NBT, Sigma^{TM}) at 0.5 mg/mL together with a bacterial extract (Stimulant, No. 840-15-Sigma^{TM}) in an equivalent concentration of McFarland Scale 2 in Lab-tek chamber slide w/cover (Nalge Nunc International, USA, Naperville, IL). After 1 h of incubation at 37°C in 5% CO_2 atmosphere, the cells were washed with PBS, then fixed with 4% paraformaldehyde and observed with an optic microscope (Kingston). One hundred cells per mouse were counted and the percentage of NBT-positive cells was determined. Every parameter was assayed in triplicate in two independent experiments.

Isolation and cytokine production by bone marrow-derived dendritic cells

Dendritic cells (DCs) were generated from bone marrow as described previously (14). Cells were seeded at a concentration of 1×10^6 (90-94% DCs) in 1 mL of culture medium without rm GM-CSF in 24-well plates (Corning, Cultek) and incubated in the presence of different stimuli at 37°C under 5% CO_2 for 24 h. To evaluate the different response to a common stimulus, we incubated DCs from different mouse groups in the presence of purified LPS from *S. enterica* serotype Typhimurium (Sigma Chemical Co, Madrid, Spain) at a concentration of 1 μg/mL. Nonstimulated peritoneal macrophages were also evaluated as controls of basal cytokine production. To evaluate the immune properties of fecal samples related to the microbiota, we incubated DCs from control mice in the presence of fecal samples (30 μL of 10-fold dilution) from the different mouse groups. Fecal samples were obtained and prepared as described above. The cell culture supernatants were collected and stored at −20°C until used for cytokine determination (TNF-α and IL-10) as described above. Every parameter was assayed in triplicate in two independent experiments.

Interactions between DCs and T CD4+ lymphocytes

CD4+ T lymphocytes were isolated from mouse spleens. To do so, spleens were excised, suspended in complete medium, and passed through a stainless steel wire mesh, and the crude cell suspension obtained was washed once. CD4+ T cells were immunomagnetically isolated by positive selection with "CD4+ (L3T4) microbeads" (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. CD4+ T cells (purity exceeded 95%) were used for mixed lymphocyte reaction.

TABLE 1 Serum glucose, cholesterol, triglyceride, leptin and insulin concentrations in mice fed either a high-fat diet or standard diet, supplemented or not with *B. pseudocatenulatum* CECT 7765

	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)	Leptin (ng/mL)	Insulin (ng/mL)
SD	127.9 ± 4.5^{a}	143.0 ± 18.9^{a}	219.8 ± 9.2^{a}	5.83 ± 1.61^{a}	0.573 ± 0.05^{a}
HFD	$160.1 \pm 9.9^{b,a'}$	$196.1 \pm 14.3^{b,a'}$	$265.5 \pm 8.1^{b,a'}$	$38.10 \pm 5.13^{b,a'}$	$1.59 \pm 0.09^{b,a'}$
SD+P	$120.9 \pm 6.9^{a,b'}$	$157.5 \pm 5.0^{a,b'}$	$220.0 \pm 11.7^{a,b'}$	$3.88 \pm 1.20^{a,b'}$	$0.53 \pm 0.05^{a,b'}$
HFD+P	$138.4 \pm 6.6^{a,b'}$	$147.5 \pm 25.0^{a,b'}$	$219.8 \pm 17.3^{a,b'}$	$32.01 \pm 4.68^{b,b'}$	$1.07 \pm 0.042^{b,b'}$

SD: standard diet group (control) ($n = 12$); SD + *P*: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$); HFD: high-fat diet group ($n = 12$); HFD + *P*: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$). Glucose (mg/dL), total cholesterol (mg/dL), triglyceride (mg/dL), leptin (ng/mL), and insulin (ng/mL) levels were determined in sera taken from animals after 7 weeks of intervention. Data are expressed as means and SD. Means in the same column with different letters are significantly different from SD (a,b) or from HFD group (a',b') at *P*-values < 0.05.

Isolated DCs were incubated for 24 h in the presence of 1 μg/mL LPS from *S. enterica* serovar *Typhimurium* (Sigma Chemical Co, Madrid, Spain). Aliquots of mature DCs from different mouse groups were plated in triplicate with allogeneic CD4+ T cells (TL) at 1:1, 1:2, 1:4, TL/DC cell ratios, in 0.2 mL culture medium in 96-well flat-bottomed plates (Corning, Cultek, Madrid, Spain) at 37°C for 72 h. Lymphocyte proliferation was measured with the cell proliferation ELISA BrdU-colorimetric assay (Roche Applied Science, Mannheim, Germany). Individual cultures of DCs, and TL stimulated with or without ConA, used as mitogen, were used as controls.

Samples and microbial analysis by quantitative PCR (qPCR)

Fecal samples were weighed, diluted 1:5 (w/v) in PBS (pH 7.2), homogenized by thorough vortex shaking, and stored at −20°C until analyzed. One aliquot of this dilution was used for DNA extraction using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany). Specific primers (Supporting Table 1) targeting different bacterial genera and species were used to characterize the composition of the microbiota by qPCR using LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany) with a an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, Warrington, UK), as described previously (15).

Statistical analyses

Statistical analyses were carried out using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Biochemical parameter data were normally distributed and significant differences were determined by applying a one-way ANOVA with post hoc Tukey's test or Fisher's least significant difference test. The rest of the data were non-normally distributed and the differences were determined by applying the Mann–Whitney *U*-tests. In every case, *P*-values <0.05 were considered statistically significant.

Results

Strain selection based on *in vitro* ability to induce cytokine production by macrophages

The results of the effects of different *Lactobacillus and Bifidobacterium* strains on induction of cytokine production by Raw 264.7 macrophages are shown in Supporting Table 2. All strains induced the production of significantly higher amounts of the pro-inflammatory cytokine TNF-α and the chemokine MCP-1 than the nonstimulated cells, although the magnitude of the effect was strain-dependent. The strain *B. pseudocatenulatum* CECT 7765 induced significantly lower TNF-α production than the other strains. *B. pseudocatenulatum* CECT 7765 and *B. longum* BB536 also induced significantly lower production of MCP 1 than the other strains studied. *B. pseudocatenulatum* CECT 7765 was finally selected on the basis of its lower pro-inflammatory potential.

