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(-)-Epicatechin mitigates anxiety-related behavior in a mouse model of high fat diet-induced obesity

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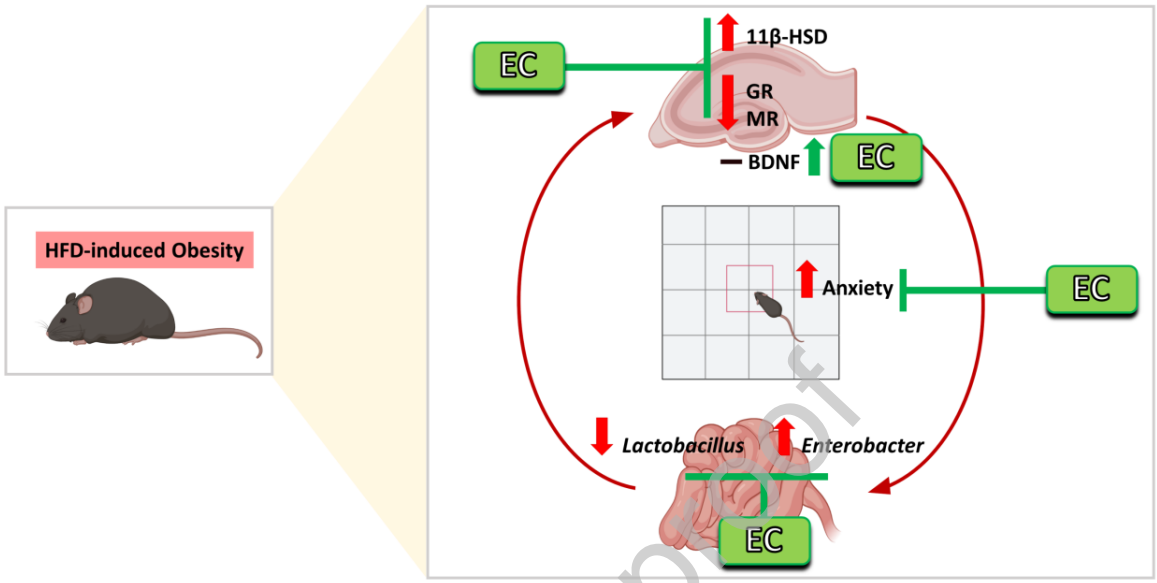
Highlights

- (-)-Epicatechin (EC) mitigates anxiety-related behavior in HFD-induced obese mice
- EC acts in part by modulating BDNF- and glucocorticoids (GC)-regulated signaling
- EC restored Lactobacillus and Enterobacter abundance altered by the HFD
- EC may improve anxiety by modulating BDNF/GC signaling and mitigating dysbiosis

Graphical abstract



Abstract:

Mounting evidence demonstrates that consumption of high fat diet (HFD) and subsequent development of obesity leads to alterations in cognition and mood. While obesity can affect brain function, consumption of select dietary bioactives may help prevent obesity-related cognitive decline. This study investigated the capacity of the dietary flavonoid (-)-epicatechin (EC) to mitigate HFD-induced obesity-associated alterations in memory and mood. Healthy 8-week old male C57BL/6J mice were maintained on either a control diet (10 kCal% from fat) or a HFD (45 kCal% from fat) and were supplemented with EC at 2 or 20 mg/kg body weight (B.W.) for a 24 week period. Between week 20 and 22, anxiety-related behavior, recognition memory, and spatial memory were measured. Underlying mechanisms were assessed by measuring the expression of selected genes in the hippocampus and by 16S rRNA sequencing and metabolomic analysis of the gut microbiota. 24 weeks of HFD feeding resulted in obesity, which was not affected by EC supplementation. HFD-associated increase in anxiety-

related behavior was mitigated by EC in a dose-response manner and was accompanied by increased hippocampal brain-derived neurotrophic factor, as well as partial or full restoration of glucocorticoid receptor, mineralocorticoid receptor and 11β-HSD1 expression. Higher EC dosage (20 mg/kg B.W.) also restored aberrant Lactobacillus and Enterobacter abundance altered by HFD and/or the associated obesity. Together, these results demonstrate how EC mitigates anxiety-related behaviors, revealing a connection between BDNF- and glucocorticoids-mediated signaling . Our findings link changes in the hippocampus and the gut microbiota in a context of HFD-induced obesity and anxiety.

Keywords: obesity, hippocampus, anxiety, memory, epicatechin, high fat diet

Abbreviations:

BDNF, brain-derived neurotrophic factor; CNS, central nervous system; EC, (-)-epicatechin; HFD, high fat diet; B.W., body weight; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type I; HPA, hypothalamic-pituitary-adrenal; OFT, open field test; NOR, novel object recognition; MWM, Morris water maze; OLM, object location memory.

1. Introduction

Obesity has reached epidemic proportions and is regarded as a major public health concern. Obesity deleteriously affects health, increasing the risk of many chronic diseases ultimately decreasing quality of life and life expectancy [1, 2]. Among them, obesity has been linked to impairments in the central nervous system (CNS) contributing to the development of neurological diseases and mood disorders such and dementia, anxiety, and depression [3-9]. Indeed, obese individuals have a 55% higher risk of developing depression over their lifetime [10]. Similarly, animal models have also demonstrated that chronic consumption of high fat diets (HFD) and subsequent obesity leads to alterations in mood, anxiety, and depressive-like behavior [11-13].

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been reported in both obesity and emotional disorders. Glucocorticoids modulate HPA activity, targeting glucocorticoid receptors in limbic forebrain circuits to mediate psychological and behavioral stress [14]. In mice, chronic HFD induces anxiety-associated behaviors, accompanied by stress-induced activation of the HPA axis [15, 16]. Excess glucocorticoids impair adult neurogenesis, resulting in hippocampal atrophy, which in turn increases anxiety-like behaviors [17, 18]. The hippocampus has a well-defined central role in memory consolidation, but it is also involved in the regulation of mood and emotion [19, 20], which can be influenced by obesity and HFD [21-25].

Changes in the gut microbiota and associated variations in derived metabolites are being intensively studied for their participation in the gut-brain crosstalk. The capacity of polyphenols to modulate the gut microbiota is also proposed as a mechanism involved in the capacity of select polyphenols to mitigate mood disorders [26]. It is currently proposed that select gut bacteria could be associated with improvements in moods, including depression and anxiety [27]. Indeed, diet-induced obese mice exhibited altered insulin and inflammatory signaling in the brain and anxiety-associated behaviors, which were improved by antibiotic treatments [11].

Plant bioactives such as flavonoids have consistently been shown to improve a range of behaviors in rodents and humans [28-30]. (-)-Epicatechin (EC) is a flavan-3-ol abundant in several fruits and vegetables, e.g. grapes, apples, berries, cocoa, tea, which is reported to beneficially influence cognition and mood. The benefits of EC upon the CNS are purportedly mediated through their capacity to modulate vascular function (increase angiogenesis/cerebral blood flow) [31, 32], modulate cell signaling (increase brain-derived neurotrophic factor (BDNF)) [29] and mitigate neuroinflammation [33].

We previously observed that EC (20 mg/g B.W.) supplementation of mice fed a high fat diet (HFD) (60 kCal% from fat) for 13 weeks improved recognition memory [33]. This effect was associated with increased BDNF levels in the hippocampus and the prevention of HFD-induced endotoxemia and

neuroinflammation. However, in this model, HFD-fed mice did not show other major behavioral changes [33] and EC did not improve HFD-induced dysbiosis [34]. To further understand the potential capacity of EC to mitigate obesity-induced changes in mood and behavior, the current study used a mouse model of obesity with a longer (24 weeks) exposure to a HFD with a level (45 kCal% from fat) more relevant to human consumption. EC was supplemented at two levels, one that can be extrapolated to average human dietary consumption (2 mg EC/kg B.W.) [35], and a higher amount (20 mg EC/kg B.W.) that could be reached in humans by supplementation [36]. Thus, the current study investigated the link between changes in the hippocampus and gut microbiota in a context of HFD-induced obesity and anxiety, and the role of EC mitigating the adverse effects associated with HFD-induced obesity in the CNS and shifts in the gut microbiota. Characterization of the microbiota and microbiome allowed investigation of the relevance of the gut-brain axis crosstalk in the beneficial effects of EC on HFD/obesity-induced alterations in behavior.

2. Materials and methods

2.1 Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee.

Healthy 8 weeks old male C57BL/6J mice (20–22 g) (2-3 mice housed together, 10 mice per group) were fed for 24 weeks either: A - a control diet containing approximately 10% total calories from fat (C) (TD.06416, Envigo, Indianapolis, IN), B - a high fat diet containing approximately 45% total calories from fat (lard) (HF) (TD.06415, Envigo, Indianapolis, IN), the control diet supplemented with C - 2 mg EC (CE2) or D - 20 mg EC (CE20) per kg B.W., or the HFD supplemented with E - 2 mg EC (HFE2) or F

- 20 mg EC (HFE20) per kg B.W.. The composition of the control and the high fat diet is listed in **Supplementary Table S1**. The EC-containing diet was prepared every two weeks to account for changes in body weight and food intake, and to prevent potential EC degradation. All diets were stored at -20°C until use.

Body weight and food intake were measured weekly throughout the study as previously described [37]. At 12 weeks, blood was collected from the submandibular vein to assess midpoint metabolic parameters. Body composition was measured at weeks 12 and 24 by EchoMRI (Echo Medical Systems, Houston, TX). After 24 weeks on the dietary treatments, and after 4 h fasting, mice were euthanized by cervical dislocation. Blood was collected from the submandibular vein into tubes containing EDTA, and plasma collected after centrifugation at 3,000 x g for 10 min at room temperature. Brains were extracted from the skulls, and the hippocampus isolated. Visceral, epididymal, retroperitoneal, subcutaneous, and brown fat pads were excised. The collected subcutaneous fat depot consisted of the posterior (dorsolumbar, inguinal and gluteal) and the anterior (cervical and axillar) subcutaneous fat. The visceral fat isolated was the mesenteric adipose tissue. Tissues were dissected and flash frozen in liquid nitrogen and then stored at -80°C for further analysis.

