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Heat-killed *Bifidobacterium animalis* subsp. *Lactis* CECT 8145 increases lean mass and ameliorates metabolic syndrome in cafeteria-fed obese rats

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ABSTRACT

To evaluate the ability of the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (Ba8145) to ameliorate Metabolic Syndrome (MetS), four groups of male Wistar rats were fed either standard chow (ST) or the obesogenic cafeteria diet (CAF) and were orally supplemented with either heat-killed Ba8145 (10^{10} CFU/day) (ST-Ba8145 and CAF-Ba8145 groups) or vehicle (ST-veh and CAF-veh groups) for 84 days. Both ST-Ba8145 and CAF-Ba8145 rats displayed increased energy expenditure (EE) and a decrease of relative mesenteric white adipose tissue (MWAT%). CAF-BA8145 animals showed decreased cumulative energy intake, increased relative lean mass, higher insulin sensitivity, elevated gene expression of adipose triacylglycerol lipase and fatty acid transporter 1 in MWAT and lower circulating levels of non-esterified free fatty acids, LDL/VLDL cholesterol and triacylglycerols than CAF-veh rats. In conclusion, heat-killed Ba8145 intake ameliorates mesenteric adiposity and dyslipidaemia, increases EE and lean mass and improves insulin sensitivity in rats induced to MetS.

1. Introduction

Metabolic syndrome (MetS), which is characterized by a combination of interconnected risk factors for cardiovascular disease (CVD), including abdominal obesity, insulin resistance, dyslipidaemia and hypertension, is increasing in prevalence and has become a leading cause of mortality and morbidity worldwide, affecting more than 20% of the global adult population (Asrih & Jornayvaz, 2015; Roberts, Hevener, & Barnard, 2013). Therefore, there is an urgent need to identify innovative strategies to prevent or ameliorate this multifactorial disorder. Among these approaches, the use of probiotics (live microorganisms, usually lactobacilli and bifidobacteria, that are used to benefit the health of the individual when consumed in adequate amounts) has gained interest in recent years (Le Barz et al., 2015; Peluso, Romanelli, & Palmery, 2014; Thushara, Gangadaran, Solati, & Moghadasian, 2016).

Different studies performed in both murine models (Aronsson et al., 2010; Huang, Korivi, Tsai, Yang, & Tsai, 2013; Kang, Yun, & Park, 2010; Kang et al., 2013; Savcheniuk et al., 2014; Stenman et al., 2014; Wang et al., 2015; Wu et al., 2015) and humans

Abbreviations: *Acc1*, acetyl CoA carboxylase 1; *Agrp*, agouti-related protein; ANOVA, analysis of variance; *Atgl*, adipose triacylglycerol lipase; β-actin, actin beta; Ba8145, *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145; *Cart*, cocaine and amphetamine-regulated transcript; *Cd36*, fatty acid translocase, homologue of CD36; *Cebpα*, CCAAT/enhancer binding protein alpha; *Cpt1β*, carnitine palmitoyltransferase 1 beta; CVD, cardiovascular disease; EWAT, epididymal white adipose tissue; *Fas*, fatty acid synthase; *Fatp1*, fatty acid transporter, member 1; *Ghsr*, ghrelin receptor; *Gpat*, glycerol-3-phosphate acyltransferase; *Had*, hydroxyacyl-CoA dehydrogenase; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Hsl*, hormone-sensitive lipase; IWAT, inguinal white adipose tissue; LDL/VLDL-C, low density lipoprotein + very-low density lipoprotein cholesterol; *Lpl*, lipoprotein lipase; MetS, metabolic syndrome; MWAT, mesenteric white adipose tissue; NEFAs, non-esterified fatty acids; *Npy*, neuropeptide Y; *ObRb*, long-form leptin receptor; *Pomc*, proopiomelanocortin; *Pparg2*, peroxisome proliferator-activated receptor gamma *2*; *Ppia*, peptidylprolyl isomerase A; RCTs, randomized controlled trials; R-QUICKI, revised quantitative insulin sensitivity check index; RWAT, retroperitoneal white adipose tissue; TC, total cholesterol; TG, triacylglycerol.

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(Barreto et al., 2014; Bernini et al., 2016; Chang et al., 2011; Kadooka et al., 2010; Kadooka et al., 2013; Sanchez et al., 2014; Stenman et al., 2016) have showed the beneficial effects of probiotic supplementation against obesity and different pathologies that comprise MetS. However, the observed effects and the mechanisms by which probiotics exerted these effects differed depending on the microorganism administered, the animal model used and the clinical features of the subjects included in the human trials. Concerning the *Bifidobacterium animalis* strains, Wang et al. showed that the supplementation with *B. animalis* subsp. *lactis I-2494* (10^8 CFU/day) to high-fat diet-fed mice for 12 weeks increased glucose tolerance, decreased body weight gain and adipocyte size, and attenuated hepatic steatosis and adipose and hepatic inflammation (Wang et al., 2015). In a similar study, the administration of the *B. animalis* subsp. *lactis* 420 (10^9 CFU/day) reduced body fat content in obese mice, attenuated glucose intolerance in both diet-induced diabetic and obese mice, and ameliorated metabolic endotoxaemia, hepatic inflammation and *Escherichia coli* adhesion in ileum and caecum of diabetic mice (Stenman et al., 2014). In humans, different randomized controlled trials (RCTs) have evidenced the effectiveness of the strain *B. animalis* subsp. *lactis* against different alterations associated with MetS, such as systemic inflammation, hypercholesterolemia and adiposity (Bernini et al., 2016; Stenman et al., 2016). As an example, in a RCT carried out with 134 overweight and obese adults, Stenman et al. demonstrated that the administration of the *B. animalis* subsp. *lactis 420* (10^{10} CFU/day) for 6 months reduced waist circumference and total, trunk and android fat mass. These effects could be mediated, at least in part, by an inhibition of daily energy intake since the probiotic group showed a lower consumption of dietary fat compared to the subjects that received the placebo (Stenman et al., 2016). Interestingly, Million et al. demonstrated, in humans, an alteration in obese gut microbiota towards lower levels of *B. animalis,* showed that higher levels of this bacterial strain were associated with a normal body weight and reported that the gut concentrations of *B. animalis* negatively correlated with body mass index (Million et al., 2012, 2013). Altogether, these results strongly suggest that the administration of probiotics containing *B. animalis* could be a useful strategy to combat MetS. Nevertheless, despite these promising results, further studies are needed to better define the beneficial effects of this bacterial strain and its mechanisms of action.

Probiotics can be provided by dietary supplements marketed in forms such as tablets and capsules, by dairy products such as yogurts and other fermented milks and by non-dairy products such as cereals, juices, soy products, baked food and fermented meat products (Vijaya Kumar, Vijayendra, & Reddy, 2015). One of the main challenges of the dairy probiotic-containing product industry is the preservation of cell viability after the application of high temperatures because heat stress produces high mortality and inactivates microorganisms (Haffner, Diab, & Pasc, 2016). In these sense, the use of heat-inactivated microorganisms that provide health-promoting effects could emerge as a useful strategy to enhance the commercial use of probiotics. In a related study, we recently demonstrated that the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* CECT 8145 (Ba8145) decreased fat content by more than 30% in the model organism *Caenorhabditis elegans*, supporting the idea that nonviable cells retain probiotic efficacy (Martorell et al., 2016). Here, we hypothesized that heat-killed Ba8145 could also exert beneficial effects on rats, attenuating obesity and its related metabolic disturbances, such as dyslipidaemia and insulin resistance.

The aim of the present study was to evaluate whether the administration of heat-killed Ba8145 ameliorated obesity and the metabolic disorders that comprise MetS. For this purpose, we used rats that were fed a cafeteria diet (CAF), which reproduces a human MetS-like phenotype more effectively than a high-fat diet (Sampey et al., 2011).

2. Materials and methods

In this study, we evaluated whether the administration of the heat-killed probiotic Ba8145 for 84 days was able to attenuate the development of MetS in rats fed the obesogenic CAF during the same experimental period. For this purpose, we carried out analyses of body composition (lean and fat masses), energy expenditure, food intake, and substrate utilization, investigated metabolic parameters related with insulin sensitivity and lipid metabolism and performed gene expression analyses in white adipose tissue and hypothalamus. Furthermore, the effects of heat-killed probiotic Ba8145 on rats fed a standard chow diet (ST) were also studied. These two groups were compared to rats that were fed either ST or CAF and supplemented with a placebo.

