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**MODULATION OF INTESTINAL MICROBIOTA AND IMMUNO-
METABOLIC PARAMETERS BY CALORIC RESTRICTION AND LACTIC
ACID BACTERIA**

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ABSTRACT

The objective of this work was to evaluate the effect of a caloric restriction diet with and without the administration of *Lactobacillus fermentum* CRL1446, *Lactobacillus casei* CRL431 and *Lactococcus lactis* CRL1434, on immune-metabolic parameters and the composition of intestinal microbiota in mice.

The supplementation of the CR diet with *L. fermentum* CRL1446 showed a bifidogenic effect and was able to maintain the abundance of the genus *Lactobacillus* over time. On the other hand, this strain showed hypocholesterolemic and hypoglycemic properties as well as inducing a decrease in plasma leptin levels.

L. casei CRL431 administration increased the abundance of the *Lactobacillus* genera in the intestinal microbiota, which would improve the absorption of nutrients from the diet. This strain restores glucose values decreased by the diet in addition to inducing an increase in leptin and cytokines.

Lac. lactis CRL1434 showed greater immunomodulatory capacity, without significantly affecting the composition of the intestinal microbiota. It had hypoglycemic properties and induced a decrease in leptin concentrations.

L. fermentum CRL1446 and *Lac. lactis* CRL1434 could be potentially probiotic strains useful to correct the immuno-metabolic alterations associated with obesity, while *L. casei* CRL431 is a more suitable strain to be used in cases of malnutrition where it is sought to improve the absorption of nutrients and protection against infections, in addition to the stimulation of the immune system.

Keywords: adipokines, probiotics, microbiota, caloric restriction

1. INTRODUCTION

Caloric restriction (CR) is defined as a reduction in energy intake below the number of calories consumed *ad libitum* ($\geq 10\%$ reduction in humans and usually $\geq 20\%$ reduction in rodents) (Bales & Kraus, 2013). Several researchs had shown that CR can extend lifespan in several animal models, ameliorating aging-related diseases (Masaro, 2009). The mechanisms by which CR exerts its effects remain controversial (Zhang et al., 2013).

The CR favours the *phyla* that correlate positively with "life expectancy" or longevity, such as species belonging to the genus *Lactobacillus* (Moschen, A. R., Wieser, V., & Tilg, H., 2012; Zhang et al., 2013). In addition, these shifts in intestinal microbiota (IM) composition with CR can be beneficial in obesity because would induce a decrease intestinal permeability and lipopolysaccharide (LPS)-related endotoxemia, and thereby reducing chronic low-grade inflammation (Zhang et al., 2013). Furthermore, the IM develops an intense metabolic activity, improving the bioavailability of nutrients and the degradation of non-digestible compounds, supplying new nutrients (short-chain fatty acids, SCFAs), or eliminating harmful anti-nutrients (Krajmalnik-Brown, Ilhan, Kang, & DiBaise, 2012). These metabolic functions have a significant impact on the nutritional status and health of the host (Ojeda et al., 2015). However, as mentioned, it depends on the composition of the gut microbiota and its complex interactions with the individual's diet and genome (Cani, 2014; Cani, 2015; Druart et al., 2015; Sanchez, Panahi, & Tremblay, 2015).

The CR can be explained, from the point of view of evolution, as a compensation mechanism that is involved in the metabolic and neuroendocrine responses that living organisms use to adapt to periods of deficiency of food (Holliday, 1989). It is well

known that a sustainable weight loss over time (between 5% and 10%) in overweight and obese individuals is associated with immuno-metabolic improvements (Wing et al., 2011). However, up to now, the pharmacological approaches have poor long-term efficacy in weight management. Thus, new alternatives should be considered to maintain the beneficial effect of weight loss efficiently over time. In this sense, several authors suggested that the modulation of the IM through different diet interventions or the administration of beneficial microorganisms are interesting strategies to alleviate the alterations caused by nutritional disorders (Sanz, Santacruz, & Gauffin, 2010). In this context, we proposed that the use of non-inflammatory lactic acid bacteria (LAB) strains with the capacity to modulate adipokines secretion could be an effective strategy for the treatment of metabolic diseases associated with immune abnormalities (Fabersani et al., 2017). Furthermore, we suggested that the supplementation of these LABs combined with a CR diet could synergize their beneficial effect in such metabolic disorders.

The aim of this work was to evaluate the oral administration of probiotic *Lactobacillus casei* CRL431, *Lactobacillus fermentum* CRL1446, and *Lactococcus lactis* CRL1434 strains in a murine caloric restriction model. These strains were previously selected (Fabersani et al., 2017) for their adipo- and immune-modulatory capacity. We analysed immune and metabolic parameters and the composition of the IM.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The strains used in this study were *L. casei* CRL431 (CRL431), *L. fermentum* CRL1446 (CRL436) and *Lac. lactis* CRL1434 (CRL1434). LAB strains were obtained from the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina) culture collection. They were isolated in our laboratory from regional products and stools of

healthy infants. The used microorganisms could potentially be considered as probiotic strains or as starter cultures for meat or milk fermentation. Probiotic properties previously determined were immunomodulation (Villena, Salva, Agüero, & Alvarez, 2011), adipose tissue modulation (Fabersani et al., 2017), production of conjugated linoleic acid (Terán et al., 2015), feruloyl esterase activity (Abeijón Mukdsi, Gauffin Cano, González, & Medina, 2012), and protection against intestinal infections (Marranzino, Villena, Salva, & Alvarez, 2012).

Bacteria were cultured in Man–Rogosa–Sharpe (MRS) broth (Britania, Buenos Aires, Argentina) at 37°C for 22 h (stationary growth phase). Cells were harvested by centrifugation (10,000 rpm for 10 min), washed twice with phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), and re-suspended in PBS containing 20% (v/v) glycerol. Aliquots of these suspensions were frozen and stored at –80°C until they were administered to the animals. One fresh aliquot was thawed for every day to avoid variability in the viability of cultures in the experiments. Cells were then resuspended in sterile drinking water to the desired concentration: 1×10^8 cells per mL. Bacterial suspensions were prepared freshly every day and changed every 12 h.

2.2. Animals and Diets

Weaned 21-day old male Balb/c mice ($n = 40$) were obtained from the closed random-bred colony maintained at CERELA. They were housed in individual cages and acclimated to $22 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle. They were then separated into the following five groups ($n=8$ each group): standard diet (SD), caloric restriction diet (CRD), CRD supplemented with CRL1446 (CRD-CRL1446), CRD supplemented with CRL1434 (CRD-CRL1434) and, CRD supplemented with CRL431 (CRD-CRL431).

The composition of SD was; 61% carbohydrates, 23% proteins, 7.5% fats, 4% raw fibre, 3.5%, total minerals (3.10 Kcal/g) (Asociación de Cooperativas Argentinas, Buenos Aires, Argentina). The SD group was *ad libitum* fed with SD and drinking water for 45 days. Animals from CRD groups were acclimated for 5 d to *ad libitum* SD and after this period they were daily fed with a restricted size of SD (25% less than the daily ration) during 45 days in order to reach a 10%–25% body weight loss compared to mice from SD Group. CRD-CRL groups were fed with CRD supplemented with CRL1446, CRL431 or CRL1434 strains in the drinking water at the dose of 10^8 cells/mL.

Body weight was measured weekly and caloric intake was accounted for daily food consumption. At the end of the study, animals were fasted for 12 h, anaesthetized, bled by aortic puncture, and sacrificed 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 $-20\text{ }^{\circ}\text{C}$. The faecal contents were collected at the end of the experimental period (day 45) for microbiological analyses. The white adipose tissue (epididymal) was processed for histological analysis.

The experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals Center of CERELA and the study protocol was approved by the Ethical Committee (approval number CRL-BIOT- EF-2012 /2A).