2.2 Determination of plasma metabolic parameters

Plasma triglyceride and cholesterol concentrations were determined using kits purchased from Wiener Lab Group (Rosario, Argentina), glucose concentrations using a kit from Sigma-Aldrich Co (St. Louis, MO), and insulin concentration using a kit purchased from Crystal Chem Inc. (Downers Grove, IL), following the manufacturer's protocols. The homeostasis model for insulin resistance (HOMA-IR) was calculated as (fasting blood glucose (mmol/L) × fasting plasma insulin (μU/ml) / 22.5) to assess insulin resistance.

2.3 RNA isolation and quantitative PCR (q-PCR)

For quantitative PCR studies, RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was generated using high-capacity cDNA Reverse Transcriptase (Applied Biosystems, Grand Island, NY). Expressions of *β-actin*, *Bdnf* (brain-derived neurotrophic factor), GR (glucocorticoid receptor; *Nr3c1*), MR (mineralocorticoid receptor; *Nr3c2*), and *11β-Hsd1* (11β-hydroxysteroid dehydrogenase type I) were assessed by quantitative real-time PCR (iCycler, Bio-Rad, Hercules, CA) with the primers listed in **Table 1**.

2.4 Animal Behavioral Test

Behavioral tests were performed between week 20 and 22 of the dietary intervention. Animals were acclimated to a behavioral testing room separate from the housing room at least 1 hour prior to all handlings and behavioral tests.

Open field test (OFT). After being exposed to the diets for 20 weeks, each animal was habituated in a white, square arena (40 × 40 cm) where the animal was naïve to. To evaluate anxiety-related behavior of mice, the amount of time traveled in the center zone and total distance traveled was measured during the first 5 minutes using EthoVision XT 13 (Noldus, Wageningen, The Netherlands). After each trial, the arena was cleaned with 70% ethanol.

Novel object recognition (NOR) and object location memory (OLM) tasks. The day after the OFT, short-term object recognition memory was evaluated using the NOR task. On the following day, short-term spatial memory was evaluated with the OLM task. For both tasks, each animal was allowed to explore two identical unfamiliar objects (A, A') in the square arena described above for 5 minutes (sample phase). After being placed in the home cage for 1 hour (retention phase), mice were

reintroduced to the arena for 5 minutes (test phase). For the NOR task, one of the objects was changed to a novel object during the test phase (A, B). For the OLM task, location of one of the objects was changed to a novel location (A, B) and each arena had spatial cues made with construction papers mounted on the north and west side of walls. The time that each animal spent directly sniffing or whisking towards the familiar and the novel objects or locations was analyzed by blinded investigators. A preference index, a ratio of the amount of time spent exploring one of the identical object (A') in the sample phase or the novel object/location (B) in the test phase over the total amount of time spent exploring both objects was used to determine preference for novelty (A'/(A + A') × 100% or B/(A + B) × 100% respectively) [38, 39]. A preference index above 50% indicates preference for novel object or location, below 50% for familiar object or location, and 50% null preference. Animals that did not spend more than 10 seconds total exploring both objects during the testing phase were excluded from analysis. After each trial, all objects and the arena were cleaned with 70% ethanol.

Morris water maze (MWM). At week 21, animals started training for the MWM to be evaluated for spatial learning and reference memory. Spatial learning and reference memory were assessed in a circular pool of 120 cm diameter containing water to a depth of 40 cm. The water temperature was controlled at 23±1°C. After every training and trial, each animal was gently scooped out of the pool, placed in a heated holding cage, and returned to the home cage. The pool was virtually divided into four quadrants: northeast (NE), northwest (NW), southeast (SE), and southwest (SW).

(1) Handling (MWM day 0): mice were introduced to water for the first time. Each animal was allowed to swim in a clear plastic cage (23.5 × 14 × 13 cm) containing water to a depth of 0.5 cm for 20 seconds. Afterwards, the animal was transferred to a cage filled with a depth of 1 cm water for 20 seconds and then to a cage filled with a depth of 2 cm water for 20 seconds.

(2) Pre-training (MWM day 1): mice were introduced to the pool described above and a plexiglass platform (10 cm top diameter). Each animal was placed on the platform, which was in the center of the pool and 1 cm above the surface of the water, for 15 seconds. Afterwards, the animal was allowed to swim freely for 30 seconds. Then, the animal was guided to climb on the platform and to stay there for 30 seconds.

(3) Visible platform task (MWM day 2-3): non-spatial training was conducted to ensure that non-cognitive effects were not interfering with upcoming water maze performance. White curtains were hung around the pool to obscure any spatial cues in the room. Both locations of starting point of mice and platform were moved to new locations in each trial. The platform was 1 cm above the surface of the water and mounted with a flag that reached a height of 13 cm. Each animal was gently placed into the pool and allowed to swim freely for 60 seconds. Once the animal located the platform, the animal was allowed to stay on there for 20 seconds. If the animal failed to locate the platform within 60 seconds, experimenters gently scooped the animals with a net and placed the animal on the platform for 20 seconds. Visible platform task was conducted 4 times daily with a 1-hour intertrial interval.

(4) Hidden platform task (MWM days 4-8): large and high-contrast geometrical patterns made with construction papers were mounted on the walls of the testing room to serve as distant spatial landmarks. The platform was hidden from the mice; it was submerged 1 cm below the surface of the water, which was rendered opaque with non-toxic, white, powdered tempera paint. Starting point was moved to a new location for each trial while the location of the platform stayed in the center of the southwest (SW) quadrant throughout all trials. Hidden platform task was conducted 4 times daily with a 1-hour intertrial interval. Learning curves of the animals were analyzed by measuring time spent to reach the platform (escape latency) using EthoVision XT 13 (Noldus, Wageningen, The Netherlands).

(5) Probe trial (MWM day 9): the testing environment for probe trial was the same as the hidden platform task except there was no platform placed in the pool. For this one-time trial, each animal was allowed to swim freely for 60 seconds. Spatial memory was analyzed by measuring the time spent by the animals in the target quadrant (SW) using EthoVision XT 13.

2.5 Genomic DNA extraction and 16S rRNA amplicon sequencing

Genomic DNA was extracted from all samples using a commercially available kit (Maxwell® RSC PureFood GMO and Authentication Kit, Cat. #AS1600). Around 50 mg of fecal pellet was used, following manufacturer's instructions, with an additional bead beating step using the FastPrep (MP Biomedicals, USA), protocol previously described by [40]. DNA concentrations of each sample were evaluated using Qubit® dsDNA High Sensitivity Assay Kit (Cat. Q32851) with Qubit® 2.0 Fluorometer, following manufacturer's instructions.

Quality assessment was performed by agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequences analysis were performed by Uparse software (Uparse v7.0.1001) [41] using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database [42]. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences.

2.6 ^{1}H NMR Metabolomic analysis

Metabolites were analyzed and quantified by ^{1}H NMR analysis. The preparation method was similar to that previously described [43]. Briefly, 500 µl NMR buffer [0.25g Na_2HPO_4, 1.44 g NaH_2PO_4, and 17 mg trimethylsilylpropanoic acid [sodium 3-(trimethysilyl)-propionate-d4] in 100 ml deuterated water (Goss Scientifics, Crewe, United Kingdom] were added to 40-60 mg of defrosted fecal materials and thoroughly mixed with a pellet pestle attached to a cordless motor grinder, followed by centrifugation (18,000 x g for 1 min). Additional NMR buffer was added to each sample, to reach a final dilution factor of 16. After vortexing, 550 µl were transferred into a 5-mm NMR tube for spectral acquisition. High resolution [^{1}H] NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton-optimized triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Coventry, England). Sample temperature was controlled at 300 K. Each spectrum consisted of 64 scans with a spectral width of 20.8 ppm (acquisition time 2.62 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 4 s) and mixing time (D8 = 0.01 s). Spectra were transformed with a 0.3-Hz line broadening and zero filling, manually phased, baseline corrected, and referenced by setting the trimethylsilylpropanoic acid methyl signal to 0 ppm. Metabolites were identified and quantified using the software Chenomx (V 8.6).

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.04 (GraphPad Software, Inc., San Diego, CA). Pearson correlation analyses were conducted to assess relationships between time spent in the center zone (%) and BDNF mRNA levels. Body weight, metabolic parameters, behaviors, and hippocampal mRNA levels were analyzed by one-way analysis of variance (ANOVA) and Fisher's Least

Significant Difference (LSD) post hoc analysis. Differences were considered statistically significant at $p < 0.05$. Data are shown as mean ± SEM.

Alpha-diversity and beta diversity were assessed using Shannon H diversity index and weighted UniFrac distances analyses respectively. Statistical significance was determined by Kruskal–Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA). Comparisons at the Phylum and Genus level were made using classical univariate analysis using Kruskal–Wallis combined with a false discovery rate (FDR) approach used to correct for multiple testing. Correlation analysis between metabolomics data and microbiome data was conducted using M2IA [44]. Missing values were filtered if present in more than 80% of samples or the relative standard deviation was smaller than 30% [45]. Remaining missing data values were handled using random forest. Data was normalized using total sum scaling. Correlation analysis between bacterial genus and metabolite profile across the different treatment groups was made using Spearman's rank-order correlation analysis [46].

Statistical analysis of metabolomics data was carried out using Metaboanalyst 5.0 [47]. Data was normalized by median, scaled by Pareto scaling and log-transformed. Univariate Analysis was carried out by one way ANOVA, followed by Tukey HSD. Dendrogram and heatmaps were created with Pearson correlation and Ward hierarchical clustering.