Body weight gain and food intake are reduced by *B. pseudocatenulatum* CECT 7765 in obese mice

The HFD-fed mice experienced significant weight gain as compared to the SD-fed mice as of the first two weeks of treatment (data not shown). In the 7th week, the relative body weight increase of HFD-fed mice was 31.0 ± 10.2%, whereas that of SD-fed mice was 17.7 ± 8.0% ($P = 0.001$). The administration of *B. pseudocatenulatum* CECT 7765 significantly reduced ($P = 0.001$) body weight gain in HDF-fed mice at the end of the intervention, but not in SD-fed mice (Figure 1A).

Caloric intake was significantly higher ($P = 0.002$) in HFD-fed mice than in SD-fed mice, as anticipated, and reduced by the administration of *B. pseudocatenulatum* CECT 7765 in HFD-fed mice ($P = 0.025$) (Figure 1B). The weight of total ($P = 0.016$) and epididymal adipose tissues were greater ($P = 0.019$) in obese mice than in lean mice, but the administration of the bifidobacteria did not exert a statistically significant effect in any of the mice groups (Figure 1C).

Serum cholesterol, triglyceride, glucose, insulin, and leptin levels are reduced by *B. pseudocatenulatum* CECT 7765 in obese mice

Serum levels of biochemical markers of metabolic relevance are shown in Table 1. The HFD induced a significant increase ($P = 0.05$) in serum cholesterol, triglyceride, glucose, and insulin levels as compared with the SD. The administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice led to a significant ($P = 0.05$) reduction in serum cholesterol, triglyceride, and glucose levels of

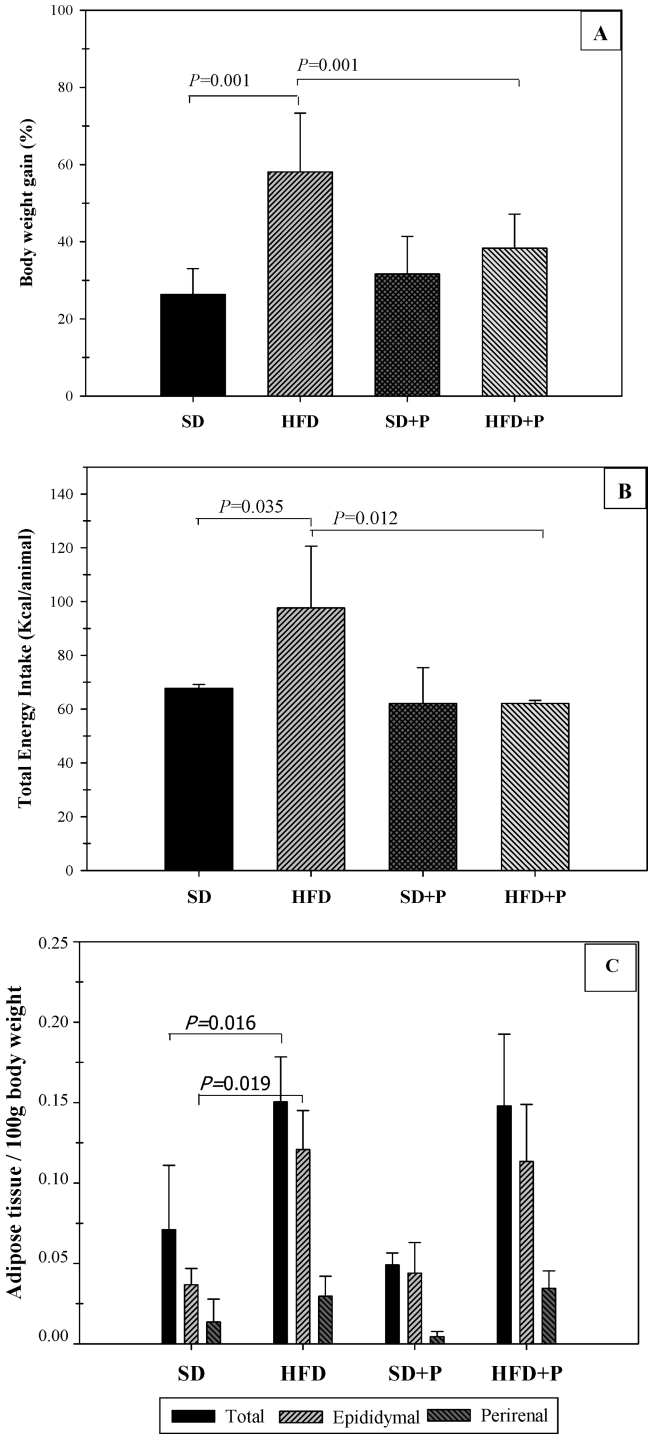

FIGURE 1 Body weight gain (**A**), total caloric energy intake (**B**), and relative adipose tissue weight (**C**) in mice fed standard diet (SD) or high-fat diet (HFD) supplemented or not with *B. pseudocatenulatum* CECT 7765. SD: standard diet group (control) ($n = 12$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU/mouse of *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 12$); HFD: high-fat diet group ($n = 12$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU/mouse of *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 12$). Mice were weighed weekly during the 7-week period and energy intake was accounted from daily food consumption. Relative adipose tissue weight was expressed as adipose tissue weight per 100 g of body weight (total adipose tissue includes epididymal and perirenal white adipose tissues). Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

14, 25, and 17%, respectively, as compared to the levels reached in the HFD-fed mouse group not receiving the bifidobacterial strain.

Mice receiving HFD became markedly hyperleptinemic ($P = 0.001$) compared to SD-fed mice (Table 1). The administration of *B. pseudocatenulatum* CECT 7765 significantly ($P = 0.035$) reduced the leptin levels in HFD-fed mice (Table 1). In addition, a significant ($P = 0.01$) increase of fasting insulin levels was observed in HFD compared to SD-fed mice (Table 1), but *B. pseudocatenulatum* CECT 7765 induced a significant decrease ($P = 0.004$) of fasting insulin levels in HFD mice.

Insulin resistance and oral glucose tolerance are improved by *B. pseudocatenulatum* CECT 7765 in obese mice

The HFD-induced insulin resistance and glucose intolerance compared with the SD, as expected. Figure 2 shows increased serum glucose concentrations in both OGTT and IPIST in HFD-fed mice compared to SD-fed mice, according to the values of the area under the glucose curve (both $P = 0.004$). The administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice significantly reduced insulin resistance and glucose intolerance as reflected in the reduced serum glucose concentrations in OGTT and IPIST compared with HFD-fed mice administered placebo ($P = 0.040$ and $P = 0.010$, respectively). The administration of the same strain to SD-fed mice did not induce significant changes in these parameters.

