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Anti-obesity properties of the strain *Bifidobacterium animalis* subsp. *lactis* CECT 8145 in Zücker fatty rats

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RESEARCH ARTICLE

Abstract

We evaluated the effect of oral administration of *Bifidobacterium animalis* subsp. *lactis* CECT 8145 strain in Zücker fatty rats. The Zücker fatty rats were randomly divided into two groups (n=10 each) and administered either *B. animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day) suspended in skim milk, or skim milk alone (control group). Each treatment was administered in drinking bottles from week 5 until week 17 of age. A lean Zücker rat group (standard group) was included to provide normal values for the Zücker strain. This group was administered skim milk in the drinking bottle for the same experimental period as Zücker fatty rats. Body weight gain was greater in the fatty control group than in the fatty rats treated daily with *B. animalis* subsp. *lactis* CECT 8145. Furthermore, dry and liquid food intake significantly decreased in the treated Zücker fatty group and these rats also showed decreased plasma ghrelin levels as compared with the Zücker fatty control group. *B. animalis* subsp. *lactis* CECT 8145 intake also decreased plasma tumour necrosis factor-α (a proinflammatory cytokine) and plasma malondialdehyde (a biomarker of oxidative stress). Moreover, the ratio plasma total cholesterol/plasma cholesterol transported by high-density lipoproteins, considered as an index for cardiovascular disease, also significantly decreased in the Zücker fatty rats treated with *B. animalis* subsp. *lactis* CECT 8145. By contrast, this bacterial strain significantly increased plasma adiponectin (an insulin-sensitising adipokine), but did not produce significant effects on triglyceride levels or glucose metabolism biomarkers. Although further research is required to confirm *B. animalis* subsp. *lactis* CECT 8145 is an efficient anti-obesity treatment in humans, the results obtained in this study are promising and point to the health and anti-obesity properties of this bacterial strain.

Keywords: *Bifidobacterium* spp., cholesterol, glucose, ghrelin, obesity

1. Introduction

Obesity is an independent risk factor for cardiovascular disease, associated with an increased risk of morbidity and mortality. In fact, prevalence of obesity has increased dramatically worldwide over the last decades and is becoming a global epidemic in both children and adults. Therefore, adipocytes cannot be considered nowadays a simply storage depot for body energy but in terms of its behaviour as an endocrine mediator, orchestrating crucial interactions with vital organs and tissues. Small adipocytes in lean individuals promote metabolic homeostasis, but the enlarged adipocytes of obese individuals recruit macrophages, promote inflammation and release a range of factors commonly known as adipokines. The latter predispose to insulin resistance, alterations in the lipid profile and atherosclerosis, changes in blood pressure and endothelial dysfunction. Dysregulated adipokine production or secretion caused by excess adipose tissue, in addition with adipose tissue dysfunction, can contribute to the development of obesity-related metabolic diseases (Bastien *et al.*, 2014; Jung and Choi, 2014; Juonala *et al.*, 2011; Reilly and Kelly, 2011; Van Gaal *et al.*, 2006).

The human distal gut harbours a vast ensemble of microbes (the microbiota), which provides important metabolic

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functions. Microbiota was identified as an additional factor contributing to the pathophysiology of obesity. Moreover, chronic inflammation induced by a dysbiotic gut microbiota linked to obesity-related metabolic disorders (Griffiths *et al.*, 2004; Turnbaugh *et al.*, 2006, 2009). In fact, gut microbes also influence the metabolism of cells in tissues outside of the intestines (in the liver and adipose tissue), thereby modulating lipid and glucose homeostasis, as well as systemic inflammation, in the host. Obesity seems to be associated with changes in the relative abundance of the two dominant bacterial divisions in the gut, namely *Bacteroidetes* and *Firmicutes*. Ley and coworkers (2005) have demonstrated that *ob*/*ob* mice have a 50% reduction in *Bacteroidetes* and a proportional increase in *Firmicutes*. They also found that obese people had a lower proportion of *Bacteroidetes*, compared to *Firmicutes*, than lean people, but that the proportion of *Bacteroidetes* increased with weight loss when subjects followed two types of low calorie diet (Ley *et al.*, 2006). Bifidobacteria are Gram positive anaerobic microorganisms that naturally colonise the human intestinal tract, improving the mucosal barrier and protecting against chronic inflammation (Griffiths *et al.*, 2004; Wang *et al.*, 2004, 2006). According to Ley and coworkers, these microorganisms also guard against obesity (Ley *et al.*, 2006). Other authors have shown that *Bifidobacterium* (B) spp. significantly decreased in high-fat-fed mice as compared to those receiving the standard high carbohydrate diet (Cani *et al.*, 2007). Furthermore, in 2010, Kondo and coworkers demonstrated the anti-obesity activity of a probiotic bifidobacterial strain (*B. breve* B-3) in a mouse model with high-fat diet induced obesity (Kondo *et al.*, 2010). The gut microbiota is therefore considered as a potential nutritional and pharmacological target in the management of obesity and obesity-related disorders (Delzenne *et al.*, 2011).

Zücker fatty rats are the best-known and most widely used experimental model of genetic obesity. Compared with their lean counterparts, Zücker fatty rats present a mutation in the leptin receptor, which is the molecular base of their characteristic phenotype (Chua *et al.*, 1996a,b; Phillips *et al.*, 1996). Zücker fatty rats are also characterised by an increased expression of ghrelin, both at the peripheral and central levels (Beck *et al.*, 2003, 2004). They present dyslipidemia, mild glucose intolerance and hyperinsulinemia, alterations similar to those in human metabolic syndrome. In fact, they can also be considered an insulin-resistant model, whereas Zücker lean rats show normal tolerance of glucose and are insulin sensitive and normoinsulinemic (Kasiske *et al.*, 1992; Stern *et al.*, 1972; Zücker and Antoniades, 1972; Zücker and Zücker, 1962). Obesity is associated with a state of chronic inflammation characterised by abnormal production of proinflammatory mediators (Ouchi *et al.*, 2003), including tumour necrosis factor-α (TNF-α) (Hotamislogil *et al.*, 1993, 1995). In this context, TNF-α, a proinflammatory cytokine, is overexpressed in obesity and likely mediates insulin resistance in animal models of obesity (Hotamislogil *et al.*, 1993), including Zücker fatty rats (Picchi *et al.*, 2006). By contrast, adiponectin expression in the visceral fat of Zücker fatty rats is supressed to basal levels, correlating with significantly reduced plasma adiponectin concentrations and increased insulin resistance in these animals (Altomonte *et al.*, 2003). In addition, in the Zücker strain, differences in the *Bacteroidetes*/*Firmicutes* ratios have been associated with the subsequent lean or obese state (Hakkak *et al.*, 2014a), and the same researchers also demonstrated increased levels of oxidative stress in Zücker fatty rats (Hakkak *et al.*, 2014b).

