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Bifidobacterium pseudocatenulatum CECT 7765 Ameliorates Neuroendocrine Alterations Associated with an Exaggerated Stress Response and Anhedonia in Obese Mice

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Abstract Obesity, besides being a problem of metabolic dysfunction, constitutes a risk factor for psychological disorders. Experimental models of diet-induced obesity have revealed that obese animals are prone to anxious and depressive-like behaviors. The present study aimed to evaluate whether *Bifidobacterium pseudocatenulatum* CECT 7765 could reverse the neurobehavioral consequences of obesity in a high-fat diet (HFD) fed mouse model via regulation of the gut–brain axis. Adult male wild-type C57BL-6 mice were fed a standard diet or HFD, supplemented with either placebo or the bifidobacterial strain for 13 weeks. Behavioral tests were performed, and immune and neuroendocrine parameters were analyzed including leptin and corticosterone and their receptors, Toll-like receptor 2 (TLR2) and neurotransmitters. We found that obese mice showed anhedonia ($p < 0.050$) indicative of a depressive-like behavior and an exaggerated hypothalamic-pituitary axis (HPA)-mediated stress response to acute physical ($p < 0.001$) and social stress ($p < 0.050$), but these alterations were ameliorated by *B. pseudocatenulatum* CECT 7765 ($p < 0.050$). These behavioral effects were parallel to reductions of the obesity-associated hyperleptinemia ($p < 0.001$) and restoration of leptin signaling ($p < 0.050$), along with fat mass loss ($p < 0.010$). *B. pseudocatenulatum* CECT 7765 administration also led to restoration of the obesity-induced reductions in adrenaline in the hypothalamus ($p < 0.010$), involved in the hypothalamic control of energy balance. Furthermore, the bifidobacterial strain reduced the obesity-induced upregulation of TLR2 protein or gene expression in the intestine ($p < 0.010$) and the hippocampus ($p < 0.050$) and restored the alterations of 5-HT levels in the hippocampus ($p < 0.050$), which could contribute to attenuating the obesity-associated depressive-like behavior ($p < 0.050$). In summary, the results indicate that *B. pseudocatenulatum* CECT 7765 could play a role in depressive behavior comorbid with obesity via regulation of endocrine and immune mediators of the gut–brain axis.

Keywords Obesity · *Bifidobacterium* · Microbiota · Depression · Stress · Serotonin · TLR2

Introduction

Obesity is more than just a problem of excessive body weight and metabolic dysfunction. Recent studies indicate that it is also a risk factor for neurobehavioral changes and psychological disorders. For example, obese individuals have about 55% increased odds of developing depression and related symptoms [1–3]. Although the biological causes and mechanisms underlying these associations are uncertain, alterations in the communication between the gastrointestinal (GI) tract and the nervous system via the so-called gut–brain axis could partly explain their high comorbidity. This axis constitutes a complex neuronal and humoral network of communication,

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providing a bidirectional interconnection between mood and food [4]. In order to maintain the homeostasis of the GI tract and the central nervous system (CNS), the gut–brain axis needs to function correctly [5]. Several studies report that mice consuming a high-fat diet (HFD) show depressive-like features, characterized by greater immobility in the forced swim task and reduced exploratory behavior in elevated plus-maze and open-field tests, which could reflect bottom–up effects [6]. Likewise, depressed individuals are more likely to gain excessive weight since they are also prone to making poor food choices and reduce their physical activity, which could reflect a top–down effect [1, 7].

Several studies demonstrated that HFD-induced obesity, per se, activates an inflammatory cascade [8]. One of the receptors involved in this inflammatory cascade is the Toll-like receptor (TLR) family. Specifically, TLR2 is located at the surface of different cellular types (epithelial cells, professional antigen-presenting cells, etc.) and is activated by lipoteichoic acids from Gram-positive bacteria and LPS from Gram-negative bacteria, acting synergistically with TLR4 [9, 10]. TLR2 is also responsive to dietary fatty acids [11] and involved in obesity [9]. Latorre et al. [10] have recently demonstrated that TLR2 activation inhibits the serotonin transporter (SERT) producing an increase of 5-HT in the intestine. Alterations in the intestinal serotonergic system have been described to be involved in gastrointestinal diseases such as inflammatory bowel disease (IBD) [12] or diarrhea [13] associated with intestinal dysbiosis. Also, alterations in the serotonergic system in the hippocampus are involved in depression since many 5-HT receptor subtypes are widely expressed in this brain region [14]. Obesity is also characterized by leptin resistance that implies alterations in the levels and functionality of leptin and its receptor LepRb; in turn, leptin and LepRb have been suggested to play a role in the pathogenesis of depression [15]. Therefore, the health problem addressed is twofold, whereby obesity and low mood (depression) provide mutual feedback, and could thus be self-perpetuating.

The gut–brain axis is also a route for the gut microbiota to impact on brain function as demonstrated in experimental models of stress and mood disorders [16, 17]. Consequently, intentional modulation of gut microbiota composition and function by dietary intervention (e.g., via administration of the so-called probiotic bacteria) is being investigated to ameliorate these disorders [18]. Bacterial fermentation products, including butyrate and propionate, influence behavior in animals [19] and humans [20]. For example, sodium butyrate is demonstrated to elicit an antidepressant effect in the murine brain [21] and high faucal concentrations of propionic acid correlated with anxiety in patients with IBS [20]. These findings indicate that microbiota-derived metabolites play a role in the depressive–anxiety spectrum. In addition, clinical trials indicate that interventions with some probiotic strains of the genera *Bifidobacterium* and *Lactobacillus* improve mood and reduce anxiety symptoms in patients with chronic fatigue syndrome and inflammatory bowel syndrome [22, 23]. Studies in rodent models of stress also suggest that *Bifidobacterium* strains act as protective dietary factors, mainly in childhood rather than in adulthood [24].

The aim of the present study was to evaluate the role of *Bifidobacterium pseudocatenulatum* CECT 7765 in the adverse endocrine and neurobehavioral consequences of obesity in adulthood using a HFD-induced obese mouse model. This strain was selected for this study owing to its pre-clinical efficacy on metabolic and immune dysfunction in the same obesity model [25–27]. The ultimate purpose of the study is to progress in our understanding of the role played by the gut–microbiota–brain axis and specific components of the indigenous human gut microbiota in regulating diet-induced psychological alterations associated with and contributing to obesity.

Methods and Materials

Bacterial Strain and Culture Conditions

B. pseudocatenulatum CECT 7765 was isolated from healthy infants and identified by sequencing the amplified 16S rRNA gene, as previously described, and selected on the basis of its anti-inflammatory properties in a macrophage cell line [27], in animal models of obesity and cirrhosis [25–28], and in macrophages of cirrhotic patients [29].

Bacteria were grown in MRS broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (*w*/*v*) cysteine (MRS-C) (Sigma, St. Louis, MO) and incubated at 37 °C for 24 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). Cells were harvested by centrifugation (10,000 rpm for 15 min), washed twice in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), and re-suspended in 10% skimmed milk for oral administration to mice.

Animals, Diets, and Experimental Design

Adult (age 6–8 weeks) male wild-type C57BL-6 mice were purchased from Charles River Laboratories (L'Arbresle Cedex, France). During the adaptation period (7 days), each animal was housed in a stainless-steel cage in a temperature-controlled (23 °C) room with a 12-h light/dark cycle and 40–50% relative humidity. Then, mice were randomly divided into four groups (n = 10 mice per group) as follows: (1) a control group, receiving a standard diet (SD) plus placebo (10% skimmed milk) by gavage; (2) an obese group, receiving a high-fat diet (HFD) plus placebo (10% skimmed milk) by gavage; (3) a group receiving a SD and a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage (SD + Bif); and (4) an obese group receiving the HFD and a daily

dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage (HFD + Bif). This regime was maintained for 14 weeks. To induce obesity in two of the experimental groups, mice were switched from the SD (CA.170481—AIN-76A Purified Diet-Rats/Mice, Harlan Laboratories, Madison, WI 53744-4220), administered during the adaptation period, to a HFD (TD.06414—Adjusted Calories Diet—60/Fat, Harlan Laboratories, Madison, WI 53744-4220) for 14 weeks. The HFD provided 18.4% kcal as protein, 21.3% kcal as carbohydrate, and 60.3% kcal as fat (5.1 kcal/g), whereas the SD provided 18.8% kcal as protein, 68.8% kcal as carbohydrate, and 12.4% kcal as fat (3.8 kcal/g). Mice had free access to water and food.