Hepatic steatosis, adipocyte size, and enterocyte fat absorption are reduced by *B. pseudocatenulatum* CECT 7765 in obese mice

Hepatic steatosis and significant increases in the number of hepatocytes with grades 3 ($P = 0.008$) and 2 ($P = 0.018$) of steatosis were detected in HFD-fed mice in comparison with SD-fed mice, whereas hepatocytes with no steatosis or grade-1 ($P = 0.013$) of steatosis decreased in HFD-fed mice in comparison with SD-fed mice (Figure 3A). *B. pseudocatenulatum* CECT 7765 significantly reduced steatosis in HFD-fed mice, but did not influence this parameter significantly in mice fed the SD. *B. pseudocatenulatum* CECT 7765 administration reduced the number of hepatocytes with grade-3 of steatosis ($P = 0.013$) and increased those with grades-1 ($P = 0.018$) and -2 ($P = 0.018$) of steatosis in HFD-fed mice.

The effects of the diets, supplemented or not with *B. pseudocatenulatum,* on adipocyte size in epididymal adipose tissue are shown in Figure 4. The HFD-induced significant increases in the adipocytes in the size ranges 2,000-4,000 ($P = 0.018$) and 4,000-6,000 μm^2 ($P = 0.018$) and reductions in those $\leq$2,000 μm^2 ($P = 0.018$) in comparison with SD-fed mice. *B. pseudocatenulatum* CECT 7765 administration in SD-fed mice did not induce significant modifications in adipocyte size, whereas in HFD-fed mice it led to a significant decrease ($P = 0.018$) in the number of larger adipocytes (2,000-4,000 μm^2) and an increase in the number of smaller adipocytes (<2,000 μm^2) (Figure 4A).

Dietary fat absorption was determined by analyzing the number of fat micelles per enterocyte in intestinal tissue sections (Figure 5). The HFD induced a significant increase ($P = 0.018$) in the number of fat micelles in the enterocytes in comparison with SD

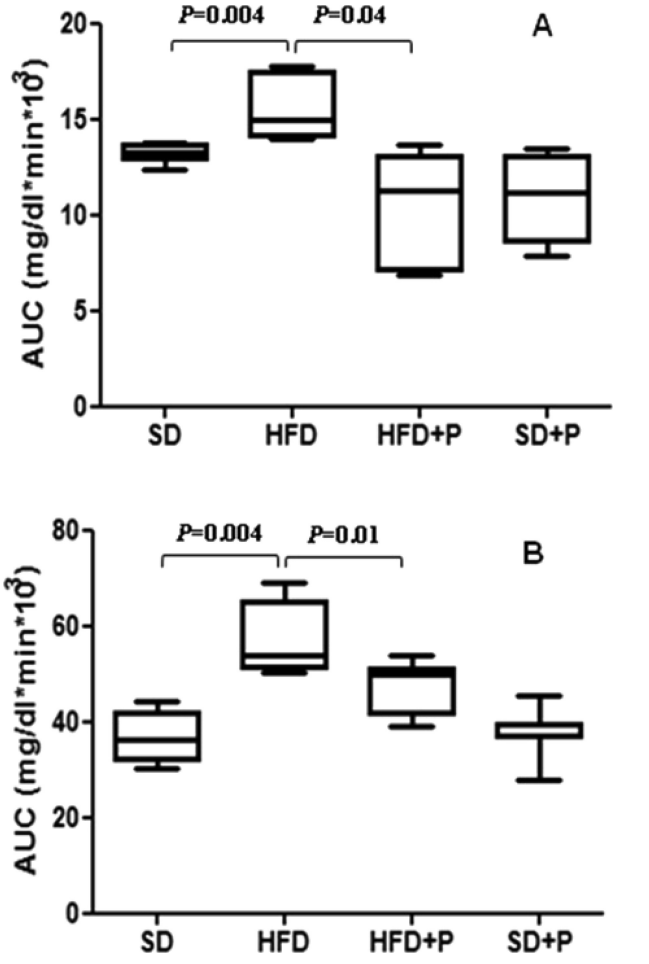

FIGURE 2 Intraperitoneal insulin sensitivity test (IPIST) (**A**) and oral glucose tolerance test (OGTT) (**B**) in mice fed a standard diet (SD) or a high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT 7765. SD: standard diet group (control) ($n = 8$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 8$); HFD: high-fat diet group ($n = 8$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 8$). Data are expressed as means of area under curve (AUC) from the plot glucose concentration (mg/gL) versus time (min) ± SD. Statistically significant differences were established at $P < 0.05$.