3. Results

3.1 Body weight and metabolic parameters

Average daily food intake was 13% lower in the HFD-fed groups (3.22 g/day/mouse) compared to the control groups (3.72 g/day/mouse) ($p < 0.01$; **Table 2**), however, caloric intake remained similar across all groups (**Figure 1A**). A significant increase in body weight emerged between the HFD-fed mice

and the controls after one week of intervention ($p < 0.001$; **Figure 1B**). Body composition analysis highlighted a significantly greater percent body fat mass in the HFD-fed mice when compared to the control mice after both 12 and 24 weeks ($p < 0.0001$; **Figure 1C**). Addition of EC had no influence upon control nor HFD associated body weight gain and percent body fat throughout the experimentation. Consistent with the findings, an increased fat pad weight (except epididymal fat) was observed in the HFD-fed and the EC supplemented HFD-fed mice (**Table 2**). Total brain and hippocampal weight remained unchanged across all groups.

Following the 24-week dietary intervention, analysis of 4h fasted plasma samples revealed significantly elevated plasma glucose, insulin and HOMA-IR (17%, 166% and 3-fold respectively) in response to the HFD when compared to the control (**Table 3**). EC Supplementation resulted in a full or partial amelioration of HFD-induced increase in plasma glucose and insulin, with HOMA-IR reduced 42% and 20% by supplementation with EC 2 and 20 mg/kg B.W., respectively. Despite this, EC had no significant impact upon the HFD-induced increase in plasma triglyceride and cholesterol levels after 24 weeks.

3.2 EC supplementation mitigates anxiety-related behavior in mice in a dose-dependent manner

The OFT was conducted to evaluate anxiety-related behaviors. The test utilizes rodents' naturally evolved behavioral preference to avoid brightly lit open areas and instead remain in close proximity to a darker less exposed protective wall [48]. Consumption of the HFD significantly decreased the percentage time spent in the center zone compared to control ($F_{(5, 53)} = 2.49$, $p < 0.05$; **Figure 2A and 2B**). A statistically significant difference in center exploration time was not found between HF and HFE2 groups; however, EC supplemented HFD groups increased the time in the center zone in a dose-dependent trend (HF vs HFE2: $p = 0.31$; HF vs HFE20: $p = 0.023$; **Figure 2B**). The total distance traveled in

the open field was significantly lower in the HF group compared to the controls ($F_{(5, 54)}$ = 6.11, p < 0.001; **Figure 2C**); however, locomotor activity does not seem to confound emotional measure in the current study as EC supplementations in both control and HFD-fed groups had no influence upon total distance traveled throughout the 5 min time frame of the experiment while the addition of EC, particularly the high dose (20 mg/kg B.W.), increased center exploration time.

3.3 EC supplementation does not affect recognition, spatial, and reference memory and spatial learning

The NOR task was conducted to assess the short-term recognition memory of mice. During the sample phase, all groups spent a comparable amount of time exploring each of the two identical objects ($F_{(5, 46)}$ = 1.10, p = 0.38; **Figure 3A**). During the test phase ($F_{(5, 46)}$ = 1.56, p = 0.19), group means revealed that control group exhibited greater novel object preference compared to the control supplemented with the higher dose of EC (CE20) as measured by the preference index (p < 0.05). This could potentially indicate detrimental rather than protective effects of the higher dose of EC on the control group on recognition memory, decreasing the ability in recognizing the novel object from the familiar object.

The OLM task was conducted to assess short-term spatial memory of mice. All groups performed similarly in both sample ($F_{(5, 32)}$ = 0.47, p = 0.80) and test ($F_{(5, 32)}$ = 0.95, p = 0.46) phases as measured by the preference index, indicating that EC did not affect the short-term spatial memory (**Figure 3B**). Spatial learning and reference memory was also evaluated in the MWM with the hidden platform task and the probe trial. Comparing the first (MWM day 4) and the last day (MWM day 8) of the hidden platform task, all groups found the hidden platform more quickly (**Figure 3C**). On the last day of the hidden platform task, all groups had similar escape latencies exhibiting comparable spatial learning ($F_{(5, 54)}$ = 1.11, p = 0.37). During the probe trial, group means revealed that the control group spent significantly more time in the target quadrant zone compared to the HF group (p < 0.05; **Figure**

3D). All the other groups spent a comparable amount of time in the target zone, indicating no differences in reference memory among the groups.

3.4 EC supplementation increases the expression of BDNF

We next measured mRNA levels of BDNF, a promoter of neuronal differentiation and survival and important mediator of synaptic plasticity in the hippocampus [49]. As previously observed [33], consumption of the HFD did not affect hippocampal BDNF mRNA content. However, BDNF mRNA levels were 32% higher in the HFE20 group compared to the HF group ($p < 0.05$; **Figure 4A**). There was a positive correlation between BDNF mRNA levels in the hippocampus and the percentage time spent in the center zone of the open field (r: 0.36, $p < 0.05$; **Figure 4B**).

3.5 EC supplementation increases the expression of the hippocampal glucocorticoid and mineralocorticoid receptors and decreases the expression of 11β-HSD1 in the HFD-fed animals

We next measured the hippocampal mRNA levels of receptors that mediate glucocorticoids action in the brain (GR and MR) and of the enzyme that catalyzes the regeneration of active glucocorticoids (11β-HSD1). Compared to the control group, mRNA levels of GR were 19% lower ($p < 0.05$) and of MR were 28% lower ($p < 0.05$) in the HFD-fed mice, and both doses of EC partially or fully prevented the decrease (**Figure 5A** and **5B**). Interestingly, the high dose of EC (20 mg/kg B.W.) significantly decreased GR mRNA levels when fed to control mice (22% decrease; $p < 0.05$) while the same dose of EC significantly increased the mRNA levels when fed to HFD mice (55% increase; $p < 0.001$). 11β-HSD1 mRNA levels were 33% higher in the HF mice compared to the control ($p < 0.05$), and this increase was mitigated by EC with a dose-dependent trend (**Figure 5C**). No significant correlations

between center exploration time in the open field and mRNA levels of GR, MR, and 11β-HSD1 were observed (data not shown).

3.6 EC supplementation affects microbiota structure and metabolism

The overall composition of the gut bacterial community in the different diet groups was assessed by 16S r RNA sequencing to investigate the degree of bacterial taxonomic similarity groups and treatments. Alpha diversity measured by the Shannon index was significantly increased following the HFD ($p < 0.04$; **Figure 6A**) indicating a greater richness and evenness within samples. EC supplementation further increased Shannon alpha diversity index with only the higher dose (EC 20 mg/kg B.W.) reaching significance ($p < 0.05$). Bacterial communities were then clustered using a principal coordinates analysis (PCoA) of weighted Unifrac distances which distinguished microbial communities based on their diet. The statistical significance of the clustering pattern was further evaluated using a permutational ANOVA (PERMANOVA). As depicted in **Figure 6B**, there was a clear separation of the diet groups along the axis 1 of the PcoA (58.6%) indicating a strong effect of the HF feeding diets versus the control diets ($p<0.001$). EC addition only seemed to have a small effect on the overall microbial communities. Comparison of relative abundance at the Phylum level identified several changes. HFD significantly reduced Verrucomicrobia, Bacteroidetes, Epsilonbacteraeota and Tenericutes while marginally increasing the abundance of Deinococcus Thermus ($p=0.06$) (**Figure 6C** and **Supplementary Table S2**). At the genera level, the HFD led to the modulation of 67 taxa including a significant increase of *Romboustsia, Solibacilus, Sporosarcina* and a decrease of *Akkermansia, Dubosiella* and *Planococcus* (**Figure 6D** and **Supplementary Table S3**). Supplementation with EC (2 and 20 mg/kg B.W.) to both control- and HFD-fed mice affected the microbiota composition (**Supplementary Figure S1**). In particular, EC 20 mg/kg B.W. significantly increased the abundance of Firmicutes, Acidobacteria, Bacteroidetes and Nitrospirae and

decreased the abundance of Actinobacteria in the HF group (**Figure 6E** and **Supplementary Table S4**). At the genera level, EC significantly modulated up to 139 taxa of which *Lechevalieria, Nitrospira, Opitutus, Sphingomonas* and *Lactobacillus* were significantly increased (**Figure 6F** and **Supplementary Table S5**).

In addition to the microbial analysis, ^{1}H-NMR metabolomic profiling was conducted on the same fecal samples to gain insights into the metabolomic environment. Consistent with the microbiota, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) of the metabolome showed clear separations of control versus HFD-fed mice (**Figure 7A**). This was further supported by hierarchical clustering using Spearman and Ward which resulted in the formation of 2 robust clusters representing each dietary intervention (**Figure 7B**). The HFD significantly increased the concentration of amino acids (histidine, ornithine, tryptophan, 5-aminopentanoate, 2-oxoisocaproate), organic acids (3-phenylpropionate, 4-hydroxybenzoate, nicotinate, tartrate, 3-methyl-2-oxovalerate, 4-hydroxyphenyllactate) along with methylamines (dimethylamine and trimethylamine), methanol, lactaldehyde and acetate (**Figure 7B** and **Supplementary Table S6**). Presence of these metabolites was negatively correlated with the abundance of Verrucomicrobia and positively correlated with Deinococcus-Thermus abundance (**Figure 7C** and **Supplementary Table S8**).