Caenorhabditis elegans is the first multicellular organism to have its whole genome sequenced. This nematode is widely used, as it provides a relatively simple and genetically tractable model to study the effects of bacterial strains as nutrients (Yilmaz and Walhout, 2014). We have previously described the use of *C. elegans* to screen a culture collection of probiotic strains and *B. animalis* subsp. *lactis* CECT 8145 was the most effective to reduce body fat deposition in this worm. Moreover, this strain also revealed *in vivo* antioxidant activity in this experimental model (Martorell *et al.*, 2016).

The aim of the present study is to evaluate the effect of oral administration of *B. animalis* subsp. *lactis* CECT 8145 in Zücker fatty rats. A group of lean rats is included as a reference standard group, in order to provide normal values in the Zücker strain. We also evaluate some important obesity-related biomarkers in all these rats, to better understand the mechanisms involved in the effect of the probiotic under study.

2. Materials and methods

Preparation of *Bifidobacterium animalis* subsp. *lactis* CECT 8145

The *B. animalis* strain used in this study was originally isolated from the faeces of healthy babies under breast-milk feeding, and was obtained from the Biopolis Culture Collection. This strain was selected by screening for probiotic properties, such as resistance to gastrointestinal stress, as well as a safety evaluation. It was identified by 16S rRNA gene sequencing according to Chenoll *et al.* (2014) and deposited in the Spanish Type Culture Collection as *B. animalis* subsp. *lactis* CECT 8145. The cultures were maintained in MRS broth with 0.05% L cysteine. The bacterial cells were collected, washed with saline and suspended in 10% skim milk (as vehicle) for experimental use.

General protocol in the rats

This study used twenty male 5-week-old Zücker fatty rats weighing 182.24±2.11 g and ten male 5-week-old Zücker lean rats weighing 139.41±1.92 g, purchased from Charles River Laboratories (Barcelona, Spain). The Zücker fatty rats were randomly divided into two groups (n=10 each) respectively administered either *B. animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day) suspended in skim milk, or skim milk alone (fatty control group), in their drinking bottles, until 17 weeks of age. To attain the dose established for the treated Zücker fatty rats, the corresponding solutions were prepared weekly, according the increase in body weight of these animals, and considering their liquid ingest in the next week as in the previous one. The lean Zücker rats (standard group) were used to provide normal values in the Zücker strain, and were administered skim milk in drinking bottles for the same experimental period as Zücker fatty rats. During the experimental period, all rats were maintained at a temperature of 23 °C, with 12 h light/dark cycles. They were fed a standard diet for rats (A04 Panlab, Barcelona, Spain) *ad libitum* and had free access to the beverage.

Food intake, liquid intake, and body weight gain were recorded on a weekly basis in the different groups of rats. In order to carry out determinations of plasma ghrelin in the different groups of animals, blood (1 ml) was extracted from the jugular vein of all the rats at 17 weeks of age. The rats were not fasted before these extractions because, according to our previous research, a period of fasting induces always a marked increase in plasma ghrelin levels that makes near impossible to observe statistical differences between groups. Nevertheless, the rats were sacrificed by decapitation, after overnight fasting, at the end of the experimental period, and blood from the sacrificed animals was used for the remaining biochemical determinations in plasma. Thus, plasma total cholesterol, plasma cholesterol transported by high-density lipoproteins (HDL cholesterol), plasma triglycerides, plasma glucose, and plasma insulin were measured in the sacrificed rats at the end of the experimental period. The standard biochemical procedures used to determine the cholesterol transported in plasma by low-density lipoproteins (LDL cholesterol) in rodents are not reliable; therefore, we calculated the ratio between total cholesterol/HDL cholesterol as a cardiovascular risk index. This ratio is a better clinical indicator of cardiovascular disease than total cholesterol. Moreover, according to Matthews and coworkers (1985), we used plasma concentrations of both glucose and insulin to calculate indices of insulin resistance (homeostasis model assessment-insulin resistance, HOMA-IR) and insulin secretion (HOMA-β) with the following formulae: HOMA-IR = fasting insulin (μU/ml) × fasting glucose (mM)/22.5; HOMA-β = 20× fasting insulin (μU/ml)/[fasting glucose (mM)-3.5]. The Quantitative Insulin Sensitivity Check Index (QUICKI) was also calculated according to Katz *et al.* (2000). QUICKI = 1/log fasting insulin (μU/ml) + log fasting glucose (mg/dl). In addition, TNF-α, a proinflammatory cytokine, adiponectin, an insulin-sensitising adipokine, and malondialdehyde (MDA), a biomarker indicating lipid peroxidation in the rats, were also determined in the plasma samples from the sacrificed animals. Moreover, epididymal fat pad from the sacrificed animals was collected and weighed.

All the above-mentioned experiments were performed as authorised for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food) (EC, 1998; MAFF, 1988). Moreover, the experiments for this study were approved by the Ethical Committee for Animal Research of the Complutense University of Madrid.

Plasma determinations

Preparation of plasma samples

Blood from the jugular vein of the 17-week-old Zücker and lean rats, and from all sacrificed animals was collected in tubes containing lithium heparin as anticoagulant. The tubes were centrifuged at 3,500×*g* for 20 min to obtain plasma samples, which were frozen and stored at -80 °C until analysis.

Cholesterol, triglyceride, glucose and insulin determination

Lipid profiles of the rats (triglycerides, total cholesterol and HDL cholesterol) were assayed using enzymatic and colourimetric methods with commercial kits (Biovision-Axxora, Milpitas, CA, USA). The different concentrations were determined spectrophotometrically using a Hitachi 911 autoanalyser (Boehringer Mannheim, Mannheim, Germany) (wavelength 700 nm). Plasma glucose was assayed using the glucose-oxidase enzymatic test with commercial kits (Roche Diagnostics S.L., San Cugat del Vallés, Spain) and plasma insulin concentration was spectrophotometrically quantified using an ultrasensitive rat insulin enzyme immunoassay kit (USCN, Wuhan, China P.R.) with a ThermoMax microplate reader (Molecular Devices LLC., San Jose, CA, USA). Absorbance was measured at 450 nm.