2.3. DNA Extraction from Intestinal Contents and High-Throughput Sequencing (HTS) of 16S rRNA Gene Amplicons

Fifty milligrams of intestinal contents were homogenized in 500 μL of 10 mg/mL lysozyme solution in Tris-Sucrose buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose, pH 8.0) and incubated for 1 h at $37\text{ }^{\circ}\text{C}$. Total DNA was purified using the

Maxwell R 16 DNA Purification Kit and the Maxwell R 16 Instrument (Promega, Madison, WI, USA), according to the manufacturer's instructions.

The bacterial V3-V4 16S rRNA regions were amplified by PCR. The primer pairs used were 343F (5'-TACGGRAGGCAGCAG-3') and 802R (5'-TACNVGGGTWTCTAATCC-3') according to Polka et al. (Polka, Rebecchi, Pisacane, Morelli, & Puglisi, 2015). The PCR product pool was sent to Fasteris SA (Geneva, Switzerland) to be sequenced using the TruSeq™ DNA sample preparation kit (Illumina Inc., San Diego, CA, USA). High-throughput sequencing was carried out in one lane MiSeq Illumina instrument (Illumina Inc.) using the V2 chemistry in a 2 × 250 configuration sufficient to cover the entire amplicon length, estimated to be ~450 bp. Paired-end assembly and quality filtering were performed by using Flash software (Magoč & Salzberg, 2011). Sample de-multiplexing was carried out using DNA barcoding information per sample and Mothur platform. The assembled and barcode/primer-free sequences were processed for chimera removal using Uchime algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011) and SILVA reference set of 16S sequences (Quast et al., 2013).

Diversity indexes were calculated with Mothur platform using default parameters and average method in the clustering step (Schloss et al., 2009). Different alpha diversity parameters (Sobs and Chao's richness, Shannon's diversity) and evenness indexes were computed from Operational Taxonomic Units (OTUs). OTUs clustered at 97% and using a normalized subset of 23,000 sequences randomly selected after multiple shuffling (10,000×) of the original dataset. Taxonomy assignment at Phylum and Genus levels of the subset of high-quality reads was performed with Bayesian RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007), using an assignment confidence cutoff 0.8. Beta

diversity was analysed by performing Unifrac-weighted analysis (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011).

2.4. Biochemical assays

Plasma triglycerides, total cholesterol, glucose and leptin were measured by enzymatic methods using commercial kits (Wiener Lab, Rosario, Argentina). The plasma leptin levels were determined by enzyme-linked immunosorbent assay (Mouse / Rat Leptin Quantikine ELISA Kit, MN, USA). The plasma IL-2, IL-4, IL-6, IFN- γ , TNF- α , IL-17A and IL-10 were quantified using the BD™ kit (CBA Th1 / Th2 / Th17 Mouse, BD Bioscience, # 560485, CA, USA). The limit of detection for this kit was 0.1 pg / mL (IL-2), 0.03 pg / mL (IL-4), 1.4 pg / mL (IL-6), 0.5 pg / mL (IFN- γ), 0.9 pg / mL (TNF- α), 0.8 pg / mL (IL-17A) and 16.8 pg / mL (IL-10). The concentration of MCP-1 (eBioscience, Inc., Mouse CCL2 (MCP-1) Ready-Set-Go!®, CA, USA)

2.5. Isolation of peritoneal macrophages to evaluate their *ex vivo* functionality

Peritoneal cells were collected by washing the peritoneal cavity of mice from the different groups (45 d), with 5 mL of sterile cold Dulbecco's Modified Eagles Medium (DMEM) (Gibco, #41965-039, CA, USA), containing 10% inactivated (56 °C for 30 min) fetal bovine serum (Gibco, 10082139, CA, USA), 100 μ g/mL streptomycin, and 100 U/mL penicillin (Sigma ALDRICH P 4333, MO, USA). Isolated macrophages were plated into the flasks (Costar® 24, NY, USA) at a concentration of 10^6 cells/mL in DMEM and incubated for 2 h at 37°C in an atmosphere containing 5% CO₂, and non-adhered cells were washed out with warm PBS.

To evaluate the functionality of macrophages, adhered macrophages were cultured overnight into 24-well flat-bottom polystyrene microtiter plates (Costar® 24 well flat, #3524, NY, USA) at the concentration of 1×10^5 cells/mL in DMEM. The media were changed before LAB stimulation, and then, macrophages were incubated in the presence

of 100 μ l of a cell suspension 1×10^7 CFU/ml of each strain for 24 h. Macrophages from different mice groups were also incubated in the presence of purified LPS from *E. coli* serotype O26:B6 (SIGMA-ALDRICH, # L2654, MO, USA) at a concentration of 1 μ g/mL. Non-stimulated peritoneal macrophages were also evaluated as controls of basal cytokine production. Cell viability was evaluated with Trypan-blue (SIGMA-ALDRICH, # T6146, MO, USA). The cell culture supernatants were collected and stored at -20°C until used for cytokine determination. The cytokines were determined in the supernatants of stimulated and non-stimulated cells as previously mentioned in point 2.4. The tests were carried out according to the manufacturer's instructions. Each parameter was determined in triplicate in two independent experiments.

2.6. Isolation of adipocytes to evaluate their *ex vivo* functionality

Adipocytes from the epididymal adipose tissue of the mice from the different groups (45 d) were isolated by collagenase digestion (Collagenase from *Clostridium histolyticum* Type II #C6885 SIGMA, USA.) as previously described (Bernstein, Hyun, Davis, Fulwyler, & Pershadsingh, 1989). Briefly, adipose tissues from 4-6 mice were pooled for adipocyte isolation. Tissue was digested during 1 h incubation period at 37°C in DMEM high glucose supplemented with 10% (v/v) fetal bovine serum, penicillin/streptomycin and containing 1,5 mg/ml collagenase II under constant agitation. The suspension was subsequently centrifuged at 400 rpm for 5 min at room temperature. The mature adipocyte floated to the surface, and the stromal-vascular cells (capillary, endothelial, mast, macrophage, and epithelial cells) were deposited at the bottom. The stromal-vascular cells were removed by aspiration, and the fat cells were washed with 10 ml of DMEM high glucose supplemented with 10% FBS and penicillin/streptomycin and centrifuged at 400 rpm for 2 min. This procedure was repeated twice. Two aliquots (10 μ l) of cell suspension were stained with Trypan-blue

and counted on a hemocytometer to estimate the concentration of adipocyte cells. 0.5 mL suspension of adipocyte cells in plating medium was inoculated into 24-well plates at a concentration of 5×10^5 cells/wells. The cells were maintained in a humidified 5% CO₂ atmosphere for 24 h at 37°C. The media were changed before LAB stimulation, and then, adipocytes were incubated in the presence of 100 µl of a cell suspension 1×10^7 CFU/ml of each strain for 24 h. Then, adipocytes from different mice groups were also incubated in the presence of purified LPS from *E. coli* serotype O26: B6 (SIGMA-ALDRICH, # L2654, MO, USA) at a concentration of 1 µg/mL. Non-stimulated adipocytes were also evaluated as controls of basal leptin production. Cell viability was evaluated with Trypan-blue (SIGMA-ALDRICH, # T6146, MO, USA). The cell culture supernatants were collected and stored at -20°C until used for cytokine determination. The leptin levels were determined in the supernatants of stimulated and non-stimulated cells by enzyme-linked immunosorbent assay (Mouse / Rat Leptin Quantikine ELISA Kit, MN, USA).