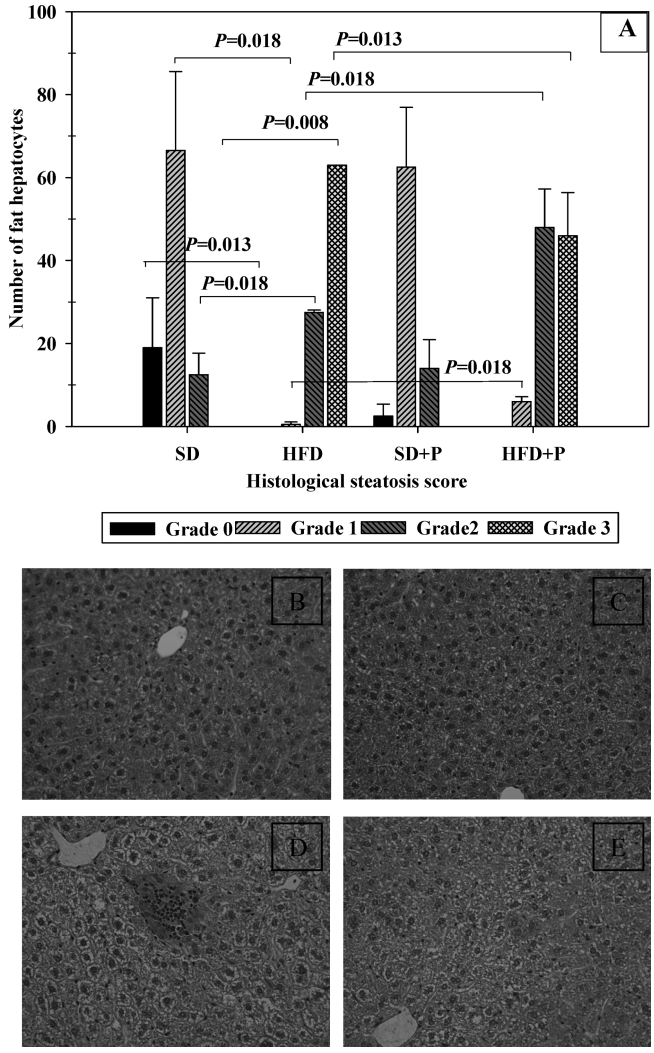

FIGURE 3 Determination of hepatic steatosis (hepatic histology) in mice fed standard diet (SD) or high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT 7765 (**A**). SD: standard diet group (control) ($n = 6$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$); HFD: high-fat diet group ($n = 6$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$).The fat vacuoles were measured in 100 hepatocytes of two liver tissue sections per mouse and scored for the severity of steatosis according to the following criteria: For grade-0 steatosis, no fatty hepatocytes; grade-1 steatosis, fat occupying less than 30% of the hepatocyte; grade-2 steatosis, fat occupying less than 30-60% of the hepatocyte; grade-3 steatosis, fat occupying more than 60% of the hepatocyte. Data are expressed as means ± SD and statistically significant differences are established at $P < 0.05$. Photomicrographs 20× of representative HE-stained slides are shown. (**B**) SD group, (**C**) SD+P group, (**D**) HFD group, and (**E**) HFD+P group.

(0.630 ± 0.100 versus 0.019 ± 0.002 number of fat micelles/enterocyte), whereas the administration of *B. pseudocatenulatum* CECT 7765 significantly reduced ($P = 0.018$) these numbers in HFD-fed mice (0.170 ± 0.040 number of fat micelles/enterocyte).

Serum inflammatory cytokine and chemokine levels are reduced by *B. pseudocatenulatum* CECT 7765 in obese mice

The HFD-fed mice showed larger serum concentrations of IL-6 and MCP-1 (329.8 ± 38.7 and 1160.0 ± 267.3 pg/mL, respectively) than the SD-fed mice (36.8 ± 16.0 pg/mL and 359.6 ± 166.9 pg/mL, respectively), as shown in Figure 6. In HFD-fed mice, IL-10 was also increased at concentration of 50.4 ± 6.8 pg/mL, whereas it was not detected in SD group. Concentrations of IP-10, TNF-α, IL-1β, and IFN-γ were similar when SD and HFD groups were compared. IL-4 was not detected in either SD- or HFD-fed mouse group.

The administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice significantly reduced IL-6 and MCP-1 serum concentrations by 60% ($P = 0.02$) and 50% ($P = 0.02$), respectively, compared to HFD-fed mice administered placebo. The administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice increased IL-4

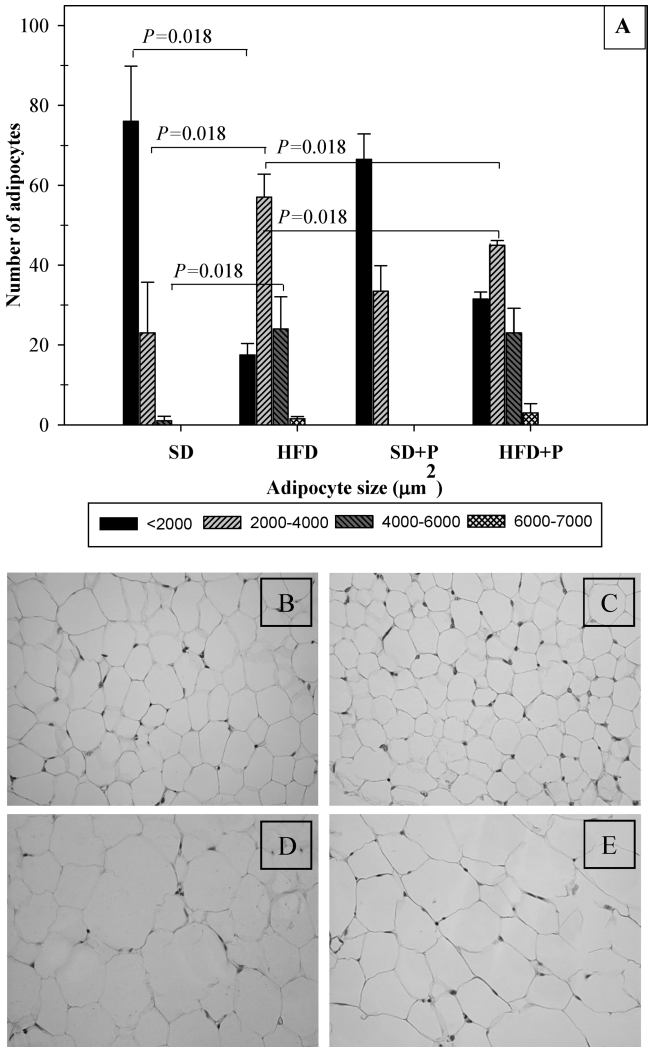

FIGURE 4 Distribution of adipocyte size in epididymal adipose tissue in mice fed a standard diet (SD) or a high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT 7765 (**A**). SD: standard diet group (control) ($n = 6$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$); HFD: high-fat diet group ($n = 6$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$). Adipocyte cell sizes are expressed as area ranges as follows: <2,000; 2,000-4,000; 4,000-6,000; and 6,000-7,000 μm^2. Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$. Photomicrographs 20× of representative HE-stained slides are shown. (B) SD group, (**C**) SD+P group, (**D**) HFD group, and (**E**) HFD+P group.