Tumour necrosis factor-α, adiponectin and ghrelin determination

TNF-α, adiponectin and ghrelin concentrations in plasma were determined using a rat TNF-α ELISA kit (ENZO, Grupo Taper SA, Alcobendas, Spain), a rat adiponectin ELISA kit (Adipogen, San Diego, CA, USA) and a rat ghrelin ELISA kit (USCN), respectively, according to the manufacturer´s instructions. Spectrophotometric

measurements were in all cases made at 450 nm using a spectrophotometer (Molecular Devices LLC). Plasma TNF-α values were expressed as pg TNF-α/ml plasma; plasma adiponectin values were expressed as µg adiponectin/ml plasma; and plasma ghrelin values were expressed as pg ghrelin/ml plasma.

Malondialdehyde determination

Plasma malondialdehyde (MDA) levels were measured by thiobarbituric acid (TBA) assay, previously described by Manso *et al.* (2008). Briefly, plasma was mixed with 20% trichloroacetic acid in 0.6 M HCl (1:1, v/v), and the tubes were kept in ice for 20 min to precipitate plasma components and avoid possible interferences. Samples were centrifuged at 1,500×*g* for 15 min before adding TBA (120 mM in Tris 260 mM, pH 7) to the supernatant in a proportion of 1:5 (v/v); then, the mixture was boiled at 97 °C for 30 min. Spectrophotometric measurements at 535 nm were made at 20 °C. Plasma MDA values were expressed as µmol MDA/ml plasma.

Statistical analysis

The results were expressed as mean values ± standard error of the mean for 10 animals, or at least 5 samples, and were analysed by a one- or two-way analysis of variance (ANOVA) using the GraphPad Prism software. Differences between groups were assessed by the Bonferroni test and were considered significant when $P<0.05$.

3. Results

Body weight of all Zücker rats increased progressively during the experimental period, and was always significantly higher in the fatty animals than in the lean rats. In addition, from the seventh week of age, the increase in body weight became more accentuated in the fatty control group than in the fatty rats receiving daily treatment with *B. animalis* subsp. *lactis* CECT 8145. Therefore, at the end of the experimental period, body weight in the treated fatty rats was 4.9% lower than the corresponding value in the fatty control rats.

Dry food intake in the fatty groups was always significantly higher than dry food intake in the lean group, but liquid intake was paradoxically higher in the lean animals than in the fatty ones. In any case, dry and liquid food intakes also increased progressively in both groups of fatty rats from the beginning of the experimental period until the animals were 9 weeks of age. As of that moment, dry food intake decreased and liquid food intake stabilised in both Zücker fatty groups. Even though liquid food intake was highly variable, importantly dry and liquid food intakes were significantly higher in the Zücker fatty control group than in the Zücker fatty rats treated daily with *B. animalis* subsp. *lactis* CECT 8145. The difference in liquid intake was particularly noticeable throughout the experimental period, but the difference in dry food intake virtually disappeared in the terminal phase of the experimental period (Figure 1).

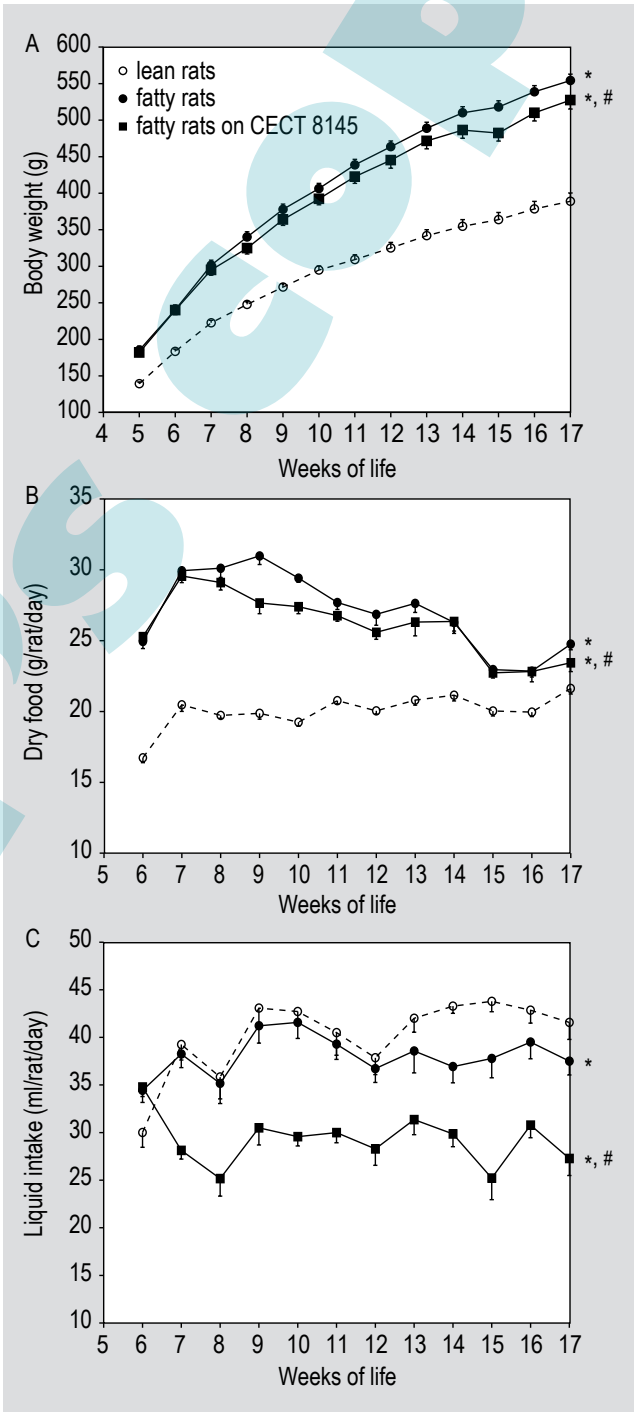

Figure 1. Evolution of body weight and intakes. (A) Body weight; (B) dry food intake; and (C) liquid intake of Zücker rats on skim milk with or without *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day). Data are expressed as mean ± standard error of the mean for 10 animals. * Statistical difference with lean rats on skim milk; # statistical difference with fatty rats on skim milk. $P<0.05$ estimated by two-way ANOVA.

Plasma total cholesterol, plasma HDL cholesterol and plasma triglycerides were higher in the fatty animals than in the lean animals, but no differences were found in these measurements between the Zücker fatty control group and the Zücker fatty treated group. Nevertheless, the ratio total cholesterol/HDL cholesterol was clearly lower in the fatty rats receiving daily treatment with *B. animalis* subsp. *lactis* CECT 8145, than in the fatty control rats (Figure 2).