2.1. Bacterial strain

The Ba8145 strain (Biópolis SL, Valencia, Spain) was isolated from faeces of healthy babies undergoing breast-milk feeding. The fresh faecal samples were collected at home by the parents and were processed directly from the nappies in the laboratory within 24 h of sample collection. The Ba8145 strain was grown anaerobically in an Applikon fermenter at 37 °C for 18 h in 1 L of the de Man, Rogosa, and Sharpe medium (MRS; Oxoid, Basingstoke, United Kingdom) supplemented with cysteine (0.05% wt/vol; Sigma, St. Louis, MO, USA; MRS-C). The final cell content was evaluated by plate counting on MRS-C agar (anaerobically grown at 37 °C for 48 h). A volume of 1 L of the culture was then inactivated by heat treatment (autoclaved at 121 °C for 20 min), harvested by centrifugation (Sorvall Lynx 6000 centrifuge, ThermoFisher Scientific, 5524*g*, 10 min), mixed with maltodextrin (5% w/vol cells) and lyophilised (Martorell et al., 2016). Once obtained, the Ba8145 powder was standardized taking into account the total CFU content obtained in culture and the grams of powder recovered by combining with maltodextrin. The Ba8145 powder used in the present study contained 10^{11} inactivated cells per gram of maltodextrin. The absence of a decrease in cell content throughout the harvesting and lyophilisation processes was checked in preliminary protocol development assays. The absence of active cells was verified in the concentrated and final powder by plate count in MRS-C agar (37 °C for 48 h, anaerobically grown). The safety of this strain was assessed according to European Food Safety Authority (EFSA) and World Health Organization (WHO) recommendations (unpublished results) and can be considered generally recognized as safe (GRAS)/qualified presumption of safety (QPS). The genome of the strain has been sequenced (Chenoll et al., 2014).

2.2. Animals, diets and treatments

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and the *Generalitat de Catalunya* approved all of the procedures (DAAM 4840). The experimental protocol followed the 'Principles of laboratory animal care', and was carried out in accordance to the European Communities Council Directive (86/609/EEC). All animals were housed individually at 22 °C under a light/dark cycle of 12 h (lights on at 09:00 am) and were given free access to food and water.

The animals used were six-week-old male Wistar rats (Envigo RMS Spain S.L, Barcelona, Spain) weighting 170 g. After an adaptation period of 4 days, the rats were randomly distributed into four experimental groups (n = 10) depending on the diet and the oral treatment received over 84 days: the ST-veh group was fed with

ST (Teklad Global 18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain) and daily supplemented with 100 mg of maltodextrin dissolved in low-fat condensed milk, which was diluted 1:2 with water (vehicle); the ST-Ba8145 group was fed with ST and daily supplemented with 100 mg of maltodextrin containing 10^{10} CFU of heat-killed Ba8145 dissolved in diluted low-fat condensed milk; the CAF-veh group was fed with CAF and supplemented with vehicle; and the CAF-Ba8145 group was fed with CAF and supplemented with 100 mg of maltodextrin containing 10^{10} CFU of heat-killed Ba8145 dissolved in diluted low-fat condensed milk. Both treatments were administrated orally with a syringe of 1 mL in a volume of 0.33 mL. Four days before the beginning of the treatments, the rats were trained to lick diluted low-fat condensed milk (0.3 mL) to ensure voluntary consumption. It was checked that each rat fully ingested the daily dose of the corresponding treatment. The caloric distribution of the ST diet (3.1 kcal/g) was 24.2% protein, 18.2% fat and 57.6% carbohydrates. The energy provided by the different types of fatty acids was: 2.6% saturated; 3.8% monounsaturated; 9.9% polyunsaturated. The CAF diet included the following components (quantity per rat): bacon (5–7 g); biscuit with pâté (13–14 g); biscuit with cheese (14–15 g); muffins (7–8 g); carrots (6–8 g); milk with sugar (220 g/l; 100 ml); and ST (10 g). The caloric distribution of the CAF diet was 10.0% protein; 31.9% fat; and 58.1% carbohydrates. The energy provided by the different types of fatty acids was: 14.4% saturated; 10.3% monounsaturated; 5.6% polyunsaturated.

Body weight was recorded once each week, food was renewed daily and food intake was documented every 10 days. On day 84, the rats were sacrificed under anaesthesia (pentobarbital sodium, 80 mg/kg body weight) after 6 h of diurnal fasting. Blood was collected by cardiac puncture, and serum was obtained by centrifugation and stored at −20 °C until analysis. Hypothalamus, liver and white adipose tissue depots (retroperitoneal (RWAT), mesenteric (MWAT), epididymal (EWAT) and inguinal (IWAT) depots) were rapidly removed, weighed, frozen in liquid nitrogen and stored at −70 °C until RNA analysis.

2.3. Body composition analyses

Lean and fat mass measurements (in grams) were performed without anaesthesia on days 0, 28, 56 and 84 using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA).

2.4. Adiposity index

The adiposity index was computed as the sum of the EWAT, IWAT, MWAT and RWAT depot weights (in grams) and was expressed as a percentage of body weight.

2.5. Serum analysis

Enzymatic colorimetric kits were used to determine serum total cholesterol, triacylglycerols and glucose (QCA, Barcelona, Spain), HDL-cholesterol and LDL/VLDL-cholesterol (Bioassay systems, CA, USA) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany). Circulating insulin levels were measured using a rat/-mouse ELISA kit (intra-assay reliability, CV%, 0.92 to 8.35; inter-assay reliability, CV%, 6.03 to 17.90) (Millipore, Barcelona, Spain). Serum leptin levels were determined with a rat ELISA kit (intra-assay reliability, CV%, 1.88 to 2.49; inter-assay reliability, CV%, 2.95 to 3.93) (Millipore, Barcelona, Spain).

2.6. Oral glucose tolerance test (OGTT)

On day 56, rats were subjected to an OGTT following a previously described procedure (Crescenti et al., 2015). Blood samples were collected under fasting conditions (6 h of diurnal fasting) and at 15, 30, 60 and 120 min after glucose gavage (2.0 g kg^{-1} of body weight, 60% glucose in tap water solution). Serum glucose levels were determined at each point, and insulin levels were analysed at baseline and at 15, 30 and 60 min.

2.7. HOMA-IR and R-QUICKI analyses

The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated following the formula: (Glucose × Insulin)/22.5 as described previously (Matthews et al., 1985).

The insulin sensitivity was assessed by the revised quantitative insulin sensitivity check index (R-QUICKI) using the following formula: 1/[log insulin (μU/mL) + log glucose (mg/dL) + log FFA (mmol/l)] (Perseghin, Caumo, Caloni, Testolin, & Luzi, 2001).

2.8. Indirect calorimetry and activity measurements

The analyses were performed on day 78 using the Oxylet Pro™ System (PANLAB, Cornellà, Spain) under *ad libitum* conditions over a period of 22 h (from 11.00 am to 09.00 am). For this purpose, at 09:00 am, the animals were transferred from their cages to an acrylic box (Oxylet LE 1305 Physiocage, PANLAB). After an initial acclimatisation period of 2 h, oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured every 9 min by an O2 and CO2 analyser (Oxylet LE 405 gas analyser, PANLAB) at a controlled flow rate of 600 ml/min (Oxylet LE 400 air supplier, PANLAB). At each point of analysis, the software program Metabolism 2.1.02 (PANLAB, Cornellà, Spain) automatically calculated the respiratory quotient (RQ) as the VCO2/VO2 ratio and the Energy Expenditure (EE) in kcal/day/$Kg^{0.75}$ as VO2 × 1.44 × [3.815 + (1.232 × RQ)], according to the Weir formula (Weir, 1949). To calculate the rates of fat and carbohydrate oxidation, the VO2 and VCO2 measures were used, and the stoichiometric equations of Frayn (Frayn, 1983), which defines the oxidation of carbohydrates ($g \cdot min^{-1}$) as 4.55 × VCO2 − 3.21 × VO2 − 2.87 n and the oxidation of fat ($g \cdot min^{-1}$) as 1.67 × VO2 − 1.67 × VCO2 − 1.92 n, were applied. According to Carraro, Stuart, Hartl, Rosenblatt, & Wolfe (1990), a nitrogen excretion rate (n) of 135 $\mu g \cdot kg^{-1} \cdot min^{-1}$ was assumed. To obtain the EE from fat and carbohydrate in $kJ \cdot min^{-1}$, the fat and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor (Bircher & Knechtle, 2004). All rats were fed with ST during the measurements to minimize the effect that the composition of the food eaten (carbohydrate-fat balance) produces on RQ (Melzer, Kayser, & Schutz, 2014). Locomotor activity was measured by continuously recording spontaneous activity using extensiometric weight transducers placed below the home cage, and the number of rearings was monitored using 2=dimensional infrared frame elements and the software program Metabolism 2.1.02 (PANLAB, Cornellà, Spain).