Experiments were carried out in strict compliance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the University of Valencia (Central Service of Support to Research [SCSIE], University of Valencia, Spain), and the protocol was approved by its Ethics Committee (Approval number 2015/VSC/PEA/00041).

A schematic representation of the intervention and assessments is shown in Fig. 1. Body weight and food ingestion were measured once a week throughout the trial. White adipose tissue (WAT) and brown adipose tissue (BAT) were determined at the end of the trial. The forced swimming test (FST), the open field test, the sucrose and saccharin preference test, and the light/dark test were carried out at weeks 8, 10, and 11 of the intervention, respectively. The resident–intruder test was performed at week 12 of the intervention and the acute stress test a few days before sacrifice. Fecal samples were also taken before and 3 h after acute stress and kept frozen at − 80 °C for further corticosterone analysis. All of these tests were evaluated as described in the following sections.

At the end of the study, animals were fasted for 4 h, anesthetized with isoflurane and sacrificed by cervical dislocation. In order to analyze hormonal and metabolic parameters, blood samples were collected in tubes, containing EDTA, and were centrifuged and the supernatant (serum) was kept at − 20 °C for further analysis. The hypothalamus and hippocampus tissues were immediately frozen in liquid nitrogen until analyzed. The small intestine was immediately frozen in PBS until analyzed. The fat was separated in WAT and BAT and weighed.

Hormonal and Metabolic Parameter Analyses

Serum leptin concentration was determined by the Assay Max Mouse Leptin ELISA Kit (Assay pro, LLC; Ireland) with a sensitivity threshold of 0.3 ng/mL. Biochemical parameters were also quantified in serum using enzymatic kits for glucose (Glucose Liquid Kit), cholesterol (Cholesterol Liquid Kit), and triglycerides (Triglyceride Liquid Kit, all from Química Analítica Aplicada SA, Spain), according to the manufacturer's instructions. Insulin was measured using a Rat/Mouse ELISA Kit (Merck Millipore, Germany) with a sensitivity threshold of 0.2 ng/mL.

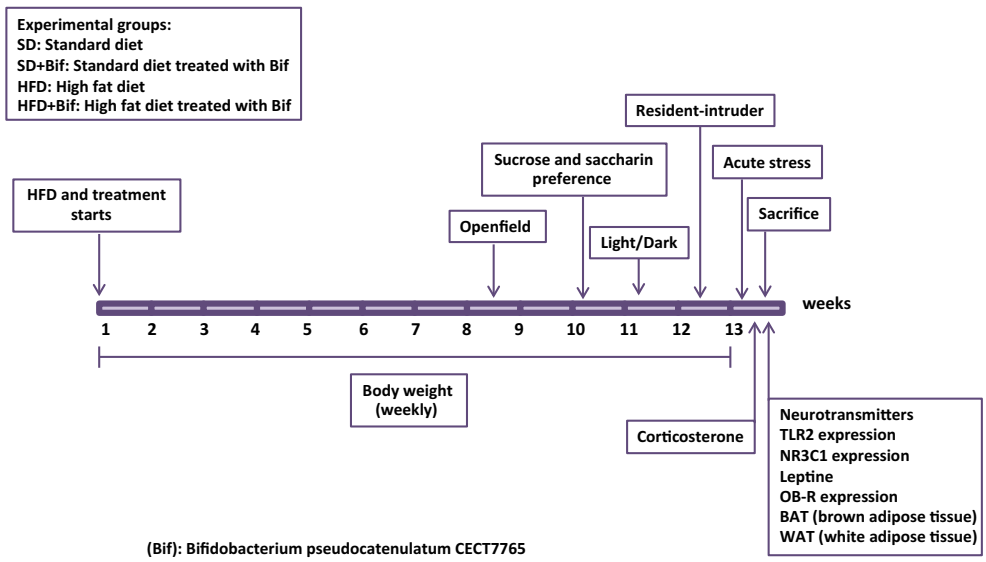

Fig. 1 Scheme of the study protocol and assessments conducted during the intervention. C57BL/6 mice were divided into four experimental groups (n = 10/each): control group fed a standard diet (SD), obese group fed a high-fat diet (HFD); group fed a HFD and a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage (HFD + Bif); and group fed a SD and a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage (SD + Bif) for 13 weeks. Body weight was monitored throughout the 13-week study period. From week 8 to week 13, animals sequentially underwent a battery of behavioral testing as indicated in the figure. After sacrifice, biological samples were used for assessments of body composition, biochemical, neural, and immune-related markers. Abbreviations: Bif: *B. pseudocatenulatum* CECT 7765; BAT: brown adipose tissue; WAT: white adipose tissue

RNA Isolation and RT-qPCR Analysis

The hippocampus and small intestine (ileum) tissues were immediately snap-frozen in liquid nitrogen. RNA was extracted from the tissues using the TRIsure Bioline Reagent (Bioline, London, UK) and a homogenization step using a UP400S ultrasonic processor (Hielscher, Teltow, Germany). RNA quality was assessed by measuring the absorbance (A) ratio A_{260}/A_{280} in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). RNA (2 μg) was subjected to reverse transcription (Applied Biosystems, Foster City, USA). An amount of 20 ng of the resulting complementary DNA (cDNA) was used as a template for real-time PCR amplifications. The messenger RNA (mRNA) levels for specific genes were determined in a LightCycler 480 instrument (Roche, Branchburg, USA). For each PCR reaction, cDNA template was added to LightCycler 480 SYBR Green I Master (Roche, Branchburg, USA) containing the primer pairs for the corresponding gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The sequence and information for the primers are shown in Supplemental Table 1. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to the content of housekeeping gene and expressed as percentage of control.

Determination of Corticosterone Levels

Corticosterone levels were determined in fecal samples taken before and 3 h after exposure to both acute stress tests and preserved at − 80 °C until analyzed. The analysis of corticosterone in stools has been reported to be a valid noninvasive method to assess physiological stress during the course of experiments without the need of collecting blood samples that will constitute an additional stress source [30, 31]. Corticosterone was removed from frozen fecal samples (0.1 g per sample) using the "Steroid Soil Extraction Protocol" (Assay Arbor, Michigan, USA). Briefly, 1 mL ethyl acetate was added to every 0.1 g of solid fecal material, centrifuged at 1000×g at 37 °C for 15 min, and the supernatant was transferred to a clean tube for evaporation at 37 °C. After extraction, corticosterone was quantified with "Enzyme Immunoassay Kit Corticosterone" (Assays Arbor, Michigan, USA). The concentration of corticosterone is expressed as picograms per gram of stool sample. The limit of detection was 16.9 pg/mL.

Behavioral Tests

Sucrose and Saccharin Preference Test

The test has been performed with sucrose and saccharin to assess hedonic behavior and monitor the effects of the chronic stress possibly associated with obesity [32]. The test consists of a 12-h period of water deprivation, after which animals are exposed to two bottles: one containing water and another containing 3% of sucrose solution or 0.3% saccharin solution. The bottles of sucrose/saccharin and water were switched around during the 2 h test period to ensure that there were no effects related to preference of place. The amount of sucrose or saccharin solution ingested during the subsequent 2 h indicates hedonic behavior, while lower sucrose or saccharin ingestion indicates anhedonia. The preference for sucrose or saccharin was calculated as the percentage of sucrose or saccharin solution ingested relative to the total amount of liquid consumed and corrected for body weight.

Light–Dark Box Test

This test is based on a conflict between the innate aversion to brightly illuminated areas and the spontaneous exploratory activity [33]. The light–dark box was made of Plexiglas (44 × 44 × 40 cm). One third of the box is the dark compartment with black walls, and two thirds is the light compartment with white walls. The box is illuminated by 400 lx. The light and dark compartments were communicated by an open door (10 × 7.5 cm). Mice were individually placed in the dark box and allowed to freely explore the whole box (light and dark) for 10 min and recorded by a video camera (Sony EXviewHAD CCD II, Cornellá (Barcelona), Spain. The latency to emerge from the dark side of the chamber (four paws in the lighted side) is a measure of anxiety [34] and was measured and analyzed comparatively (Panlab, Barcelona, Spain).