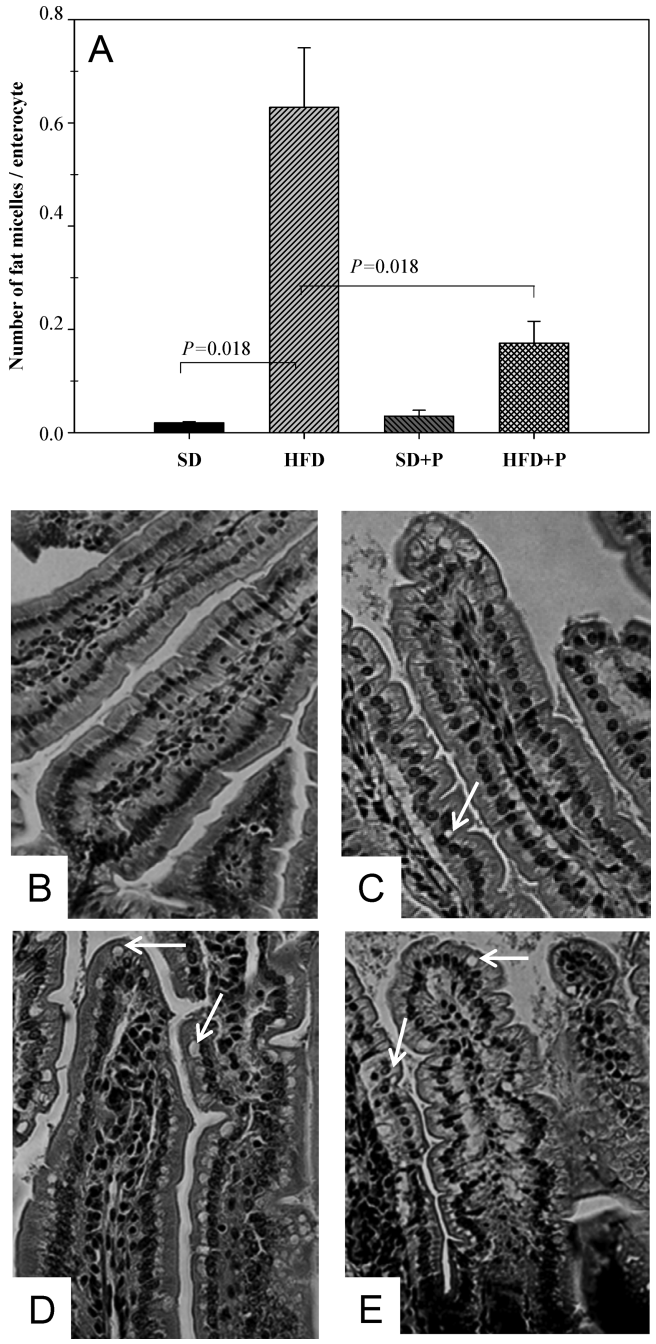

FIGURE 5 Determination of the number of fat micelles per enterocyte in intestinal tissue sections from mice fed standard diet (SD) or high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT 7765. (**A**) SD: standard diet group (control) ($n = 6$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$); HFD: high-fat diet group ($n = 6$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$). The ratio of fat micelles to enterocyte was determined in 100 cells from two sections of small intestine tissue of each mouse by counting ten 100× light microscope fields in a NIKON Eclipse 90i Microscopic, using the NIS Elements BR 2.3 basic research software (Kingston). Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$. Photomicrographs 20× of representative HE-stained slides are shown in: (**B**) SD group, (**C**) SD+P group, (**D**) HFD group, and (**E**) HFD+P group. Fat micelles in enterocytes are shown with arrows.

concentration (37.9 ± 2.3 pg/mL) while IL-10 was reduced (28.6 ± 1.8 pg/mL) compared to HFD-fed mice ($P = 0.02$).

LPS-induced cytokine production and phagocytosis of macrophages are improved by *B. pseudocatenulatum* CECT 7765 in obese mice

The results of cytokine production by LPS-stimulated peritoneal macrophages of SD- and HFD-fed mice with and without *B. pseudocatenulatum* CECT 7765 supplementation are shown in Figure 7A. Cytokine TNF-α production by peritoneal macrophages stimulated

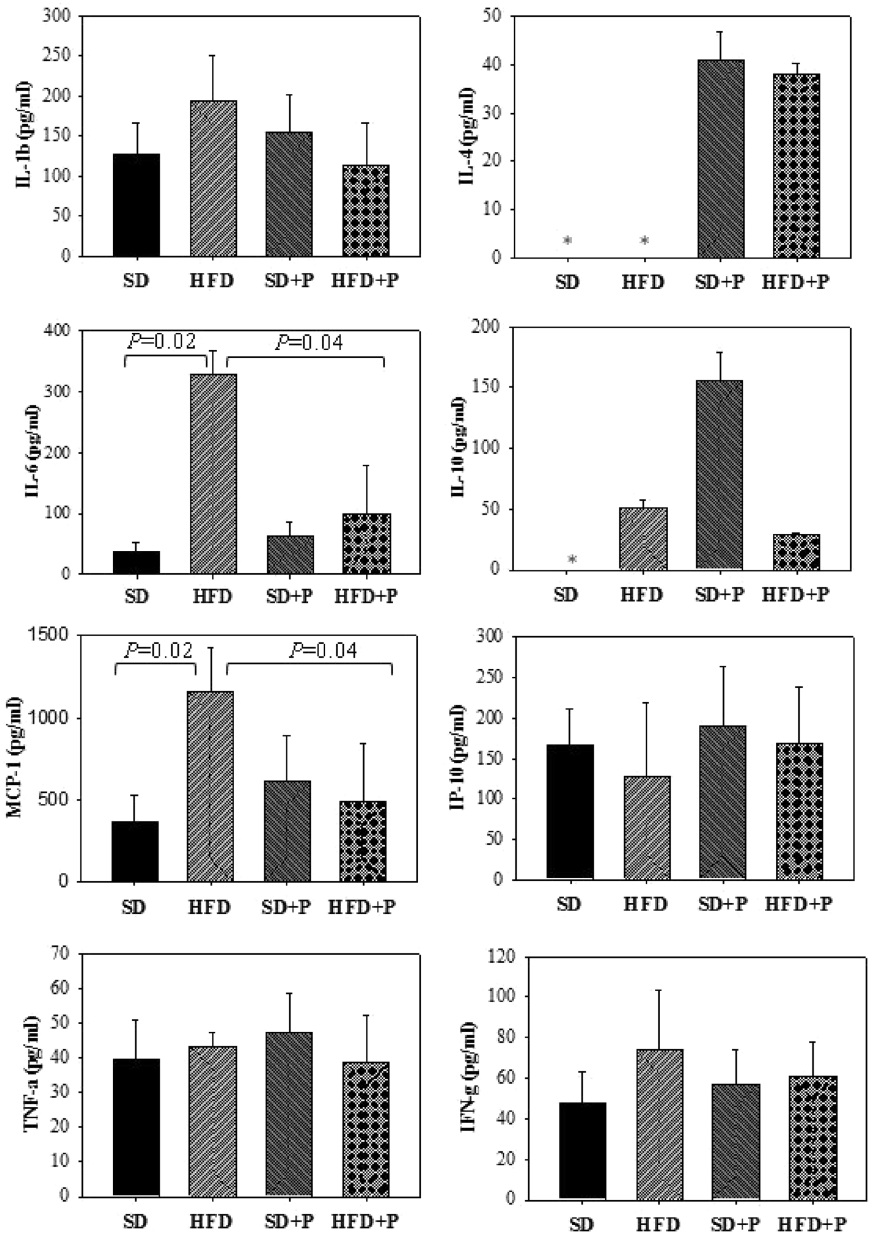

FIGURE 6 Inflammatory (IL-1β, IL-6, TNF-α, IFN-γ) anti-inflammatory (IL-4, IL-10) cytokine and chemokine (MCP-1, IP-10) concentration in serum from mice fed a standard diet or a high-fat diet, supplemented or not with *B. pseudocatenulatum* CECT 7765. SD: standard diet group (control) ($n = 8$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 8$); HFD: high-fat diet group ($n = 8$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 8$). Data are expressed as means ± SD and statistically significant differences are established at $P < 0.05$. *Nondetected.