2.9. Lipid extraction and quantification

Briefly, lipids were extracted from the liver (100–120 mg) and oven-dried faeces (100–120 mg) with 1 mL of hexane/isopropanol (3:2, vol/vol), degassed with nitrogen before left overnight under orbital agitation at room temperature protected from light. After an extraction with 0.3 ml of Na2SO4 (0.47 M), the lipid phase was dried and total lipids quantified gravimetrically before emulsifying as described previously (Rodríguez-Sureda & Peinado-Onsurbe, 2005). In the liver, triglycerides and cholesterol were assayed with commercial enzymatic kits (QCA, Barcelona, Spain).

2.10. Gene expression analysis

MWAT and hypothalamus total RNA was extracted using Tripure Reagent (Roche Diagnostic Barcelona, Spain) and purified with Qiagen RNeasy Mini Kit spin columns (Izasa, Barcelona, Spain). The cDNA was synthesised using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain), and was subjected to quantitative RT-PCR amplification using the Power SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain) in the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain). The primers for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA was calculated as a percentage of the ST-veh group, using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) with hypoxanthine guanine phosphoribosyl transferase (*Hprt*), peptidylprolyl isomerase A (*Ppia*) and actin beta (*β-actin*) used as reference genes. These genes were selected because they are well-accepted reference genes (Martínez-Beamonte et al., 2011) and showed equal expression among the four groups included in the study (i.e. their expression did not change as a consequence of CAF feeding and heat-killed Ba814 treatment).

2.11. Statistical analysis

Statistical analyses were performed using SPSS (SPSS, Inc., Chicago, IL, USA). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. The assumption of normality was determined using the Kolmogorov-Smirnov test, and the homoscedasticity among groups was assessed using Levene's test. When one or both of these conditions were not accomplished, data was transformed to a base-10 logarithm to obtain normal distribution and/or similar variances before statistical testing.

Two-way ANOVA analysis (2×2 factorial designs: diet (ST or CAF) × treatment (vehicle or heat-killed Ba8145)) was used to evaluate differences in nutrient consumptions, energy intake, indirect calorimetry and activity measurements, liver and faecal lipid content, biometric and serum parameters and gene expression data. This statistical analysis was also used to evaluate, at each study point, differences in the body composition measurements (days 0, 28, 56 and 84) and the OGTT data (day 56). When one or both main effects were statistically significant and no significant interaction was found between both factors, one-way ANOVA followed by the least significance difference (LSD) test was used to determine treatment differences between groups. When the interaction between diet and treatment was statistically significant under the two-way ANOVA, Student's *t* test was used to compute pairwise comparisons between groups (i.e., the effect of treatment within diet groups and the effect of diet within treatment groups). The evolution of body weight, fat mass and lean mass during the study, and the evolution of the circulating levels of glucose and insulin during the OGTT were analysed by a repeated measures (RM-) ANOVA with time as a within-subject factor and diet and treatment as between-subject factors. Student's *t*-test was used for single statistical comparisons. Data are presented as means ± SEM (n = 9–10). The level of statistical significance was set at bilateral 5%.

3. Results

3.1. CAF feeding induced a MetS-like phenotype

CAF-fed groups displayed higher body weight than their ST-fed counterparts from day 21 of the study onwards (Fig. 1a). As expected (Cigarroa et al., 2016; Lalanza et al., 2014; Reynés, García-Ruiz, Díaz-Rúa, Palou, & Oliver, 2014), both groups of CAF-fed rats showed significant increases of cumulative energy intake, body weight gain and adiposity as well as a reduction of lean mass in comparison with ST-fed counterparts (Fig. 1b and c and Tables 1 and 2). The OGGT performed at day 56 revealed that these animals also showed a slight, but significant, higher glucose area under the curve (AUC) than their lean counterparts and a clear increase in insulin AUC, indicating that both CAF-fed groups required a greater release of insulin than the ST-fed animals to clear the glucose load (Fig. 2a and b). In addition, regardless of the treatment received, CAF-fed animals developed dyslipidaemia, hyperinsulinemia and insulin resistance as previously described (Cigarroa et al., 2016; Lalanza et al., 2014; Sampey et al., 2011) (Table 1 and Fig. 2). Furthermore, these animals also showed a significant decrease of feed efficiency, which gives an estimation of the efficiency of conversion of energy intake into body weight gain (Steiner, Sciarretta, Pasquali, & Jenck, 2013) (Table 2).

3.2. Heat-killed Ba8145 administration increased lean mass and decreased mesenteric adiposity in CAF-fed rats

CAF-Ba8145 rats attained a slightly lower body weight than their non-treated counterparts throughout the study (Fig. 1a), although no significant changes were observed either in this parameter (Fig. 1a) or in the body weight gain at the end of the experimental period (Table 1).

Although the two-way ANOVA analysis revealed that the heat-killed Ba8145 supplementation during the first 56 days produced a significant increase of relative lean mass (expressed as a percentage of the body weight) in both ST-fed and CAF-fed animals, the LSD test indicated that the effect was more evident in CAF-Ba8145 rats (4.3% higher than CAF-veh animals) (Fig. 1c). Furthermore, at the end point, this effect was only observed in this group of animals, which attained a 5.5% increase in relative lean mass when compared with CAF-veh rats (Fig. 1c). Compared with CAF-veh rats, CAF-Ba8145 animals displayed a progressive decrease in relative fat mass content and showed a non-significant decrease of 8.2% for this parameter at day 28 and a non-significant decrease of 12.2% at the end point (Fig. 1b). The dissection of specific WAT depots at sacrifice revealed a significant decrease of relative MWAT weight in both ST-Ba8145 and CAF-Ba8145 animals, and the reduction of this abdominal fat depot was higher than 13% in both groups as compared with that for the non-supplemented groups (Table 1). A very similar pattern in response to heat-killed Ba8145 administration was observed for relative RWAT weight and adiposity index in both groups of animals [RWAT: ST-Ba8145 (10.7% lower), CAF-Ba8145 (11.9% lower); adiposity index: ST-Ba8145 (8.8% lower), CAF-Ba8145 (12.0% lower)], although the differences did not reach statistical significance ($p = 0.074$ and $p=0.064$ for RWAT and the adiposity index, respectively) (Table 1).

3.3. Heat-killed Ba8145 intake decreased energy intake in CAF-fed rats

Heat-killed Ba8145 administration significantly reduced cumulative energy intake in CAF-fed rats. This effect was mainly caused by a significant decrease in carbohydrate intake, which can be attributed to the lower consumption of milk with sugar that was observed in CAF-Ba8145 animals in comparison with CAF-veh rats (Table 2).

3.4. Heat-killed Ba8145 supplementation ameliorated the hyperlipidaemia induced by CAF diet intake

Heat-killed Ba8145 intake partially counteracted the dyslipidaemia developed by CAF-veh rats, producing a significant drop in the blood levels of VLDL/LDL-cholesterol (27.7% lower) (Table 1) and a residual decrease of triacylglycerols (30.8% lower, $p = 0.050$ versus CAF-veh rats, Student's *t* test). Conversely, ST-BA8145