Supplementation with EC 20 mg/kg B.W. to HFD-fed mice had a profound impact on the metabolomic profile. In particular organic acids (3-(3-hydroxyphenyl)propanoate and 4-hydroxyphenylacetate), nucleotides (2'-deoxyguanosine, 2'-deoxyinosine, 2'-deoxyuridine, uridine) along with the fatty acid isobutyrate were significantly increased in the EC group. Alanine, valerate, cytosine and citrate were decreased in this diet group (**Figure 7B** and **Supplementary Table S7**). Correlation analysis between the microbiome and metabolome indicated that nucleotides and organic acids increases were strongly correlated to increased abundances of Nitrospirae, Firmicutes, Acidobactaeia, Elusimicorbia, Latescibacteria, Entotheonellaeota and Rokubacteria and a decrease in

Actinobacteria (**Figure 7D** and **Supplementary Table S9**). Further analysis revealed that the fecal content of cytosine, a metabolite strongly associated with the reported microbiome shift, was significantly and negatively correlated with center exploration time in the OFT (r: -0.4652, p = 0.0096).

4. Discussion

EC has been shown to influence cognition and behavior in both humans and rodents. Among the described mechanisms, increased brain BDNF concentration has been consistently described [29, 33] along with the promotion of vasodilation [31, 50-52], mitigation of neuroinflammation [33] and activation of ERK1/2/CREB [53]. The present study supports a potential role for EC in the mitigation of anxiety-related behaviors, which is in part mediated through BDNF, GR, and MR upregulation and 11β-HSD1 downregulation in the hippocampus and mitigation of HFD-mediated dysbiosis.

The HFD induced significant increase in body weight and percent body fat in mice after 24 weeks which was not prevented by EC supplementation. The increase in adiposity in the HFD-fed animals was reflected by a higher weight of fat pads, except the epididymal fat. Consistent with our finding, previous studies have shown that obese mice fed a HFD for 20 weeks have a reduced epididymal fat mass compared to controls [54, 55]. This decrease in mass was attributed to increased deaths of adipocytes and associated immune cell infiltration and activity. Thus, the rate of adipocyte death caused by chronic HFD consumption would exceed the rate of tissue repair resulting in net loss of epididymal fat pads.

Obesity is associated with increased risk of neuropsychiatric conditions, including cognitive impairment and mood disorders [56, 57]. This is also apparent in preclinical models of obesity in which HFD diet-induced obesity results in deterioration of learning and memory [22, 58], as well as anxiety and depression [11, 12]. Like obesity, type 2 diabetes (a prevalent comorbidity of obesity), results in an

increased risk of neuropsychiatric disorders [59, 60]. This is consistent with the present evidence, in which HFD-induced obese and insulin resistant mice spent less time in the center of the OF maze (increased anxiety) and less time in the target quadrant of the MWM (impaired spatial memory). Although not significant, there was an additional drop in object location performance (spatial memory) in response to HFD, while NOR (recognition memory) remained unaffected. This may suggest that spatial memory performance, and therefore specific brain regions such as hippocampus are particularly sensitive to diet-induced metabolic changes. Surprisingly, supplementation with EC had no beneficial impact upon learning and memory and did not mitigate HFD-induced spatial memory deficits. In fact, control animals receiving EC supplementation displayed even poorer performance on the NOR task, particularly at 20 mg EC/kg B.W.. Interestingly this decline was absent in HFD animals suggesting that high doses of EC are better tolerated by mice in combination with a high fat meal. Despite this, EC ameliorated the HFD-induced increase in anxiety in a dose-dependent manner with the high EC dose (20 mg/kg B.W.) restoring center exploration time (anxiety measure) back to control levels. This suggests that the mechanisms leading to learning and memory impairment are uncoupled from those associated with anxiety. The lack of spatial memory improvement following EC supplementation may potentially relate to EC's inability to mitigate the HFD-induced insulin resistance. As such, the contrasting improvement in anxiety observed with 20 mg EC/kg B.W., must therefore relate to an alternative mechanism.

Gut microbial composition has been suggested as a potential contributor to the neurobehavioral abnormalities associated with HFD consumption. Changes in gut microbiota and derived metabolites are being intensively studied for their participation in the gut-brain crosstalk that could lead to the improvements of behavior, including depression and anxiety [27]. In this regard, the capacity of polyphenols to modulate the microbiota is proposed as a mechanism in which polyphenols may mitigate mood disorders [26]. In the present study, the HFD surprisingly increased species richness as assessed

via Shannon α-diversity when compared to the control diet. Although not expected, this phenomenon has been described by others and has recently been reported to arise from the higher fiber content (cellulose) present in HFD [61]. This additionally explains some of the unexpected increases of bacterial phyla and genera considered to be beneficial. Despite this, evidence of HFD-induced dysbiosis was also apparent with several genera including *Akkermansia*, *Lactobacillus* and *Lachnochlostridium*, that were altered by the HFD. In agreement with our findings, which show a decrease in *Akkermansia* abundance in the HFD-fed mice, previous studies have shown that the colonization of *Akkermansia muciniphila* in the gut has protective effect in diet-induced obesity [62, 63]. Similarly, *Akkermansia* has been identified as a key player in the metabolic disorders and can influence glucose metabolism. Indeed, an inverse association between *Akkermansia* and insulin resistance is well established [64]. EC did not increase/restore *Akkermansia* which may account for EC's inability to improve HFD-induced insulin resistance and subsequent cognitive decline. In contrast to *Akkermansia*, *Lactobacillus* and *Enterobacter* were restored through EC supplementation. *Enterobacter*, which is linked to HFD-induced obesity and hepatic damage [65], was reduced by EC supplementation. *Enterobacter* has been linked to bipolar disorders and depression [66, 67] in which higher abundance leads to greater risk. Furthermore, *Lactobacillus* has been consistently recognized for its role in HFD-induced anxiety [68], with ingestion of *Lactobacillus* strains linked to gamma-aminobutyric acid (GABA) and acetylcholine production [69]. In our experiments, HFD-mediated *Lactobacillus* decrease was mitigated by EC (20 mg/kg B.W.). Thus, modulation of microbial species, such as *Enterobacter* and *Lactobacillus* may in part explain EC-mediated improvement of HFD-mediated anxiety-related behavior.

Supplementation with EC 20 mg/kg B.W. to HFD-fed mice also had a profound impact on the metabolomic profile. Cytosine, a metabolite associated with the reported microbiome shift, was significantly decreased in the HFE20 group compared to the HF group. Further analysis revealed that cytosine levels correlated with the anxiety-associated behavior observed in the OFT. In agreement with

this finding, changes in cytosine levels were observed in a mouse model of anxiety, which were proposed to reflect changes in oxidative stress-related pathways and mitochondrial function [70]. In addition, oral administration of an EC-rich grape seed polyphenol extract (GSPE) significantly increased the brain content of the gut derived 3-(3'-hydroxyphenyl) propionic acid (3-HPP). Accumulation of this metabolite was also observed to interfere with the assembly of β-amyloid (Aβ) peptides into neurotoxic Aβ aggregates [71]. Our finding of increased fecal 3-HPP concentration in HFE20 compared to HF mice, may in part contribute to the capacity of EC to modulate anxiety. While these changes in cytosine and 3-HPP are interesting, related evidence is limited, and further research is needed to confirm such connections.

Obesity is associated with altered BDNF expression, which has been proposed to be in part mediated by dysbiosis [72]. Dysregulation of BDNF has been linked to anxiety disorders [73, 74], and circulating BDNF indeed represents a potential biomarker for several psychiatric disorders [75]. Consumption of flavanols increase circulating and hippocampal BDNF levels in humans and animal models. High serum levels of BDNF were found in a group of subjects aged between 62 and 75 years consuming a high-flavanol cocoa drink daily for 12 weeks [76]. EC supplementation mitigated anxiety-related behavior which was associated with increased hippocampal BDNF levels in adult male mice [29]. Consistent with our previous finding [33], EC significantly increased BDNF mRNA levels in the hippocampus of both control and HFD-fed animals. Although HFD-induced alterations of BDNF levels were not observed, hippocampal BNDF levels were positively correlated with center exploration time in the OFT, which suggests that EC may in part mitigate HFD-induced anxiety by promoting BDNF upregulation.

Dysregulation of neural glucocorticoid signaling has been also suggested to be a potential mediator of the adverse neurological consequences of obesity and associated pathologies [77-79]. Glucocorticoids exert multiple effects within the CNS via MR and GR, which are located in different brain

regions, including the hippocampus [80]. The present study found that consumption of the HFD decreased hippocampal mRNA levels of MR and GR while EC reversed the decreases. Consistent with these findings, high hippocampal MR expressions have been linked to low-anxiety phenotype [81]. Conversely, inhibition of MR is linked to anxiety-like behavior, which is accompanied by decreased adult hippocampal cell proliferation [82]. The currently observed anxiety-related behavior observed in HFD-fed mice could also be explained by decreased hippocampal cell proliferation. Indeed, consumption of a HFD reduced cell proliferation in the hippocampus of preclinical models of obesity [83]. On the other hand, EC supplementation upregulate proteins involved in neurogenesis, i.e. NeuN, DCX, NGF, and MAP2 [84]. Current evidence on the role of the hippocampal GR on anxiety-related behavior is conflicting. The present study showed that EC consumption increased mRNA levels of GR in the hippocampus. In agreement with our finding, upregulation of GR expression has been correlated with decreased anxiety-related behavior [85], and increased resistance to stress and inflammation [86, 87]. On the other hand, transgenic mice with disrupted brain GR expression showed anxiety-related behaviors [88]. These conflicting results suggest that either too little or too much GR activity or expression could be detrimental to mood regulation [89]. Interestingly, supplementation with 20 mg EC/kg B.W. to mice fed the control diet showed significantly decreased mRNA levels of GR while did not show mood alterations. As shown by the NOR data, it is possible that long-term consumption of the higher dose of EC tested may be toxic to mice fed the control diet, while the same dosage is well tolerated by HFD-fed animals. As the relationship between levels of GR and cognition/mood regulation is not clear, further research on the potential neurotoxicity of high EC doses is warranted.