2.7. Histological analysis of adipocytes

Paraffin-embedded epididymal adipose tissues were sectioned to a thickness of 4-5 µm and fixed to glass slides. Slides were deparaffinised and stained with hematoxylin-eosin. The adipocyte number and sizes of each group were determined according to the methodology described by Gauffin-Cano et al. (Gauffin Cano, Santacruz, Trejo, & Sanz, 2013) using the Carl Zeiss-Axio Vision software, Release 4.8. Adipocyte number and sizes were measured in 100 cells of two sections of epididymal adipose tissue per mouse (n=8 each group). Adipocyte sizes were expressed as area ranges using the following ranges areas: <500; 500-1000; 1,000-2,000; 2,000-4,000; and > 4,000 µm² to be able to compare between the different groups.

2.8. Statistical analysis

The experiences in animals were performed twice (40 mice each experience), and the determinations were repeated in triplicate. The results were expressed as means \pm standard error mean (SEM). The data were analysed with the SPSS program version 17.0 (SPSS Inc., Chicago, IL, USA). The data were normally distributed and significant differences were determined by applying One and Two-way ANOVA with post hoc Tukey's test or Fisher's least significant difference test. In every case, P -values <0.05 were considered statistically significant.

3. Results and discussion

In this work, we used a caloric restriction model that is achieved with a restriction of 25% of the daily food consumed, which induced an approximately 24% loss of body weight at 45 d of treatment. Usually, the loss of body weight can be related to moderate (26-39%) or severe ($> 40\%$) malnutrition; the latter, which may have a high mortality (Gómez et al., 2000). Unlike these, our caloric restriction model could be considered a model of CR "without malnutrition". There are many epidemiological and clinical studies that suggest that a CR diet without malnutrition may be beneficial, decreasing the incidence of cardiovascular disease, type 2 diabetes, and different types of cancer in humans (Anderson and Weindruch, 2010). Various studies have shown that CR without malnutrition delays the aging process and prolongs the half-life of various species (Spindler, 2010). However, the mechanisms by which this occurs are still not completely known. The inverse linear relationship between calorie intake and half-life suggests that the regulators of energy metabolism are important for the effects attributing to CR (Anderson and Weindruch, 2010). Nevertheless, the evaluation of the metabolic response to the CRD is a field that is not completely clarified and requires additional studies. In order to provide more knowledge about the effect of that

“beneficial” CR on health status prevalence, we evaluate the effects in mice of a CRD with and without the oral administration of different “adipokine-modulatory” LAB (bacteria that has the indirect ability to stimulate the secretion of adipokines): *L. fermentum* CRL1446, *L. casei* CRL431 and *Lac. lactis* CRL1434 (Fabersani et al., 2017). Effects on metabolic and immunological parameters and the structure of the intestinal microbiota were evaluated in two periods of time, at days 20 and 45, with the additional purpose of analysing the impact of time as a variable.

3.1. Effect of CRD supplemented with LAB on body weight gain (BWG) and feed conversion ratio (FCR)

The **Fig 1A** shows the effect of the different dietary treatments on body weight gain (BWG), at days 7, 20 and 45. We observed that CRD induced a significant reduction in BWG compared to the SD. The supplementation of the CRD with LAB also produced significant effects on the BWG. CRL431 strain induced a significant increase in BWG compared with CRD group (24% at day 20 and 21% at day 45), while the supplementation with CRL1446 and CRL1434 strains significantly reduced the BWG. On the other hand, at day 7 (when CRD still does not induce significantly change on BWG), CRL1446 and CRL431 strains showed a different effect with respect to the CRD. Although CRL1446 reduced BWG, CRL431 increased it. The food intake allows evaluating the capacity of an organism to transform grams of food consumed in grams of body weight. This is defined as feed conversion or feed conversion ratio (FCR). The **Fig 1B** shows the FCR, at days 7, 20 and 45. Our results indicate that the CRD significantly decreases the FCR after 20 days of treatment. The administration of LAB also induced significant changes in this parameter. CRL1446 and CRL1434 strains significantly reduced FCR in comparison with the CRD group. On the other hand, the supplementation of CRD with CRL431 significantly increased FCR at all studies times

compared with the CRD group. A strong strain-dependent effect on the BWG and FCR was demonstrated, that suggests a modulatory effect on the energetic metabolism (since all the animals of the CRD groups consumed the same amount of feed). These results suggest a different energy extraction capacity by each group of animals. The efficiency to extract energy from the diet is closely related to the structure of the microbial community (Bäckhed et al., 2004; Turnbaugh et al., 2009). Various effects of the administration of probiotics have been reported on the BWG and the accumulation of body fat. Raoult *et al.* suggest that probiotics, mostly microorganisms from the *phylum* Firmicutes, and particularly *Lactobacillus* spp genera, could be related to both the control of body weight and the development of obesity (Raoult, 2009). These authors, also described in children under diarrhoea treatment, an increase of BWG after received *Lactobacillus* spp. Contrary, Ehrlich and Delzenne and Reid affirm that probiotics are not related to the development of obesity since the humanity has been consuming probiotics without inducing obesity (Delzenne & Reid, 2009; Ehrlich, 2009). In this regard, Kang et al. demonstrated that the administration of *Lactobacillus gasseri* BNR17 induced a reduction in body and white adipose tissue weight in overweight rats (Kang et al., 2013). In line with this, other authors reported beneficial effects of *Lactobacillus* in diet-induced obesity models (Novotny Núñez, Maldonado Galdeano, de Moreno de LeBlanc, & Perdigón, 2015; Tomaro-Duchesneau et al., 2014; Toshimitsu, Mochizuki, Ikegami, & Itou, 2016; Yoo et al., 2013)

3.2. Effect of the CR diet supplemented with LAB on the structure of adipose tissue (AT)

We analysed the AT weight (**Fig. 2A**), and the number of adipocytes into different area ranges (distribution of adipocyte size) (**Fig. 2B and 2C**) in all the studied experimental groups. Significant differences in the AT weight were observed. The CRD induced a

significant reduction in AT weight (**Fig.2A**) of 18% at day 20 and 24% at day 45 with respect to the SD group. Supplementation of the CRD with CRL431 completely restored the AT weight at day 20 and partially at day 45. While the supplementation with CRL1446 and CRL1434, induced a reduction of TA weight of about 22% at day 20, in comparison with the CRD groups. Regarding the analysis of adipocyte area, the CRD favoured a significant increase in the number of adipocytes of a smaller area (between 0-500 and 500-1000 μm^2) and the reduction in the number of adipocytes of a greater area (between 1000-2000 and ≥ 2000 μm^2) with respect to the SD (**Fig. 2B and 2C**). Supplementation with CRL1446 induced significant changes at day 45 (**Fig.2C**), causing a large increase of small adipocytes with an area of 0-500 μm^2 .

The changes observed in the BWG were associated with changes in the size of the AT weight and the area of adipocytes. We suggest that the effect on the BWG is strain dependent, and it is mainly due to changes in the structure of the AT induced by the diet and/or the synergism between LAB and diet. Sato et al already mentioned a close relationship between the administration of a product containing a *Lactobacillus* and the decrease in the size of adipocytes (Sato et al., 2008). According these authors, the decrease in adipocyte size could be one mechanisms for preventing obesity due to the inhibition of hypertrophy and hyperplasia. Futhermore, previous research suggested that adipocyte size is a significant indicator for the future development of metabolic disease (Weyer, Foley, Bogardus, Tataranni, & Pratley, 2000). Adipose tissue is not only an energy storage organ, but it is also an endocrine and immune organ able to secrete hormones and cytokines, known as adipokines, with implications in immune function and energetic homeostasis (Antuna-Puente, Feve, Fellahi, & Bastard, 2008; Grant & Dixit, 2015; Sam & Mazzone, 2014)

3.3. Effect of the CRD supplemented with LAB on plasma metabolic parameters and cytokines.

In the **Fig. 3A, B, C, D** we showed the effects of CRD and LAB in the glucose, total cholesterol, triglycerides and leptin levels. Our results revealed that the CRD significantly reduce the levels of these metabolites in plasma at days 20 and 45, compared with SD animals.