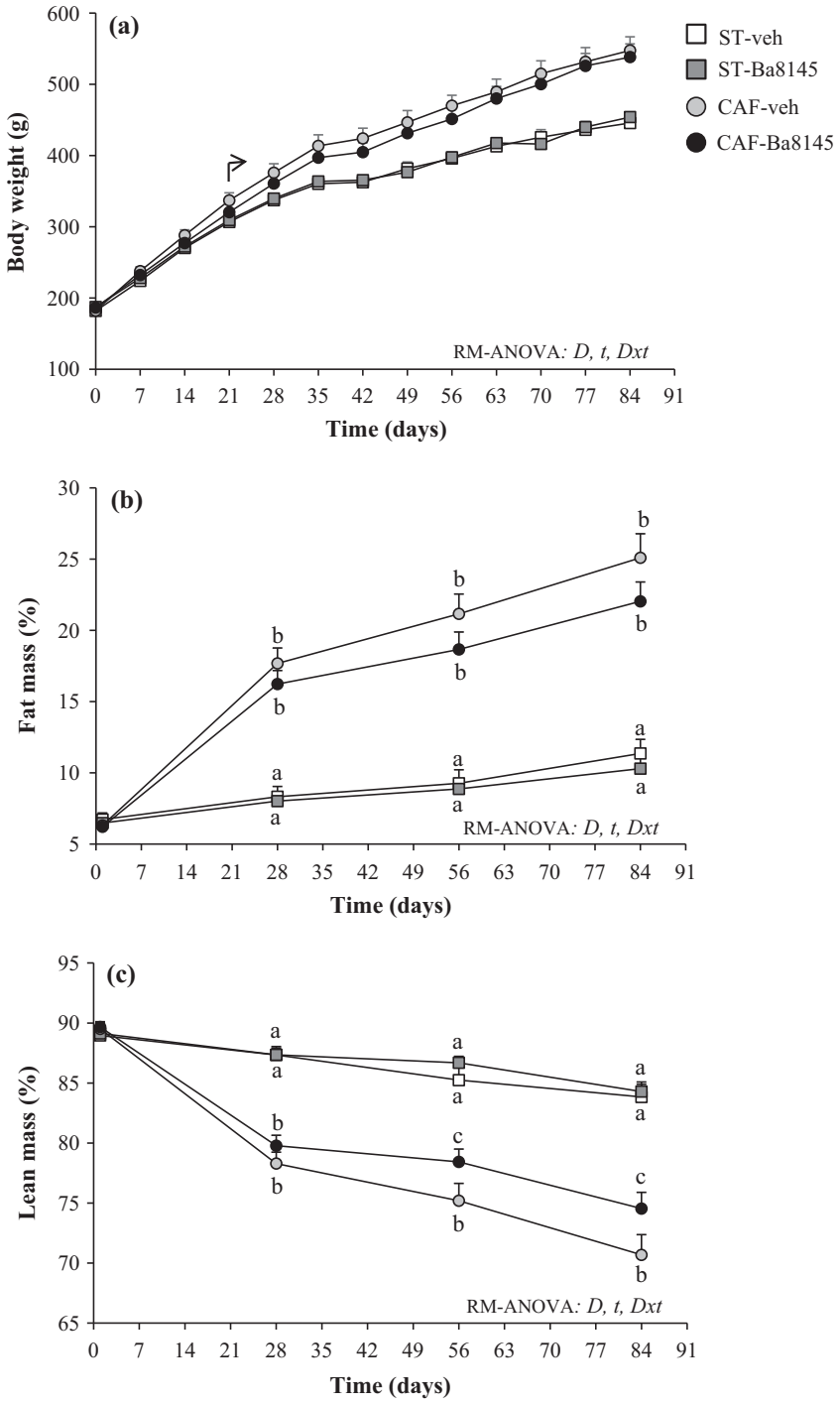

Fig. 1. The evolution of body weight, fat mass and lean mass of male Wistar rats that were fed with a standard diet (ST) or a cafeteria diet (CAF) for 84 days and received a daily oral dose of the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145 (Ba8145, 10^{10} CFU/day) or vehicle (veh) during the same period. Body weight was recorded weekly, whereas fat and lean masses were documented every 28 days. Relative fat and lean mass weights (%) were calculated according to the formula (100* fat or lean weight/body weight) and are expressed as a percentage of body weight. Data are given as the mean ± SEM (n = 9–10). *D*: the effect of diet type, *t*: the effect of time, *Dxt*: the interaction between diet type and time (RM-ANOVA, $p < 0.05$). [abc]Mean values with unlike letters differ significantly among groups (one-way ANOVA and LSD post hoc comparison, $p < 0.05$). The arrow indicates the day from which significant differences in body weight between the CAF and ST groups were found, i.e., the effect of diet type (two-way ANOVA, $p < 0.05$).

animals displayed higher levels of VLDL/LDL-cholesterol than their counterparts (Table 1).

3.5. Heat-Killed Ba8145 intake increased insulin sensitivity in CAF-fed rats

At day 56, the two-way ANOVA analysis revealed that both groups of heat-killed Ba8145-treated animals displayed significantly lower circulating levels of insulin under fasting conditions, although the pairwise comparisons showed that there was no significant difference between ST-veh and ST-Ba8145 animals, nor between CAF-veh and CAF-Ba8145 rats (Fig. 2b). No changes were found in HOMA-IR and R-QUICKI indexes at this point (Fig. 2c and 2e). There was no overall effect of the heat-killed Ba814 supplementation on glucose and insulin AUC (Fig. 2a and b). However, the decomposition of the significant

Table 1
Biometric parameters and serum concentrations of metabolites in rats fed a standard diet (ST) or a cafeteria diet (CAF) and supplemented with heat-killed Ba8145 or vehicle during 84 days.

	ST-veh	ST-Ba8145	CAF-veh	CAF-Ba8145	
Body weight					
Initial body weight (g)	182 ± 5	187 ± 4	182 ± 5	186 ± 5	
Final body weight (g)	446 ± 11[a]	454 ± 6[a]	548 ± 18[a]	538 ± 19[a]	*D*
Body weight gain (g)	264 ± 8[a]	267 ± 7[a]	365 ± 18[a]	352 ± 18[a]	*D*
Tissue weights (%)					
Liver (%)	2.55 ± 0.06	2.62 ± 0.07	2.75 ± 0.05	2.76 ± 0.10	*D*
RWAT (%)	2.62 ± 0.23[a]	2.34 ± 0.16[a]	5.32 ± 0.26[a]	4.69 ± 0.33[a]	*D*
IWAT (%)	2.32 ± 0.14[a]	2.06 ± 0.15[a]	4.60 ± 0.39[a]	3.98 ± 0.32[a]	*D*
MWAT (%)	1.75 ± 0.11[a]	1.52 ± 0.07[a]	2.77 ± 0.17[a]	2.41 ± 0.14[a]	*D, T*
EWAT (%)	2.49 ± 0.18[a]	2.44 ± 0.15[a]	4.60 ± 0.29[a]	4.13 ± 0.29[a]	*D*
Adiposity index (%)	9.18 ± 0.58[a]	8.37 ± 0.50[a]	17.29 ± 0.98[a]	15.21 ± 0.89[a]	*D*
Serum parameters					
Glucose (mmol/L)	8.47 ± 0.35	9.76 ± 0.26[Δ]	10.68 ± 0.41[*]	9.60 ± 0.34	*D, DxT*
TG (mmol/L)	0.82 ± 0.10[a]	0.79 ± 0.13[a]	2.66 ± 0.30[a]	1.84 ± 0.23[a]	*D*
TC (mmol/L)	1.95 ± 0.10	2.13 ± 0.11	2.16 ± 0.12	1.93 ± 0.09	
HDL-C (mmol/L)	1.33 ± 0.10[a]	1.37 ± 0.06[a]	1.02 ± 0.07[a]	0.99 ± 0.11[a]	*D*
LDL/VLDL-C (mmol/L)	0.49 ± 0.04	0.69 ± 0.08[Δ]	1.01 ± 0.11[*]	0.73 ± 0.08[$]	*D, DxT*
NEFAs (mmol/L)	0.32 ± 0.04[a]	0.31 ± 0.02[a]	0.67 ± 0.08[a]	0.43 ± 0.03[a]	*D, T*
Leptin (ng/mL)	11.1 ± 0.3[a]	10.9 ± 0.2[a]	41.7 ± 7.7[a]	35.9 ± 12.3[a]	*D*
Insulin (ng/mL)	10.9 ± 1.3[a]	10.7 ± 1.6[a]	18.9 ± 1.3[a]	16.1 ± 1.9[a]	*D*
HOMA-IR	105 ± 15[a]	115 ± 17[a]	222 ± 17[a]	173 ± 24[a]	*D*
R-QUICKI	0.247 ± 0.006[a]	0.246 ± 0.004[a]	0.211 ± 0.003[a]	0.227 ± 0.005[a]	*D*

Relative tissue weights (%) were calculated according to the formula (100* tissue weight/body weight) and were expressed as a percentage of body weight. The adiposity index was computed as the sum of the EWAT, IWAT, MWAT and RWAT depot weights (in grams) and is expressed as a percentage of body weight. Data are given as the mean ± SEM (n = 9–10). *D*: the effect of diet type, *T*: the effect of heat-killed Ba8145 treatment, *DxT*: the interaction of diet type and heat-killed Ba8145 treatment (two-way ANOVA, p<0.05).
[a] Mean values with unlike letters significantly differed among the groups (one-way ANOVA and LSD post hoc comparison, p<0.05).
[*] The effect of diet within vehicle groups;
[Δ] the effect of heat-killed Ba8145 treatment within ST groups;
[$] the effect of heat-killed Ba8145 treatment within CAF groups (Student's *t* test, p<0.05). Ba8145: probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145; RWAT: retroperitoneal white adipose tissue; IWAT: inguinal white adipose tissue; MWAT: mesenteric white adipose tissue; EWAT: epididymal white adipose tissue; TG: triacylglycerol; TC: total cholesterol; LDL/VLDL-C: low density lipoprotein + very-low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; NEFAs: non-esterified fatty acids; HOMA-IR: homeostasis model assessment-estimated insulin resistance; R-QUICKI: revised quantitative insulin sensitivity check index.

Table 2
Cumulative nutrient and energy intake and feed efficiency in rats fed a standard diet (ST) or a cafeteria diet (CAF) and supplemented with heat-killed Ba8145 or vehicle during 84 days.