Plasma glucose and plasma insulin were higher in the fatty animals than in the lean animals. Moreover, the plasma values of these variables were quite similar in both groups of fatty animals and the same occurred for HOMA-IR values. On the contrary, QUICKI values were higher in the lean rats than in the fatty rats. No differences were observed in QUICKI values on comparing both groups of fatty animals. In addition, HOMA-β values were similar in all the Zücker rats (Figure 3).

Plasma TNF-α was significantly higher in the fatty control group than in the lean or treated fatty animals. Moreover, no differences were observed in the plasma levels of this cytokine between treated fatty and lean rats. On the contrary, plasma adiponectin was significantly higher in the treated fatty group than in the fatty control animals. The level of this adipokine was also higher in the plasma obtained from the treated fatty group than in the plasma obtained from the lean rats (Figure 4).

Plasma MDA was significantly higher in the fatty control group than in the lean or treated fatty rats. Moreover, the treated fatty rats reached lower plasma MDA values than the corresponding values in lean rats (Figure 4). No significant differences were observed in the plasma ghrelin levels of untreated fatty and lean rats, but the former showed slightly higher plasma values of this peptide. In any case, the treated fatty rats showed very low plasma ghrelin levels, which were clearly lower compared with the corresponding values in the other groups (Figure 4).

Weight of the epididymal fat increased in all fatty rats compared with lean rats (4.75±0.28 g). Significant differences were not observed in the weight epididymal fat between treated fatty (11.82±0.33 g) and fatty control (12.07±0.33 g) rats, even if this value was slightly lower in the treated ones.

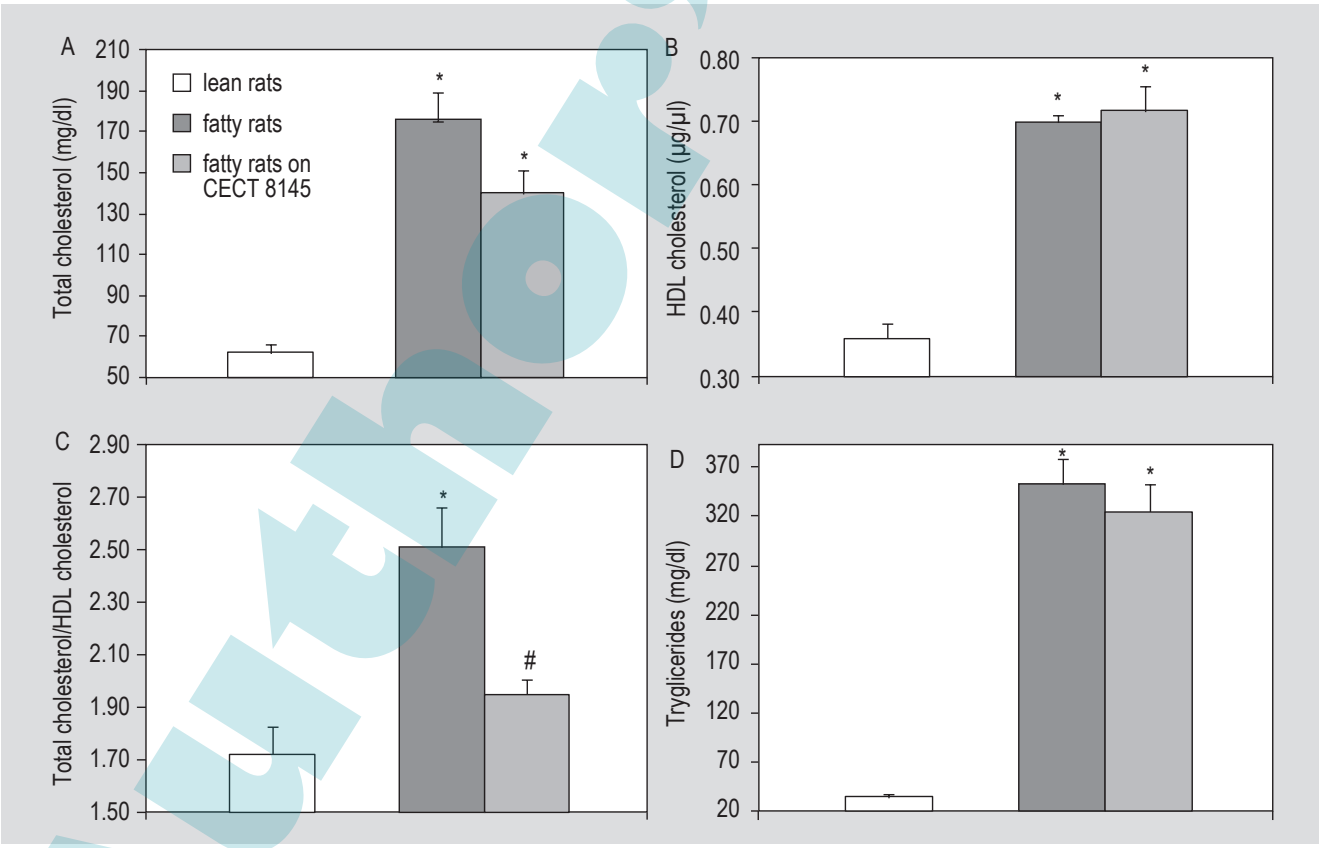

Figure 2. Lipid profile at final point. (A) Plasma total cholesterol; (B) plasma high-density lipoprotein (HDL) cholesterol; (C) plasma total cholesterol/plasma HDL cholesterol ratio; and (D) plasma triglycerides of Zücker rats on skim milk with or without *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day). Data are expressed as mean ± standard error of the mean for a minimum of 5 animals. * Statistical difference with lean rats on skim milk; # statistical difference with fatty rats on skim milk. *P*<0.05 estimated by one-way ANOVA.