3.3.1. Glucose

The supplementation of the CRD with CRL1446 and CRL1434 at day 45 induced a significant decrease in glucose with respect to the CRD group (**Fig. 3A**). The administration of the CRL431 strain for 20 days increase glucose levels to values similar to those of SD groups. (**Fig. 3A**). These results show the modulatory effect of LAB on glucose metabolism and may be related to the modulation of leptin levels, which affects the sensitivity of insulin and therefore glucose levels (Al-Jada & Ahmad, 2014).

3.3.2. Leptin and triglyceride

We showed that the CRD was adipokine-modulatory by itself, since it significantly reduced leptin levels (about 70% reduction) compared with the SD (**Fig. 3D**). The administration of CRL431 induced a significant increase in leptin levels (about 32%) with respect to the CRD group. In contrast, supplementation with CRL1446 and CRL1434 only had an effect at day 45, where they induced a significant reduction in leptin levels (about 50%) compared with the CRD control.

The CRD diet was the only treatment responsible for the decrease in triglycerides. On the other hand, we did not observe significant effects with any strain on triglyceride levels (**Fig. 2C**). However, previously we showed that the administration of other beneficial bacteria, as *Bifidobacterium pseudocatenulatum* CECT7765 and *Bacteroides*

uniformis CECT7771, were able to reduce plasma triglycerides levels in obese mice. This effect was not observed in lean mice (Gauffin Cano et al., 2013; Gauffin Cano, Santacruz, Moya, & Sanz, 2012).

Leptin is a pleiotropic hormone and is regulated by numerous factors. One of them is the size of the adipocytes, which depends on the plasma levels of triglycerides, either coming from the diet or synthesized *de novo* (liver). Strains CRL1446 and CRL1434 were considered hypoleptinemic; however, we did not observe changes in triglyceride levels in addition to those induced by the CRD. But, we observed changes in the size of adipocytes (**Fig 2B and 2C**), probably due to changes in adipocytes turnover. Furthermore, the modulation of leptin in these experimental conditions could be also related to changes in its secretion by other cells.

3.3.3. Cholesterol

When we analysed the total cholesterol values, we observed that only CRL1446 strain significantly reduced it compared to the control of CRD group (**Fig. 3B**). Some authors (Fontana, Meyer, Klein, & Holloszy, 2004) showed that CRD has beneficial effects on cardiovascular risk factors such as atherosclerosis, serum cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride (which usually increase with advancing age). These authors also showed that CRD provides a powerful protective effect against obesity and insulin resistance. Approximately, up to 1 g of dietary cholesterol enters the colon, and despite the enormous variations observed among individuals, only half of this dietary cholesterol is absorbed on average, mainly in the duodenum and the proximal jejunum (Gérard, 2013). In addition to the cholesterol from the diet, serum levels can be maintained by *de novo* synthesis that occurs mainly in the liver. Since CRD also affects the structure of the intestinal microbiota (**Fig 6**), it could indirectly alter the metabolism of hepatic cholesterol and the *de novo* synthesis of this lipid (Caesar, Nygren, Orešič, &

Bäckhed, 2016). Changes in the predominant *phyla* (i.g., prevalence of Firmicutes and Bacteroidetes) has been shown to affect the extraction of energy from the diet and therefore the levels of metabolites in the blood (Bäckhed et al., 2004; Jumpertz et al., 2011). In our experimental model, CRL1446 had a hypocholesterolemic effect and could be acting synergistically with the CRD. We previously demonstrated that CRL1446 has been shown to have a high feruloyl esterase activity (Abeijón Mukdsi et al., 2012) and could probably be the mechanism of action by which this strain exerts its hypocholesterolemic effect, in addition to the modifications that induces on the gut microbiota. In concordance, Bhatena *et al.* reported that the feruloyl esterase producing strain, *L. fermentum* LF11976, decreased serum lipids in hypercholesterolemic hamsters (Bhatena, 2013). This assertion is based on the fact that the ferulic acid has hypocholesterolemic properties, since it can inhibit hydroxymethylglutaryl CoA reductase (a limiting enzyme in the biosynthesis of cholesterol) and cholesterol acyltransferase (enzyme that esterifies cholesterol in the tissues, mainly in the liver), and can increase the excretion of acid sterols (Balasubashini, Rukkumani, & Menon, 2003).

3.3.4. Cytokines

Concerning the immune status, the levels of the following cytokines were determined in plasma at day 45 of intervention: IL-2, IL-4, IL-6, IL-10, IL-17, IFN- γ , TNF- α and MCP-1. However, we only observed significant changes in the levels of TNF- α and IL-10 (**Fig. 4A and B**). The CRD significantly reduced TNF- α levels compared to SD. The supplementation with CRL1434 and CRL431 induced a significant increase in the levels of IL-10 compared to SD and CRD groups. These strains had an important modulatory capacity since they were able to induce a significant increase of IL-10 levels. IL-10 is a regulatory cytokine, which inhibits the expression of pro-inflammatory cytokines such

as TNF- α or IL-6 (Opal & Depalo, 2000). Some authors demonstrated a relationship between insulin resistance and levels of this cytokine (Kim et al., 2004). Also, that higher IL-10 levels can protect from high-fat-diet-induced obesity and glucose intolerance in mice (Gao et al., 2013). Cytokines act in concert to regulate the human immune and metabolic response. If we analyse together plasma levels of leptin and TNF- α (two pro-inflammatory molecules), we could infer that the reduction of these adipokines by CRD are associated with the decrease in AT weight and plasma lipid. Similar results were previously reported by other authors (Fontana, Klein, & Holloszy, 2010; Fontana et al., 2004). The multiple effects of leptin on immune system suggest that immunomodulation by leptin may have therapeutic potential in a variety of diseases (Farooqi & O'Rahilly, 2014). Thus, leptin and TNF- α are positively correlated with the increase of AT mass (Faggioni, Feingold, & Grunfeld, 2001), inflammation, and the development of chronic diseases linked to diet such as obesity and type 2 diabetes (Rose, Komninou, & Stephenson, 2004).

Therefore, we can suggest that the capacity of CRD to modulate these adipokines secretion (such as leptin and TNF- α) could be one of the main mechanisms by which this diet (CR) prolongs the half-life of different species (Colman et al., 2009). It has been established that the main adipokines that influence the adipo-insular axis (relationship between AT and pancreatic β -cells), are leptin, adiponectin and TNF- α (Dunmore & Brown, 2013). Most studies suggest that leptin and TNF- α induce a decrease on the synthesis and secretion of insulin by β -pancreatic cells (Andreev, Paz-Filho, Wong, & Licinio, 2009; Dunmore & Brown, 2013). Then, this adipo-insular axis may participate in the inhibitory feedback mediated by leptin on insulin secretion to decrease adipogenesis.

3.4. Effect of the CRD and LAB administration on the functionality of murine adipocytes and peritoneal macrophages

3.4.1. Adipocytes

The functionality of *ex vivo* adipocytes was evaluated through the production of leptin (**Fig. 5A and B**). The adipocytes obtained from the epididymal AT of the different groups showed a different profile of leptin production. The amount of leptin produced by unstimulated adipocytes was considered as basal values (DMEM, basal control). While, adipocytes stimulated with LPS were considered as control of the stimulation (LPS, control (+)). Our results showed that the CRD induced a reduction on basal leptin levels, and including those measured in LPS-stimulated adipocytes. Previously, several authors have been reported that leptin levels are directly proportional to the size of adipocytes (Skurk et al., 2007). Thus, reduction in leptin values may be related to the reduction in the adipocytes size of CRD group.