Concentrations of glucocorticoids are also determined by intracellular 11β-Hydroxysteroid dehydrogenases, which regenerate active glucocorticoids from inert 11-keto forms [90]. The type 1 isozyme, 11β-HSD1, is widely expressed throughout the adult CNS, and its increase in the hippocampus has been associated with cognitive decline [91]. Thus, inhibition of 11β-HSD1 has been proposed to

provide neuroprotective effects. Indeed, carbenoxolone, an effective inhibitor of 11β-HSDs, improved cognitive function in healthy elderly men and T2D patients [79]. Although not significant, a trend for reduced anxiety score with carbenoxolone treatment was reported. The present study showed that EC consumption significantly mitigated HFD-induced increase in 11β-HSD1 levels in the hippocampus. The potential mechanism of EC in decreasing the levels of 11β-HSD1, and its role in mood regulation in obesity is an interesting prospective to further investigate as inhibition of 11β-HSD1 hold therapeutic potential for obesity, T2D, and neuropsychiatric decline [77-79].

It is suggested that EC can exert neuroprotective effects both directly, inside of the brain [29, 92], and indirectly, by improving cerebral blood flow [31, 32] and/or affecting select receptors present at the blood-brain barrier (BBB) [29, 33, 93]. Ingested EC is highly bioavailable and extensively metabolized into a wide range of metabolites [94], and some metabolites were shown to cross the BBB and detected in the brain [29, 92, 95]. However, the primary route by which EC metabolites cross the BBB and their further metabolism in the brain is not yet completely understood [96]. EC can also have indirect neuroprotective actions. For instance, EC mitigated neuroinflammation and improved recognition memory in mice in part by reducing metabolic endotoxemia and preventing the hippocampal upregulation of TLR4, an innate immune receptor for endotoxin [33]. As suggested by other authors, it is also possible that EC may act via an specific receptor expressed in the brain, similar to the one described in arterial endothelial cell membrane [29, 93]. Further studies investigating both direct and indirect effects of EC are needed to fully understand the mechanisms underlying the neuroprotective benefits of EC.

In summary, EC supplementation mitigated anxiety-related behavior in a model of diet (HFD)-induced obesity in mice, which can be in part mediated through the modulation of BDNF- and glucocorticoids-mediated signaling. The reported findings on BDNF and glucocorticoid signaling are entirely based on gene expression analyses. Thus, further studies should evaluate the protein and

activation levels of these pathways. Additionally, EC modulated select microbial species, i.e. *Enterobacter* and *Lactobacillus,* altered by the consumption of the HFD and/or the associated obesity. This mechanism may also be involved in EC-mediated improvement of anxiety-related behavior in HFD-fed obese mice. Clinical studies will be essential to support the concept that consumption of EC-rich foods could contribute to mood improvement in obesity. Moreover, as the safety of long-term supplementation with high EC doses and its effects on the CNS is not clear, further research on EC potential neurotoxicity is warranted.

Credit Author statement

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Conflicts of interest

There are no conflicts to declare.

Legend to Figures

Figure 1. Effects of supplementation with EC on body weight gain and body fat mass. A- Calorie intake, **B-** body weight gain, and **C-** % fat mass. Mice were fed a control diet (empty circles), the control diet supplemented with 2 mg EC/kg (light blue circles) or 20 mg EC/kg (dark blue circles) B.W., a HFD (empty triangles), or the HFD supplemented with 2 mg EC/kg (pink triangles) or 20 mg EC/kg B.W. (red triangles). Body weight was measured weekly, and body composition was measured at weeks 12 and 24. Results are shown as mean ± SEM of 9-10 animals/group. *Differences between the HF and control body weight gain and % body fat values are significant ($p < 0.05$, one-way ANOVA with Fisher's LSD).

Figure 1

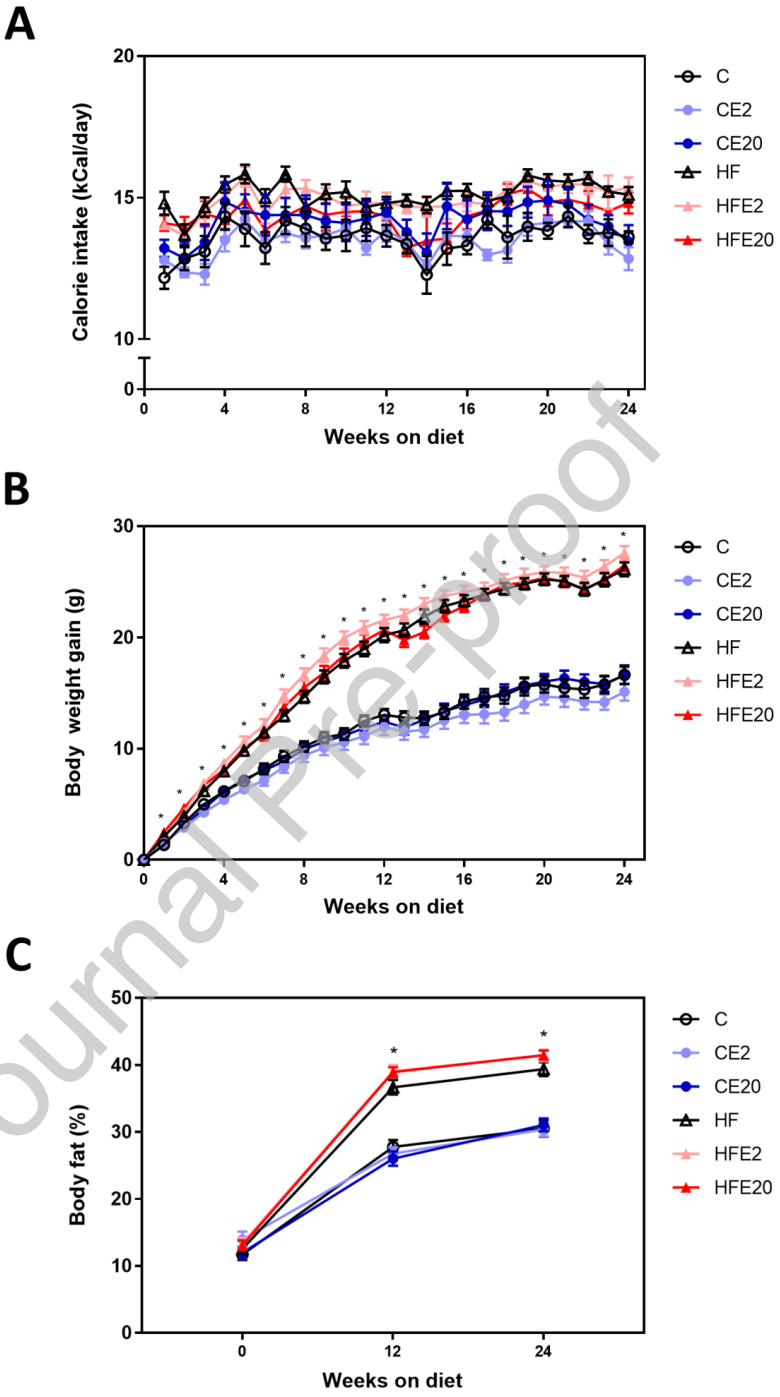


Figure 2. EC supplementation mitigates anxiety-related behavior in mice in a dose-dependent manner. A- Representative tracks of C, CE2, CE20, HF, HFE2, and HFE20 mice in the open field arena over 5 min. **B-** EC supplemented mice exhibit reduced anxiety-like behavior, spending significantly more time in the center zone of the open field apparatus. **C-** Total distance traveled during the first 5 min in the arena. Results are shown as mean ± SEM of 9-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD).