with LPS was lower ($P = 0.021$) in HFD-fed mice than in SD-fed mice (Figure 7A). However, *B. pseudocatenulatum* CECT 7765 administration significantly increased the ability of LPS-stimulated macrophages to produce TNF-α in HFD ($P = 0.021$) while did not exert a significant effect in SD-fed mice (Figure 7A). The HFD did not exert a significant effect on the ability of LPS-stimulated macrophages to produce the anti-inflammatory cytokine IL-10 (Figure 7A). *B. pseudocatenulatum* CECT 7765 administration induced a significant increase in IL-10 production by stimulated macrophages in SD-fed mice ($P = 0.019$) but not in those fed a HFD (Figure 7A).

The oxidative burst in peritoneal macrophages after *B. pseudocatenulatum* CECT 7765 intake was also studied to analyses effects on

phagocytosis function (Figure 7C), with results indicating this function was boosted in macrophages of both SD- ($P = 0.01$) and HFD-fed mice ($P = 0.001$) (Figure 7C).

LPS-induced cytokine production and ability to induce T-cell proliferation by DCs are improved by *B. pseudocatenulatum* CECT 7765 in obese mice

In obese mice, *B. pseudocatenulatum* CECT 7765 administration increased LPS-stimulated TNF-α production in DCs ($P = 0.021$), which was reduced by the HFD ($P = 0.021$) (Figure 7D). In HFD-fed mice, production of IL-10 by LPS-stimulated DCs was significantly increased ($P = 0.01$), whereas this effect was partially reversed by the administration of *B. pseudocatenulatum* CECT 7765 ($P = 0.034$) (Figure 7D). In SD-fed mice, the administration of *B. pseudocatenulatum* CECT 7765 also reduced ($P = 0.034$) the ability of LPS-stimulated DCs to produced IL-10.

The influence of HFD-induced obesity and oral administration of *B. pseudocatenulatum* CECT 7765 on the ability of matured DCs to prime a T-cell proliferative response are shown in Figure 7F. DCs from SD-fed mice were able to induce a significant increase in T-cell proliferation compared to T cells alone (data not shown) for all the DC:T-cell ratios examined. The HFD impaired the capacity of DCs to induce a T-cell proliferation response ($P = 0.043$-0.046), but this function was restored ($P = 0.046$) by *B. pseudocatenulatum* CECT 7765 administration, and the highest effects were obtained at 1:4 DC:T-cell ratio ($P = 0.050$).

Shifts in the intestinal microbiota composition and inflammatory properties are attenuated by *B. pseudocatenulatum* CECT 7765 in obese mice

The composition of the fecal microbiota in SD- and HFD-fed mice is shown in Supporting Table 3. The HFD led to reductions in numbers of *Lactobacillus* group ($P = 0.025$), *C. coccoides* ($P = 0.013$) group, and *C. leptum* ($P = 0.004$) group and *Bifidobacterium* spp. ($P = 0.004$), and to increases in *Enterobacteriaceae* ($P = 0.025$). In HFD-fed mice, *B. pseudocatenulatum* CECT 7765 administration increased the numbers of total *Bifidobacterium* spp. ($P = 0.011$) and reduced those of *Enterobacteriaceae* ($P = 0.007$), whereas in SD-fed mice only total numbers of *Bifidobacterium* spp. ($P = 0.05$) increased.

To evaluate whether these changes in the microbiota composition could modify the inflammatory signals coming from the gut in the different mice groups, we assessed the fecal samples ability to induce cytokine production by immunocompetent cells *in vitro* (Figure 7B and E). Fecal samples from HFD-fed mice induced higher TNF-α production ($P = 0.021$) than those from SD-fed mice by macrophages (Figure 7B) and DCs from control mice ($P = 0.021$) (Figure 7E), indicating that the HFD induced an increase in the pro-inflammatory signals coming from the intestine. Meanwhile, *B. pseudocatenulatum* CECT 7765 administration significantly reduced the production of this pro-inflammatory cytokine by studied immune cells ($P = 0.043$ in macrophage, $P = 0.021$ in DCs), demonstrating its ability to reduce the inflammatory properties of the intestinal content (Figure 7B and E). Fecal samples of HFD-fed mice also triggered greater production ($P = 0.050$) of the anti-inflammatory cytokine IL-10 by macrophages and DCs than those from SD-fed mice, which could be due to the activation of regulatory mechanisms to counteract an inflammatory response, whereas *B. pseudocatenulatum* CECT 7765 administration reduced ($P = 0.020$) this effect only in DCs (Figure 7B and E). For mice fed SD supplemented with *B. pseudocatenulatum* CECT 7765, fecal samples significantly increased ($P = 0.031$) the synthesis of IL-10 by DCs in comparison with control mice.

Discussion

This study supports the hypothesis that intervention in the gut ecosystem by administration of specific bacterial strains, such as *B. pseudocatenulatum* CECT 7765, could ameliorate both metabolic and immunological dysfunctions underlying obesity.

The oral administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice induced a decrease in body-weight gain and adipocyte size, which could help to curb obesity development. The largest adipocytes are those that produce the growth factors inducing adipogenesis and maturation of pre-adipocytes into adipocytes, and a higher proportion of pro-inflammatory mediators contributing to obesity development (16). Both HFD-induced liver steatosis and obesity have previously been reported (17), predisposing hepatocytes to oxidative stress and activating inflammatory pathways (18). *B. pseudocatenulatum* CECT 7765 administration was also able to reduce fat accumulation in hepatocytes. Moreover, this strain significantly reduced the fat micelles inside enterocytes, which is a potential mechanism whereby adipocyte hypertrophy and liver steatosis could be reduced. The caloric intake was also reduced by *B. pseudocatenulatum* CECT 7765 administration in HFD mice, which could partially explain the physiological changes described above as well as those detected on metabolic parameters described below.