Our study demonstrated that adipocyte capacity to release leptin was strain-dependent. At day 45, the administration of CRL1446 and CRD-CRL1434 groups induced a significant reduction in the leptin production by LPS-stimulated adipocytes, with respect to the CRD group. In addition, at day 45 of treatment, the LPS-stimulated adipocytes from CRD-CRL431 group produced significantly high levels of leptin in comparison with LPS-stimulated adipocytes from CRD-CRL1446 and CRD-CRL1434 groups (**Fig. 5B**). From the immunological point of view, the leptin is an adipokine which can elicit a pro-inflammatory immune response against pathogenic bacterial stimulus (like as LPS). The different studies showed an extensive actions of leptin on immune responses (Lago, Gómez, Lago, Gómez-Reino, & Gualillo, 2008). The CRD associated to CRL431 could promote the ability of adipocyte cells to produce appropriate leptin response and, consequently, may contribute to the immune response

against an infection (Gauffin Cano & Perdigon, 2003). While, with the other strains (CRL1446 and CRL1434), the leptin decrease could be reinforce their hypocholesterolemic and hypoglycemic effects.

3.4.2. Peritoneal macrophages

The functionality of *ex vivo* peritoneal macrophages was assessed by the production of the TNF- α , IL-6 and IL-10 cytokines and MCP-1 chemokine (**Fig. 5C – J**). We observed that the CRD induced a reduction of both, the basal (DMEM) and the LPS-stimulated cytokines and chemokine production compared with SD group. In general, the supplementation with CRL1434 and CRL431 favour the basal production of TNF- α , IL-6, (pro-inflammatory molecules), and IL-10 (regulatory cytokine). While CRL1446 only induce change in IL-10 after LPS stimulus with respect to the CRD group at day 45 (**Fig. 5C, D, E, F, I and J**).

Although there was a reduction in the levels of cytokines induced by CRD, peritoneal macrophages may respond appropriately to a pro-inflammatory stimulus such as LPS, which indicates that they maintained their functionality under this regimen (**Fig. 5C, D, E, F, I and J**). After LPS stimulus at day 20 of treatment the levels of TNF- α , IL-6 and IL-10 were significantly higher in the groups supplemented with LAB (CRD-CRL1446, CRD-CRL1434 and CRD-CRL431) compared with CRD group. In this regard, the CRL431 showed the highest capability of induction of TNF- α , and CRL1434 of IL-10. On the other hand, the levels of MCP-1 were significantly higher in the groups supplemented with CRL431 and CRL1434 strains, compared with the CRD group at day 20, this strains reached levels similar to the SD control.

On the other hand, the levels of MCP-1 were significantly lower in the groups supplemented with CRL1446 and CRL1434 strains, compared with the CRD group, while in the CRD-CRL431 group the levels were similar to the SD control.

After LPS stimulus at days 20 and 45 of treatment, groups CR-CRL1434 and CR-CRL431 had the higher IL-10 levels. Regarding TNF- α levels, both the CR-CRL431 and CR-CRL1434 groups showed a significant increase in this cytokine at day 45. While for IL-6, only the CR-CRL431 group differed significantly with respect to CRD group. Finally, we only observed significant changes with CR-CRL1434 and CR-CRL431 at day 20.

These results demonstrate a strong immune- and adipo-modulatory effect of LAB on macrophages and adipocytes, which was also strain dependent. We observe increases in the levels of IL-10 (mainly in CR-CRL1434 group), TNF- α , and IL-6 (mainly in CR-CRL431 group).

3.5. Effect of the CR diet and LAB administration on the composition of the intestinal microbiota (IM)

The microbial communities present in the mice faecal samples of the different groups were analysed by massive and parallel sequencing of 16S rDNA amplicons (**Fig.6**). Our results represent an evidence of the LAB effect associated to CRD on IM, which was also strain-dependent and it may not be generalized at the genus level. Some alpha diversity parameters were showed in **Table 1**. The observed richness (rarefaction analysis) shows that there were not great differences in the IM from the different groups. However, all CRD groups showed a tendency to a smaller number of gut microbial species when compared with the SD group (Sobs index). Interestingly, the CRD seems to induce a diversity gain in terms of homogeneity of the microbial communities, since it increased the Shannon index over time. This was supported by the inverse of the Simpson index (1/Simpson index indicates microbial diversity), since we determined that the CRD for day 45 induce an increase of almost twice (11.8) of the microbial diversity observed at day 20 (6.21). The supplementation of the CRD with

CRL1446 and CRL431 reduced the biodiversity, mainly due to the increase of the dominance of specific species in the microbial community. This assessment was demonstrated by the augment of the Simpson index and decline of the Shannon index, with respect to the other treatments.

Our results demonstrate important changes in the composition of the IM (both *phyla* and genus) in the study groups (**Fig. 6 A-D**). The CRD induce changes in the relative abundance of specific groups of bacteria in the faecal content of the mice. Thus, this diet favorous a reduction of the *phyla* related to pathogens, such as Proteobacteria and TM7 compared with the SD. In addition, the CRD induced an increase of Bacteroidetes and Actinobacteria *phyla* (but this last *phylum* decreased over time). Firmicutes showed no significant changes between the SD and CRD groups. Consequently, CRD significantly reduced the Firmicutes/Bacteroidetes ratio (F/B) (**Fig. 6E and 6F**) with respect to the SD. This is in agreement with other authors, who used other restrictive treatments, such as bariatric surgery (Damms-Machado et al., 2015). This reduction in the index F/B is probably related to the energy extraction by the host (Jumpertz et al., 2011). Previous research have demonstrated that the Firmicutes *phylum* favorous the extraction of energy from the diet (Flint, Scott, Duncan, Louis, & Forano, 2012; Krajmalnik-Brown et al., 2012), this reduction in Firmicutes *phylum* was greater when we supplemented the CRD with CRL1446 (day 20), whereas CRL431 induced an opposite effect, increasing the levels of the F/B index (day 20) (**Fig. 6E and 6F**). The species belonging to the *phylum* Firmicutes have demonstrated a great capacity to degrade the polysaccharides of the diet and favour the absorption of sugars and other metabolites (Flint et al., 2012; Krajmalnik-Brown et al., 2012), thus maximizing the energy supply provided by the diet (Jumpertz et al., 2011). We could suggest that the

effect of the dietary treatments on the F/B index partially explain the changes on the AT, the profile of adipokines, BWG, and the biochemical parameters observed.

The CRD induced an increase of *Bifidobacterium* and *Lactobacillus* genera, which accounted between 60% and 70% of the total microbiota of this group. *Barnesiella*, is another genus that showed an increase due to CRD. Zhang et al., reported that the CRD enriches the IM with filotypes that correlate positively with the half-life, for example the *Lactobacillus* genus, and decrease those that correlate negatively, such as opportunistic pathogens (Zhang et al., 2013).

In CRD-CRL1446 group, we observed a F/B ratio reduction associated to a reduction of the *phylum* Firmicutes and *Lactobacillus*. However, it was the only group that no change the levels of *Lactobacillus* during the treatment. In another groups, with the exception of CRD-CRL431 group, a decrease of the *Lactobacillus* number was observed at days 20 and 45. Contrariwise, the supplementation with *L. casei* CRL431 induced higher levels of this genus at both days 20 and 45. In addition, the CR-CRL431 group showed the highest value of the F/B ratio at day 20.

The increase of Actinobacteria *phylum* was observed in CR-CRL1446 group with a significant increase in the relative abundance of the *Bifidobacterium* genus ($\approx 50\%$ of the microbial community at day 45). As was previously reported, this results demonstrate a bifidogenic effect for the CRD combined with the supplementation of CRL1446 (Russo et al., 2016). Zhang et al. (2013) suggested that under conditions of restricted nutrient availability, as occurs in CRD, the organism can extract nutrients more efficiently from proteins and fats, leaving an abundant proportion of indigestible plant and polysaccharides in relation to other sources of energy in the colon (Zhang et al., 2013). This statement could explain the increase in the abundance of bifidobacteria

by CRD, that relies on the bifidogenic effect reported for dietary fiber (Davis, Martínez, Walter, Goin, & Hutkins, 2011).