	ST-veh	ST-Ba8145	CAF-veh	CAF-Ba8145	
Protein (g)	35.1 ± 0.9[a]	35.0 ± 0.6[a]	28.3 ± 1.4[a]	26.0 ± 1.2[a]	*D*
Fat (g)	11.7 ± 0.3[a]	11.7 ± 0.2[a]	42.0 ± 2.4[a]	39.3 ± 1.8[a]	*D*
Carbohydrate (g)	85.0 ± 2.2	84.6 ± 1.5	200 ± 10[*]	169 ± 7[# $]	*D, T, DxT*
Fibre (g)	27.6 ± 0.8[a]	27.7 ± 0.5[a]	5.71 ± 0.53[a]	5.74 ± 0.37[a]	*D*
Milk with sugar (mL)	—	—	512 ± 23	394 ± 23[$]	
Energy intake (kcal)	586 ± 15[a]	583 ± 11[a]	1285 ± 67[a]	1132 ± 45[a]	*D*
Feed efficiency (%)	44.0 ± 0.9[a]	45.9 ± 1.5[a]	28.8 ± 1.5[a]	31.1 ± 1.3[a]	*D*

Feed efficiency (%) was calculated as the quotient between final body weight gain in grams and the total kilocalories consumed during the study, and is expressed as a percentage of total caloric intake. The CAF diet included the following components (quantity per rat): bacon (5–7 g); biscuit with pâté (13–14 g); biscuit with cheese (14–15 g); muffins (7–8 g); carrots (6–8 g); milk with sugar (220 g/l; 100 ml); and ST (10 g). The caloric distribution of the CAF diet was 10.0% protein; 31.9% fat; and 58.1% carbohydrates. The caloric distribution of the ST diet (3.1 kcal/g) was 24.2% protein, 18.2% fat and 57.6% carbohydrates. The animals were fed *ad libitum*, the food was renewed daily, and the amounts of each component that were eaten were determined every ten days (nine times during the experiment). *D*: the effect of diet type, *T*: the effect of BA8145 treatment, *DxT*: the interaction of diet type and heat-killed Ba8145 treatment (two-way ANOVA, p < 0.05).
[a] Mean values with unlike letters significantly differed among groups (one-way ANOVA and LSD post hoc comparison, p < 0.05).
[*] The effect of diet within vehicle groups.
[#] the effect of diet within Ba8145 groups.
[$] the effect of heat-killed Ba8145 treatment within CAF groups (Student's *t* test, p < 0.05). Ba8145: probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145.

interaction between diet and treatment (two-way ANOVA, p < 0.05) obtained at 60 min revealed that CAF-Ba8145 rats showed lower circulating levels of glucose than CAF-veh animals (student's *t* test, p < 0.05). Furthermore, CAF-Ba8145 rats presented numerically lower levels of insulin than their non-treated counterparts 60 min after the glucose load (Fig. 2b).

At the end of the study, CAF-Ba8145 rats displayed significantly lower levels of NEFAs (35.8% lower) and a clear trend towards lower fasting glucose levels (10.1% lower, p = 0.061, Student's *t* test) than CAF-veh animals. These metabolic changes resulted in a significant increase of R-QUICKI, which indicated an improvement of insulin sensitivity (Table 1).

3.6. Heat-killed Ba8145 treatment enhanced EE in both ST and CAF-fed rats

As expected (García-Díaz et al., 2007), CAF-fed animals exhibited a lower RQ than ST-fed animals, which indicates that a shift occurred towards higher lipid oxidation and a lower use of carbohydrates as an energy source.

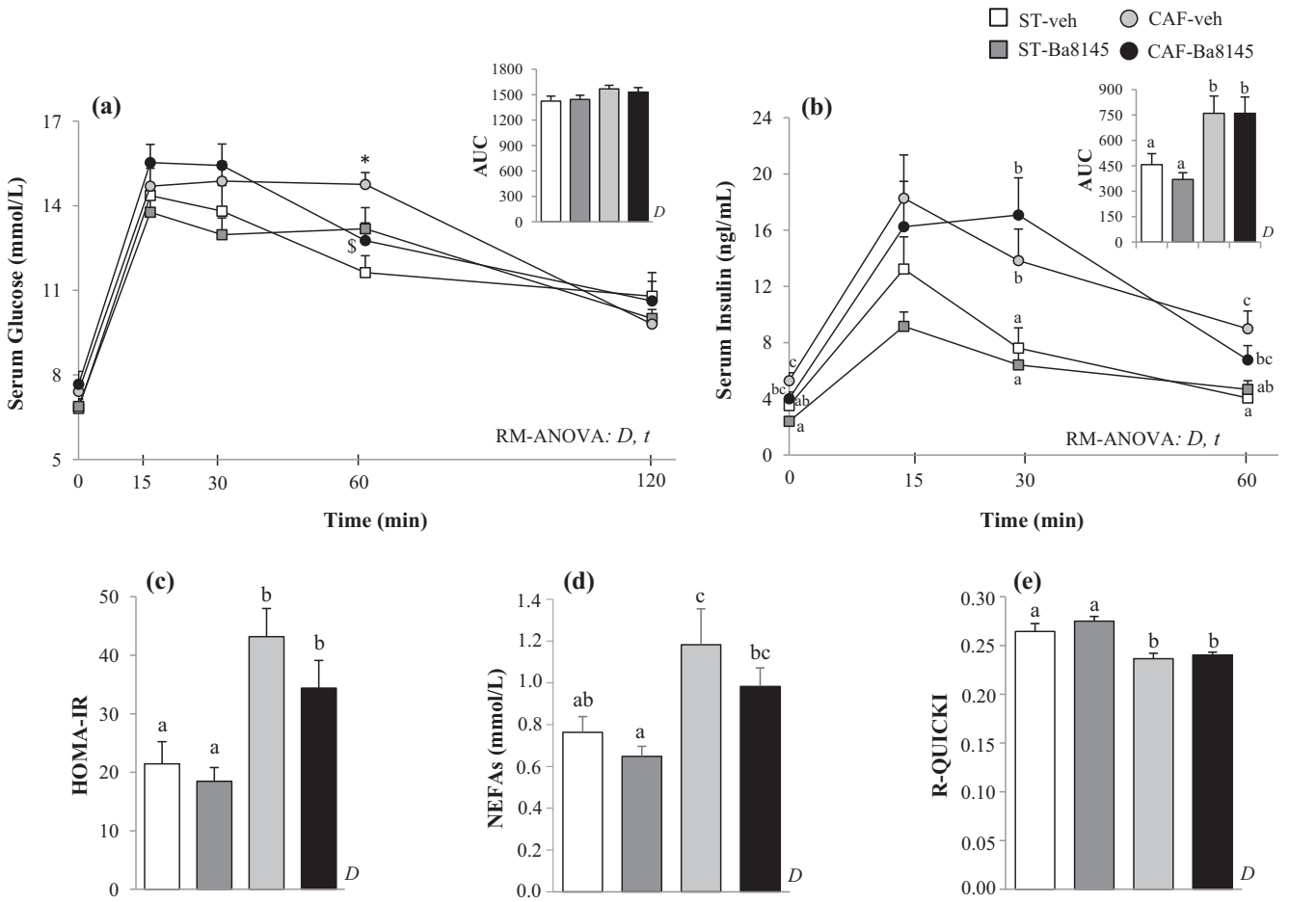

Fig. 2. Serum levels of glucose (a) and insulin (b) after an OGTT (2 g kg^{-1} of body weight) performed on day 56 of the study in male Wistar rats that were fed with a standard diet (ST) or a cafeteria diet (CAF) for 84 days and received a daily oral dose of the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145 (Ba8145, 10^{10} CFU/day) or vehicle (veh) during the same period. The integrated area under the curve (AUC) was determined for glucose and insulin circulating levels using the software GraphPad Prism (GraphPad Software, Inc., La Joya, CA, USA). HOMA-IR (c), circulating levels of NEFAs (d) and R-QUICKI (e) at time 0 of the OGTT (baseline) are also shown. Data are given as the mean ± SEM (n = 9–10). *D*: the effect of diet type, *t*: the effect of time (RM-ANOVA or two-way ANOVA, $p < 0.05$). [abc]Mean values with unlike letters significantly differed among groups (one-way ANOVA and LSD post hoc comparison, $p < 0.05$). *The effect of diet within vehicle groups; [$] the effect of heat-killed Ba8145 treatment within CAF groups (Student's *t* test, $p < 0.05$). HOMA-IR: homeostasis model assessment-estimated insulin resistance; NEFAs: non-esterified fatty acids; R-QUICKI: revised quantitative insulin sensitivity check index.

Heat-killed Ba8145 intake did not affect RQ; consequently, no changes in fat and carbohydrate oxidation were reported (Fig. 3a, c and d). Both ST-Ba8145 and CAF-Ba8145 animals showed a significant increase in EE (Fig. 3b). This effect cannot be attributed to higher locomotor activity since no changes were found either in this parameter (Fig. 3e) or in the number of rearings (Fig. 3f).

3.7. Heat-killed Ba8145 administration did not affect liver and faecal lipid content

Lipid concentrations (mg/g) were increased in the liver and faeces of CAF-fed animals. These parameters were not affected by heat-killed Ba8145 treatment (Supplementary Table 2).