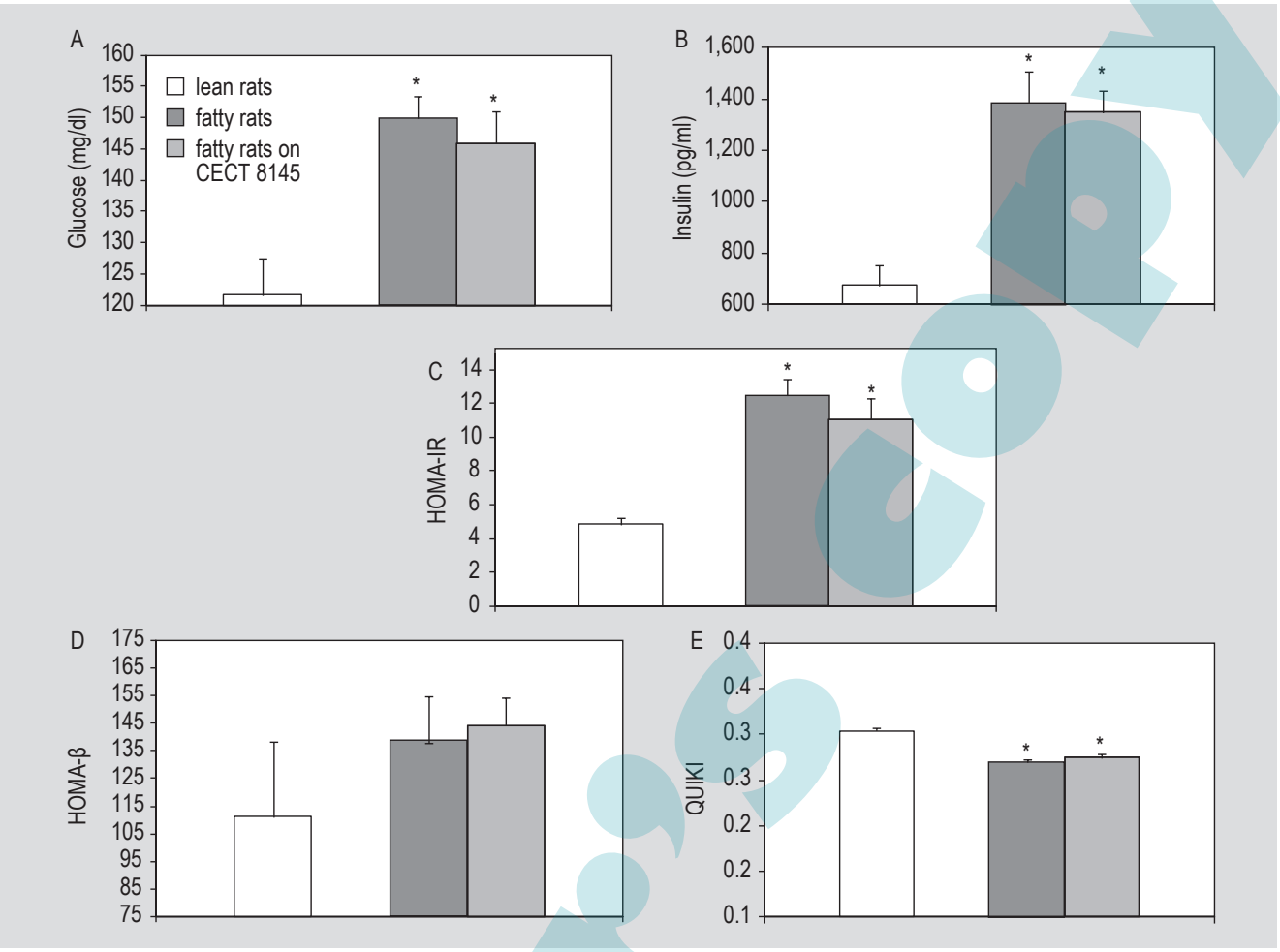

Figure 3. Glucose metabolism data at final point. (A) Plasma glucose; (B) plasma insulin; (C) homeostasis model assessment of insulin resistance (HOMA-IR) values; (D) homeostasis model assessment of insulin secretion (HOMA-β) values; and (E) quantitative insulin sensitivity check index (QUICKI) values of Zücker rats on skim milk with or without *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day). Data are expressed as mean ± standard error of the mean for a minimum of 5 animals. * Statistical difference with lean rats on skim milk; # statistical difference with fatty rats on skim milk. *P*<0.05 estimated by one-way ANOVA.

4. Discussion

The gut microbiota is an environmental factor involved in body weight control. Zücker fatty rats, the animals selected for this study, show clear alterations in the gut microbiota that may influence their obese state (Hakkak *et al.*, 2014a). This rat strain is a very good experimental model for obesity and constitutes an appropriate approach to investigate the anti-obesity properties of *B. animalis* subsp. *lactis* CECT 8145. As expected, Zücker lean rats showed very different characteristics to those of the Zücker fatty rats, enabling us to demonstrate the association between *B. animalis* subsp. *lactis* CECT 8145 intake and the decrease in body weight of obese Zücker animals. Even though the decrease in body weight in the rats treated with the bacteria did not surpass 5% of the corresponding value in the Zücker fatty control rats ingesting skim milk, body weight loss caused by efficient anti-obesity treatments neither surpass this percentage. Moreover, drugs can only be prescribed for obesity when their benefits outweigh the risks, and unfortunately anti-obesity medication is approved with difficulty because of potential side effects. Natural products or probiotics can offer a more appropriate and less dangerous way to control this disease, and the efficiency of these treatments should be considered key to characterise their clinical utility. Preclinical evidence supporting the 'anti-obesity' effects of probiotics mainly comes from studies on probiotics belonging to the genus *Lactobacillus*. Thus, we can mention that probiotic supplementation with *Lactobacillus curvatus* HY7601, or *L. curvatus* HY7601 in combination with *Lactobacillus plantarum* KY1032, effectively suppressed body weight gain and reduced the adipose tissue weight in mice fed a high-fat high-cholesterol diet for 9 weeks (Yoo *et al.*, 2013). Nevertheless, some other studies have also focused on the use of bifidobacterial strains against obesity, and, in this context, Kondo *et al.* (2010) evaluated the effect of *B. breve* B-3 in a mouse model with obesity induced by a high-fat diet, revealing an important suppression in body weight gain in the group of animals treated with this bifidobacterial