The supplementation of the CRD with *L. casei* CRL431 induced a significant increase of the *phylum* Firmicutes at day 20 of treatment, accompanied with an increase of the *Lactobacillus* genus (70% of the IM). The genera *Lactobacillus* and *Bifidobacterium* are responsible for beneficial effects on the host (Saez-Lara, Gomez-Llorente, Plaza-Diaz, & Gil, 2015), could represent another mechanism by which the CR diet extends the average life of an individual, this due to the known capacity of these LAB to inhibit the adhesion of pathogens to the intestinal wall, protect the alteration of the intestinal barrier, improve intestinal transit, among others.

Finally, the CR-CRL1434 group did not show significant differences in the composition of the microbiota (*phylum* and genus) with respect to the CRD group.

4. Conclusions

The CRD had a significant effect on IM composition. In addition, the supplementation of the CRD with specific LAB can modify the effects of this diet on the IM composition. This study reports a bifidogenic effect associated with the CRD, which was higher after the supplementation with *L. fermentum* CRL1446. Thereby, the CRD supplemented with LAB could represent an effective treatment to promote the growth of intestinal beneficial bacteria and improve the health of the host. *L. fermentum* CRL1446 and *Lac. lactis* CRL1434 could be potentially probiotic strains to improve the immunometabolic alterations associated with obesity. While, *L. casei* CRL431 could be used in cases of undernourishment, where this strain could improve the absorption of nutrients and protection against infections, further the stimulation of the immune system.

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FIGURE LEYENDS

Figure 1. Body weight gain (A) and feed conversion ratio (FCR) (B) in mice at days 7, 20 and 45 after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. Controls mice were fed with standard diet (SD) or CRD without bacterial supplementation. The FCR is the relation between the food consumed (g) and the body weight gain (g) expressed as a percentage. Each group of data is represented by the mean \pm SEM (standard error mean). In the same period of treatment (day 7, 20 or 45), data with different lowercase letters (a–d), data with different lowercase letter with apostrophe (a'–e') and data with different uppercase letters (A–E) are significantly different ($P < 0.05$) according to ANOVA statistical analysis.

Figure 2. Relative weight of adipose tissue (AT) at days 20 and 45 (A), distribution of adipocyte size at day 20 (B), and distribution of adipocyte size at day 45 (C) after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. Adipocyte cell sizes are expressed as area ranges as follows: <500 , 500–1000, 1000–2000, and 2000–4000 μm^2 . Photomicrographs 40X of representative HE-stained slides of AT at day 45 are shown. Data are represented by the mean \pm SEM (standard error mean). In Figure A, data with different lowercase letters (a–c) and data with different uppercase letters (A–C) at the same period of treatment (day 20 or 45) are significantly different ($P < 0.05$). In Figure B and C, data with different lowercase letters (a,b) in the same adipocyte cell sizes (area ranges) are significantly different ($P < 0.05$). These evaluations were carried out using ANOVA statistical analysis.

Figure 3. Plasma levels of glucose (A), total cholesterol (B), triglycerides (C) and leptin (D) at days 20 and 45 after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. Data are represented by the mean \pm SEM (standard error mean). In the same period of treatment (day 20 or 45), data with different lowercase letters (a–c), and data with different uppercase letters (A–D) are significantly different ($P < 0.05$) according to ANOVA statistical analysis.

Figure 4. Plasma levels of pro-inflammatory TNF- α (A) and anti-inflammatory IL-10 (B) cytokines at days 20 and 45 after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. Data are represented by the mean \pm SEM (standard error mean). Data with different lowercase letters (a–d) at day 45 are significantly different ($P < 0.05$) according to ANOVA statistical analysis.

Figure 5. Levels of leptin (A, B) produced by LPS-stimulated or non-stimulated *ex vivo* epididymal adipocytes at days 20 and 45; and levels of TNF- α (C, D), IL-6 (E, F), MCP-1 (G, H) and IL-10 (I, J) produced by LPS-stimulated or non-stimulated *ex vivo* peritoneal macrophages at days 20 and 45. The cells were obtained from mice after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. DEMEN or control (-): basal levels of leptin, TNF- α , IL-6, MCP-1 IL-10 produced by epididymal adipocytes or peritoneal macrophages without any stimulus. LPS or control

(+): epididymal adipocytes or peritoneal macrophages stimulated with LPS. Data are represented by the mean \pm SEM (standard error mean). At day 20 or 45, in cells without stimulus (DEMEN) and cells with stimulus (LPS), data with different lowercase letters (a–c), and data with different uppercase letters (A–E) are significantly different ($P < 0.05$) according to ANOVA statistical analysis.

Figure 6 Relative abundance (%) of the *phyla* (A, B) and *genera* (C, D) and F/B index (Firmicutes /Bacteroidetes ratio) (E, F) in the intestinal faecal content of mice after the dietary intervention with a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL. The microbial communities present in the mice faecal samples of the different groups were analysed by massive and parallel sequencing of 16S rDNA amplicons. Each group of data represents the average of 6 mice and the 20 main genera that represent more than 99% of the intestinal microbiota are presented.

Table 1 Common ecological parameters of alpha diversity for the microbial communities analysed.

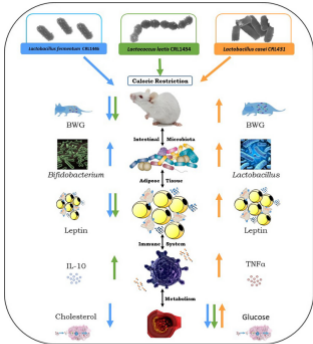
| Experimental groups | days | Alpha diversity coefficients | | | |
|---------------------|------|------------------------------|------|---------|---------|
| | | Distance | Sobs | Shannon | Simpson |
| SD | 20 | 0,0300 | 1374 | 3,6777 | 0,0836 |
| | 45 | 0,0300 | 1400 | 3,4771 | 0,1963 |
| CR | 20 | 0,0300 | 1275 | 3,0224 | 0,1609 |
| | 45 | 0,0300 | 1399 | 3,6284 | 0,0848 |
| CR-CRL1446 | 20 | 0,0300 | 1269 | 2,7711 | 0,2049 |
| | 45 | 0,0300 | 1193 | 2,2196 | 0,2742 |
| CR-CRL1434 | 20 | 0,0300 | 1235 | 3,4516 | 0,0988 |
| | 45 | 0,0300 | 1474 | 3,7409 | 0,0740 |
| CR-CRL431 | 20 | 0,0300 | 1133 | 2,3185 | 0,2650 |
| | 45 | 0,0300 | 1249 | 2,7095 | 0,2454 |

The data were obtained from mice after the dietary intervention at 20 and 45 days of standard diet (SD), or a caloric restriction diet (CRD) with and without administration of *L. fermentum* CRL1446 (CRD-CRL1446), *Lac. lactis* CRL1434 (CRD-CRL1434) and *L. casei* CRL431 (CRD-CRL431) at a dose of 1×10^8 CFU/mL.