3.8. Heat-killed Ba8145 intake altered gene expression in MWAT

In MWAT, the administration of heat-killed Ba8145 to CAF-fed rats fully counteracted the drop in the mRNA levels of the fatty acid transporter 1 gene (*Fatp1*) that were produced by CAF feeding, and the same pattern was observed for the key lipolytic gene adipose triacylglycerol lipase (*Atgl*) (Fig. 4a). Furthermore, compared with ST-veh animals, ST-Ba8145 rats displayed lower mRNA levels of the gene encoding glycerol-3-phosphate acyltransferase (GPAT), a key enzyme involved in triacylglycerol synthesis, and a significant decrease of the fatty acid uptake-related gene fatty acid translocase, homologue of CD36 (*CD36*) (Fig. 4a). In this tissue, heat-killed Ba8145 supplementation did not alter the expression of key genes involved in β-oxidation (carnitine palmitoyltransferase 1 beta -*Cpt1β*- and hydroxyacyl-CoA dehydrogenase –*Had*-), fatty acid synthesis (acetyl CoA carboxylase 1 -*Acc1*- and fatty acid synthase –*Fas*-) and adipogenesis (CCAAT/enhancer binding protein alpha –*Cebpα*- and peroxisome proliferator-activated receptor gamma 2 –*Pparg2*-) either in ST-fed or in CAF-fed animals (Fig. 4a).

The hypothalamic mRNA levels of the genes encoding leptin and ghrelin receptors (ObRb and GSHR), which play a key role in the control of food intake (Sánchez, Cladera, Llopis, Palou, & Picó, 2010), did not change in response to heat-killed Ba8145 treatment (Fig. 4b). Furthermore, no changes among groups were found in the gene expression of the orexigenic (neuropeptide Y –NPY-, Agouti-related protein –AGRP-), and anorexigenic (pro-opiomelanocortin –POMC- and amphetamine-regulated transcript -CART) neuropeptides in this tissue (García et al., 2010; Sánchez et al., 2010) (Fig. 4b).

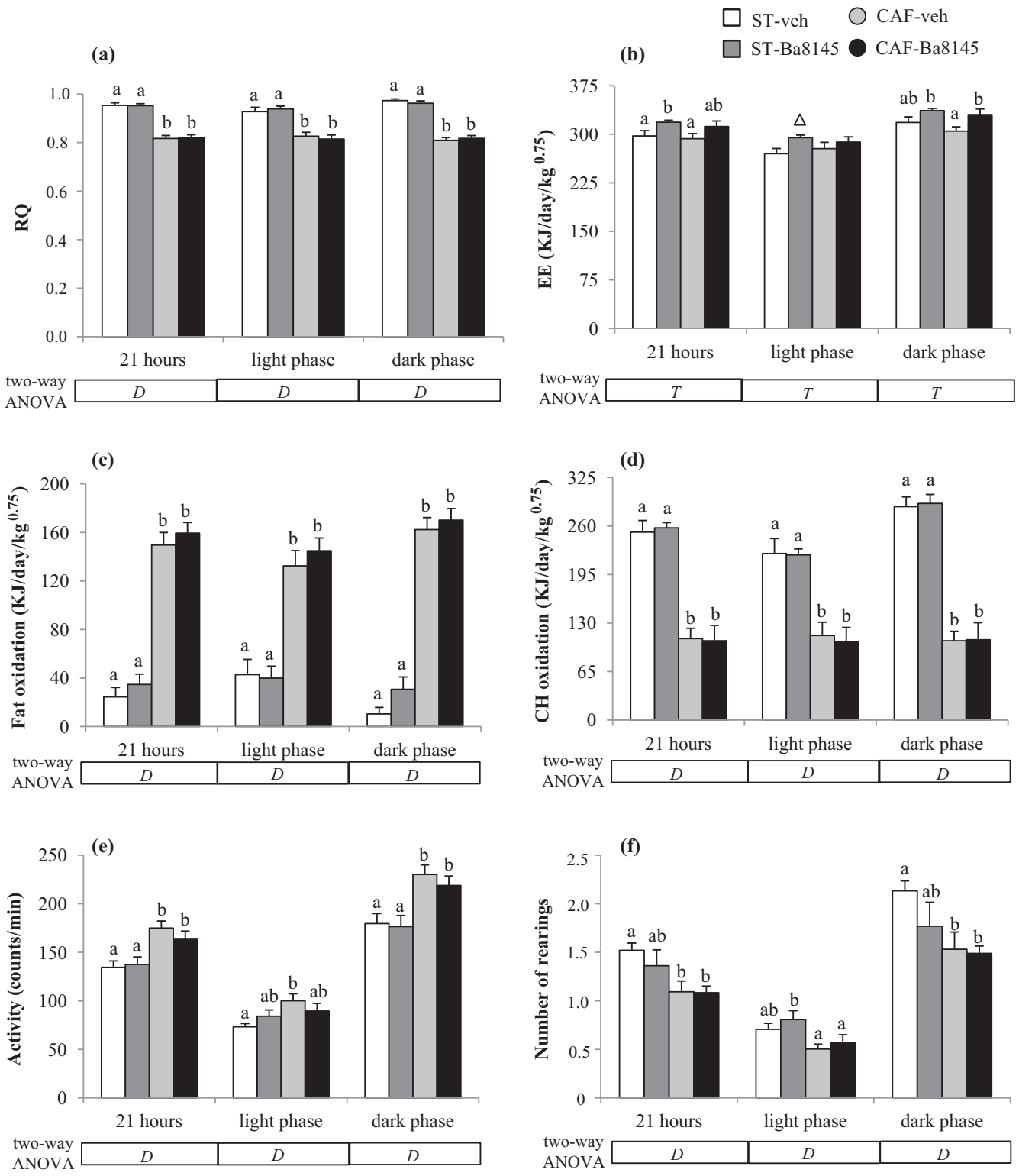

Fig. 3. Respiratory quotient (RQ) (a), energy expenditure (EE) (b), fat oxidation (c), carbohydrate oxidation (d), spontaneous locomotor activity (e) and number of rearings (f) in male Wistar rats that were fed with a standard diet (ST) or a cafeteria diet (CAF) for 84 days and that received a daily oral dose of the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145 (Ba8145, 10^{10} CFU/day) or vehicle (veh) during the same period. Indirect calorimetric measurements were performed on day 78 under *ad libitum* conditions and during 22 h (from 11:00 am to 09:00 am). The data obtained during the first hour was discarded for final analyses. All rats were fed with ST during the measurements. Data are given as the mean ± SEM (n = 9–10). *D*: the effect of diet type, *T*: the effect of heat-killed Ba8145 treatment (two-way ANOVA, $p < 0.05$). ^{ab}Mean values with unlike letters significantly differed among groups (one-way ANOVA and LSD post hoc comparison, $p < 0.05$). $^{\blacktriangle}$ the effect of heat-killed Ba8145 treatment within ST groups (Student's *t* test, $p < 0.05$).