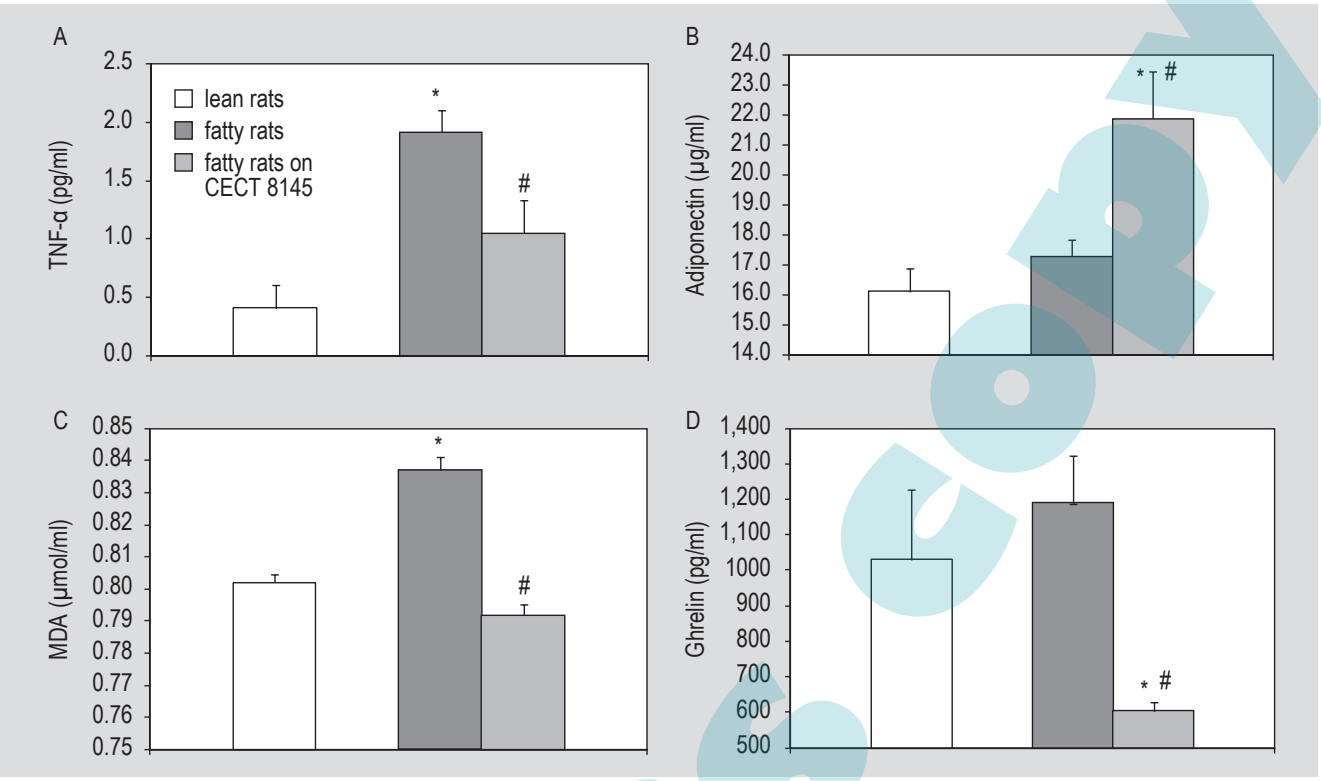

Figure 4. Adipokines, oxidative stress and ghrelin at final point. (A) Plasma adiponectin; (B) plasma tumour necrosis factor-α (TNF-α); (C) plasma malonildialdehyde (MDA); and (D) plasma ghrelin of Zücker rats of Zücker rats on skim milk with or without *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (10^{10} cfu/day). Data are expressed as mean ± standard error of the mean for a minimum of 5 animals. * Statistical difference with lean rats on skim milk; # statistical difference with fatty rats on skim milk. *P*<0.05 estimated by one-way ANOVA.

strain. In the present study, the treated fatty rats showed a clear decrease in body weight and a slight decrease in the weight of their epididymal fat, as compared with the control fatty animals. All these experimental results show that bifidobacteria are good candidates for use as an anti-obesity probiotic.

The experiments carried out in the Zücker fatty rats also suggest some other health properties for *B. animalis* subsp. *lactis* CECT 8145, beyond body weight loss. This indicates that this specific strain is a good candidate to prevent obesity and related disorders. In fact, the improvement in obesity related biomarkers shown in the treated obese rats could also be attributed to the presence of *B. animalis* subsp. *lactis* CECT 8145 in their gut. Even if plasma levels of total cholesterol or HDL-cholesterol in the treated Zücker fatty rats were only slightly different from the corresponding values in the Zücker fatty control group, the rats that received daily treatment with *B. animalis* subsp. *lactis* CECT 8145 showed a marked and significant decrease in the ratio total cholesterol/HDL-cholesterol, that is considered as an indicator of cardiovascular disease. In the treated fatty animals, this index attains somewhat higher values than those of the lean animals, but despite this, it seems important to highlight that significant differences did not exist between these groups of rats when the corresponding indexes were compared. It is true that daily treatment with *B. animalis* subsp. *lactis* CECT 8145 did not produce any effect on triglyceride levels, but we want to underscore that the cholesterol ratio that we have mentioned above has been extensively used as a monitoring tool by some health care specialists. LDL cholesterol collects in the walls of blood vessels, causing the blockages of atherosclerosis and dramatically increasing the chance of developing heart disease and heart attack, but the variation in the total cholesterol/HDL cholesterol ratio may be associated with more substantial alterations in metabolic processes, predictive of ischemic heart risk and insulin resistance syndrome, than variation in the LDL cholesterol/HDL-cholesterol ratio (Lemieux *et al.*, 2001). In any case, there are controversial results regarding the effect of probiotics on the lipid profile. Several studies established the hypocholesterolemic effects of some bacterial strains, including *Lactobacillus acidophilus* (Park *et al.*, 2008) and *Bifidobacterium longum* (Xiao *et al.*, 2003). Yin *et al.* (2010) compared the effects of four *Bifidobacterium* strains (L66-5, L75-4, M13-4 and FS31-12), originating from normal human intestine, on lipid metabolism in an obese murine model induced by high-fat diet, and all the four strains reduced serum and liver triglyceride and significantly alleviated the lipid deposition in liver. All strains showed a trend of decreasing serum and liver total cholesterol

in the rats, while strains L66-5 and FS31-12 did so more significantly. By contrast, Kang *et al.* (2013) reported that there were no significant changes in the lipid profile of rats supplemented with *Lactobacillus gasseri* BNR17.

Zücker fatty rats are not considered strictly as diabetic rats, but may be considered as a model of resistance to insulin. According to Ionescu *et al.* (1985), in control conditions, Zücker fatty rats exhibited similar basal fasting glucose levels, but impaired glucose tolerance, as compared with lean Zücker rats. In our study, plasma glucose and insulin levels were higher in the Zücker fatty rats than in the Zücker lean animals. The obese animals treated with *B. animalis* subsp. *lactis* CECT 8145 also showed abnormal values for these variables. In addition, significant differences were not attained when comparing these values, or the derived indexes, in both groups of Zücker fatty rats. Nevertheless, probiotics like *Lactobacillus rhamnosus* GG exert beneficial effects on glucose homeostasis (Kim *et al.*, 2013).