Higher serum leptin concentrations were observed in HFD than in SD-fed mice. Under certain physiological conditions, leptin, a hormone produced predominantly in adipocytes, reduces food intake, increases energy expenditure and inhibits lipogenesis (19,20). However, obesity often manifests with leptin resistance-associated hyperleptinemia, leading to increased hunger and reduced energy expenditure (21). *B. pseudocatenulatum* CECT 7765 administration decreased leptin levels in obese mice. Reduced leptin levels in obese mice might be beneficial and indicate improved leptin function, which could contribute to the regulation of body-fat distribution and prevention of excessive triglyceride accumulation in peripheral tissues (22) In the obesity model, it also is likely that the greater proportion of smaller adipocytes induced by *B. pseudocatenulatum* CECT 7765 administration could be responsible for reducing plasma leptin concentrations since leptin expression and release may partially depend on adipocyte size (23). Changes in leptin induced by *B. pseudocatenulatum* CECT 7765 in HFD-fed mice were paralleled to reductions in total caloric intake, which could also indicate an improvement in leptin function. *B. pseudocatenulatum* CECT 7765 supplementation also reduced the HFD-induced increase in fasting serum levels of cholesterol, triglycerides, insulin and glucose. Specific probiotic strains have been shown to ameliorate obesity-related metabolic alterations, such as insulin resistance (17,22) and increased cholesterol (24,25) as well as triglyceride levels in rodents and also humans in some cases (26); however, reported effects seem to be strain dependent (5,27). The most likely mechanism by which

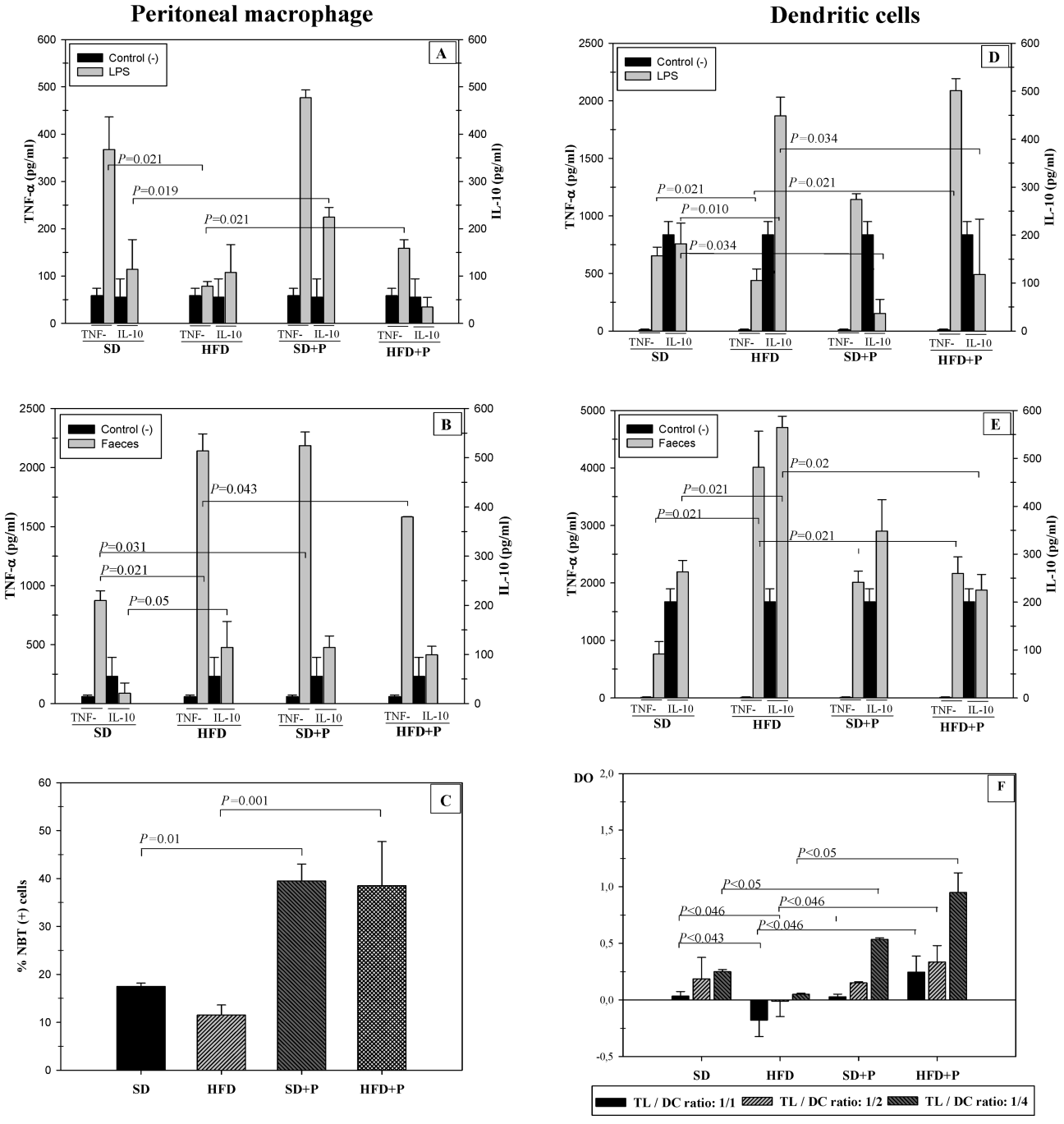

FIGURE 7 Influence of different stimuli on cytokine production and phagocytosis function in peritoneal macrophages and of different stimuli on cytokine production and activation of T-lymphocyte proliferation by dendritic cells (DCs) of mice fed standard diet or high-fat diet supplemented or not with *B. pseudocatenulatum* CECT 7765. SD: standard diet group (control) ($n = 6$); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 7 weeks ($n = 6$); HFD: high-fat diet group ($n = 6$); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU *Bifidobacterium* CECT 7765 by gavage for 7 weeks ($n = 6$). In the cytokine production study, peritoneal macrophages and DCs from different mouse groups were stimulated with purified lipopolysaccharide (LPS) from *Salmonella enterica* serotype Typhimurium (Figure 7**A** and **D**) and macrophages and DCs from control mice were stimulated with fecal samples from different mouse groups (Figure 7**B** and **E**). Nonstimulated peritoneal macrophages and DCs were evaluated as controls of basal cytokine levels. In the phagocytosis study (Figure 7**C**), evidence of oxygen-radical production by macrophages from different mouse groups was determined by the NBT test after *in vitro* interaction with a bacterial extract. In the lymphocyte proliferation study (Figure 7**F**), matured DCs from different mouse groups were used for priming a T-cell proliferative response at the following LT/CD ratios: 1:1, 1:2, 1:4. Lymphocyte proliferation was measured with the cell proliferation ELISA BrdU-colorimetric assay. Data are expressed as mean ± SD of duplicate measurements determined in two independent experiments. Statistically significant differences were established at $P < 0.05$.