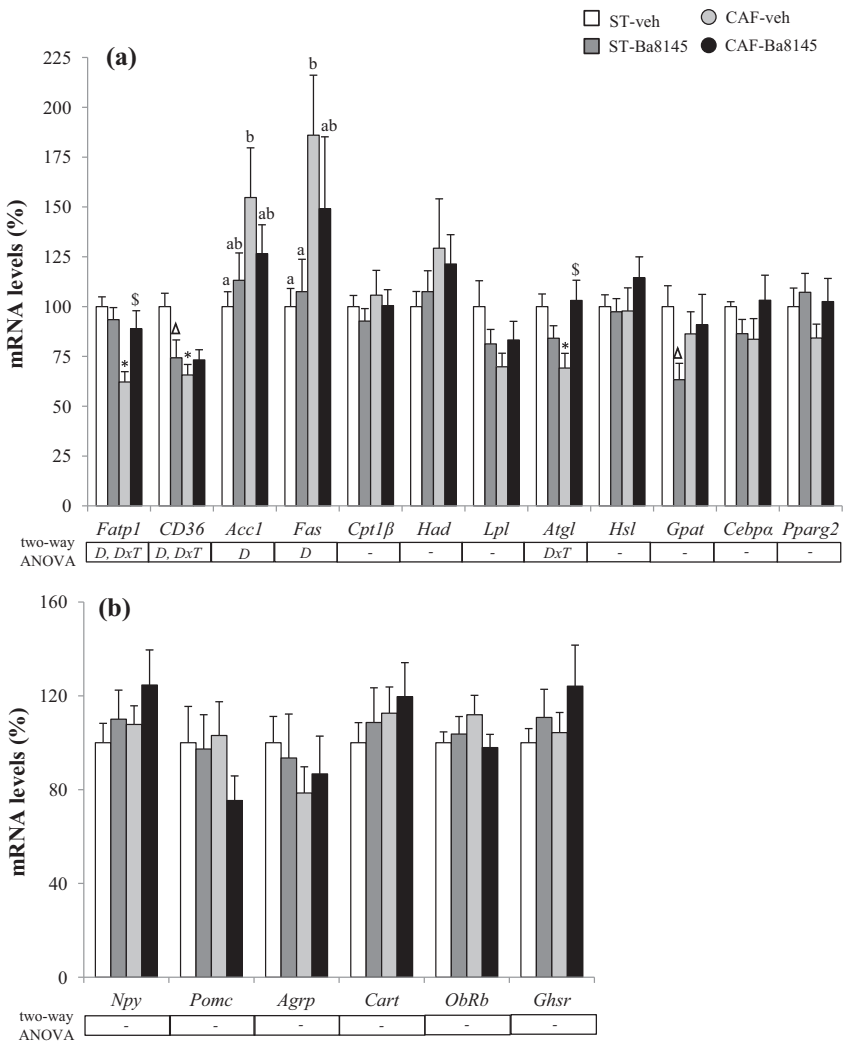

Fig. 4. The mRNA expression levels of genes related to lipid metabolism (MWAT, a) and food intake control (hypothalamus, b) of male Wistar rats that were fed with a standard (ST) or cafeteria (CAF) diet for 84 days and received a daily oral dose of the heat-killed probiotic *Bifidobacterium animalis* subsp. *lactis* strain CECT 8145 (Ba8145, 10^{10} CFU/day) or vehicle (veh) during the same period. Data are given as the mean ± SEM (n = 9–10). *D*: the effect of diet type, *DxT*: the interaction of diet type and heat-killed Ba8145 treatment. *The effect of diet within vehicle groups; $^{\Delta}$the effect of heat-killed Ba8145 treatment within ST groups; $^{\$}$the effect of heat-killed Ba8145 treatment within CAF groups (Student's *t* test, $p < 0.05$). *Acc1*, acetyl CoA carboxylase 1; *Agrp*, agouti-related protein; *Atgl*, adipose triacylglycerol lipase; β-actin, actin beta; *Cart*, cocaine and amphetamine-regulated transcript; *Cd36*, fatty acid translocase, homologue of CD36; *Cebpα*, CCAAT/enhancer binding protein alpha; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Fas*, fatty acid synthase; *Fatp1*, fatty acid transporter, member 1; *Ghsr*, ghrelin receptor; *Gpat*, glycerol-3-phosphate acyltransferase; *Had*, hydroxyacyl-CoA dehydrogenase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Hsl*, hormone-sensitive lipase; *Lpl*, lipoprotein lipase; *Npy*, neuropeptide Y; *ObRb*, long-form leptin receptor; *Pomc*, proopiomelanocortin; *Pparg2*, peroxisome proliferator-activated receptor gamma 2; *Ppia*, peptidylprolyl isomerase A.

4. Discussion

In this study, we reported that the heat-killed probiotic Ba8145 was able to exert beneficial effects against MetS in CAF-fed obese rats, including (1) decreasing mesenteric adiposity, (2) up-regulating key genes involved in fatty acid uptake (*Fatp1*) and lipolysis (*Atgl*) in MWAT, (3) enhancing EE and lean body mass accretion, (4) increasing insulin sensitivity, and (5) ameliorating dyslipidaemia. Interestingly, we also demonstrated that the supplementation with these inactivated microorganisms also positively affected normoweight rats, attenuating mesenteric fat accretion, down-regulating the triacylglycerol synthesis-related gene *Gpat1* in MWAT and increasing EE.

To the best of our knowledge, few data are available regarding the impact of the administration of heat-inactivated probiotics on obesity and the metabolic disturbances that are linked to this pathology (Matsuzaki, Nagata et al., 1997; Matsuzaki, Yamazaki, Hashimoto, & Yokokura, 1997; Sakai et al., 2013; Ting, Kuo, Hsieh et al., 2015; Ting, Kuo, Kuo et al., 2015). As far as we know, only one study has previously demonstrated the anti-adiposity properties of a heat-killed probiotic (*L. reuteri* GMNL-263), which significantly decreased EWAT weight in hamsters that were fed a high-fat diet after 8 weeks of supplementation (Ting, Kuo, Hsieh et al., 2015). Here, we report that the intake of the heat-killed probiotic Ba8145 for 84 days significantly reduced mesenteric adiposity in CAF-fed obese rats, an animal model that resembles the MetS that occurs in humans (Sampey et al., 2011). Furthermore, this anti-adiposity effect of heat-killed Ba8145 was also observed in the MWAT of ST-fed rats, and both normoweight and obese groups supplemented with the probiotic showed a clear trend towards lower RWAT weight and adiposity index. Remarkably, this beneficial effect of this heat-killed probiotic against fat mass accretion was less evident in the other white adipose depots studied (EWAT and IWAT). This depot-specific response to the heat-killed probiotic supplementation could be partly attributed to the heterogeneity in terms of vascularization and innervation that has been

described among the different white adipose depots, which would indicate different regulatory mechanisms in these tissues (Cinti, 2005; Pond & Mattacks, 1991).

Three mechanisms involved in the anti-obesity effects of bioactives are the enhancement of EE, the inhibition of food intake and the regulation of lipid metabolism (Torres-Fuentes, Schellekens, Dinan, & Cryan, 2015). Recently, Shirouchi et al. (Shirouchi et al., 2016) demonstrated that, in rats, the anti-obesity effects of *L. gasseri* SBT2055 are mediated by enhanced carbohydrate oxidation, which increases EE. In the present study, the adiposity-lowering effects of heat-killed Ba8145 might also be associated with the increased EE that was observed in both ST-Ba8145 and CAF-Ba8145 animals, although no changes were found in lipid and carbohydrate oxidation or in locomotor activity. In this sense, the shift to a ST carried out in the CAF-fed animals during these measurements could have obscured the effects of the heat-killed probiotic on RQ and, consequently, on the utilization of lipid and carbohydrates as energy sources. Additional studies using CAF would be needed to elucidate whether the supplementation with this bioactive compound is able to modulate the substrate oxidation preferences in obese animals. The enhancement of EE in the rats that were supplemented with heat-killed Ba8145 might be related to improved mitochondrial function and to the activation of thermogenesis in brown adipose tissue, which is, in rodents, the major contributor of energy dissipation through the production of heat (Lagouge et al., 2006). Nevertheless, the collection of this tissue would have been needed to shed more light on this issue. On the other hand, although some studies have demonstrated that probiotics are able to inhibit energy intake acutely by modulating gastrointestinal hormones involved in satiety, such as peptide YY and glucagon-like peptide 1 (Bjerg et al., 2014; Forssten et al., 2013), as far as we know, the adiposity-reducing effects of these microorganisms (both viable (Kang et al., 2013, 2010; Wu et al., 2015) and non-viable (Ting, Kuo, Hsieh et al., 2015)) have not yet been associated with decreased food intake. Here, the significantly lower cumulative energy intake displayed by CAF-Ba8145 animals would also contribute to the anti-adiposity effect of heat-killed Ba8145 and would explain the slightly greater body fat-lowering effects observed in these rats in response to heat-killed Ba8145 supplementation as compared with those for their lean counterparts. This finding was not associated with changes in the mRNA levels of genes that are involved in the leptin and ghrelin systems, which play essential roles in the control of food intake at the hypothalamus (Sánchez et al., 2010). However, one limitation of the gene expression data is the fact that they do not always match protein levels. Therefore, further research focused on the hypothalamic protein levels of leptin and ghrelin receptors, and the orexigenic and anorexigenic neuropeptides, including NPY, AgRP, POMC and CART, would be of value to elucidate the molecular mechanisms that are responsible for the observed effects. The lower MWAT depot size observed in CAF-Ba8145 rats can also be tentatively associated with the increased mRNA levels of *Atgl*, the gene encoding the enzyme that hydrolyses triacylglycerols in the first step of lipolysis (Caimari, Oliver, & Palou, 2008; Antoni Caimari, Oliver, & Palou, 2012); in ST-BA8145 rats, the down-regulation of the gene that encodes GPAT (which catalyses the initial step in triacylglycerol synthesis (Caimari, del Bas, Crescenti, & Arola, 2013)) might also be responsible for the observed effects. Additional mechanisms, such as decreased conversion of energy intake into body weight gain and diminished lipid absorption appear not to be involved in the adiposity-lowering effects of heat-killed Ba8145 since no changes were found in feed efficiency and in the lipid content of faeces. Furthermore, the activation of β-oxidation and/or the inhibition of adipogenesis and fatty acid synthesis were not evident at the transcriptional level in the MWAT, suggesting that the heat-killed probiotic did not decrease adiposity through the modulation of these pathways. Nevertheless, further studies of gene and protein expression performed in different white adipose depots, liver and skeletal muscle could contribute to shed more light on this issue.