Figure 2

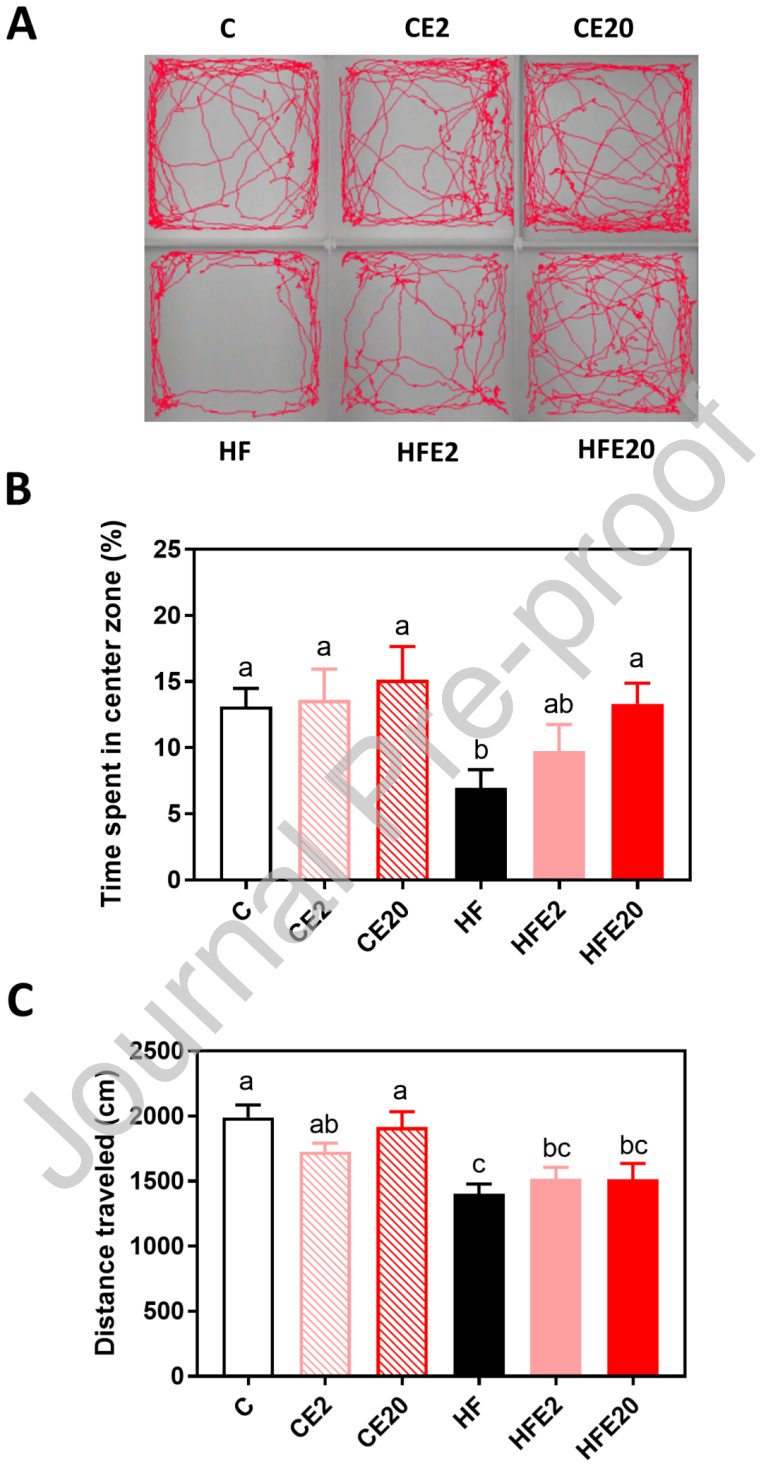


Figure 3. Effects of EC supplementation on short-term recognition, spatial, and reference memory and spatial learning. For both NOR and OLM tasks, animals explored two identical unfamiliar objects for 5 minutes (sample phase). After being placed in the home cage for 1 hour (retention phase), they were reintroduced to the arena for 5 minutes (test phase). **A-** Control group supplemented with the highest dose of EC (CE20) exhibited decreased novel object preference compared to the C group as measured by the preference index. All the other groups performed similarly in both sample and test phases as measured by the preference index. **B-** All groups performed similarly in both sample and test phases as measured by the preference index. Dashed lines delineate 50% null preference. Results are shown as mean ± SEM of 4-10 animals/group. **C-** Learning curves of mice in the hidden platform task and **D-** time spent in the target quadrant during the probe trial. Results are shown as mean ± SEM of 10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD).

Figure 3

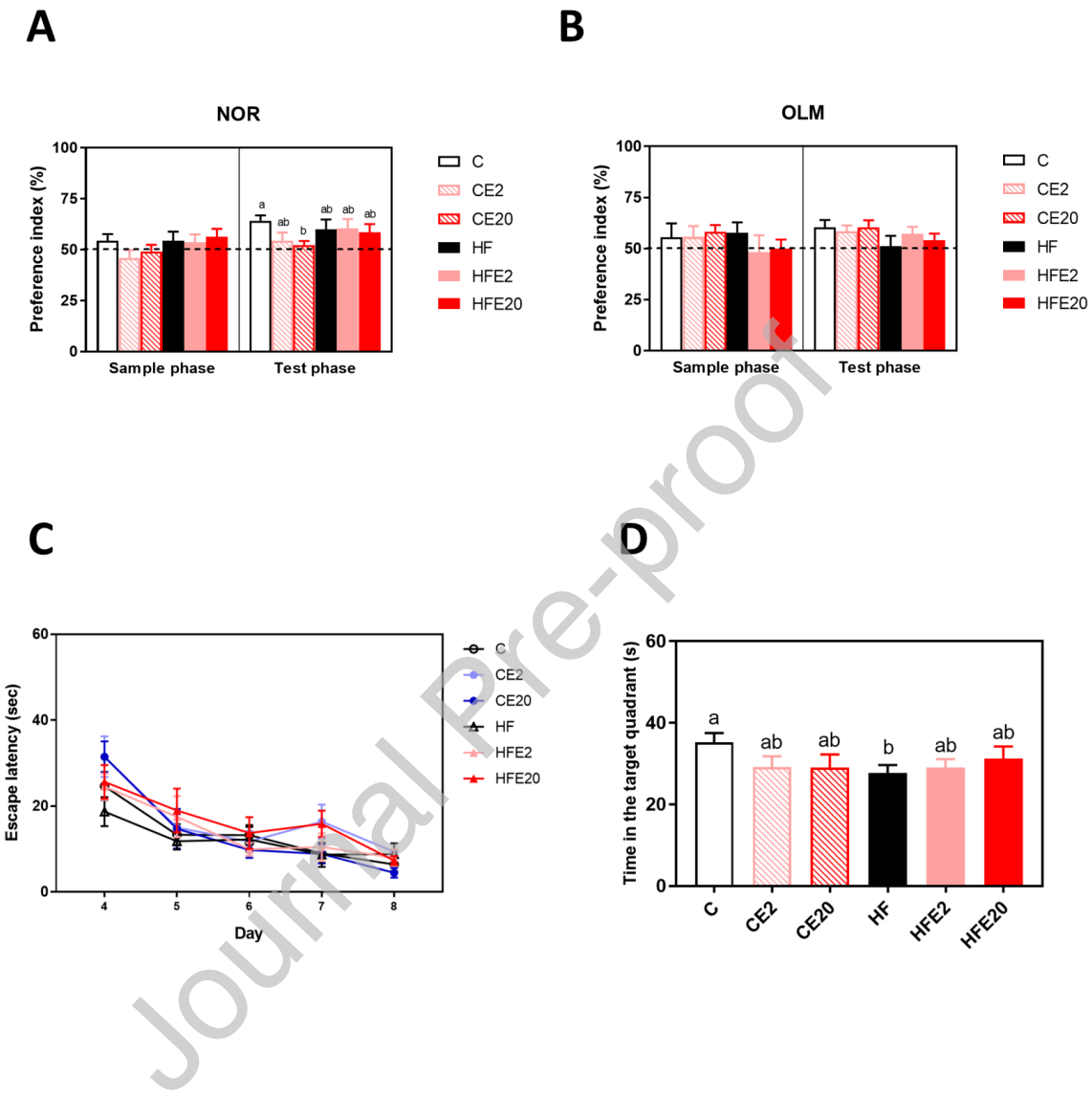


Figure 4. Effects of EC supplementation on the anxiety-related behavior and its correlation with BDNF levels. A- BDNF mRNA levels in the hippocampus were determined by q-PCR and the relative gene expression was normalized to β-actin as housekeeping gene. Determinations were done after 24 weeks on the respective diets. Results are shown as mean ± SEM of 6-9 animals/group. Data were normalized to control values. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD). **B-** Correlations between the time spent in the center zone (%) and BDNF mRNA levels. The solid line represents the regression line and the gray area delineates the 95% confidence band.

Figure 4

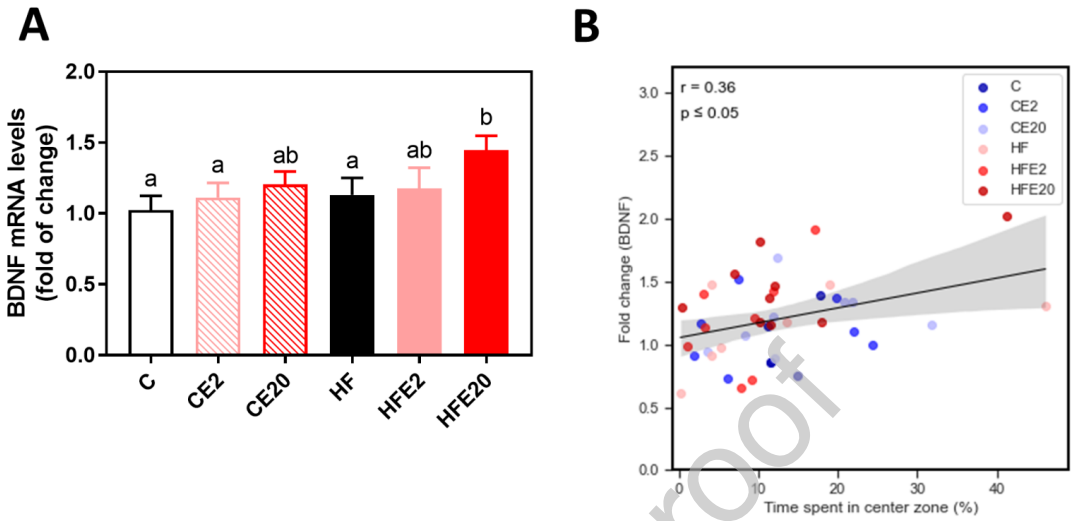


Figure 5. Effects of EC consumption on the mRNA levels of the glucocorticoid receptors (GR; Nr3c1), the mineralocorticoids receptor (MR; Nr3c2), and 11β-HSD1. The mRNA levels of the **A-** GR, **B-** MR, and **C-** 11β-HSD1 in the hippocampus were determined by q-PCR and the relative gene expression was normalized to β-actin as housekeeping gene. Determinations were done after 24 weeks on the respective diets. Results are shown as mean ± SEM of 4-6 animals/group. Data were normalized to control values. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD).