Open Field Test

To evaluate the locomotor activity, an open field test was performed. It consists of a square shaped, grey open field, measuring 44 × 44 cm^2. Mice were placed individually into the arena for 20 min and recorded by a video camera (Sony EXviewHAD CCD II). Data were measured and analyzed using the Videotracking Smart 3.0 (Panlab). The parameters assessed were the distance traveled (cm) and the time spent in the center of the open field.

FST

This paradigm was performed as described originally [35]. In the FST, mice swim under conditions in which escape is not possible. On the first day, mice were habituated to a dark

Plexiglas cylinder 35 cm tall, 30 cm diameter, filled to 21.5 ± 1.5 cm with water at 24 ± 0.5 °C. After 5 min, mice were removed from the water, dried with towels and placed in a warm enclosure. The next test session (5 min) was conducted 24 h later and was videotaped in a room dimly illuminated (an indirect 40 W white fluorescent bulb) for evaluation purposes. Behavioral measures were scored and included latency to immobility and total time of immobility.

Acute Immobilization Stress

Physical immobilization was used as a model of acute stress as previously described [36]. Mice were placed inside a hemicylindrical plastic tube and were physically immobilized for 1 h.

Acute Social Stress

We used the resident-intruder model to induce social stress [37] in our experimental mice. These mice were used as intruders that were exposed to an isolated adult antagonist resident male. In the resident–intruder paradigm, one of the animals is allowed to establish a territory (the resident) in its home cage. Subsequently, another animal is placed inside the residents' home cage and the two animals are allowed to interact with each other for a short period of time. During the interaction, the intruder, in particular, experiences a high degree of social stress (indicated by an increase in corticosterone) due to being introduced to an unknown animal [38]. Antagonist encounters took place in a separate room (outside the habitual animal facility) and were performed just once for 5 min. After the test, experimental mice were single housed for 3 h and stool samples were collected after this period in order to measure corticosterone levels to evaluate the stress level.

Neurotransmitter Analysis

Monoamines (dopamine, noradrenaline, adrenaline, and serotonin) were measured in dissected tissue from the hypothalamus and hippocampus and in small intestine (ileum) using high-pressure liquid chromatography (HPLC). The hypothalamic samples were frozen in liquid nitrogen, and the small intestine was frozen in PBS and kept at − 80 °C until analyzed. Samples were thawed and 200 μL of trifluoroacetic (TFA) acid were added per 100 mg of tissue. Samples were homogenized using a Tissue Micro pestle (Labbox, Vilassar de dalt, Barcelona, Spain). The samples were centrifuged 10,000×*g* for 15 min at 4 °C. The supernatant was filtered through 0.45 μm filters (Millipore) and collected.

Chromatographic separation was performed in an Agilent 1220 infinity series HPLC system (Agilent, Waldbronn, Germany), equipped with a degasser, a binary pump, and an auto-sampler. Tissue homogenates were analyzed using a Poroshell 120 EC-C18 column (4.6 × 50 mm, 2.7 μm i.d.). The mobile phase was composed of (A) 0.1% FA in deionized water (18 MΩcm) at pH = 2.2 and (B) 0.1% FA in deionized water/acetonitrile (20:80, *v*/*v*) using the following gradient program: 0–9 min, isocratic period at 100% (A); 9–14 min, linear gradient to 75% (B); 14.1–15 min, isocratic period at 100% (B); and 15–18 min, isocratic period at 100% (A). A 5-min pre-equilibration period was used between each run. The flow rate was 0.8 mL/min; the column temperature was 25 °C; the detector was set at a 254-nm wavelength; and the injection volume was 10 μL.

Neurotransmitters were identified by their retention times as determined by using commercially available standards (Sigma-Aldrich. St Louis, MO, USA). Results are expressed as nanograms of neurotransmitter per gram of fresh tissue weight.

TLR2 Immunostaining Analysis by FACS

Membrane surface expression of TLR2 (FITC anti-mouse TLR2, eBioscience, San Diego, USA) in hippocampus and small intestine tissues was assessed by FACS. The mechanically digested tissues were passed through 40 μm mesh filters and washed in FACS buffer (PBS with 2 mM EDTA and 0.5% BSA), then centrifuged at 2000 rpm for 5 min, and the pelleted cells were stained and analyzed. Fluorescence intensity was measured in a BD LSRFortessa flow cytometer, and the results were analyzed using BD FACS DIVA Software v.7.0. (Becton Dickinson, Franklin Lakes, USA). Mean fluorescence intensity (MFI) of 10^4 counted events was measured in each sample. Expression levels were presented as MFI corrected for nonspecific binding of isotype control antibodies.

Statistical Analyses

All data was analyzed using GraphPad Prism (La Jolla, CA, USA). Before the statistical analysis, all data were normalized. Statistical significance was evaluated using one-way or two-way ANOVA (when necessary) with Tukey's post hoc test for physiological and behavioral measures. Differences were considered statistically significant at $p \leq 0.050$. Data are presented as mean ± S.E.M.

Results

Body Weight Gain, Increased Fat Mass, and Metabolic Alterations Are Ameliorated by *B. pseudocatenulatum* CECT 7765 in Obese Mice

The effects of the HFD on body weight gain, fat mass, and metabolic alterations associated with obesity were confirmed as described previously [25]. As shown in Fig. 2a, b, body

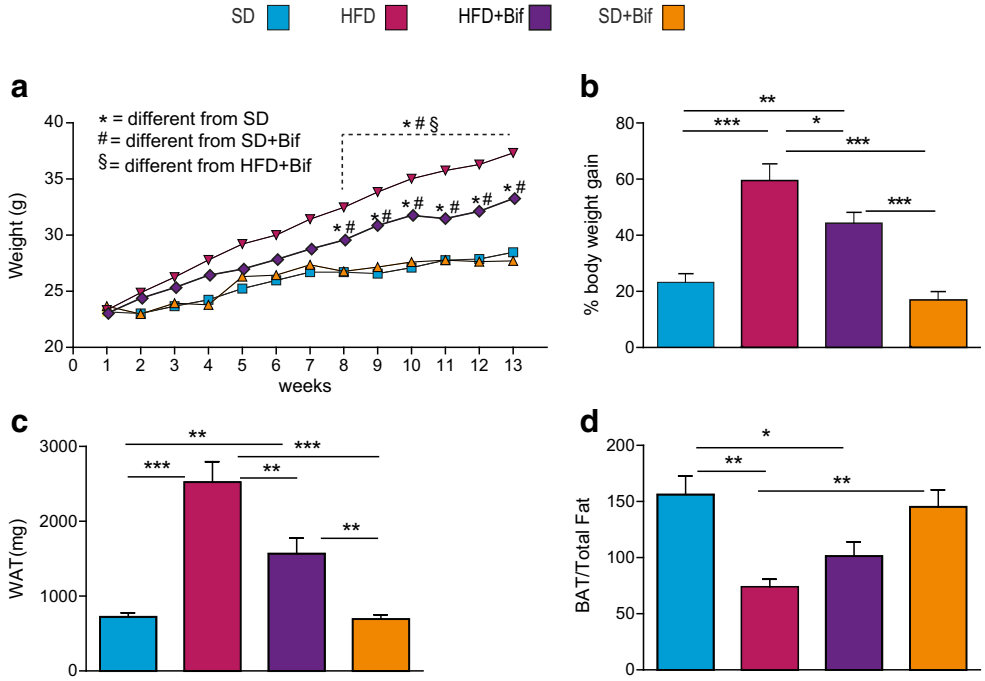

Fig. 2 Effects of *B. pseudocatenulatum* CECT 7765 on body weight and fat mass in obese and control mice. Weekly body weight (g) (**a**), body weight gain (%) (**b**), white fat (WAT) (mg) (**c**), and brown fat (BAT) relative to the total fat (**d**) in mice fed with SD or HFD and treated or not with *B. pseudocatenulatum* CECT 7765. Abbreviations of experimental groups: SD, standard diet group ($n = 10$); HFD, high-fat diet group ($n = 10$); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Mice were weighed every week during the 13-week period. Values significantly different from the SD group are indicated by an asterisk (*), different from SD + Bif are indicated by "#" and different from HFD + Bif by "$". $^{*}p < 0.050$, $^{**}p < 0.010$, $^{***}p < 0.001$, $^{\#}p < 0.050$, $^{\$}p < 0.05$

weight increased by 59.51% (SEM 5.49) in HFD-fed mice, which corresponds to an absolute increase of 13.98 g (SEM 1.40), whereas body weight increased only by 23.20% (SEM 3.01) in SD-fed mice, which corresponds to a 5.32-g (SEM 0.64) increase, after 13 weeks of dietary intervention. The administration of *B. pseudocatenulatum* CECT 7765 to HFD-fed mice significantly reduced ($p = 0.05$) the relative body weight gain by about 26%, showing a relative weight gain of 44.29% (SEM 3.54) which corresponds to 10.20 g (SEM 0.72) at the end of the intervention, without inducing alterations in SD-fed mice. Effects on fat mass are shown in Fig. 2c, d. WAT (Fig. 2c) was increased in obese mice to values of 2522 mg (SEM 273) ($p < 0.001$) but significantly reduced to 1567 mg (SEM 210) ($p < 0.010$) by the administration of *B. pseudocatenulatum* CECT 7765 in the HFD-fed group. BAT (Fig. 2d) relative to total fat mass was also significantly reduced to 73.75 mg (SEM 7.05) ($p < 0.010$) in the HFD-fed group and tended ($p = 0.080$) to be increased by the administration of *B. pseudocatenulatum* CECT 7765.

The mean of total energy intake per mouse on the last week (13th week) was higher ($p < 0.05$) in HFD mice (55.70 kcal/mouse, SEM 2.85) than SD mice (40.60 kcal/mouse, SEM 0.96). Administration of *B. pseudocatenulatum* CECT 7765 decreased the energy intake in HFD mice close to control values ($p < 0.050$) as previously reported [27].

Obesity-Induced Alterations in Serum Metabolic Parameters and Leptin and Glucocorticoid Receptors Are Ameliorated by *B. pseudocatenulatum* CECT 7765 in Obese Mice

Obese mice showed significantly higher plasma concentrations of leptin ($p < 0.001$) (Fig. 3a), cholesterol ($p < 0.001$), triglyceride ($p = 0.022$), and glucose ($p < 0.001$) than SD-fed mice (data not shown). The administration of *B. pseudocatenulatum* CECT 7765 led to a significant reduction ($p < 0.050$) in plasma leptin of HFD-fed mice and also in all other parameters as described previously [25]. We further investigated the influence of HFD with or without the simultaneous administration of *B. pseudocatenulatum* CECT 7765 on leptin receptor (OB-R) mRNA expression in small intestine and hippocampus (Fig. 3b, c). The OB-R mRNA levels were significantly decreased in small intestine ($p < 0.050$) and hippocampus ($p < 0.010$) in HFD-fed mice compared to SD-fed mice, which could explain leptin resistance being characteristic of obesity. The administration of *B. pseudocatenulatum* tended to upregulate the OB-R mRNA expression in intestine and hippocampus ($p = 0.150$ and $p < 0.050$, respectively) in obese mice.

NR3C1 mRNA levels were slightly decreased in the hippocampus ($p = 0.070$) of obese mice compared to controls, which could affect the mediating role of this receptor in the

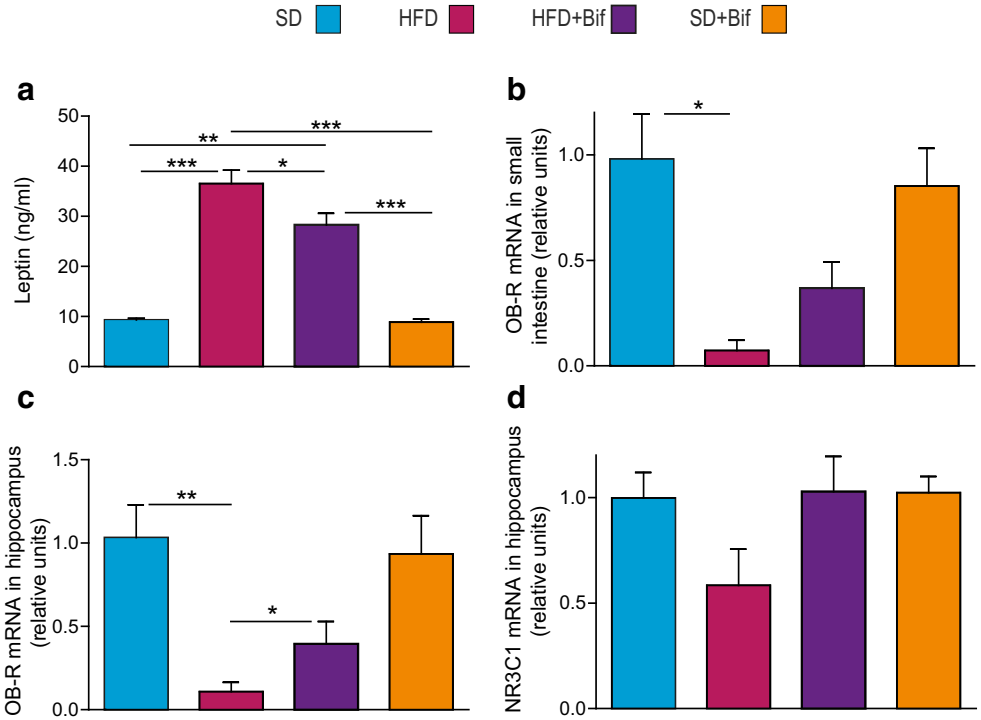

Fig. 3 Effects of *B. pseudocatenulatum* CECT 7765 on serum leptin levels in plasma and on leptin receptors and glucocorticoid receptors in small intestine and/or hippocampus. Leptin in serum (ng/mL) (**a**), relative gene expression of leptin receptor (OB-R) in small intestine (**b**) and hippocampus (**c**), and relative gene expression of glucocorticoid receptor (NR3C1) in hippocampus (**d**). Abbreviations of experimental groups: SD, standard diet group (n = 10); HFD, high-fat diet group (n = 10); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Statistically significant differences at $^*p \leq 0.050$, $^{**}p < 0.010$, $^{***}p < 0.001$

negative feedback of corticosterone on the hypothalamic-pituitary-adrenal (HPA) axis following stress [39]. The administration of *B. pseudocatenulatum* CECT 7765 also tended to increase (p = 0.090) the NR3C1 mRNA expression, but the effect did not reach statistical significance (Fig. 3d).

Obesity-Increased Responsiveness to Stress Is Ameliorated by *B. pseudocatenulatum* CECT 7765

Corticosterone concentration was assessed before and after acute stress exposure as this is the primary glucocorticoid produced in the adrenal cortex in response to hypothalamic-pituitary stimulation. Basal corticosterone levels in stools were significantly higher in HFD-fed mice than in any other group (SD vs HFD, p = 0.007; HFD vs HFD + Bif, p = 0.030; HFD vs SD + Bif, p = 0.004; Fig. 4a). Stressful situations increased corticosterone in all the groups. However, when obese mice were subjected to acute stressful situations, either by physical immobilization (Fig. 4b) or by social stress (Fig. 4c), they showed higher levels of stool corticosterone than control mice, suggesting that obese mice are more susceptible to these acute stressful situations. Obese mice treated with the bifidobacteria showed a reduced increase in concentrations of stool corticosterone after both immobilization (Fig. 4b) and acute social stress exposure (Fig. 4c), indicating that the bacterial strain tested is able to reverse the anxiogenic obesity profile. Social stress caused the highest increases in corticosterone levels in all the groups (Fig. 4d), indicating that this was the most stressful situation.

Obesity-Induced Anhedonia Was Ameliorated by *B. pseudocatenulatum* CECT 7765

Obese animals consumed significantly less sucrose solution in the sucrose preference test in relation to their body weight compared with SD-fed mice (p = 0.047; Fig. 5a). This has been described as anhedonic depressive-like behavior [40]. However, the percentage of sucrose solution ingested by HFD-fed mice receiving the bifidobacterial strain was substantially higher (p = 0.050) (Fig. 5a) compared with the HFD-fed mice group receiving placebo and similar to the percentage ingested by the control mouse group fed a SD and placebo.

To check whether differences in the sucrose preference test could be due to differences in the caloric demand of HFD- and SD-fed mice, we performed the same test with

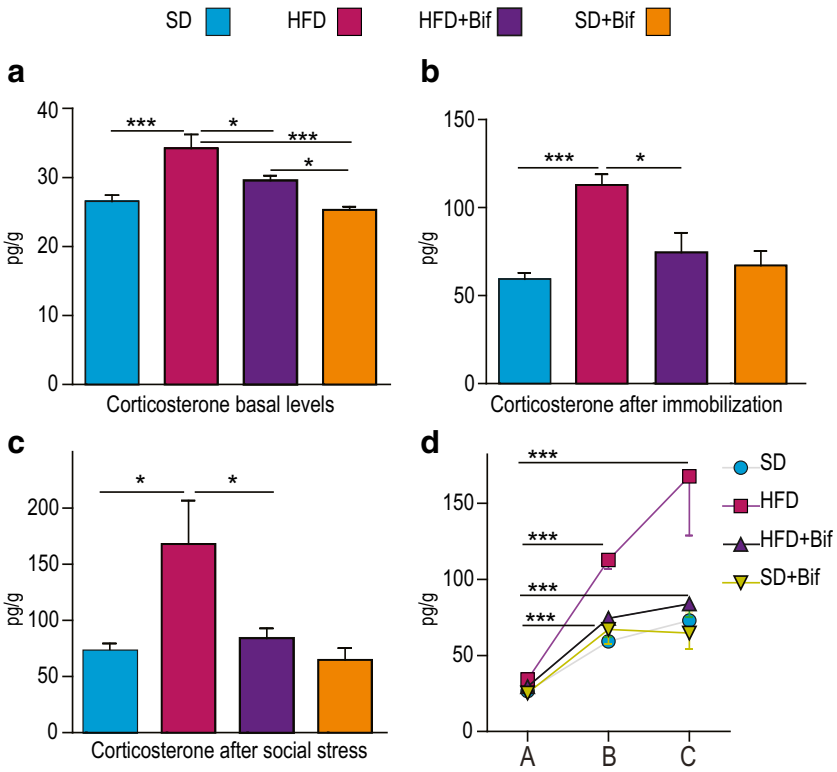

Fig. 4 Effects of *B. pseudocatenulatum* CECT 7765 on stress and fecal corticosterone levels. Fecal corticosterone level (pg/g) at baseline (**a**), after immobilization (**b**), and after intruder-resident social stress (**c**); changes from baseline (**a**) to **b** and to **c**. Abbreviations of experimental groups: SD, standard diet group ($n = 10$); HFD, high-fat diet group ($n = 10$); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif: high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage. Statistically significant differences at $^*p < 0.050$, $^{***}p < 0.001$

a saccharin solution showing the same trend, regarding the effects of obesity and the *B. pseudocatenulatum* administration to obese mice (data not shown). Importantly, *B. pseudocatenulatum* CECT 7765 did not change the levels of sucrose or saccharin intake in SD mice, showing that it is not inducing a reward-seeking effect per se.

Obese Mice Showed Anxious Behavior in the Light-Dark Box

The HFD diet significantly increased the latency (Fig. 5b) in moving from the dark box to the light box (25.3 ± 3.97 s, $p = 0.049$) compared with the SD fed-mice (14.05 ± 3.41 s) in the light-dark test. This result indicates that HFD fed-mice were more anxious than SD fed-mice [33] since elevated levels of anxiety decrease or delay the spontaneous exploratory activity that is innate in mice. The intervention with the *B. pseudocatenulatum* did not significantly decrease the latency in moving from the dark box to the light box in obese mice (20.42 ± 3.08 s, $p = 0.280$; Fig. 5b).

Obesity Does Not Reduce the Locomotor Activity in the Open Field Test

The possible effect of obesity on locomotor activity was measured with an open field test. According to the results, the HFD-fed mice did not show significant differences in the total distance traveled compared to control mice (8695 ± 339 and 9885 ± 379 cm, respectively; Fig. 5c). The administration of *B. pseudocatenulatum* CECT 7765 did not affect locomotor activity, neither in obese nor in non-obese animals. Curiously, when we evaluated the percentage of time spent in the center of the open field (Fig. 5e), which has been classically associated with an anxious behavior called thigmotaxis (preference for "protective" walls versus "nonprotective" open areas) [41, 42], we found that the obese group remained for the same amount of time in the center (SD vs HFD, $p = 0.030$; and SD + Bif vs HFD, $p = 0.008$). These results suggest that obese mice are not affected in locomotor variables, but they do express anxiety in test performance. The HFD + Bif mouse group did not show a significant change in this sign of anxiety in the open field.

Obese Mice Showed Hyperactivity and Anxious Behavior in the FST

HFD-fed mice displayed significantly increased latency to immobility in the FST compared to SD-fed mice ($p = 0.002$; Fig. 5d), which has been considered as indicative of anxious behavior and hyperactivity [43, 44]. Delayed immobility has been interpreted as anxious behavior and susceptibility to antidepressant drugs [45]. This effect was slightly diminished by the bifidobacteria administration, although statistical significance was not reached. No difference was observed between the study groups in immobility duration (141.66 ± 8.50, 136.36 ± 13.10 s,

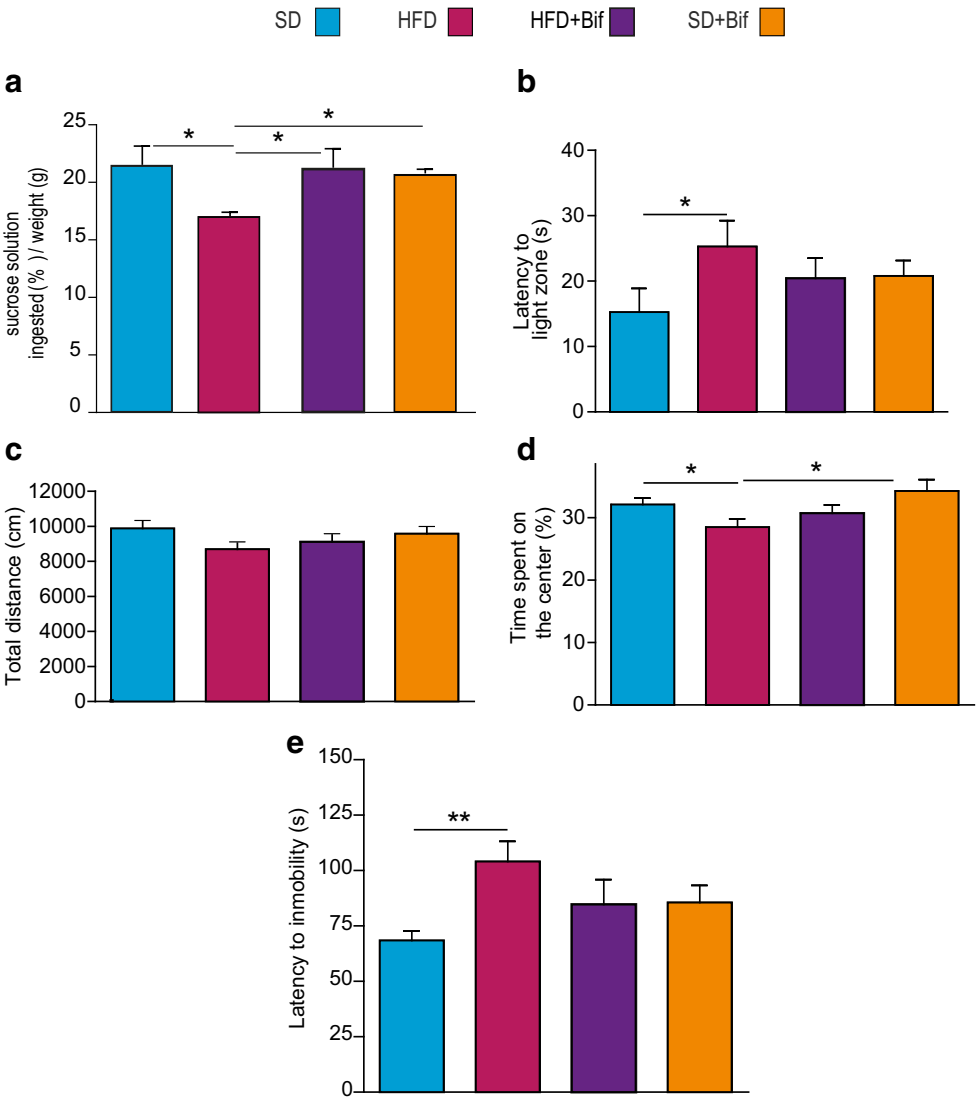

Fig. 5 Effects of *B. pseudocatenulatum* CECT 7765 on depressive and anxiety-like behaviors in obese and control mice. Sucrose preference rates (%) relative to the weight (**a**); latency in moving to the light zone in the light-dark box (s) (**b**); total distance traveled in the open field (cm) (**c**); latency to immobility in the forced swim test (s) (**d**); and time spent in the center during the open field (%) (**e**). Abbreviations of experimental groups: SD, standard diet group ($n = 10$); HFD, high-fat diet group ($n = 10$); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Statistically significant differences at $^*p < 0.050$, $^{**}p < 0.010$

143.45 ± 8.00, 141.30 ± 5.70, for the SD, HFD, HFD + Bif, and SD + Bif groups, respectively, $p = 0.900$).

Catecholamine Levels in the Hypothalamus, Hippocampus, and Small Intestine Are Modified by Both Obesity and *B. pseudocatenulatum* CECT 7765

Previous behavioral tests suggested that HFD-fed mice display anxious depressive-like behavior and that the intervention with *B. pseudocatenulatum* CECT 7765 slightly ameliorates some features of this phenotype. In order to verify whether this behavior correlated with neurochemical alterations in the gut–brain axis, we analyzed neurotransmitters in the hypothalamus, hippocampus, and small intestine (Fig. 6). Obese mice showed an increased catecholamine conversion evidenced by reduced concentrations of dopamine and noradrenaline in the small intestine (Fig. 6g, h) and of adrenaline in the hypothalamus (Fig. 6c). Remarkably, *B. pseudocatenulatum* CECT 7765 partially restored the reduced adrenaline concentrations in the hypothalamus of obese mice (Fig. 6c), which could partly explain its ability to restore metabolic and behavioral alterations related to a depression-like phenotype. *B. pseudocatenulatum* CECT 7765 also exerted marked effects on neurotransmitter levels in the intestine and central nervous systems regardless of obesity. The effects of *B. pseudocatenulatum* CECT 7771 on catecholamine conversions were especially evident in the hippocampus where *B. pseudocatenulatum* CECT 7771 significantly reduced the concentration of dopamine (Fig. 6d) and noradrenaline (Fig. 6e) and increased the end product adrenaline (Fig. 6f) in control mice. *B. pseudocatenulatum* CECT 7765 also reduced the dopamine levels in small intestine (Fig. 6g) and hypothalamus (Fig. 6a) and adrenaline levels in the hypothalamus of control mice (Fig. 6c).

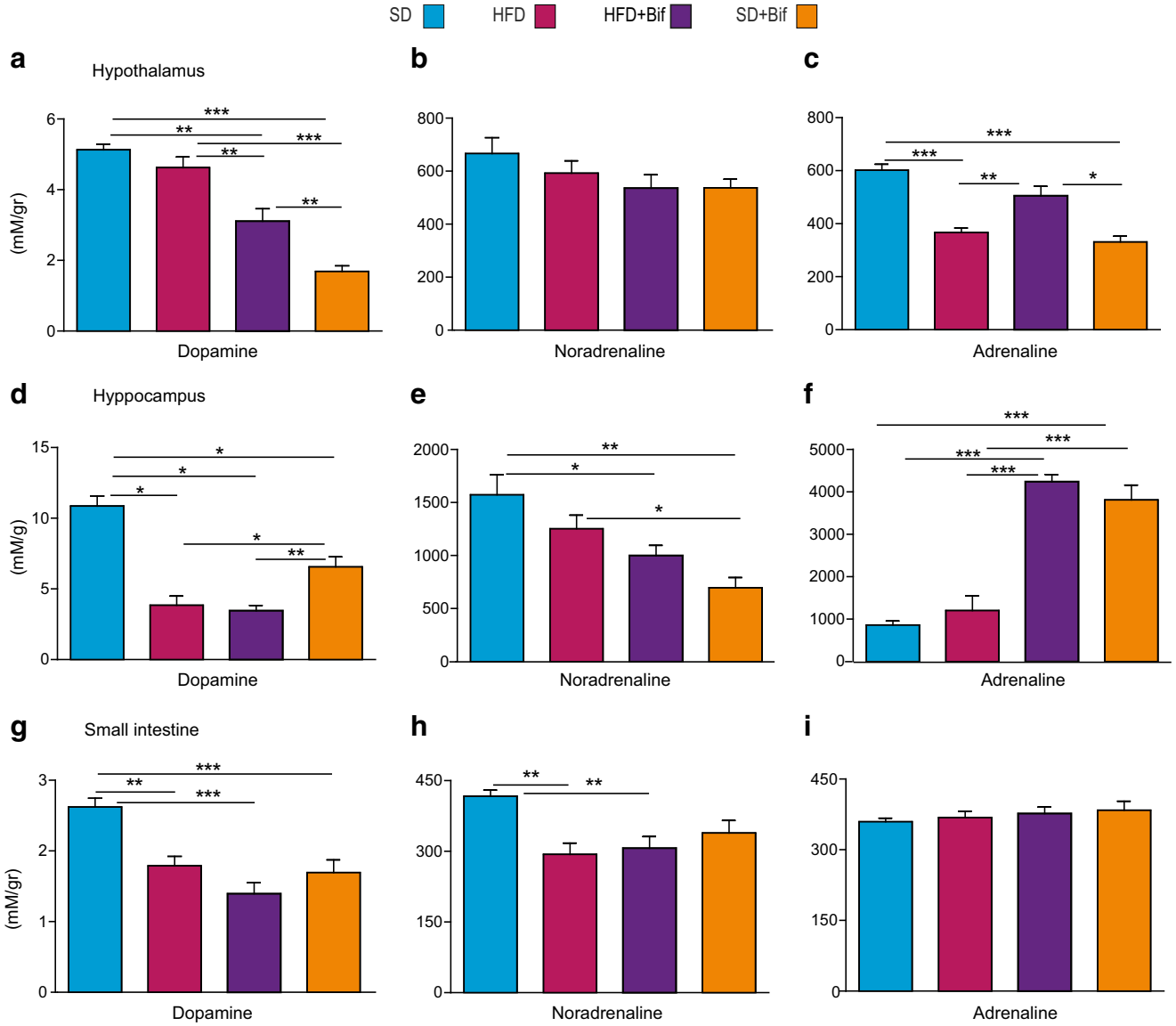

Fig. 6 Effects of *B. pseudocatenulatum* CECT 7765 on the catecholamines dopamine, noradrenaline, and adrenaline in hypothalamus, hippocampus, and small intestine. Catecholamines (mM/g) were measured by HPLC in homogenated tissues of the hypothalamus (**a**, **b**, **c**), hippocampus (**d**, **e**, **f**), and small intestine (**g**, **h**, **i**). Abbreviations of experimental groups: SD, standard diet group (n = 10); HFD, high-fat diet group (n = 10); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Statistically significant differences at $^*p < 0.050$, $^{**}p < 0.010$,$^{***}p < 0.001$

Obesity-Induced Alterations in the Serotonergic System Are Attenuated by *B. pseudocatenulatum* CECT 7765 in the Hippocampus and Small Intestine

As the serotonergic system plays an important role in emotional and cognitive processing and depression [14], we also evaluated effects of the intervention on serotonin (5-HT) levels in different brain regions. Our study revealed that HFD significantly decreased ($p < 0.05$) 5-HT concentrations in the mouse hippocampus (Fig. 7a) compared to the SD. Administration of *B. pseudocatenulatum* CECT 7765 significantly increased ($p < 0.05$) 5-HT levels in the hippocampus of HFD-fed mice, suggesting a possible positive effect on the depressive-like phenotype via this mechanism. In contrast, obese mice showed increased 5-HT concentrations in the hypothalamus ($p < 0.05$) and the bifidobacteria did not restore this alteration (Fig. 7b).

Considering that 5-HT is mainly synthetized in the intestine (90% of the total) and that microbiota plays an important role in regulating 5-HT metabolism [46], we also analyzed the effects of the intervention on the 5-HT levels locally in the small intestine (Fig. 7c). According to our study, 5-HT was

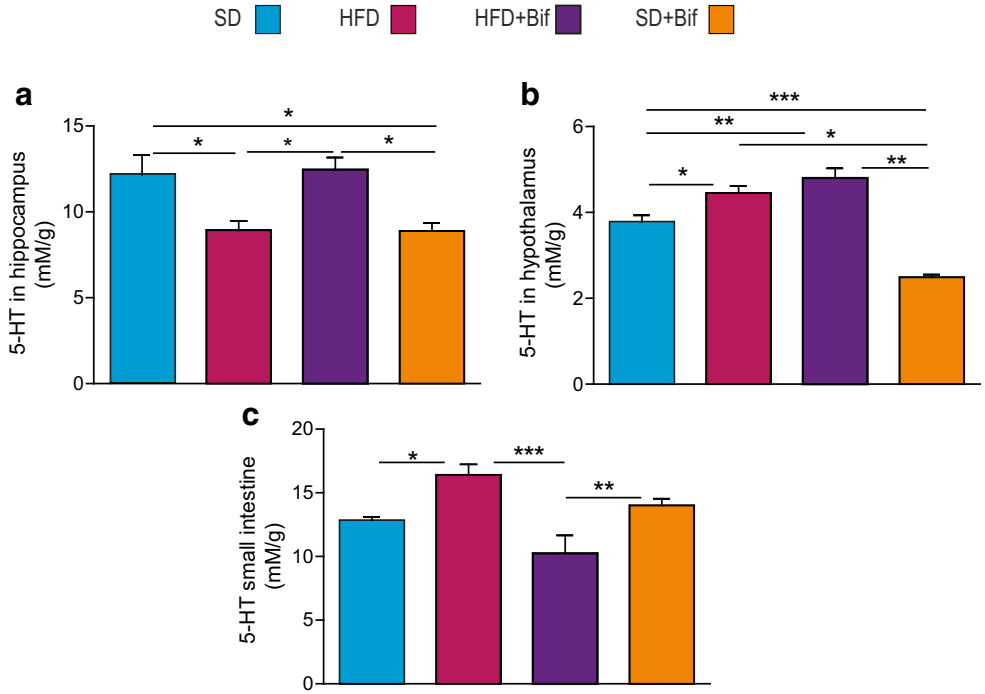

Fig. 7 Effects of *B. pseudocatenulatum* CECT 7765 on serotonin (5-HT) in hippocampus, hypothalamus, and small intestine. 5-HT (mM/g) in hippocampus (**a**), hypothalamus (**b**), and small intestine (**c**). 5-HT was measured by HPLC in homogenated tissues. Abbreviations of experimental groups: SD, standard diet group (n = 10); HFD, high-fat diet group (n = 10); SD + Bif, standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Statistically significant differences at $*p < 0.050$, $**p < 0.010$, $***p < 0.001$

significantly increased ($p < 0.050$) in HFD-fed mice compared to SD-fed mice. However, *B. pseudocatenulatum* CECT 7765 administration decreased the 5-HT levels to control values in the small intestine of obese mice ($p < 0.001$).

Obesity-Induced TLR2 Expression Was Attenuated by *B. pseudocatenulatum* CECT 7765

In order to understand the possible connection between the bifidobacterial effects and innate immunity, we analyzed the expression of TLR2 which is recognized by Gram-positive bacteria such as bifidobacteria. We found a significant upregulation of TLR2 gene expression in small intestine ($p < 0.010$) and hippocampus ($p < 0.050$) in obese mice (Fig. 8a, b), and this was significantly reduced ($p < 0.050$) by *B. pseudocatenulatum* CECT 7765 in the hippocampus although in small intestine the reduction did not reach statistical significance ($p = 0.078$). HFD-fed mice also showed increased TLR2 protein levels in small intestine ($p = 0.010$) and a similar but not significant trend was observed in the hippocampus ($p > 0.050$). *B. pseudocatenulatum* CECT 7765 also lowered the obesity-induced increase of TLR2 protein levels in small intestine ($p < 0.010$) and tended to do so in the hippocampus ($p = 0.070$) (Fig. 8c, d).

Discussion

The present study supports the notion that the gut microbiota may be one of the factors mediating associations between obesity and psychiatric disorders as reported in epidemiological studies [47], since intervention with *B. pseudocatenulatum* CECT 7765 is sufficient to restore part of the neurochemical and behavioral alterations found in diet-induced obese mice. The present study focused on this bacterial strain because it was previously demonstrated to be able to reduce the obesity-associated metabolic and immune alterations in a diet-induced obesity model [25] and the adverse consequences of chronic psychological stress in the maternal separation mouse model [48].

Although our understanding of the psychological consequences of obesity is still in its infancy, human studies suggest that chronic HFD feeding promotes negative emotional states and enhances sensitivity to stress, including that triggered by dieting, that perpetuate a vicious cycle of overeating, weight gain, and depressive mood [49]. Importantly, obese individuals classified as "metabolically healthy," defined as those not having associated cardiometabolic risk factors (e.g., high blood pressure, reduced high-density lipoprotein cholesterol and increased, glycated hemoglobin, and C-reactive protein), do not appear to have a heightened risk of depression [50], indicating that it is not

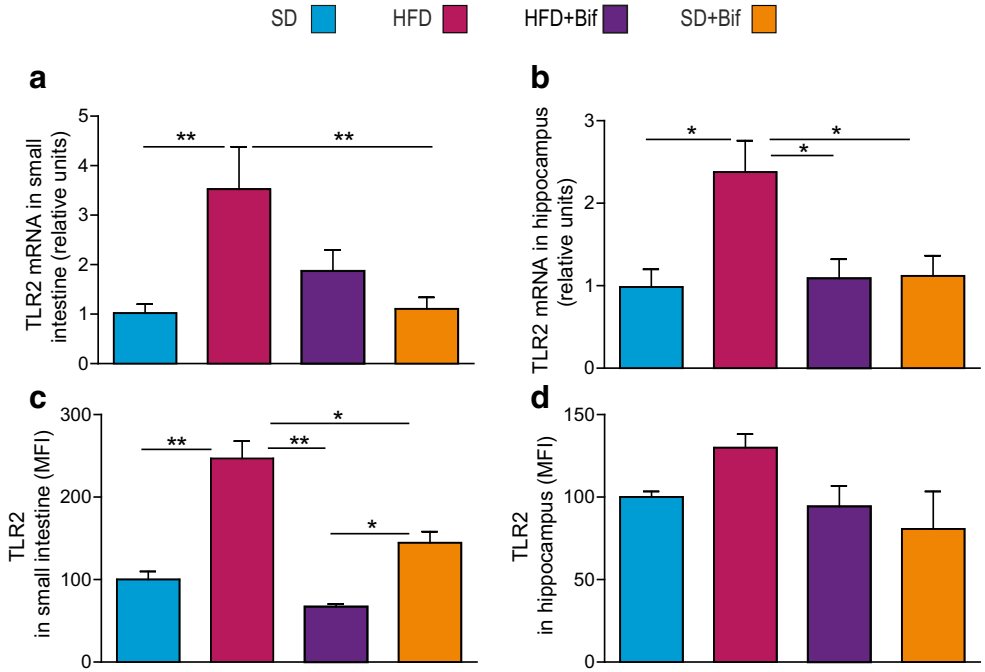

Fig. 8 Effects of *B. pseudocatenulatum* CECT 7765 on TLR2 expression in small intestine and hippocampus in obese and control mice. Detection of mRNA TLR2 in small intestine (**a**) and hippocampus (**b**) related to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by RT-qPCR and expressed in relative units. Detection of TLR2 in small intestine (**c**) and hippocampus (**d**) by flow cytometry (FACS) and expressed as mean fluorescence intensity (MFI). Abbreviations of experimental groups: SD, standard diet group (n = 10); HFD, high-fat diet group (n = 10); SD + Bif: standard diet group receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage; HFD + Bif, high-fat diet receiving a daily dose of 1×10^9 CFU *B. pseudocatenulatum* CECT 7765 by gavage for 13 weeks. Statistically significant differences at $^*p < 0.05$; $^{**}p < 0.01$

only the effect of weight gain but also the deep configurational changes caused by obesity in metabolism, immunity, and, presumably, in the nervous system what constitutes a risk factor for depression and anxiety. In this context, Slyepchenco et al. [51] specifically suggested that intestinal dysbiosis and a leaky gut could be important links between major depressive disorder (MDD) and its medical comorbidities including, among others, obesity and type 2 diabetes mellitus.

In our study, HFD-induced obesity was associated with several behavioral alterations that are indicative of a depression–anxiety-like phenotype, suggesting a causal link. These include reduced sucrose intake in the sucrose preference test [52], the increased latency to immobility in the FST [45], and increased latency in moving away from the dark zone in the light/dark test [53]. Among these alterations, the administration of the *Bifidobacterium* strain was able to reduce anhedonia in the sucrose preference test, which seems to be the most sensitive indicator of depressive-like behavior compared to other tests like the FST [54].

To progress in the understanding of mediators and pathways involved in obesity-induced emotional behavioral changes and especially in those regulated by the *B. pseudocatenulatum* CECT 7765, we investigated, among other factors, the role of leptin and its receptor. Leptin is an adipokine, mainly produced by the adipose tissue, whose concentrations in serum are proportional to the size of fat mass [55]. Apart from its actions on multiple physiological processes such as the regulation of appetite, fat oxidation, and energy expenditure, leptin seems to be linked to vulnerability to human depression [56]. Although a recent meta-analysis showed that leptin and adiponectin levels were not good biomarkers to generally predict depression [57], metabolic depression has been defined as a subtype of MDD where leptin and chronic inflammation may play a role in aggravating depression and reducing recovery rates [58, 59]. This phenotype of metabolic depression could theoretically be represented by our study model of obesity, in which a significant increase of leptin and the inflammatory tone was accompanied by anhedonic-like behavior. In fact, lack of leptin [16] or its receptor in rodents is related to increased behavioral despair in the FST [60, 61], which is indicative of depressive behavior. In normal weight rodents, leptin is shown to have antidepressant and anxiolytic effects [61, 62]. In contrast, both obesity and depression have been associated with hyperleptinemia in peripheral circulation in rodents [62]. This can be explained by the development of leptin resistance in peripheral tissues and the blood–brain barrier (BBB), which could contribute to the development of depression/anxiety-like phenotypes in our obese mice, due to malfunctioning of the overproduced leptin. In fact, the obesity-associated leptin changes were opposite to those observed on the leptin receptor expression, demonstrating that obesity causes hyperleptinemia due to alterations in

leptin signaling via its receptor. In particular, we detected a significant reduction of OB-R expression in the hippocampus of obese mice along with anhedonic behavior in the sucrose preference test [52] and anxious behavior in the light/dark test [53] of obese mice as reported by other authors. In this context, Buyse et al. [63] demonstrated that deletion of the leptin receptor (OB-R) in the hippocampus of adult mice could induce depressive behavior. Yang et al. [15] also reported that decreased OB-R expression in the hippocampus correlated to a depressive/anxiety-like behavior in an animal model of obesity and chronic stress. This decreased OB-R expression was proposed to be due to necrosis of regions CA (Cornu Ammonis) 1 and CA3 of the hippocampus. However, *B. pseudocatenulatum* CECT 7765 normalized alterations in leptin levels and the same trend was observed for the OB-R receptor expression, suggesting that this is one of the key mediating roles of this strain in obesity and the associated behavioral phenotype.

The association between obesity and depression/anxiety-like phenotypes has also been related to the obesity-associated systemic inflammation that could lead to neuroinflammation in the hypothalamus [64] and other brain areas like the hippocampus [65, 66]. Inflammation and neuroinflammation are also known to be linked since peripheral immune and inflammatory cells can cross the BBB, proliferate and enhance neuroinflammation by activating glial cells and neurons [67]. In a previous study, we reported that HFD-induced obesity in mice increased intestinal and serum inflammatory cytokines (IL-17a, TNF-α) that were reduced by *B. pseudocatenulatum* CECT 7765 administration parallel to reductions in plasma endotoxin (LPS) levels [25]. The present study intended to investigate the mechanism by which this *Bifidobacterium* strain could mediate antiinflammatory effects in the gut and beyond. We focused on TLR2 signaling because this innate immunity receptor has been related to obesity in a microbiota-dependent fashion and to neuroinflammation [65, 66]. Our results showed increased TLR2 expression in obese mouse hippocampus, where it can mediate signaling cascades through myeloid differentiation factor-88 (MyD-88) and NF-κB, triggering cerebral inflammation [68, 69] and neuronal loss [67]. In turn, neuronal loss could be causally related to reduced OB-R protein expression and downregulation of leptin/LepRb signaling, leading to a depressive-like behavior as reported in a model of obesity and chronic mild stress [15]. Similarly, this could explain the reduction of 5-HT levels in the hippocampus of our obese mice, which could also contribute to a depressive/anxious-like behavior. In our obese mice, TLR2 was also over produced in the small intestine, where it could be activated by HFD-induced intestinal dysbiosis leading to overgrowth of potential pathogens, including LPS-producing Proteobacteria and Firmicutes as reported in our previous study [25], and by the dietary saturated fatty acids [70]. Therefore, we speculate that *B. pseudocatenulatum* CECT 7765 could restore leptin signaling and 5-HT levels in the hippocampus due to its ability to attenuate intestinal and neural inflammation via a TLR-2-dependent signaling mechanism.

The finding that obesity leads to overexpression of TLR2 in the small intestine and to increased 5-HT local levels is in agreement with a recent study reporting that TLR2 activation inhibits the intestinal 5-HT transporter (SERT) producing, as a consequence, an increase of intestinal 5-HT levels [10]. Therefore, obesity-associated intestinal inflammation demonstrated in our previous study [25] seems to be associated with alterations in 5-HT levels in the intestine as reported for chronic inflammatory bowel diseases like IBD [12, 13]. *B. pseudocatenulatum* CECT 7765 also seems to play an important role in reducing TLR2 activation in the intestine with further effects on 5-HT levels.

Persistent activation of the HPA axis is associated with both mood alterations and metabolic imbalances that are perceived as stressors in rodents [71]. In our study, physical immobilization and social stress resulted in a dramatic increase in corticosterone levels in HFD-fed mice compared to control mice, indicating that obesity increases the endocrine response to stress; however, treatment with the *Bifidobacterium* strain reverses these alterations. These findings suggest a special proclivity of obese mice to develop maladaptive responses under stressful situations and indicate possible alterations in the HPA axis. Aiming to understand the possible mediating factors, we investigated the expression of hippocampal glucocorticoids receptors (NR3C1) in obese mice and in obese mice fed *B. pseudocatenulatum* CECT 7765. The exaggerated stress response of obese mice reflected by increased corticosterone levels in response to acute stress is in agreement with a slight but not significant reduction of the hippocampal expression of the glucocorticoid receptor, which could reduce its efficiency in the negative feed-back control of the HPA axis. In other study models, chronic stress conditions have been shown to downregulate this receptor [72]. However, the role of *B. pseudocatenulatum* CECT 7765 in attenuating the overproduction of corticosterone in response to stress could not be associated significantly with its ability to restore the glucocorticoid receptor expression in the hippocampus.

Finally, we also investigated changes in the brain and intestinal catecholamines for their possible role in depression and anxiety disorders associated with obesity. In this context, recent studies demonstrated that there is an adrenomedullary dysfunction in obesity that leads to decreased adrenaline secretion [73]. In our study, we observed reduced adrenaline levels in the hypothalamus of obese mice while the administration of the bifidobacteria increased its levels significantly, suggesting that this bacterial strain contributes to restoring the observed alterations in this brain section. Consistent with our findings, a recent study in rodents also reported a role of increased medial hypothalamic adrenaline levels in body weight loss and glucose tolerance in response to intermittent fasting [74].

All in all, the present study points towards a role of *B. pseudocatenulatum* CECT 7765 in the regulation of secondary effects of obesity on the HPA axis and the associated depressive-like behavior. The administration of bifidobacterial strain leads to changes in endocrine factors (leptin and glucocorticoids) and neurotransmitters involved in the stress response and the hypothalamic energy control (adrenaline). In addition, the administration of this bacterial strain influences the interaction between innate immunity (TLR2) and the serotonergic system in the gut and the hippocampus, presumably reducing neuroinflammation and restoring the 5-HT function, thereby attenuating the obesity associated depressive-like behavior. Further studies would be warranted to confirm the precise pathways by which interventions with specific intestinal bacteria may help to beneficially modulate obesity-induced alterations in both metabolism and mood. Further investigations will also be necessary to confirm whether or not interventions with specific bacteria, like *B. pseudocatenulatum* CECT 7765, could ameliorate depressive features associated with obesity and metabolic dysfunction in humans phenotyped with metabolic depression.

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Compliance with Ethical Standards Experiments were carried out in strict compliance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the University of Valencia (Central Service of Support to Research [SCSIE], University of Valencia, Spain), and the protocol was approved by its Ethics Committee (Approval number 2015/VSC/PEA/00041).

Conflict of Interest The authors declare that they have no conflict of interest.

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