The reduced adiposity showed in both ST-Ba8145 and CAF-Ba8145 rats was not accompanied by lower body weight gain, probably due to the progressive increase of lean mass that was observed along the study, especially in CAF-Ba8145 animals, which, at sacrifice, displayed a substantial increase in lean mass when compared with CAF-veh rats. Chen et al. (2016) recently showed that the administration of *L. plantarum* TWK10 to mice produced a significant increase in relative muscle mass and that this was accompanied by enhanced exercise performance. Lean mass as measured by quantitative magnetic resonance provides an accurate measurement of muscle mass (Taicher, Tinsley, Reiderman, & Heiman, 2003); therefore, it is plausible to speculate that the higher lean body mass observed in the CAF-Ba8145 rats might also be the result of muscle mass accretion. However, in our study, the dissection of specific muscles, such as the gastrocnemius, soleus and quadriceps, would have been needed to corroborate this hypothesis. The increase of lean mass observed in the CAF-Ba8145 animals does not appear to be due to a higher protein intake and can be tentatively associated with higher rates of protein synthesis or lower rates of protein degradation together with changes in amino acid bioavailability or with a lower pro-inflammatory state (Bindels & Delzenne, 2013; Bond, 2016).

Various lines of evidence suggest that the accretion of MWAT and omental visceral fat depots is more closely related to metabolic disturbances that are linked to obesity than to subcutaneous fat accumulation due to the higher susceptibility to lipolysis, the lower sensitivity to the anti-lipolytic effects of insulin and the direct release of NEFAs, glycerol and pro-inflammatory cytokines to the liver through the portal vein of these visceral depots (Foster & Pagliassotti, 2012; Tewari, Awad, Macdonald, & Lobo, 2015). In agreement with this hypothesis, several studies carried out in both rodents (Borst, Conover, & Bagby, 2005; Gabriely et al., 2002; Pitombo et al., 2006) and humans (Milleo et al., 2011; Pedersen et al., 2015; Pedersen et al., 2014; Thörne, Lönnqvist, Apelman, Hellers, & Arner, 2002) have shown that the specific reduction of visceral fat attenuates hyperglycaemia and hyperinsulinemia and improves insulin sensitivity. According to these observations, the reduction of MWAT observed in CAF-Ba8145 animals was accompanied by higher insulin sensitivity (measured as the R-QUICKI index), an effect that might be attributable to the decrease in serum NEFA levels and to the clear trend towards lower glucose levels observed in these rats when compared with the CAF-veh group. Furthermore, the decrease in the fasting serum insulin levels that were observed at day 56 in both ST-Ba8145 and CAF-Ba8145 rats and the higher glucose clearance 60 min after the glucose load that was displayed by CAF-Ba8145 rats reinforce the idea that heat-killed Ba8145 exerts beneficial effects on glucose and insulin metabolism. Our findings agree with those reported by Matsuzaki, Yamazaki et al. (1997), which demonstrated the glucose and insulin-lowering effects of heat-killed cells of *L. casei* when administered for 8 weeks to genetically obese-diabetic KK-A^Y mice and also with the results obtained by Sakai and collaborators (Sakai et al., 2013), which showed a significant decrease in blood glucose levels 30, 60, 90 and 120 min after insulin administration and lower NEFAs levels in mice that were fed a high-fat diet and supplemented with heat-killed *L. plantarum* OLL2712 for 12 weeks. Elevated circulating levels of NEFAs are strongly associated with the appearance of insulin resistance due to their deleterious effects on insulin signalling in both liver and muscle, which result in lower muscle glucose uptake, increased hepatic gluconeogenesis, hyperinsulinemia and ectopic lipid accumulation (Foster & Pagliassotti, 2012; Samuel & Shulman, 2016;

Tewari et al., 2015). Therefore, it is plausible to hypothesize that the beneficial effects of heat-killed Ba8145 on insulin sensitivity that were observed in CAF-fed rats might be mediated, at least in part, by the significant decrease in NEFA serum levels that were displayed by the CAF-Ba8145 rats. White adipose tissue is the major contributor of NEFAs to the bloodstream; therefore, it is reasonable to expect that heat-killed Ba8145 exerts its NEFA-lowering effects through the inhibition of lipolysis in fat depots. However, the higher mRNA levels of the gene encoding ATGL that were observed in the MWAT of CAF-Ba8145 animals compared with CAF-veh rats would not support this hypothesis; nevertheless, as previously mentioned, this might help to explain the lower adiposity observed in this group of rats. On the other hand, the CAF-Ba8145 animals also displayed an increase in the gene expression of *Fatp1* in MWAT, which suggests that fatty acid uptake was increased in white adipose tissue; if this were true, this would partially explain the lower circulating levels of NEFAs that were observed in these animals.

Another relevant finding of our experiment is the amelioration of dyslipidaemia that was observed in the CAF-Ba8145 rats. As far as we know, only one study has previously demonstrated this effect when animals were supplemented with non-viable cells (Ting, Kuo, Kuo et al., 2015). In our experiment, it appears plausible to speculate that the lower carbohydrate intake that was observed in the CAF-Ba8145 animals could have decreased the supply of glucose to the liver, which would in turn limit *de novo* lipogenesis, resulting in decreased hepatic triacylglycerol synthesis and VLDL assembly (Quesada et al., 2009). However, no differences in hepatic total lipids and triacylglycerols were observed in the CAF-Ba8145 animals when compared with their obese counterparts, suggesting that heat-killed Ba8145 did not decrease circulating levels of triacylglycerols by inhibiting hepatic lipogenesis and triacylglycerol secretion. Two additional mechanisms that might contribute to the anti-hypertriacylglycerolemic effects of heat-killed Ba8145 are the increased activity of LPL (which is essential for promoting adipose tissue lipid uptake from the bloodstream (Caimari et al., 2013; Samuel & Shulman, 2016)) and the inhibition of fat absorption. Nevertheless, the CAF-Ba8145 rats did not display greater mRNA levels of *Lpl* in MWAT and/or increased lipid content in faeces compared with the CAF-veh animals, indicating that these two mechanisms are not apparently involved in the observed effects. Additional studies focussing on key genes and proteins involved in hepatic lipogenesis and VLDL secretion as well as in triacylglycerol clearance in adipose tissue depots and muscle would be of value for shedding light on the molecular mechanisms by which heat-killed Ba8145 exerts its hypotriacylglycerolemic effects. Regarding cholesterol, the decrease in the circulating levels of VLDL/LDL-C that were observed in the CAF-Ba8145 animals is consistent with the findings obtained recently by Ting and collaborators, who showed that the administration of heat-killed *L. reuteri* GMNL-263 ameliorated hypercholesterolemia in hamsters that were fed a high-fat diet (Ting, Kuo, Kuo et al., 2015). This effect might be produced by the disruption of lipid absorption and/or the hepatic up-regulation of the mRNA levels of the LDL receptor and cholesterol 7α-hydroxylase, two key genes that are involved in cholesterol uptake and bile acid synthesis, respectively (Ting, Kuo, Hsieh et al., 2015). In the present study, no changes were found in faecal lipid content in the stool, and further studies are needed to elucidate whether this non-viable probiotic is able to enhance bile acid synthesis and its faecal excretion.

In conclusion, we demonstrate here that supplementation with the heat-killed probiotic Ba8145 over 12 weeks reduces MWAT depot weight in ST-fed animals and attenuates mesenteric adiposity, increases lean mass, improves insulin sensitivity and ameliorates dyslipidaemia in CAF-fed rats. The higher overall response to this inactivated probiotic observed in CAF-fed animals could be understood as an adaptive mechanism addressed to counteract the alteration of energy homeostasis produced by CAF consumption. The lower energy intake, higher EE and up-regulation of key genes involved in MWAT fatty acid uptake (*Fatp1*) and lipolysis (*Atgl*) that were observed in the CAF-Ba8145 animals might contribute to the beneficial effects of heat-killed Ba8145. Further RCT studies focused on the effectiveness of heat-killed Ba8145 in ameliorating MetS are planned. These results will be of interest to the food and nutraceutical industries over the short- and mid-term and will contribute to promoting the use of heat-killed probiotics, which are stable, easy to handle and can be used in a broad array of products that are designed to improve human health.

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Authors' contributions

Antoni Caimari (TC), Francesc Puiggròs (FP), Empar Chenoll (EC), Patricia Martorell (PM), Daniel Ramón (DR), Salvador Genovés (SG) and Lluís Arola (LA) designed the research; TC, EC, PM, Josep del Bas (JdB), Noemí Boqué (NB), and Anna Crescenti (AC) conducted the research and analysed the data; TC and LA drafted the manuscript and had the primary responsibility for the final content. All the authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.09.029.

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