Figure 5

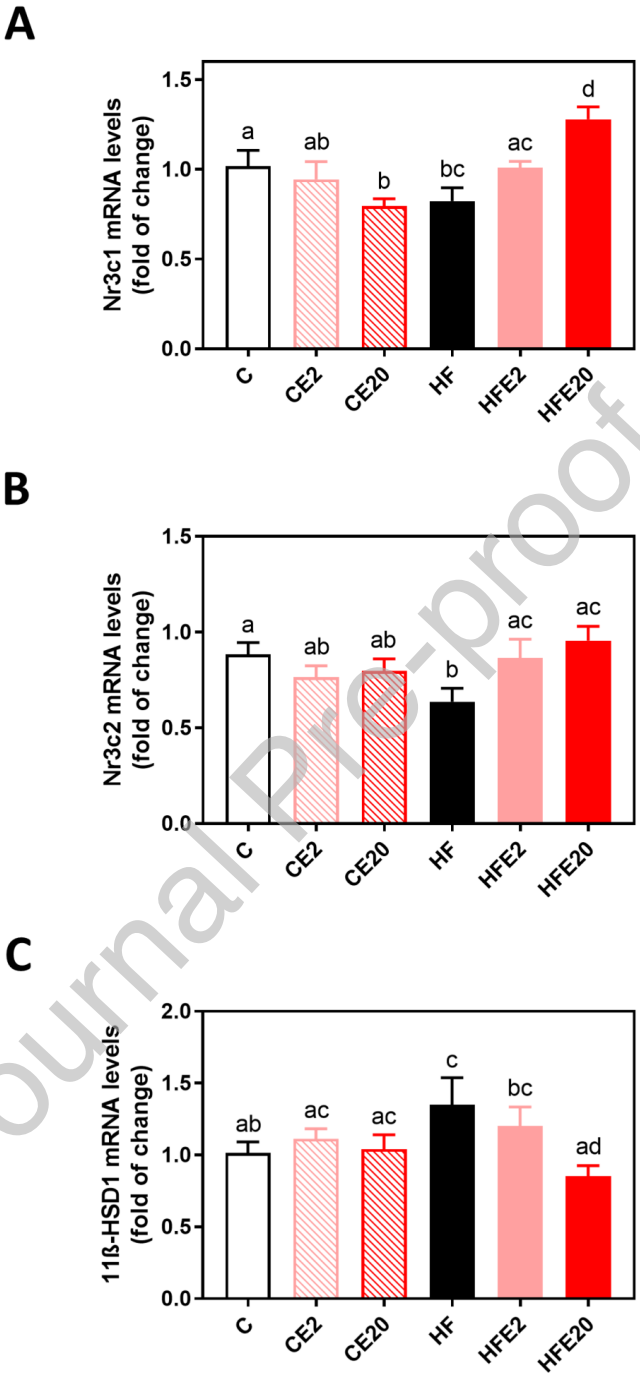


Figure 6. Effect of EC supplementation on microbiota diversity. A- α diversity as assessed by Shannon index showed a higher diversity in HF and HF treated with EC 20 mg/kg B.W.. **B-** β diversity, assessed using weighted Unifrac distance and PERMANOVA analyses showed a robust separation of control versus high-fat dietary groups. EC addition to either dietary treatment had subtle effect on the microbiota diversity. **C, D-** Classical univariate analysis highlighted key differences at the phylum (C) and genera levels (D) in control and high fat fed groups. **E, F-** Classical univariate analysis highlighted key differences at the phylum (E) and genera levels (F) in high fat and high fat supplemented with EC 20 mg/kg B.W. fed groups.

Figure 6

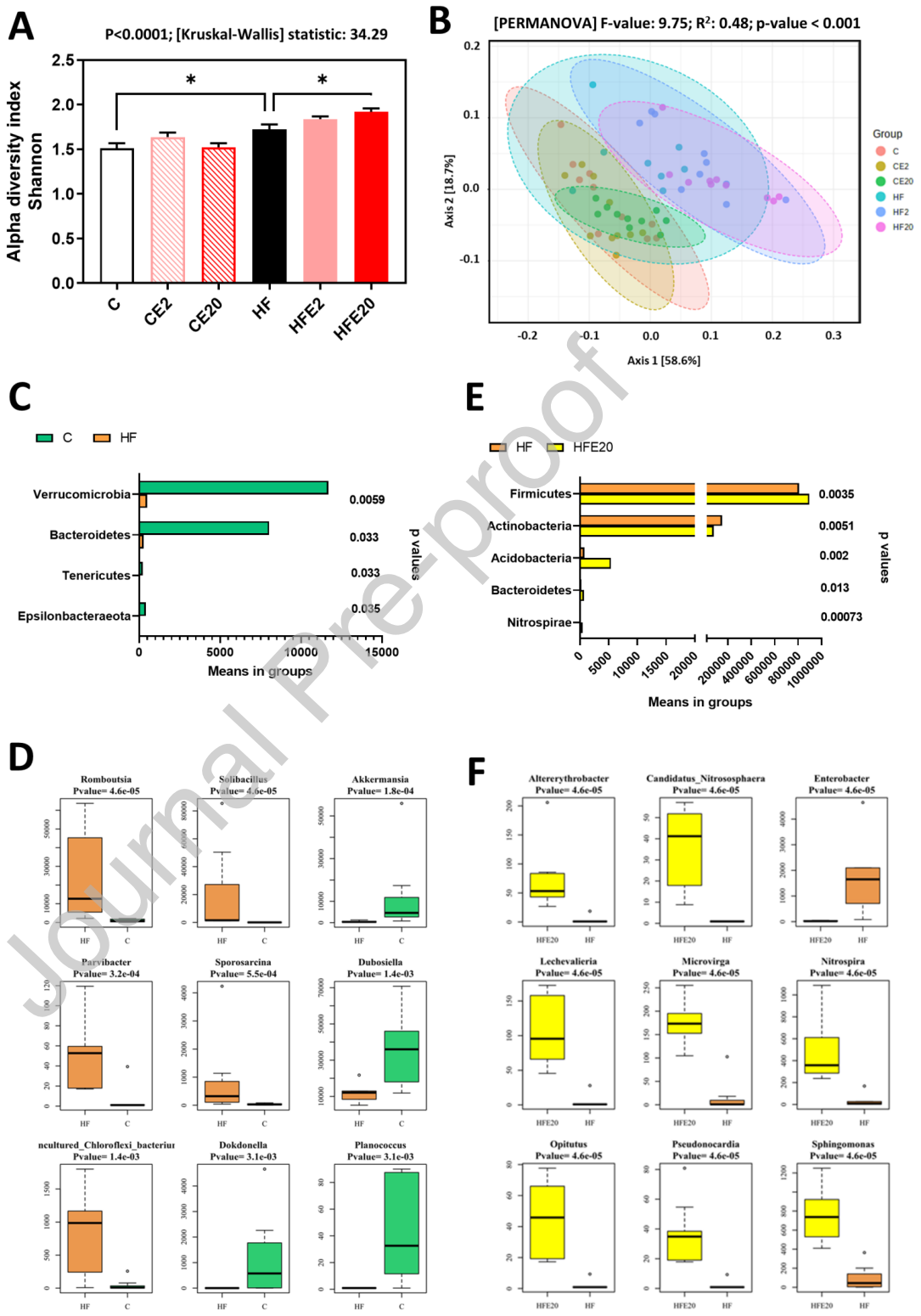


Figure 7. Effect of EC supplementation on the fecal metabolome. A- Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot of all metabolite features showed a clear separation of the fecal metabolites in the different treatment groups. **B-** Clustering result shown as heatmap (distance measure using Spearman, and clustering algorithm using Ward). **C-** Interactions between the metabolome and microbiome (Phylum) of control and high fat diet groups were made using Spearman correlation analysis, and highlighted key changes in organic acids, nucleotides and amino acids. **D-** Interactions between the metabolome and microbiome (Phylum) of high fat and high fat supplemented with EC 20 mg/kg B.W. groups were made using Spearman correlation analysis, and highlighted key changes in organic acids, nucleotides and carbohydrates.