some *Lactobacillus* and *Bifidobacterium* strains reduce serum cholesterol is by deconjugation of bile acids. This could lead to a reduction in serum cholesterol either by increasing its utilization in *de novo* synthesis of bile acids or by reducing cholesterol solubility and thereby their intestinal absorption and uptake by the low-density lipoprotein receptor pathway in the liver (28). However, the observed effects could also be due to inhibition of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver through the action of short-chain fatty acids, the end products of carbohydrate fermentation by the microbiota in the gut (29). The effects of reduced triglyceride levels could also be due to the liporegulatory effects of leptin. Direct peripheral actions by leptin have been implicated in depleting fat content through increased fatty acid oxidation as well as suppressed lipogenesis in peripheral tissues (30).

B. pseudocatenulatum CECT 7765 supplementation also reduced serum inflammatory cytokine and chemokine (IL-6 and MCP-1) concentrations, increased in mice under the HFD, and also increased production of the anti-inflammatory cytokine IL-4. These changes in inflammatory markers were parallel to improvements in glucose tolerance and insulin sensibility in HFD-fed mice. In humans and animals, serum inflammatory markers, including IL-6 and MCP-1, are increased in obesity and related to chronic metabolic disorders, such as insulin resistance, type-2 diabetes and cardiovascular disease (31,32). In particular, most studies suggest a role for adipose tissue-derived IL-6, which correlates with serum IL-6, in pathogenesis of hepatic insulin resistance, via modification of liver suppressor of cytokine signaling 3 expression (33). MCP-1 is also a key chemokine that stimulate recruitment of macrophages into the adipose tissue, which is a major contributor to systemic inflammation in obesity (34). MCP-1 serum levels correlate, in fact, with macrophage tissue macrophage infiltration in mice (34). Adipose tissue macrophages have been classified into two subpopulations phenotypically different: (i) classically activated M1 macrophages that secrete pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) and (ii) alternatively-activated M2 macrophages, whose differentiation is stimulated by IL-4 and IL-13, and secrete the anti-inflammatory cytokine IL-10. In the adipose tissue, IL-4 and IL-13 helps to control inflammation and promote normal insulin sensitivity (35). Therefore, *B. pseudocatenulatum* CECT 7765 could also contribute to down-regulate inflammation associated with a HFD by increasing one of the key cytokines that shifts phenotypic differentiation of macrophages to M2 type and, thereby, insulin sensitivity. In *ob*/*ob* mice fed a normal diet prebiotic administration, associated with increased intestinal bifidobacteria, also reduced several serum inflammatory and anti-inflammatory markers, including MCP-1 and cytokines (IL-1β, TNF-α, IL-18, and IL-15), but effects on IL-6 were not significant and those on IL-4 were not reported (36). Serum IL-10 levels were also reduced by *B. pseudocatenulatum* CECT 7765 in our model of HFD-induced obesity, which could be due to parallel down-regulation of regulatory mechanisms, as in the case of ob/ob mice fed prebiotics (36), but its relationship with IL-4 should be further explored.

This study is the first to demonstrate that the bifidobacterial strain administration improves the dysfunction of immunocompetent cells associated with obesity in mice. Oral administration of *B. pseudocatenulatum* CECT 7765 increased the oxidative burst not only in the control but also in HFD fed mice, indicating an improvement in this defense mechanism against pathogens in the context of obesity. It is known that macrophage function is impaired in obese mice, with reduced phagocytic capacity and oxidative burst, which has been linked to increased susceptibility to infections in obese subjects (37). Our study also demonstrated that cytokine production (TNF-α) by peritoneal macrophages and DCs in response to a pathogenic bacterial stimulus (LPS) is boosted by *B. pseudocatenulatum* CECT 7765, which could improve the ability of immunocompetent cells to produce appropriate inflammatory responses in face to infection. This study also confirms that a HFD impairs DC function and that *B. pseudocatenulatum* CECT7765 could restore it, improving the ability of DCs to present antigens and stimulate T-lymphocyte proliferation. Macia *et al.* (38) also demonstrated a functional deficiency of DCs in *ob*/*ob* mice, suggesting that this damage could be generalized to the more frequent forms of obesity. This DC dysfunction could lead to impaired antigen-specific immune responses of adaptive immunity, which could be involved in the high incidence of infection reported in obese patients and in hypo-responsiveness to vaccination (2).

This study also reports differences in the gut microbiota between nonobese and obese HFD-fed mice, which are in agreement with previous studies (39), and may partly be related to diet (40). Our results show that obese mice harboured a significant reduction in gene copy numbers of *Lactobacillus* and *Clostridium* groups and, particularly, *Bifidobacterium* spp., which is partially in agreement with previous studies (8,39). In contrast, other authors found reductions in *Bacteroidetes* abundance and increases in *Firmicutes* in *ob*/*ob* mice (46). *B. pseudocatenulatum* CECT 7765 administration increased total numbers of *Bifidobacterium* spp. and reduced those of *Enterobacteriaceae* These changes in the microbiota composition could contribute to reducing the inflammatory signals coming from the gut, as reflected in the lower inflammatory properties of the gut content, which could affect other peripheral tissues involved in obesity.

Taking the results as a whole, this preclinical study supports the notion that dietary strategies targeting the gut ecosystem with specific bacterial strains could be effective to control metabolic disorders and the associated immunological dysfunction, although definitive evidence should be provided by human trials. O

Acknowledgments

The scholarships of A. Santacruz from CONACYT (México) and P. Gauffin Cano from CONICET (Argentina) and the contract of F. Trejo from MICIIN (Spain) are fully acknowledged.

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