The most important data obtained in this study probably correspond to those related to adipokines, oxidative stress and ghrelin of the treated Zücker fatty rats. Obesity leads to infiltration of the expanded adipose tissue by macrophages and increased levels in proinflammatory cytokines. The first indication of increased cytokine release in obesity was provided in the early 1990s by the identification of increased expression of TNF-α, a proinflammatory cytokine, in the adipose tissue of obese mice (Tzanavari *et al.*, 2010). In fact, TNF-α is overexpressed in the adipose tissues of rodents (Hotamislogil *et al.*, 1993), including Zücker fatty (Hotamislogil *et al.*, 1993; Picchi *et al.*, 2006) and Zücker diabetic fatty (Gao *et al.*, 2010) rats, and humans (Hotamisligil *et al.*, 1995, 1997; Kern *et al.*, 1995). Upregulation of TNF-α even contributed to endothelial dysfunction in Zücker diabetic rats (Gao *et al.*, 2010). In the present study, the *B. animalis* subsp. *lactis* CECT 8145 treated Zücker fatty rats showed significantly lower plasma levels of this proinflammatory cytokine than the corresponding levels in the non-treated Zücker fatty animals. Moreover, plasma levels of TNF-α in the treated fatty animals were somewhat higher than the corresponding values in the lean rats, but no significant differences existed in the plasma levels of this cytokine between these two groups of rats. It was recently shown that TNF-α also decreased in the Zücker fatty rats that received treatment with other probiotic strains, such as *B. breve* CNCM I-4035 or *L. rhamnosus* CNCM I-4036 (Plaza-Diaz *et al.*, 2014). The immunomodulatory effects of a probiotic mixture, including *Lactobacillus* and bifidobacterial species, have also been studied by Karamese *et al.* (2016). In that study, probiotics led again to a decrease in the serum levels of TNF-α.

Our hypothalamus ultimately controls our hunger response, but the interrelations between the different factors that can act in this part of the brain are not at all clear. Inflammatory cytokines, such as TNF-α can cross into the hypothalamus and then can lead to weight gain. Therefore, the observed effect of *B. animalis* subsp. *lactis* CECT 8145 in the Zücker fatty rats may not simply be due to a direct effect on food and liquid intake. This probiotic strain probably decreases body weight in these animals, at least in part, by controlling their altered levels of TNF-α. Nevertheless, evidence from animals also suggests that TNF-α exerts a central effect on the regulation of body weight, either by decreasing energy intake or by inducing thermogenesis (Coombes *et al.*, 1987; Plata-Salaman *et al.*, 1988; Rothwell, 1988).

In any case, it is important to have in mind that leptin and TNF-α are among the most abundant adipocytokines produced by adipocytes, and the interrelations between both are complex. Leptin upregulates the secretion of TNF-α (Shen *et al.*, 2005), and TNF-α increases, in turn, the expression of leptin mRNA in the adipose tissue (Landman *et al.*, 2003). In humans, TNF-α also increases serum leptin levels (Zumbach *et al.*, 1997), and this creates a loop whose components influence each other in promoting inflammation. In fact, leptin expression is not only regulated by the intake of food, but also by various hormones, as well as by several inflammatory mediators, as TNF-α (Gualillo *et al.*, 2000; Sarraf *et al.*, 1997). TNF-α really acts directly on adipocytes to increase production of the lipostatic factor leptin and contributes to obesity-related hyperleptinemia (Kirchgessner *et al.*, 1999). The implicated TNF receptor has been also investigated (Finck and Johnson, 2000). Nevertheless, obesity promotes multiple cellular processes that attenuate leptin signaling, and this pathology is associated with leptin resistance (Myers *et al.*, 2010). TNF-α also facilitates insulin resistance (Hotamisligil, 1999; Nieto-Vazquez *et al.*, 2008). Therefore, to distinguish the mechanisms that predispose to weight gain from those that result from it is really difficult, and, consequently, to establish the mechanisms implicated in the weight loss caused by a concrete product is also complicated.

As we know, adiponectin is an adipocyte-derived protein that is also abundantly expressed and secreted from adipocytes. The close correlation between plasma adiponectin levels and insulin sensitivity has led to consider this protein as an insulin-sensitizing adipokine (Berg *et al.*, 2002; Tsao *et al.*, 2002). In Zücker fatty rats, adiponectin expression in visceral fat was suppressed to basal levels, which correlated with significantly reduced plasma adiponectin concentrations and increased insulin resistance (Altomonte *et al.*, 2003). In the present study, the Zücker fatty rats that received daily treatment with *B. animalis* subsp. *lactis* CECT 8145 showed clearly higher plasma adiponectin values than those of the other groups of rats. The increase of this adipokine in the treated rats may be probably linked to the slimming effect of the studied bacteria, since weight loss significantly elevates

plasma adiponectin levels in humans (Yadav *et al.*, 2013). Moreover, we know that benefits on body weight translate into favourable metabolic effects also when other probiotics different from bifidobacterial strains were used, and, in this context, it was demonstrated that the probiotic *L. rhamnosus* GG, consistently improved insulin sensitivity and reduced lipid accumulation by stimulating adiponectin secretion in mice during a high-fat diet (Kim *et al.*, 2013). Other studies also showed that probiotic supplementation could improve adiponectin secretion or expression (Kadooka *et al.*, 2010; Luoto *et al.*, 2012; Nerstedt *et al.*, 2007). In any case, the above mentioned results confirm the health properties of the assayed bacterial strain and guarantee that this strain prevents from insulin resistance. It should be noted that plasma adiponectin levels in the treated Zücker fatty rats exceed the corresponding values in the Zücker lean rats, but it is also true that the lean animals showed slightly lower plasma adiponectin levels than those of the Zücker control rats.

Oxidative stress results in an imbalance between the production of reactive oxygen species and the biological systems ability to detoxify the reactive intermediates or to repair the resulting damages (Le Lay *et al.*, 2014). Oxidative stress is actually enhanced as body weight increases (Mittal and Kant, 2009), and obesity represents a state of chronic oxidative stress that has been associated with enhanced reactive oxygen or nitrogen species. Moreover, recent evidence suggests that oxidative stress may be the mechanistic link between obesity and related complications (Savini *et al.*, 2013). Some researchers found specific elevation of lipid peroxidation in adipose tissue from obese KKAy mice (Furukawa *et al.*, 2004), and increased oxidative stress has been further described in white adipose tissue of other models of obesity, such as ob/ob (Houstis *et al.*, 2006) or high-fat diet (Curtis *et al.*, 2010) mice. Increased oxidative stress was also observed in Zücker fatty rats (Soltys *et al.*, 2001; Wergedahl *et al.*, 2008). Moreover, increased reactive oxygen species in aorta could be involved in endothelial dysfunction and vascular lipotoxicity in Zücker diabetic fatty (Chinen *et al.*, 2007) and Zücker fatty (Pung *et al.*, 2012) rats. In fact, abnormal endothelium dependent antioxidant activity has been described in Zücker fatty rats (Katakam *et al.*, 2006), and also there is evidence of diminished neurogenic contractions due to endothelial dysfunction in femoral arterial preparations from these animals (Martinez *et al.*, 2014). Therefore, Zücker fatty rats show a pro-oxidant status linked to their characteristic obesity and their vascular alterations.

Free radicals generate the lipid peroxidation process in an organism and MDA is one of the final products of polyunsaturated fatty acid peroxidation in the cells. An increase in free radicals causes overproduction of MDA, one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products. For these reasons, MDA level is commonly known as a marker of oxidative stress and the antioxidant status (Gaweł *et al.*, 2004; Nielsen *et al.*, 1997). At low pH and elevated temperature, MDA readily participates in a nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red, fluorescent 1:2 MDA:TBA adduct (Janero *et al.*, 1990). These facts, along with the availability of facile and sensitive methods to quantify MDA (as the free aldehyde or its TBA derivative), have led to the routine use of MDA determination and, particularly, the 'TBA test' to detect and quantify lipid peroxidation in a wide array of sample types. Having in mind the prooxidant status of the Zücker fatty rats, we have used the TBA method to quantify the MDA plasma levels in the different groups of rats of this study, and we could observe that the fatty ones that received daily treatment with *B. animalis* subsp. *lactis* CECT 8145 showed clearly lower levels of this metabolite in plasma than the fatty control ones. The antioxidant effect of the probiotic strain cannot be refuted since the treated fatty animals showed even somewhat lower values of MDA in their plasma than the Zücker lean rats. Other bifidobacterial strains have also shown antioxidant effects. Thus, oral supplementation of *Bifidobacterium adolescentis* for 12 weeks protected C57BL/6 mice against diet-induced liver damage, which also associated with prevention from lipid peroxidation (Reichold *et al.*, 2014). Antioxidant therapies are of particular interest nowadays for different pathologies, related or unrelated, with obesity. Therefore, the above mentioned results undoubtedly support the idea that the health properties of the assayed bacteria may also be useful in non-obese subjects.

Ghrelin is an orexigenic peptide, primarily produced by the stomach, but also present in the hypothalamus. It has adipogenic effects when is chronically injected in rodents, but, in obese humans, its plasma concentration is decreased (Beck *et al.*, 2003). The Zücker fa/fa rat, a genetic model of obesity related to a default in the leptin receptor, is characterised by increases in the stomach ghrelin mRNA expression and in ghrelin release in the circulation. Moreover, according to Beck *et al.* (2003), these rats clearly support a role for ghrelin in the development of obesity in the absence of leptin signalling. These researchers also established that in 60-week old fa/fa Zücker rats, with well-established obesity, the plasma ghrelin concentration was 35% higher than the corresponding value in the lean homozygous rats. In addition, Zücker fatty rats ate significantly more than the Zücker lean rats (Beck *et al.*, 2004). The same researchers also showed that, after fasting, plasma ghrelin concentrations increased significantly in lean and fatty Zücker rats (Beck *et al.*, 2003). Nevertheless, our experiments to determine plasma ghrelin levels in these animals were carried out with non-fasted rats. In these conditions, we clearly appreciated a marked decrease in the plasma levels of this peptide when the fatty rats received

daily treatment with *B. animalis* subsp. *lactis* CECT 8145. The ghrelin data for these animals are in accordance with their reduced dry and solid food intake. Moreover, *B. animalis* subsp. *lactis* CECT 8145 led to a decrease in this peptide in the fatty rats with lower values than those in lean rats. Liquid intake was also lower in the fatty treated rats than in the other groups of rats, and the marked decrease in the fluid intake that showed the fatty rats that received daily treatment with *B. animalis* subsp. *lactis* CECT 8145, could reflect the pronounced decrease that these animals also showed in their plasma ghrelin levels. Nevertheless, the organoleptic properties of the bacterial solution may also influence the scarce interest of the rats in this fluid. Additionally, it is important to note that 12-week dietary supplementation with either *Lactobacillus paracasei* CNCM I-4270, *L. rhamnosus* I-3690 or *B. animalis* subsp. *lactis* I-2494, significantly attenuated high fat diet-induced weight gain, despite no reductions in food intake in mice (Wang *et al.,* 2015). Similar results were obtained in studies where *Bifidobacterium* spp. was added to a high fat diet in rats (An *et al.,* 2011; Chen *et al.*, 2012). With respect to ghrelin levels, the experiments of our study indicated that the plasma concentration of this peptide in fatty animals was only about 10% higher than the corresponding value in the lean animals, but it should be pointed out that the ghrelin values in our study were obtained in 17-week-old Zücker rats, and not in 60-week-old Zücker rats, as in the experiments by Beck *et al.* (2004).

In conclusion, we have shown that the development of obesity in Zücker fatty rats can be attenuated by daily treatment of these animals with *B. animalis* subsp. *lactis* CECT 8145. We have also shown that this bacterial strain clearly attenuates the ratio total cholesterol/HDL-cholesterol in the obese rats. In addition, the mentioned bacterial strain improves oxidative stress and the inflammatory process linked to the obesity of the Zücker fatty rats. Moreover, it causes a very important decrease in plasma ghrelin levels in the Zücker fatty rats, which may justify their decreased food ingestion. According to our results, glucose metabolism biomarkers also slightly improved in the Zücker fatty rats treated with *B. animalis* subsp. *lactis* CECT 8145. All in all, our results show important health properties of *B. animalis* subsp. *lactis* CECT 8145. Nevertheless, further studies are recommended to better clarify the exact mechanism involved in the effects of this bacteria; above all because the said effects probably entail complicated interrelationships among the different hormones and adipokines implicated in the control of body weight and appetite. As in our study, the majority of the animal scientific studies use males to avoid the influence of hormonal changes along the research, but, within this field of interest, we suggest to investigate also the effect of *B. animalis* subsp. *lactis* CECT 8145 in female Zücker fatty rats. Moreover, additional research is needed to establish the possible functional effects of the studied bacteria and its probiotic efficiency to prevent obesity and related disorders in humans, even though our results are highly promising in this context.

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