Highlights

- Caloric restriction and lactic bacteria change gut microbiota composition in mice
- Caloric restriction and lactic bacteria modulate immune-metabolic parameters in mice
- The immune-metabolic effects of the lactic bacteria are strain-dependent
- *L. fermentum* CRL1446 has bifidogenic effect.
- *L. fermentum* CRL1446 displays hypocholesterolemic and hypoglycemic properties

ACCEPTED MANUSCRIPT



Graphics Abstract

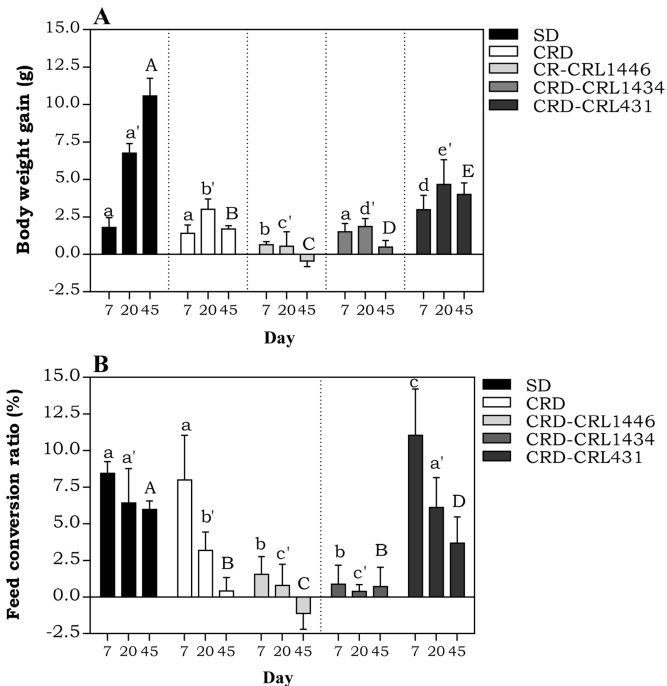


Figure 1

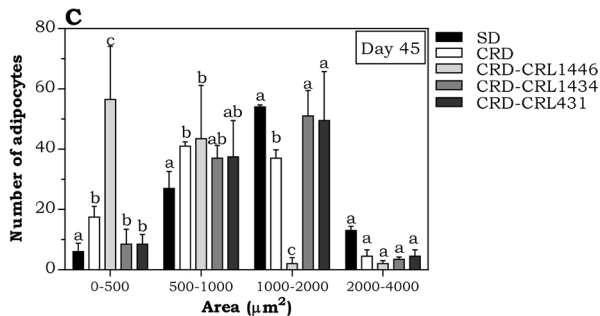
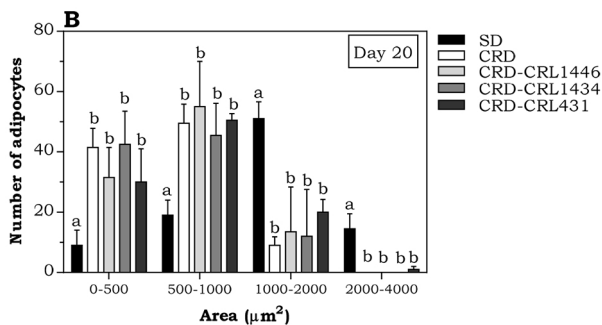
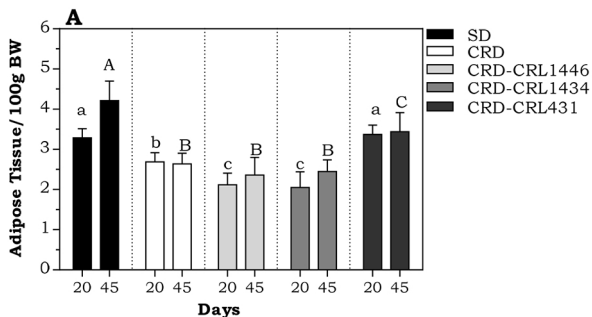
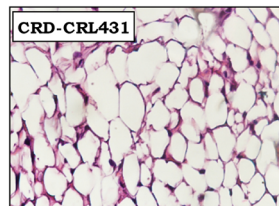
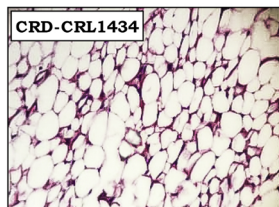
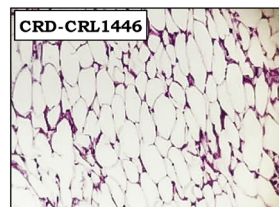
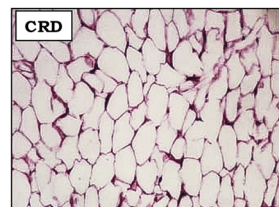
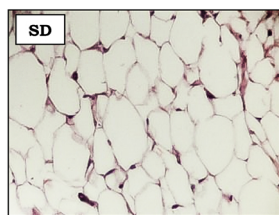


Figure 2

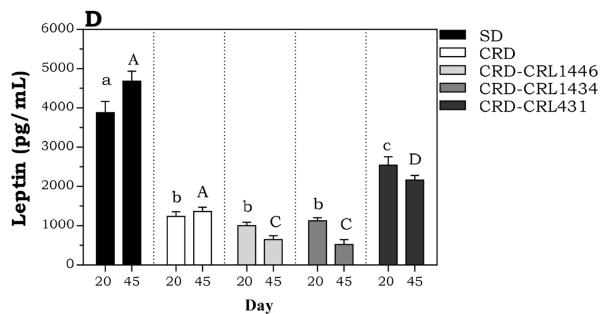
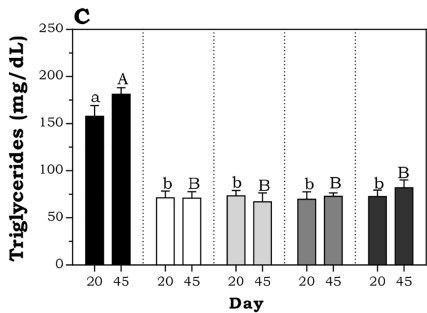
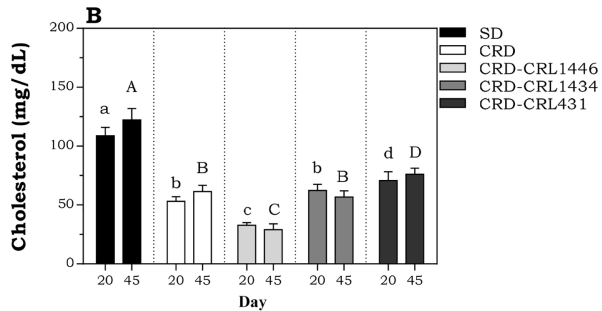
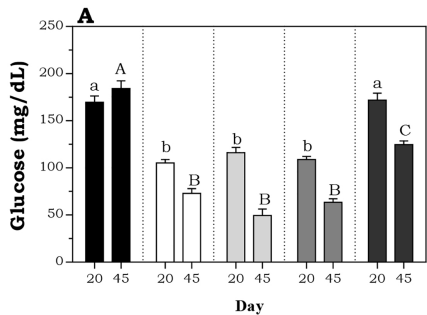


Figure 3

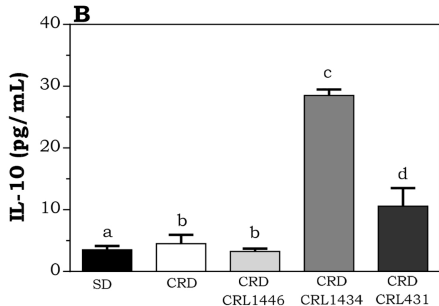
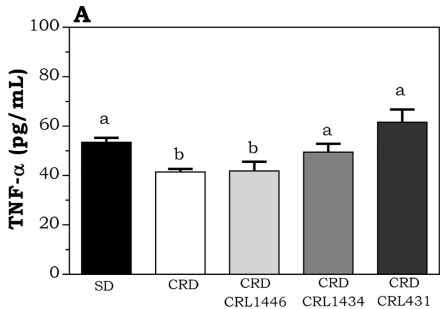


Figure 4

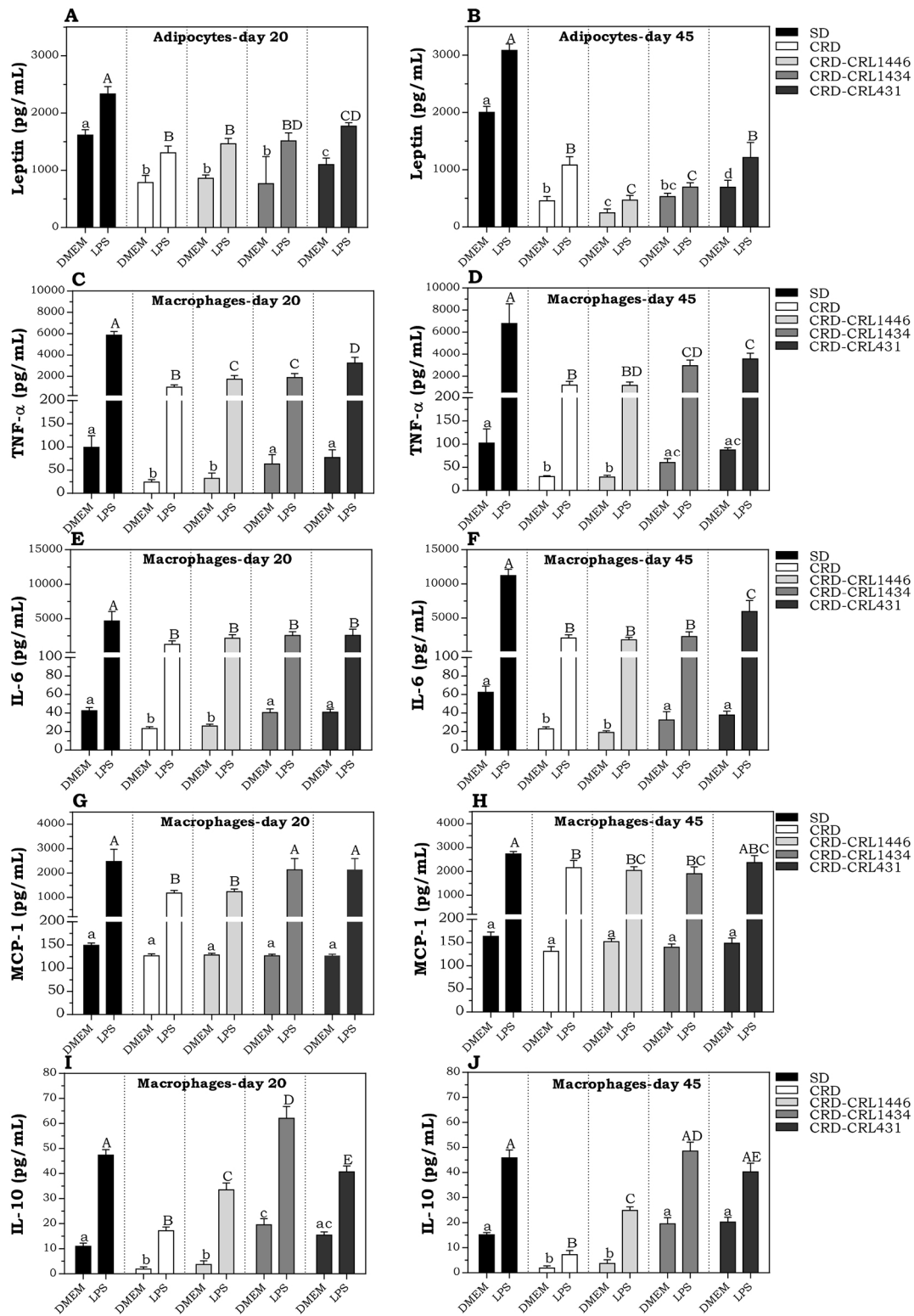


Figure 5

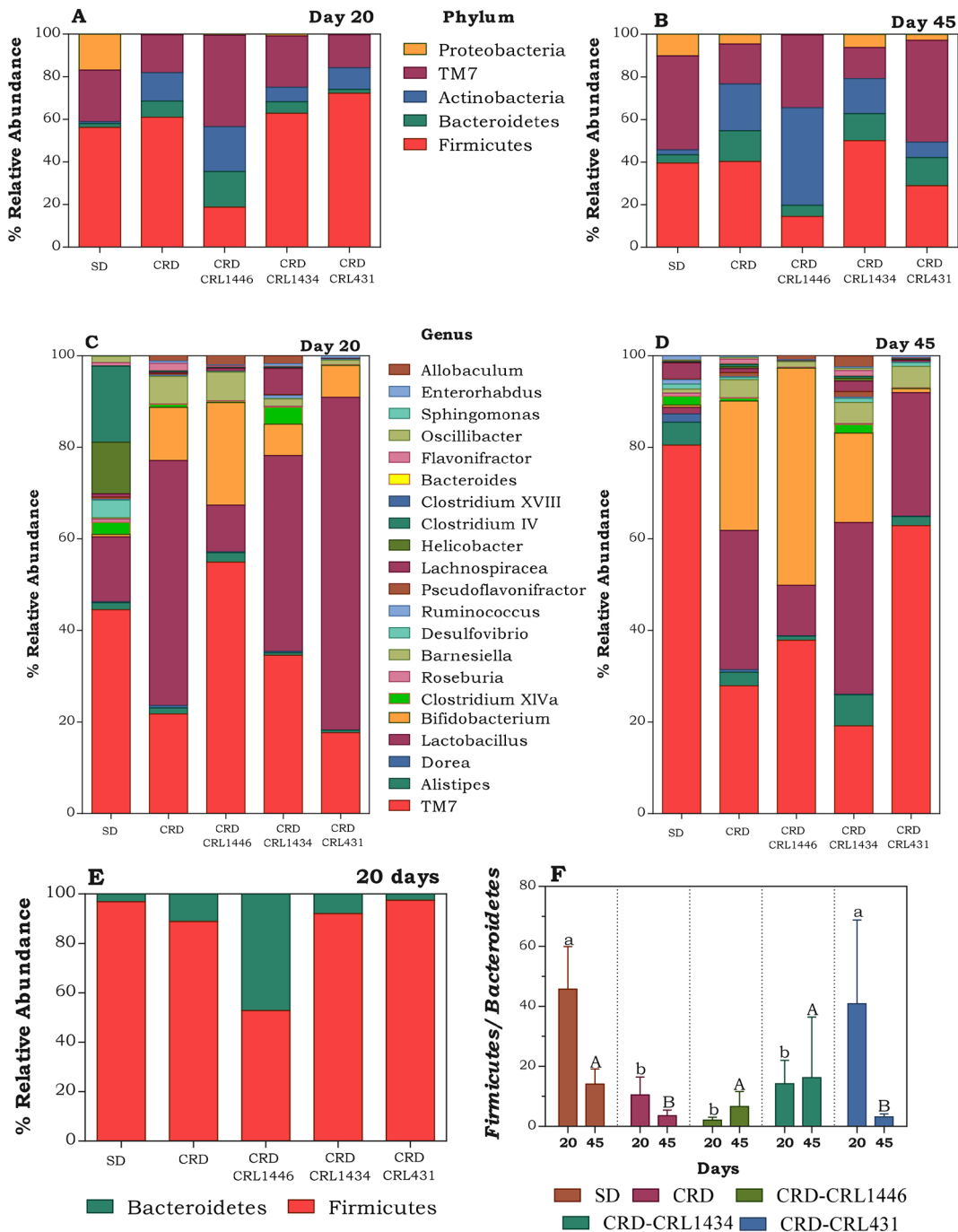


Figure 6