Figure 7

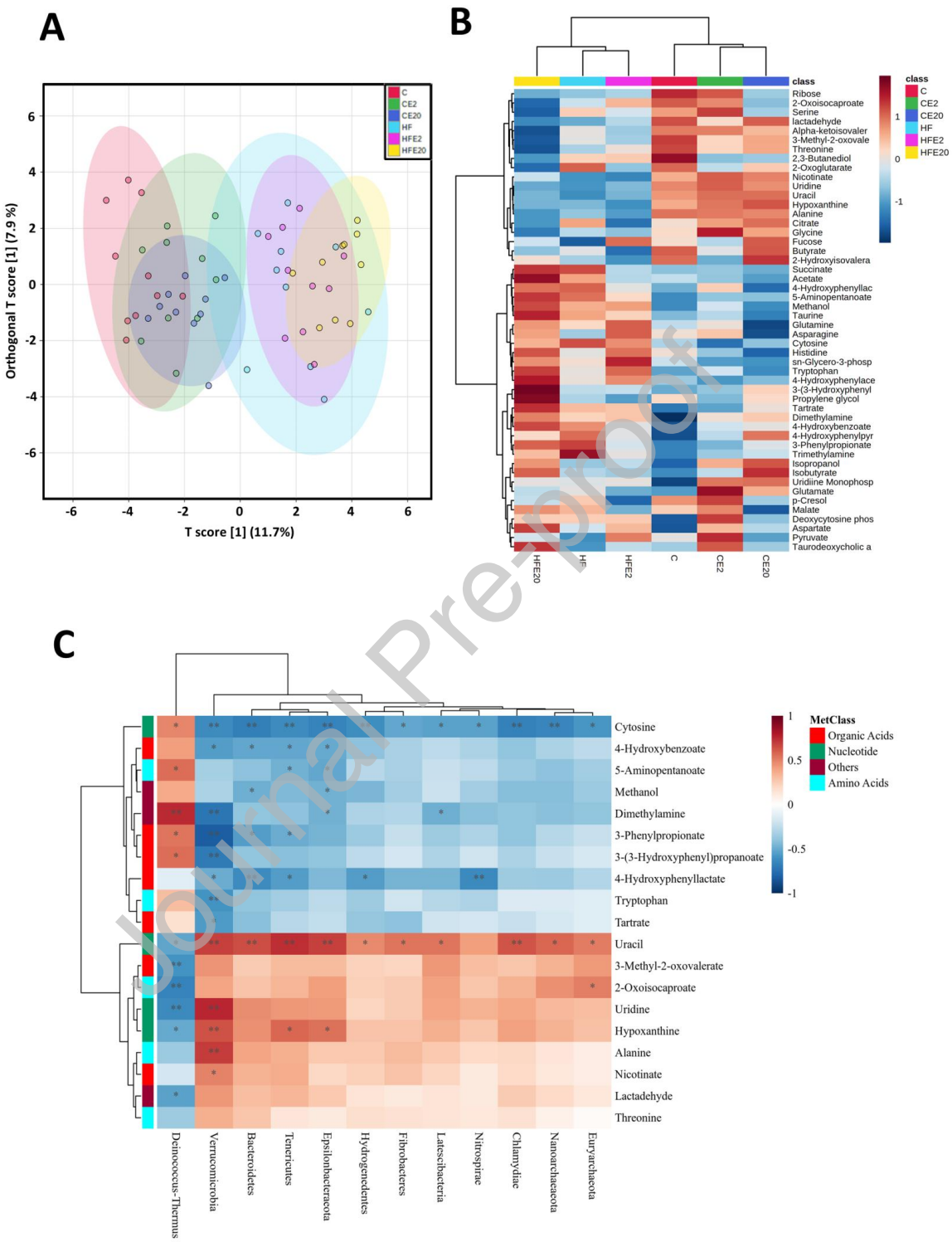
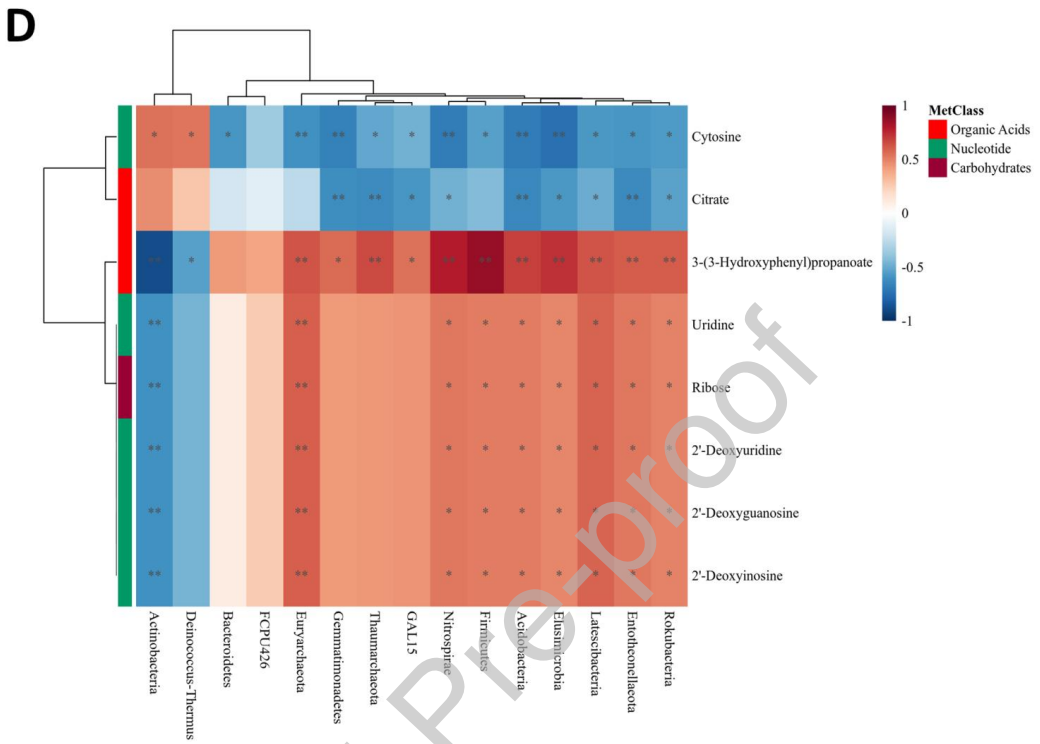


Figure 7



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Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
β-actin	TCATGAAGTGTGACGTGGACATCCGC	CCTAGAAGCATTTGCGGTGCACGATG
Bdnf	ATGGGACTCTGGAGAGCCTGAA	CGCCAGCCAATTCTCTTTTTGC
Nr3c1 (GR)	TGGAGAGGACAACCTGACTTCC	ACGGAGGAGAACTCACATCTGG
Nr3c2 (MR)	TGTGTGGAGATGAGGC	GGACAGTTCTTTCTCCGAAT
11β-Hsd1	GGGATAATTAACGCCCAAGC	TCAGGCAGGACTGTTCTAAG

Table 1. Primers used in the study.

Parameter	C	CE 2	CE 20	HF	HFE 2	HFE 20
Food intake (g/d)	3.67 ± 0.10^{a}	3.65 ± 0.05^{a}	3.83 ± 0.15^{a}	3.28 ± 0.05^{b}	3.25 ± 0.06^{b}	3.13 ± 0.09^{b}
Body weight (g)	40.9 ± 1.0^{a}	39.3 ± 1.0^{a}	40.9 ± 1.1^{a}	50.51 ± 0.6^{b}	52.34 ± 0.6^{b}	51.17 ± 0.7^{b}
Fat Mass (%)	30.5 ± 1.2^{a}	30.4 ± 1.0^{a}	31.1 ± 1.0^{a}	39.4 ± 1.0^{b}	41.4 ± 0.9^{b}	41.5 ± 0.7^{b}
Brain (mg)	433 ± 7	444 ± 5	438 ± 4	443 ± 4	443 ± 5	445 ± 6
Hippocampus (mg)	30.3 ± 4.1	28.2 ± 1.6	30.0 ± 1.5	30.6 ± 1.3	31.0 ± 3.1	27.5 ± 2.2
Visceral Fat (g)	0.68 ± 0.11^{a}	0.54 ± 0.07^{a}	0.64 ± 0.05^{a}	1.15 ± 0.04^{b}	1.25 ± 0.04^{b}	1.30 ± 0.05^{b}
Epididymal Fat (g)	1.81 ± 0.08^{a}	1.45 ± 0.17^{b}	1.76 ± 0.07^{ac}	1.38 ± 0.07^{b}	1.29 ± 0.04^{b}	1.50 ± 0.08^{bc}
Retroperitoneal Fat (g)	0.78 ± 0.08^{a}	0.66 ± 0.08^{a}	0.73 ± 0.06^{a}	1.61 ± 0.08^{b}	1.71 ± 0.09^{b}	1.57 ± 0.09^{b}
Subcutaneous fat (g)	2.9 ± 0.2^{a}	2.5 ± 0.3^{a}	2.6 ± 0.1^{a}	4.8 ± 0.3^{b}	5.1 ± 0.2^{b}	5.2 ± 0.3^{b}
Brown Fat (g)	0.27 ± 0.03^{a}	0.21 ± 0.03^{a}	0.25 ± 0.02^{a}	0.35 ± 0.03^{b}	0.44 ± 0.02^{c}	0.42 ± 0.03^{bc}

Table 2. Body and tissue weights after 24 weeks on the diets. Results are shown as means ± SEM and are the average of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD).

Parameter	C	CE 2	CE 20	HF	HFE 2	HFE 20
Week 12						
Glucose (mg/dL)	187.9 ± 13.2^{a}	173.9 ± 18.2^{ab}	138.7 ± 6.5^{b}	205.3 ± 13.4^{a}	205.0 ± 8.4^{a}	198.9 ± 13.9^{a}
Insulin (ng/mL)	1.64 ± 0.21^{a}	1.40 ± 0.10^{a}	1.66 ± 0.35^{a}	4.13 ± 1.03^{bc}	4.31 ± 0.77^{c}	2.67 ± 0.56^{ab}
HOMA-IR	19.29 ± 2.71^{ab}	15.36 ± 1.78^{a}	14.30 ± 2.18^{a}	45.53 ± 13.12^{cd}	55.62 ± 12.20^{c}	35.11 ± 6.49^{bd}
Total Cholesterol (mg/dL)	190.6 ± 6.4^{a}	183.4 ± 5.5^{a}	181.8 ± 6.4^{a}	241.6 ± 11.1^{b}	237.1 ± 12.2^{b}	194.8 ± 10.0^{a}
Triglyceride (mg/dL)	83.52 ± 7.31	71.14 ± 5.57	80.55 ± 3.45	74.91 ± 6.62	79.45 ± 6.10	70.82 ± 5.86
Week 24						
Glucose (mg/dL)	178.1 ± 6.4^{a}	186.3 ± 11.5^{ab}	182.8 ± 5.4^{ab}	208.1 ± 6.1^{c}	173.2 ± 6.6^{a}	200.6 ± 6.8^{bc}
Insulin (ng/mL)	2.02 ± 0.24^{a}	1.76 ± 0.26^{a}	1.70 ± 0.22^{a}	5.38 ± 0.47^{b}	3.72 ± 0.21^{c}	4.44 ± 0.44^{c}
HOMA-IR	22.38 ± 2.84^{a}	20.48 ± 3.51^{a}	19.16 ± 2.38^{a}	69.30 ± 6.95^{b}	40.02 ± 3.07^{c}	54.86 ± 5.61^{d}
Total Cholesterol (mg/dL)	215.7 ± 6.9^{a}	221 ± 8.2^{a}	209.3 ± 6.5^{a}	319.5 ± 12.6^{b}	301.1 ± 12.5^{b}	309.1 ± 13.2^{b}
Triglyceride (mg/dL)	70.54 ± 4.34	71.77 ± 3.87	72.08 ± 2.80	79.24 ± 4.34	75.16 ± 5.07	70.12 ± 3.31

Table 3. Blood parameters after 12 and 24 weeks on the diets. Results are shown as means ± SEM and are the average of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD).