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Effects of (-)-Epicatechin on Memory and Anxiety in High Fat Diet (HFD)-induced Obese Mice



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Effects of (-)-Epicatechin on Memory and Anxiety in High Fat Diet (HFD)-induced Obese Mice

By

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DISSERTATION

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**ABSTRACT**

Obesity is characterized by a condition of low-grade chronic inflammation and is associated with increased occurrence of cognitive and mood disorders. While consumption of high-fat diets (HFD) and associated obesity could have a detrimental impact on the brain structure and function, consumption of select dietary bioactives may help prevent these harmful effects. The overall aim of this thesis was to investigate the neuroprotective potential of the dietary flavan-3-ol (-)-epicatechin (EC) in the context of HFD and associated obesity.

Our first study investigated the capacity of dietary EC to mitigate hippocampal inflammation and impaired cognition in HFD-fed obese mice. Healthy 6 weeks old male C57BL/6J mice were fed for 13 weeks either a control diet (10% total calories from fat), a high fat diet (60% total calories from fat), or the control and high fat diets supplemented with 20 mg EC/kg body weight. Between week 10 and 12 of the dietary intervention, object recognition memory and spatial memory were evaluated. Gene expressions related to inflammation, oxidative stress, and neurotrophic factor were analyzed in the hippocampus. Impaired recognition memory was observed in HFD-fed mice, which was prevented by EC supplementation. Neither HFD consumption nor EC supplementation affected mouse spatial memory. After 13 weeks of the dietary intervention, HFD-fed mice developed obesity, endotoxemia, and showed increased parameters of hippocampal inflammation. While not affecting body weight gain, EC supplementation prevented all the HFD-induced changes. Taken together, EC supplementation prevented short-term recognition memory in HFD-induced obese mice, which could be in part due to the capacity of EC to mitigate HFD-induced metabolic endotoxemia, inflammation and oxidative stress in the hippocampus.

To further understand the capacity of EC to mitigate obesity-induced changes in cognition and mood, we conducted a longer dietary intervention study (24 weeks) with a HFD that is more comparable

to the amount of fat consumed by humans. Healthy 8-week old male C57BL/6J mice were maintained on either a control diet (10% total calories from fat) or a HFD (45% total calories from fat), and were supplemented with EC at 2 or 20 mg/kg body weight. 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. After 24 weeks of HFD feeding, mice developed 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 (BDNF), as well as partial or full restoration of glucocorticoid receptor, mineralocorticoid receptor and 11β-HSD1 expression. Higher EC supplementation (20 mg/kg body weight) also restored aberrant *Lactobacillus* and *Enterobacter* abundance altered by the HFD and/or the associated obesity. Together, EC mitigated HFD-induced anxiety-related behavior partly by modulating BDNF- and glucocorticoids-mediated signaling and mitigating HFD-associated dysbiosis.

Next we conducted an untargeted hippocampal transcriptomic analysis that included mRNAs, miRNAs, and long non-coding RNAs to further understand the underlying neuroprotective mechanisms of EC against HFD-induced obesity. EC reversed the gene expression profile induced by the HFD, counteracting the effects of the HFD. Genes involved in neurofunction-related pathways including Alzheimer's disease and neurodegeneration and cellular pathways such as insulin signaling pathway were dysregulated by the HFD, and EC counteracted the dysregulation. Functionality analysis revealed that the differentially expressed genes upon EC supplementation regulate processes involved in neurofunction, inflammation, endothelial function, and cell-cell adhesion. Taken together, the effect of EC to mitigate anxiety-related behavior in the HFD-induced obese mice can be, in part, explained by its capacity to exert complex genomic modifications in the hippocampus, counteracting changes driven by the consumption of the HFD and subsequent obesity.

In summary, this thesis contributes to the explanation of mechanisms related to the neuroprotective potential of EC in context of HFD and associated obesity. Our results suggest that EC could mitigate HFD-induced alterations in memory and anxiety, in part, by ameliorating neuroinflammation, modulating BDNF-and glucocorticoids-regulated signaling, mitigating dysbiosis, and counteracting the effects of HFD on the hippocampus at a multi-genomic level.

# Chapter 1

# Literature Review

## 1. Introduction

Obesity is a global phenomenon with its prevalence growing at an alarming rate [1]. Since 1975, the worldwide prevalence of obesity has nearly tripled [1]. Obesity is a major public health concern as it substantially increases the risk of developing metabolic diseases (type 2 diabetes, insulin resistance, and non-alcoholic fatty liver diseases), cardiovascular diseases (CVD) (hypertension, stroke, and myocardial infarction), and some types of cancer (colon, kidney, pancreas, uterus, and esophagus) [2]. Moreover, obesity and poor-quality diets have been associated with increased occurrence of cognitive and mood dysfunctions such as dementia and Alzheimer's diseases (AD) [3-7]. Chronic high fat diet (HFD) consumption and associated obesity can induce low-grade systemic inflammation and metabolic disorders such as insulin resistance and dyslipidemia [8-11]. Obesity is also associated with metabolic endotoxemia, which triggers pro-inflammatory immune responses [12, 13]. Obesity-induced systemic inflammation, metabolic disorders, and metabolic endotoxemia are characterized by elevated concentrations of circulating pro-inflammatory molecules (i.e., cytokines, chemokines, lipopolysaccharides (LPS), free fatty acids (FFA) etc.) which all can in part contribute to the development of obesity-associated alterations in cognition and mood [14-18]. While obesity-derived neurological complications have been shown to affect different brain regions, the hippocampus – a region critical for learning and memory [19] and the regulation of mood and emotion [20, 21] – is particularly vulnerable to HFD-/obesity-induced changes [22-26]. Indeed, obesity is associated with a decrease in hippocampal volume [27-29] and impairment in hippocampus-dependent function as the inflammatory mediators can be transported across the blood brain barrier (BBB) and/or compromise its barrier function and subsequently infiltrate into different brain regions [26, 30].

While obesity can lead to negative health implications including cognitive and mood dysfunctions, dietary changes towards a higher intake of fruits and vegetables, and their derived products could mitigate these harmful effects [31]. Flavanols are a subgroup of polyphenols, which are naturally occurring

secondary metabolites found abundantly in fruits, vegetables, and cereals. They have been extensively studied because of their putative benefits on human health [32-34]. (-)-Epicatechin (EC) is one of the most widely consumed flavanols by humans which mainly derives from cocoa and tea products, and fruits such as grapes, berries, and apples [34, 35]. Epidemiological and dietary intervention studies suggest that EC-rich cocoa flavanols can improve cognitive function primarily by increasing cerebral blood flow [36-39]. While evidence supports a relationship between obesity and brain dysfunction; and points to a neuroprotective potential for EC, the capacity of EC to exert neuroprotective actions in the context of diet-induced obesity has not yet been investigated. Moreover, although several studies have reported that EC exerts anti-inflammatory actions in various tissues/organs [40-44], its capacity to mitigate obesity-induced neuroinflammation and the associated alterations in cognition and mood has not yet been explored.

This dissertation project assessed the neuroprotective potential of EC, particularly its capacity to mitigate obesity-induced neuroinflammation in the hippocampus and the associated alterations in cognition and mood using preclinical models of obesity. This section will highlight some of the key health benefits of EC – its role in promoting vascular, gastrointestinal (GI) and metabolic health, and anti-inflammatory action – and discuss how these effects could be translated into the neuroprotective potential of EC in the context of HFD consumption and subsequent obesity. Although the mechanisms affecting the central nervous system (CNS) are mostly likely to be multifactorial, some of the potential mechanisms underlying the neurological alterations caused by consumption of HFD and/or obesity will be outlined, with a particular focus on the effects on the hippocampus.

## 2. (-)-Epicatechin: structure, bioavailability, and metabolism

Flavonoids are a large family of polyphenolic compounds. The general structure (C6–C3–C6) has two aromatic rings (A and B rings) connected by a three-carbon bridge, which gives rise to an oxygenated

heterocycle in between (C ring) [45]. The basic flavonoid structure can be substituted with different chemical groups, such as hydroxy, glycosidic, and methyl groups. These chemical arrangements define the different subgroups of flavonoids [45, 46] **(Figure 1)**.

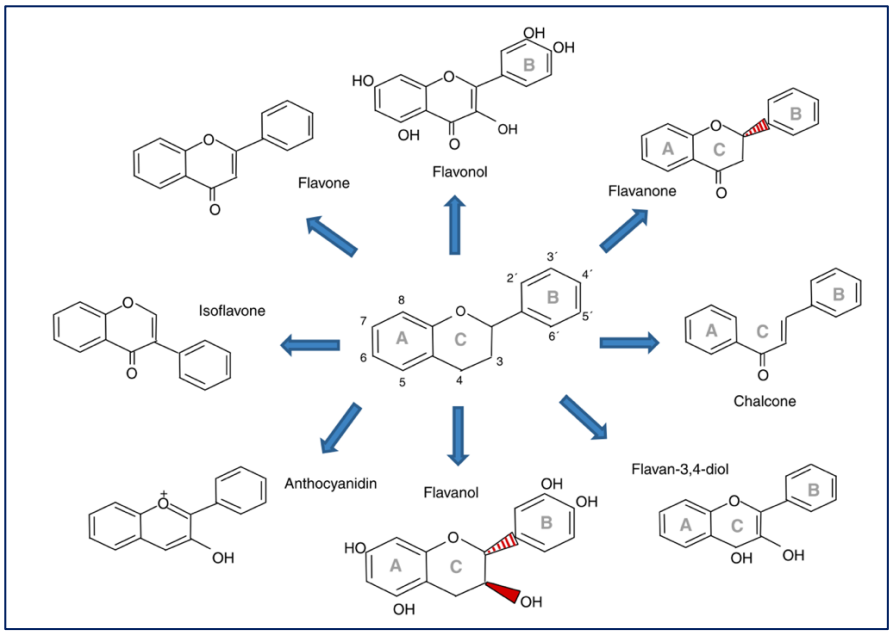


**Figure 1. Chemical structures of the flavonoid subgroups.** Adapted from [46].

EC is a monomer that belongs to the flavan-3-ols subgroups. It has hydroxyl residues at C5 and C7 (ring A), C3 (ring C), and C3' and C4' (ring B). Flavan-3-ols have two chiral centers at C2 and C3 (ring C) and thus produce four stereoisomers: (+)-catechin, (-)-catechin, (-)-epicatechin, and (+)-epicatechin **(Figure 2)**. Among them, (+)-catechin (*trans*) and (-)-epicatechin (*cis*), are commonly found in nature [45].

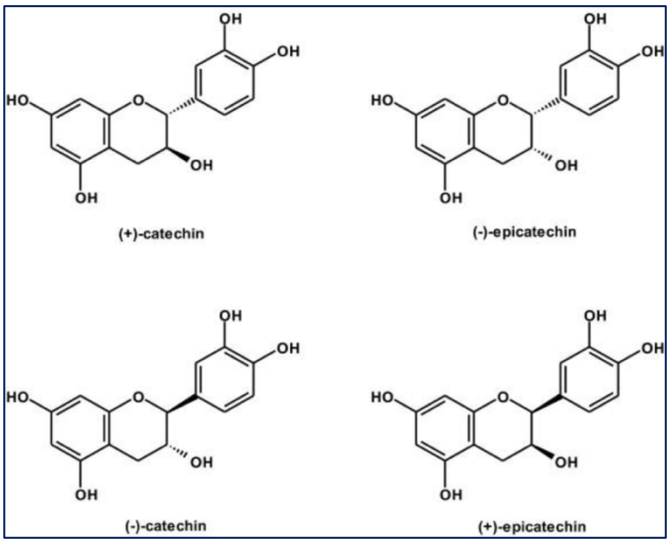


**Figure 2. Flavan-3-ol stereoisomers: (+)-catechin, (-)-catechin, (-)-epicatechin, and (+)-epicatechin.** Adapted from [47].

The stereochemical characteristics of the flavan-3-ol monomers could also influence their bioavailability. EC reaches the highest postprandial level in the circulation when equimolar amounts of the flavan-3-ol monomers are ingested by human subjects [45, 48]. The high bioavailability of EC is also evidenced by urinary and feces recovery data, analyzed after ingestion of 300 mCi (207 mmol) of radiolabeled, stereochemically pure [2-$^{14}$C]EC by human subjects (equivalent to the ingestion of 60 mg of EC) which indicate that approximately 95% of ingested EC is absorbed and excreted within 72 h, with the majority being excreted within 24 h [49]. Moreover, EC is extensively metabolized and conjugated upon ingestion. EC is glucuronidated, methylated, and sulfated in the small intestine and can be further conjugated in the liver [45, 46]. Further metabolism of unconjugated and conjugated EC can occur in the colon by the gut microflora [50, 51]. In fact, more than 20 $^{14}$C-EC-derived metabolites were detected in urine and plasma which are classified as structurally related EC metabolites (SREM), 5-carbon side chain ring fission metabolites (5C-RFM), and 3- and 1-carbon-side chain ring fission metabolites (3/1C-RFM) [49]. The mean plasma concentration of SREMs peaks about 1 h after the ingestion which represents EC absorption in the small intestine **(Table 1** and **Figure 3)**. On the other hand, the mean plasma concentration of 5C-ring fission metabolites (5C-RFMs) reaches its peak after about 6 h post consumption, which derives from the microbiota-derived catabolism of EC in the large intestine **(Table 1** and **Figure 3)**.

The absorption in the small intestine corresponds to approximately 20% of EC intake whereas the majority, approximately 70%, of EC absorption occurs in the colon [49].

| [2-$^{14}$C](−)-Epicatechin metabolites *(number of isomers)* | $C_{max}$ (nM) | $T_{max}$ (h) | $AUC_{0\text{-}24h}$ (nM/h) | $T_{1/2}$ (h) |
|---|---|---|---|---|
| (−)-Epicatechin-3′-*O*-glucuronide | 359 ± 23 | 0.8 ± 0.1 | 1624 ± 332 | 2.0 ± 1.1 |
| (−)-Epicatechin-7-*O*-glucuronide† | 22 ± 7 | 0.8 ± 0.1 | 45 ± 14 | 1.7 ± 0.3 |
| (−)-Epicatechin-3′-sulfate | 191 ± 17 | 1.1 ± 0.1 | 633 ± 135 | 2.0 ± 0.3 |
| (−)-Epicatechin-5-sulfate | 32 ± 4 | 0.8 ± 0.1 | 92 ± 25 | 1.9 ± 0.3 |
| (−)-Epicatechin-7-sulfate† | 19 ± 3 | 1.1 ± 0.2 | 43 ± 16 | 2.2 ± 0.4 |
| 3′-*O*-Methyl-(−)-epicatechin-4′-sulfate | 53 ± 13 | 0.9 ± 0.1 | 203 ± 69 | 2.3 ± 0.6 |
| 3′-*O*-Methyl-(−)-epicatechin-5-sulfate | 240 ± 24 | 0.9 ± 0.1 | 915 ± 178 | 1.6 ± 0.1 |
| 3′-*O*-Methyl-(−)-epicatechin-7-sulfate | 159 ± 20 | 1.1 ± 0.1 | 1026 ± 260 | 2.4 ± 0.2 |
| 4′-*O*-Methyl-(−)-epicatechin-5-sulfate | 53 ± 14 | 0.9 ± 0.1 | 128 ± 45 | 1.4 ± 0.3 |
| 4′-*O*-Methyl-(−)-epicatechin-7-sulfate | 33 ± 5 | 1.4 ± 0.2 | 166 ± 54 | 2.6 ± 0.5 |
| 3′-*O*-Methyl-(−)-epicatechin-5-*O*-glucuronide† | 23 ± 5 | 0.8 ± 0.2 | 25 ± 10 | 1.3 ± 0.2 |
| 3′-*O*-Methyl-(−)-epicatechin-7-*O*-glucuronide† | 39 ± 5 | 0.9 ± 0.1 | 77 ± 31 | 1.3 ± 0.3 |
| **Sum of SREMs** | **1223 ± 104** | **1.0 ± 0.1** | **4943 ± 471** | **1.9 ± 0.1** |
| 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate | 272 ± 56 | 6.4 ± 1.0 | 7595 ± 2684 | 6.3 ± 1.7 |
| 5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-*O*-glucuronide | 52 ± 9 | 6.1 ± 0.8 | 1329 ± 396 | 4.4 ± 1.3 |
| 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-*O*-glucuronide | 125 ± 30 | 6.8 ± 0.8 | 1908 ± 787 | 3.1 ± 0.6 |
| 5-(Phenyl)-γ-valerolactone-*O*-sulfate-*O*-glucuronide *(2)* | 39 ± 9 | 5.5 ± 1.1 | 1017 ± 481 | 6.4 ± 2.2 |
| 5-(Hydroxyphenyl)-γ-hydroxyvaleric acid-sulfate (2) | 56 ± 9 | 5.9 ± 0.6 | 1492 ± 475 | 7.6 ± 3.5 |
| 5-(3′-Hydroxyphenyl)-γ-hydroxyvaleric acid-4′-*O*-glucuronide | 54 ± 14 | 4.9 ± 1.3 | 835 ± 355 | 6.5 ± 0.8 |
| **Sum of 5C-RFMs** | **588 ± 102** | **5.8 ± 0.4** | **14352 ± 2264** | **5.7 ± 0.7** |

**Table 1. Human [2-14C](−)-epicatechin metabolites present in plasma and their pharmacokinetic profiles.** Adapted from [49].

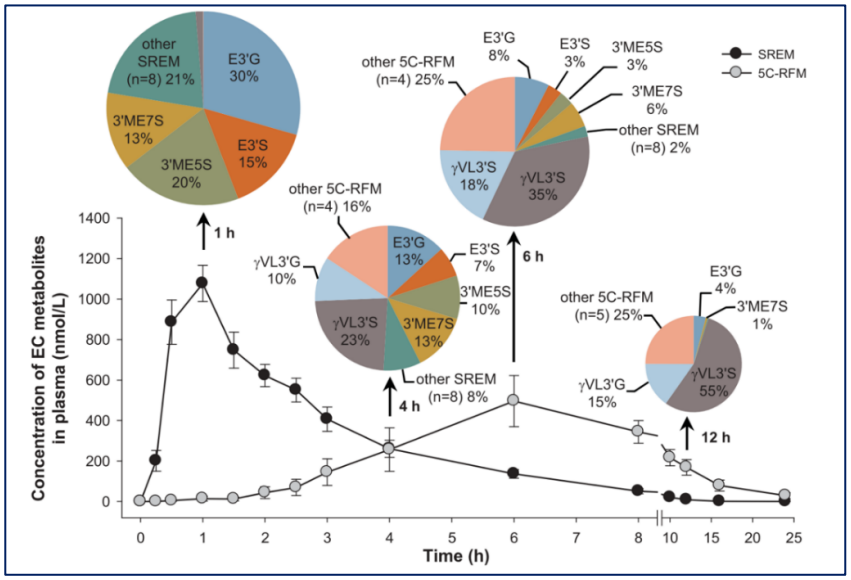


**Figure 3. Concentration and pharmacokinetic profiles of [2-14C](−)-epicatechin metabolites as a function of time.** Adapted from [49].

The high urinary and fecal recovery rates indicate that most of the metabolites are accumulated in the circulation with very low tissue accumulation upon acute EC consumption [49, 52]. EC metabolites reach high-nanomolar to low-micromolar concentration in the blood, and lower concentrations are expected to be found in tissues/organs with an exception of the GI tract where high-micromolar concentration of EC and EC metabolites can have direct contact with upon EC consumption [46]. In the brain, some EC metabolites have been detected at measurable amounts, indicating that peripheral EC metabolites could cross the BBB and may directly modulate cell signaling events inside the brain [53, 54]. Supplementation with a monomeric mixture of (+)-catechin and EC (17 mg/kg body weight) for 10 days resulted in accumulation of free EC and ~400 nM of glucuronidated EC metabolites in rat brain [54]. Moreover, the EC colonic metabolite 5-(hydroxyphenyl)-γ-valerolactone-sulfate was detected in the brain of rats after either intraperitoneal (IP) injection of the metabolite (2 mg/kg body weight) or upon grape consumption (100 mg/ kg body weight). 5-(hydroxyphenyl)-γ-valerolactone-sulfate was also detected in the brain of pigs following a cocoa powder consumption (410 mg flavan-3-ol) [53]. However, the primary route of EC metabolites crossing the BBB and their subsequent metabolism in the brain is not yet fully understood [55].

## 3. Brain health benefits of (-)-epicatechin in context of HFD and/or associated obesity

### 3.1. Regulation of vascular function

Obesity is a known independent risk factor for the development of CVD [56, 57]. Evidence support that obesity is associated with endothelial dysfunction, which is a key early event in the pathogenesis of atherosclerosis [58]. Endothelial dysfunction is characterized by decreased bioavailability of vasodilators, particularly nitric oxide (NO), which inhibits key events in the pathogenesis of atherosclerosis such as adhesion and aggregation of platelets and leukocytes [59]. While compelling evidence suggest that

obesity is associated with endothelial dysfunction and the development of CVD [56, 57, 60, 61], consumption of flavanol rich foods can be beneficial on vascular functions. Recently, a large clinical trial (COcoa Supplement and Multivitamin Outcomes Study (COSMOS)), reported that daily supplementation of a cocoa flavanol (500 mg flavanols including 80 mg of EC) significantly reduces CVD-related death by 27% during a median follow-up of 3.6 years [33]. Another clinical trial reported that consumption of an EC-rich cocoa product elicited NO dependent vasodilation whereas consumption of flavanol-poor cocoa did not induce the same response [62]. Consistently, ingestion of a flavanol-rich cocoa drink increased flow-mediated vasodilation (FMD) and circulating NO species, and improved microvascular function in human subjects [63]. Similar effects were emulated in the same individuals who ingested pure EC (1 or 2 mg/kg body weight) [63], suggesting that the beneficial vascular effects of flavanol-rich cocoa products are largely attributed to the presence of EC.

The capacity of EC to promote vascular health can also benefit the brain function as the brain is a highly vascular organ [64]. Epidemiological and dietary intervention studies suggest that EC-rich cocoa flavanols exert positive effects on cerebral blood flow and improve cognitive functions, implicating that the observed peripheral vascular effects are also present in the cerebral vascular system [36-39, 65]. For instance, in healthy adults (50-75 years), consumption of EC-rich cocoa flavanols (770 mg flavanols including 135 mg EC) for 12 weeks improved hippocampal-dependent learning suggesting that EC consumption may be associated with increased memory function in age-associated cognitive decline [36]. Consistently, intake of acute high-flavanol cocoa (681.4 mg flavanols including 150 mg EC) improved efficiency in blood oxygenation during hypercapnia in frontal cortical areas of young healthy subjects (mean age of 23.9 years) which was associated with higher performance during cognitive challenges [37]. In a genetic rat model of hypertension and ADHD, daily intake of pure EC for 2 weeks (100 mg/kg body weight) mitigated the development of hypertension and locomotor hyperactivity [66]. EC (1 mg/kg body weight) upregulated neurogenesis markers by increasing capillary density and nitrate/nitrite production

via endothelial NO synthase (eNOS) activation in mouse frontal cortex in parallel with an improvement in spatial working memory [67].

The EC-mediated improvements in vascular function can be explained by its capacity to increase eNOS expression [68], induce eNOS activation [69, 70], and inhibit enzymatic activity of arginase which competes with NOS for the same substrate, L-arginine [71]. Additionally, EC can promote vasodilation by reducing plasma endothelin-1 (ET-1), a potent vasoconstrictor that stimulates superoxide production and vasoconstriction via activation of NADPH oxidases (NOX) [72, 73]. EC can also inhibit NOX expression/activity and subsequently limits production of superoxide and increases NO bioavailability [74-76].

**3.2. Promotion of gastrointestinal health**

The GI tract provides one of the largest barriers in the body that plays a pivotal role in health and disease [13]. The intestinal barrier has a semipermeable structure that selectively allows entry of fluids, ions, and nutrients while restricts the passage of pathogenic molecules and bacteria [77]. Disturbances in GI barrier function increase paracellular translocation of pathogenic molecules such as bacterial LPS from the lumen to the circulation [13]. LPS is a major components of the outer membrane in gram-negative bacteria that initiates a strong innate immune response [78]. Toll-like receptor 4 (TLR4) is a pattern recognition receptor (PRR) that recognizes LPS, and its activation initiates downstream signaling cascades that lead to production of pro-inflammatory cytokines – such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 [78, 79] – and systemic inflammation [80].

Obesity has been associated with alterations in the intestinal barrier function [81-84] and metabolic endotoxemia [12, 85], which is characterized by low-grade elevation in plasma LPS [80]. Considering that high-micromolar concentration of EC and EC metabolites can reach the lumen of the GI

tract [46], consumption of EC can participate in maintaining GI tract homeostasis. In fact, EC supplementation (2-20 mg/kg body weight) mitigated intestinal permeabilization and endotoxemia in HFD-fed obese mice [42]. These effects were attributed to the capacity of EC to mitigate HFD-induced alterations of the tight junction (TJ), a complex transmembrane protein structure that seals spaces between intestinal epithelial cells and regulates the permeability of the intestinal barrier [42]. EC preserves TJ structure and function in part by downregulating NOXs and the redox-sensitive activation of extracellular signal-regulated protein kinases (ERK) 1/2 and nuclear factor kappa B (NF-κB) [42, 86, 87] and by increasing plasma glucagon-like peptide 2 (GLP-2) [42], a gut hormone involved in the preservation of GI mucosal trophism and function [88]. Although HFD- and obesity-induced alterations of the gut microbiota has been associated with an increase in intestinal permeability [85, 89], current evidence suggests that the EC-mediated barrier protection is not primarily due to the modulation of HFD-induced dysbiosis [42], and therefore further studies are warranted to confirm the role of EC in HFD-induced alternations of the gut microbiota.

The capacity of EC to preserve intestinal integrity and limit the entry of LPS is also relevant for the brain since LPS is a neurotoxic molecule that can induce inflammation and alterations of brain function [16, 90, 91]. LPS induces microglial activation via the TLR4-dependent pathway leading to the production and release of pro-inflammatory cytokines in the brain [16]. Cytokines can trigger the activation of brain-resident glial cells, microglia and astrocytes, and produce more pro-inflammatory cytokines, exacerbating brain inflammation [91]. Additionally, LPS can compromise the function of the BBB [92, 93], which tightly regulates the exchange of molecules between the peripheral blood and the CNS [94]. In a LPS-induced mouse model of sepsis, EC (50 mg/kg by gavage) decreased microglia activation and levels of pro-inflammatory cytokines in the hippocampus [95]. Similarly, EC (0.01-0.3 μmol/L) significantly decreased the release of TNFα in LPS/interferon-gamma (IFN-γ)-challenged glial cells [96].

Taken together, the capacity of EC to protect the intestinal barrier and mitigate metabolic endotoxemia could be beneficial to the brain structure and function in the context of obesity and other conditions that affect GI barrier function, since this action can limit the concentration of LPS reaching the brain. Interestingly, similar TJ structures to those found in the GI tract are also found in the cerebral endothelial cells of the BBB [97], which are also vulnerable to HFD [25, 98]. Although the physiological concentration of EC and EC metabolites reaching the BBB might be lower compared to the concentration reaching the intestinal barrier, EC may potentially have a direct protective effect on BBB TJ structure and function, similar those observed in the intestinal barrier [42].

### 3.3. Anti-inflammatory action

Obesity is characterized by excess adipose tissue and chronic low-grade systemic inflammation, including elevated circulating levels of pro-inflammatory molecules [99]. As discussed in section 3.2., LPS is one of the contributing factors of HFD- and/or obesity-associated systemic inflammation. Obesity-induced systemic inflammation is also thought to derive from white adipose tissue (WAT), especially from the visceral adipose tissue. The visceral depot harbors numerous immune cells, and their composition drastically change during the development of obesity [100]. HFD- and/or obesity induce a shift in WAT adipokine production profile – from producing adiponectin to leptin and monocyte chemoattractant protein-1 (MCP-1) – and an increase in the number of immune cells in the adipose tissue, including macrophages, neutrophils, and natural killer (NK) cells. The immune cells produce pro-inflammatory molecules, recruiting more pro-inflammatory cells into the visceral adipose tissue and amplifying the immune response [101].

On the other hand, EC can exert anti-inflammatory actions in the immunologically active adipose tissues [40, 41, 102]. For instance, EC mitigated HFD-induced recruitment of macrophages to the visceral

adipose tissue and decreased the elevated expression of TNFα and MCP-1 [40]. In differentiated 3T3-L1 adipocytes exposed to palmitate, EC and EC metabolites decreased the secretion of IL-6, TNFα, and MCP-1, and increased the secretion of adiponectin [40]. Additionally, EC decreased blood levels of FFA as its anti-inflammatory action reduced WAT lipolysis and the release of FFA into the circulation [103]. EC can also exert similar anti-inflammatory actions in different tissues/organs such as the liver [41] and ileum [42]. These effects can be in part explained by the capacity of EC to decrease elevated concentrations of LPS, pro-inflammatory cytokines, and FFA, and thus mitigating HFD-mediated upregulation of NOXs, protein oxidation, endoplasmic reticulum (ER) stress, and activation of NF-κB signaling pathway [41, 104].

The brain is also vulnerable to the altered levels of such circulating inflammatory mediators [15, 18]. For instance, circulating pro-inflammatory cytokines can elicit immune responses in the brain as select cytokines can be transported across the BBB into the brain via distinctive unidirectional saturable transport systems and/or via compromised BBB [18, 30]. By decreasing release of the inflammatory mediators and limiting their infiltration into the brain, EC could potentially exert neuroprotective effects and mitigate HFD-induced alteration in cognition and mood. Although the anti-inflammatory capacity of EC supplementation in the brain in context of obesity remains to be investigated, its anti-inflammatory potential has been previously described in the brain of doxorubicin-treated rats [105], aged mice [106], LPS-induced mouse model of sepsis [95], and LPS/IFN-γ-challenged glial cells [96].

### 3.4. Regulation of metabolic health

Obesity is associated to an increased risk of developing metabolic diseases such as insulin resistance and type 2 diabetes [8, 9], dyslipidemia [10, 11], and non-alcoholic fatty liver disease [107]. Substantial increases in adipose depots, especially of visceral tissues, promote low-grade inflammation that underlies metabolic dysfunction. The increased levels of FFA, LPS, and pro-inflammatory chemokines

and cytokines in obese state are involved in the development of insulin resistance [8, 108] as they can activate c-Jun N-terminal kinase (JNK), inhibitor of NF-κB (IκB) kinase (IKK), and select protein kinase C (PKC) isoforms, which phosphorylate the insulin receptor substrate 1 (IRS1) peptide in serine residues, inactivating downstream events in the insulin signaling cascade [108]. Moreover, activation of NF-κB upregulates protein-tyrosine phosphatase 1B (PTP1B), which dephosphorylates the insulin receptor (IR) and subsequently desensitizes the insulin signaling pathway [109].

Obesity and insulin resistance also contribute to alterations in lipid metabolism that can ultimately result in the development of fatty liver disease and CVD [108, 110, 111]. Pro-inflammatory molecules such as LPS, TNFα, and IL-6 stimulate adipose tissue lipolysis and lead to an increase in circulating level of FFA, a substrate for hepatic triglyceride (TG) synthesis [112]. In addition, insulin resistance blunts the lipogenic action of insulin in the adipose tissue and further induces release of FFAs into the circulation, exacerbating the dysregulation of lipid homeostasis [112]. Subsequently, these events increase TG synthesis and availability in the liver which stimulate overproduction of TG-enriched very low density lipoprotein (VLDL) particles, which contributes to an elevation in blood TG levels and hepatic lipid accumulation [112, 113].

A growing body of evidence supports protective effects of EC and EC-rich foods against alteration in lipid profiles and insulin resistance [114, 115]. For instance, a randomized, double-blind, placebo-controlled, crossover trial reported that a daily supplementation of 100 mg of pure EC for 4 weeks improved insulin sensitivity in pre-hypertensive subjects [116]. In a similar experimental design, EC supplementation improved the TG/HDL-C ratio, an indicator for insulin resistance and cardiometabolic risk, in hypertriglyceridemic subjects [117]. A cross-over study also reported the capacity of pure EC (1 mg/kg body weight) to decrease postprandial plasma TG and glucose levels in healthy subjects [118]. Interestingly, the observed beneficial effects of EC were more pronounced in overweight subjects [118]. On the contrary, a relatively lower EC amount and shorter intervention duration (25 mg/day for 2 weeks) did not have any influence upon the metabolic parameters in overweight and obese subjects [119].

The beneficial effects of EC on metabolic parameters were also demonstrated in a number of preclinical models of obesity in which EC supplementations mitigate HFD-mediated increase in plasma TG, FFA, and glucose [42, 103, 120-122]. These effects are in part attributed to the capacity of EC to downregulate select inhibitory molecules in the insulin pathway – including JNK, IKK, PKC, and PTP1B -- and mitigate HFD-induced impairment of IR and IRS1 activating and inhibitory phosphorylation in the liver and adipose tissue [103]. EC also restores HFD-/palmitate-induced decrease in secretion of adiponectin [40, 121], which regulates glucose and lipid metabolism [123]. The improvement in insulin sensitivity can also be attributed to the action of EC to protect pancreatic β-cells [124, 125] and stimulate insulin secretion [126].

The capacity of EC to promote metabolic health may be relevant for neuroprotective effect as metabolic diseases have been related to the development of cognitive and mood disorders [14, 17]. For example, oxidized low-density lipoprotein (oxLDL) induced neurotoxicity in striatal neurons while EC and 3-O-methyl-EC inhibited oxLDL-induced neuronal cell death in part by inhibiting JNK and caspase-3 activation [127]. In elderly adults with mild cognitive impairment, consumption of a high-flavanols (993 mg flavanols including 185 mg EC) and an intermediate-flavanols cocoa drink (520 mg flavanols including 95 mg EC) for 8 weeks improved cognitive function [128]. Interestingly, this improvement was associated with an improved insulin sensitivity [128], further supporting the potential role of EC in mitigating obesity-associated cognitive and mood disorders by attenuating metabolic dysfunction.

### 3.5. Neuroprotective action

A number of randomized control trials reported beneficial effects on human cognition upon EC administration as part of cocoa flavanols [39]. However, there is no study yet conducted to investigate the neuroprotective effect of pure EC in humans. Most of the clinical trials attribute the improvement in

cognitive function to the capacity of cocoa flavanols to improve cerebrovascular function [39]. *In vivo* and *in vitro* studies investigating the effects of pure EC on cognition and mood suggest additional mechanisms that may explain the neuroprotective effects of EC. These mechanisms include the capacity of EC to modulate gene expression, protein synthesis, and cell signaling events relevant to synaptic plasticity [129, 130], neurotrophic factor and monoaminergic system [131, 132], neurodegeneration, and neuroinflammation [54, 67, 95, 106, 133, 134]. For instance, daily dietary supplementation of 2.5 mg EC for 6 weeks enhanced the retention of spatial memory, hippocampal angiogenesis, and neuronal spine density in mice [129]. These effects were accompanied by increased expression of genes associated with learning, synaptic plasticity, and angiogenesis and decreased expression of genes related to learning deficits and neurodegeneration in the hippocampus [129]. In agreement with these finding, EC (100-300 nmol/L) activated cAMP-response element binding protein (CREB), a regulator of neuronal viability and synaptic plasticity, and subsequently increased CREB-mediated gene expression in cortical neurons [130]. EC also mitigated the decrease in synaptic proteins, neuronal loss, and neuroinflammation in the hippocampus of LPS-treated mice, improving recognition memory and spatial learning and memory [95].

Moreover, interventions with EC have been shown to ameliorate the pathogenesis of AD in rodents. Addition of EC to drinking water (approximately 15-20 mg of daily EC ingestion) for 3 weeks inhibited amyloid precursor protein (APP) processing [135] and reduced hyperphosphorylation of tau in mouse brain [133], mitigating the amyloid beta (Aβ) burden and AD progression. Daily ingestion of 40 mg EC/kg body weight for 9 months decreased Aβ accumulation in the brain and serum, and reduced circulating level of TNFα [136]; while daily intake of 50 mg EC/kg body weight for 4 months alleviated deficits in spatial learning and memory and upregulated brain-derived neurotrophic factor (BDNF) level in the hippocampus of APP/PS1 transgenic mice [132].

EC appears to play a role in not only preserving memory and learning but also improving mood. EC (1 mg/kg body weight) oral gavage twice daily for 5 weeks induced resilience to stress-associated

depression in mice [137]. Four weeks of daily EC oral gavage (1 mg/kg body weight) improved object recognition memory as well as anxiety in aged mice [106]. The improvements in cognition and mood were in parallel with the decreases in systemic inflammation, neuroinflammation, and hyperphosphorylation of tau in the hippocampus [106]. Consistently, daily consumption of EC (4 mg in water) for 14 weeks reduced anxiety in mice, which is explained by the capacity of EC to upregulate tyrosine hydroxylase and BDNF and downregulate monoamine oxidase-A levels in the hippocampus [131].

The neuroprotective effects of EC can also be in part explained by its capacity to induce global genomic modifications [138, 139]. SREMs and gut microbiome-derived EC metabolites simultaneously modulated the expression of protein-coding genes (mRNA) and non-coding genes, including microRNAs (miRNA) and long non-coding RNAs (lncRNA) involved in cell adhesion and endothelial permeability in human brain vascular endothelial cells (HBMEC) [138, 139]. EC metabolites counteracted lipid stress- [138] and TNFα-induced [139] alterations of gene expression profiles in an *in vitro* model of the BBB, suggesting a role for EC in protecting the BBB barrier function via multi-genomic regulations.

### 3.6. Mechanisms responsible for the neuroprotective effects of EC

Although the exact mechanisms responsible for the neuroprotective effects of EC have not yet been fully elucidated, both direct and indirect effects of EC on the brain have been suggested. If the peripheral EC metabolites can cross the BBB, they could directly modulate cell signaling events inside of the brain. Indeed, some EC metabolites have been detected in the brain at measurable amounts supporting their direct actions in the CNS [53, 54]. The indirect neuroprotective mechanisms of EC could include its capacity to 1- improve cerebral blood flow, 2- reduce systemic inflammation and infiltration of pro-inflammatory molecules (i.e. cytokines, chemokines, LPS, FFA) into the CNS, 3- preserve the integrity of the BBB, 4- regulate levels and/or the activity of receptors at the BBB and brain structures

(i.e. EC mitigates overexpression of TLR4 receptor in the kidneys [44] and proanthocyanidins, oligomer of monomeric flavan-3-ols, block the binding of LPS to TLR4 in human embryonic kidney cells (HEK 293) [140] and 5-induce multi-genomic modifications.

## 4. High fat diet and obesity: cognition and mood

### 4.1. Inflammation

Growing evidence support that HFD and/or associated obesity contribute to the development of neuroinflammation, which may underlie obesity-associated cognitive and mood dysfunction [141]. Studies with animal models provide evidence that the hypothalamus, a structure critical for regulating food intake and energy expenditure, is the first brain region affected by HFD consumption [142, 143]. The hypothalamus senses circulating nutrients and hormones important for energy homeostasis via the median eminence, an interface between the neural and peripheral endocrine systems that lacks the BBB [144, 145], which in part explains the early vulnerability of this structure HFD. Indeed, after only 1 to 3 days of consuming a HFD, hypothalamic neuroinflammation is already observed along with activation of microglia and astrocytes in both rats and mice without any substantial weight gain [143]. Hypothalamic inflammation was transient upon acute HFD consumption; however, upon chronic consumption and body weight gain, inflammation and reactive gliosis were permanent [143]. In agreement with these findings in the animal model, hypothalamic gliosis was evident in obese human subjects [143].

Chronic HFD consumption and the development of obesity can induce neuroinflammation and affect the structure and functions of other brain regions [141]. The hippocampus is particularly vulnerable to HFD-/obesity-induced alterations [22-26]. As discussed in the previous sections, obesity chronically alters the levels of numerous mediators (i.e. cytokines, chemokines, LPS, FFA, leptin, adiponectin, etc.) which can contribute to the development of neuroinflammation [14-18]. Long-term consumption of a HFD

(60% kcal from fat) induced insulin resistance and increases blood TNFα, alongside hippocampal inflammation and diminished spatial learning and memory in mice [22]. Similarly, HFD consumption (60% kcal from fat) induced hyperglycemia and increased levels of pro-inflammatory cytokines, IL-6, IL-1β, and TNFα, in the hippocampus of obese rats which was accompanied by anxiety- and depression-related behaviors [23]. HFD-induced obesity also upregulated TLR4 and downstream signaling molecules (myeloid differentiation primary response protein 88 (MyD88), transforming growth factor-β activated kinase 1 (TAK1) and IκB) in rat hippocampus, in parallel with hippocampal neuroinflammation and impairment in working memory [146].

Obesity-induced systemic inflammation and metabolic dysfunctions can also compromise hippocampal function as inflammatory mediators can be transported across the BBB and/or compromise its barrier function and subsequently infiltrate into the brain [30, 146]. The BBB in the hippocampus was shown to be compromised in rats chronically challenged with a HFD (40% kcal from fat), which was associated with an impairment in a hippocampus-dependent cognitive function [26]. In addition, the chronic low-grade inflammatory state contributes to the development of metabolic disorders, which can also contribute to the neuroinflammatory status and alterations in cognition and mood. Indeed, insulin resistance is associated with the development of cognitive impairment, neurodegeneration [147-149], and mood disorders [150-152]. Finally, hypertriglyceridemia also represents one of the potential mechanisms by which obesity can induce cognitive impairment [153, 154].

**4.2. Glucocorticoids signaling**

The hypothalamic–pituitary–adrenal (HPA) axis is a key neuroendocrine system that is critical during the stress response [155]. The HPA axis activity is regulated by the secretion of adrenocorticotrophic hormone-releasing factor (CRF) and vasopressin (AVP) from the hypothalamus,

which in turn stimulate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland. ACTH then signals the adrenal cortex to release the glucocorticoids (cortisol in humans, corticosterone in rodents) [155]. Two glucocorticoids receptors mediate the majority of the effects of cortisol and corticosterone in the brain to support adaptation to stress: high-affinity mineralocorticoid receptors (MR) and lower-affinity glucocorticoid receptors (GR) [156]. Both receptors are present in multiple target tissues including the hippocampus, which plays a significant role in negative feedback inhibition of the HPA axis [157]. Dysregulation of the feedback inhibition and subsequent hypersecretion of glucocorticoids are linked to impaired cognitive function and alterations in mood [158, 159].

It has been suggested that obesity is associated with hyperactivity of the HPA axis [28, 160, 161], suggesting that the dysregulation of the axis may be involved in obesity-associated functional and structural alterations of the brain. For instance, obese individuals with type 2 diabetes showed impaired HPA axis feedback control in parallel with verbal memory deficits and reduction in hippocampal and prefrontal volumes [28]. Additional studies report a link between HFD/obesity and elevated levels of glucocorticoids [162-165], which is associated with decreased hippocampal neurogenesis [166], impairment in cognitive function [167], and alteration in mood [168]. In addition, altered levels of GR and MR has been observed upon HFD consumption. In female rats, HFD consumption (60% kcal from fat) for 10 weeks induced anxiety-like behavior, which was associated with decreased MR and GR expression in the hippocampus [169]. Although the mechanisms that lead to the downregulation of the receptors in obesity are not fully understood, it is possible that it may be associated with increased secretion of glucocorticoids [170, 171]. In fact, administration of corticosterone significantly decreased both GR and MR in multiple brain areas of rats including the hippocampus [170, 171], which may represent a mechanism to compensate for glucocorticoid overexposure [172]. Prolonged downregulation of GR could be detrimental to the brain as deletion or deficit of GR in the multiple brain regions including the hippocampus was shown to be associated with impaired negative feedback regulation of the HPA axis and

increased anxiety- and depression-related behavior [173, 174]. Moreover, inhibition or genetic disruption of MR decreased adult rat hippocampal cell proliferation [175] and mouse neurogenesis [176].

The HFD-/obesity-associated alteration in the brain function could be, in part, explained by the capacity of glucocorticoids to potentiate inflammatory responses in the hippocampus [161, 177, 178]. For example, a short-term HFD (60% kcal from fat) consumption elevated hippocampal corticosterone and upregulated the expression of neuroinflammatory priming signals [161]. A low-dose LPS immune challenge potentiated neuroinflammatory responses in the hippocampus of the rats and induced a memory decline [161]. Blocking the corticosterone action with mifepristone, a glucocorticoid receptor antagonist, prevented the priming, pro-inflammatory response to the LPS, and memory impairment, implying that these events are mediated by glucocorticoids signaling [161]. Consistently, hippocampal neuroinflammation manifested in obese and diabetic *db/db* mice was prevented by a pharmacological inhibition of corticosterone synthesis, which in turn mitigated microglial reactivity and accumulation of pro-inflammatory cytokines [162].

### 4.3. Brain-derived neurotrophic factor (BDNF)

The hippocampus has a critical role in learning and memory consolidation [19], as well as in regulation of mood and emotion [20, 21]. BDNF is abundantly expressed in the hippocampus and plays a pivotal role in the development, survival, and differentiation of many types of neurons [179]. BDNF expression is regulated by multiple signaling pathways, including CREB signaling, which regulates the expression of genes that promote synaptic and neural plasticity [180]. Activated CREB promotes the expression of BDNF, which can in turn leads to the activation of CREB through tropomyosin receptor kinase B receptors (TrkB) [180]. BDNF also plays a critical role in hippocampus-dependent learning and mood regulation as it promotes neurogenesis and facilitates long-term potentiation in the hippocampus

[20, 181, 182]. Indeed, a decrease in the hippocampal BDNF expression is associated with aging, neurodegeneration, and psychiatric disease [182-184].

HFD consumption and obesity have been associated with downregulation of BDNF in the hippocampus, which may induce impairment in learning and memory [185-188], as well as alterations in mood [189, 190] by interfering with hippocampal function. For instance, rats fed with a high-fat/high-glucose diet for 8 months exhibited impaired learning and long-term potentiation, and reduction in dendritic spine density with a significant decrease in hippocampal BDNF levels compared to the controls [185]. Similarly, two months on a diet rich in saturated fat and refined sugar reduced hippocampal BDNF levels and spatial learning performance. These alterations occurred along with a decrease in levels of hippocampal genes and protein important for neurotransmitter release (synapsin I), neuronal viability and synaptic plasticity (CREB), and axonal growth and neurotransmitter release (growth-associated protein 43 (GAP-43)) [188]. In humans, patients with rare genetic disorders that cause BDNF haploinsufficiency or mutations inactivating the BDNF receptor exhibit severe early-onset of obesity, hyperphagia, intellectual disabilities, and hyperactivity [191].

Although the mechanism underlying HFD-/obesity-induced decrease in the hippocampal BDNF is not fully understood, evidence suggests that inflammation can partially explain the reduction in BDNF and associated alterations in cognition and mood [187, 192]. For instance, serum concentrations of BDNF decreases in patients undergoing IFN-α therapy [193]. Low BDNF and high pro-inflammatory cytokine levels are independently associated with the development of depressive symptoms in the patients [193]. Long-term activation of microglia induced by LPS in rats results in impairment in learning and memory along with increase in IL-1β and TNFα level and decrease in expression of BDNF and its receptor, TrkB in the hippocampus [194]. An *in vitro* study also showed that IL-1β inhibits the neuroprotective effects of BDNF by altering PI3-K/Akt and MAPK/ERK signaling pathways and decreasing the activity of the CREB transcription factor in neuronal cells [195].

### 4.4. Gut microbiota

The GI tract harbors a complex and dynamic population of microorganisms, collectively termed the gut microbiota, which have significant influence on the host during homeostasis and disease states [196, 197]. A mounting body of evidence suggests that the gut microbiota mediates the host's physiology including immune [198, 199], metabolic [200, 201], and neural [202, 203] function. Numerous factors contribute to shifts in gut microbiota composition such as diet, exercise, antibiotics, infection, and disease [204]. Studies suggest that alterations in gut microbiota diversity and composition are associated with metabolic abnormalities including obesity and insulin resistance [205-208]. For instance, colonization of germ-free wild-type mice with a gut microbiota from obese (*ob/ob*) mice [205] and humans [209] resulted in a significant increase in adiposity than colonization with a microbiota from lean subjects.

The role of the gut microbiota is also implicated in obesity-associated cognitive dysfunctions [27, 210, 211]. The gut microbiota and host nervous system communicates bidirectionally via neural, hormonal and immunological routes, and dysfunction of this brain-gut axis can lead to neuropathological consequences [206]. A recent study reported that a specific gut microbiome profile is linked to several memory domains and to the volume of hippocampus and prefrontal regions differentially in human subjects with and without obesity [27]. Mice receiving microbiota transplantation from obese subjects exhibited memory impairments similar to the impairment manifested in obese subjects [27]. Additionally, HFD-fed (60% kcal from fat) obese mice developed anxiety- and depression-like behaviors as well as central insulin resistance and neuroinflammation [210]. These HFD-induced alterations were transferable to germ-free mice by fecal transplantation and reversible with treatment with antibiotics [210], suggesting a consequential role of obese microbiome in the CNS.

The gut microbiome can exert neural effects via several mechanisms [202]. Some gut microbes and their metabolites may target the brain through neuroendocrine mechanisms [206]. For example,

some gut bacteria produce neuroactive metabolites, such as serotonin and γ-aminobutyric acid (GABA), which plays significant role in mood regulation, cognitive functions, and appetite control [212]. Indeed, the HFD consumption significantly altered the levels of select metabolites (tryptophan, GABA, amino acids, and multiple acylcarnitines) as well as of BDNF in the brain and the plasma of the obese mice [210]. The intestinal microbiome can also act on the brain directly as some metabolites derived from gut microbes or neurotransmitters have the potential to stimulate the vagus nerve, which connects the enteric nervous system to the CNS, and subsequently influence brain function [202, 213]. Further research identifying the role of gut microbiota in the brain function in context of obesity and microbiota-targeted interventions for HFD- and obesity-associated alterations in the brain function is warranted.

## 5. Summary of current knowledge

Obesity is associated with increased occurrence of cognitive and mood disorders. While HFD and subsequent obesity can have detrimental impact on the brain [3-7, 15, 141], dietary bioactives may mitigate some of these harmful effects. EC is one of the most widely consumed flavanols by humans, and its beneficial effects in mitigating comorbidities of obesity have been reported [104]. The underlying mechanisms of the beneficial effects of EC in obesity are explained in part by its capacity to mitigate inflammation, oxidative and ER stress. EC also has a neuroprotective potential; EC-rich cocoa flavanols have shown to improve cognitive function by increasing cerebrovascular function. Additionally, EC may exert neuroprotective effects by mitigating endotoxemia and systemic inflammation and attenuating metabolic disorders in obesity.

Although there are studies implicating relationship between obesity and cognitive impairment [3-7, 15, 141]; and indicating beneficial neuroprotective effects of EC [39, 54, 67, 95, 106, 129-137], the anti-inflammatory capacity of EC in the brain in obesity remains to be explored. Therefore, investigating the

capacity of EC to mitigate HFD- and/or obesity-associated neuroinflammation and alteration in cognition and mood; and understanding the underlying neuroprotective mechanisms will be of utmost relevant as EC has potential to intervene the neuropathological consequences of obesity.

This dissertation project investigated the neuroprotective potential of EC, particularly its capacity to mitigate HFD-/obesity-associated alterations in cognition and mood using preclinical models of obesity. The specific objectives are to investigate 1- the effects of EC on memory and learning and its capacity to mitigate neuroinflammation in the hippocampus of HFD-fed obese mice, 2- long-term effects (24 weeks) of EC on HFD-induced alteration in memory and mood and gut-microbiota in obese mice, and 3- the underlying mechanisms of EC actions at the hippocampus using a multi-genomic and bioinformatic approach. This research work will contribute to the explanation of mechanisms related to the neuroprotective potential of EC in context of HFD and associated obesity.

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## Chapter 2

# (-)-Epicatechin mitigates high fat diet-induced neuroinflammation and altered behavior in mice

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# (–)-Epicatechin mitigates high fat diet-induced neuroinflammation and altered behavior in mice†

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Obesity is characterized by a condition of low-level chronic inflammation that can lead to altered cognition and behavior. The flavanol (–)-epicatechin (EC) has been shown to have anti-inflammatory actions in mouse models of diet-induced obesity. This study investigated the capacity of dietary EC to mitigate hippocampal inflammation and impaired memory in high fat diet (HFD)-fed mice. Healthy 6 weeks old male C57BL/6J mice (10 mice per group) were fed for 13 weeks either: a control diet (10% total calories from fat), a high fat diet (60% total calories from fat), or the control and high fat diets supplemented with 20 mg EC per kg body weight. Short-term object recognition memory was evaluated by the novel object recognition (NOR) task and spatial memory by the object location memory (OLM) task and the Morris water maze (MWM). After 13 weeks on the dietary treatments, HFD-fed mice developed obesity, which was not affected by EC supplementation. HFD consumption caused metabolic endotoxemia, and increases in parameters of hippocampal inflammation, *i.e.* mRNA levels of TLR4, Iba-1, and NOX4. All these changes were mitigated by EC supplementation. EC supplementation also significantly improved recognition memory in HFD-fed mice while neither HFD consumption nor EC supplementation affected mouse spatial memory. Overall, EC supplementation prevented short-term recognition memory impairment in HFD-induced obese mice, which could be in part due to the capacity of EC to mitigate metabolic endotoxemia and associated hippocampal inflammation and oxidative stress.

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## Introduction

Obesity has become a worldwide epidemic, and its incidence is rising at an alarming rate.[1] Chronic low-grade inflammation is an important characteristic of obesity. This chronic inflammatory condition contributes to the development of obesity-associated comorbidities, including cardiovascular disease, type 2 diabetes, insulin resistance, and cancer, resulting in serious health burdens and incalculable social and medical costs.[2–5] Moreover, in humans and rodents, obesity has been associated with increased occurrence of disorders in the central nervous system (CNS), such as mild cognitive impairment, dementia, and Alzheimer's disease.[6–11] Altered structure and function of the hippocampus, a region of the brain critical for learning and memory, is observed in obese humans and rodents.[12–16] For instance, a higher body mass index (BMI) was linked to hippocampal atrophy in males and females (60–64 years age), with those with higher BMI experiencing greater hippocampal atrophy upon an 8 years follow up.[14]

Obesity and consumption of high fat/high sugar diets are contributing factors for metabolic endotoxemia, which is defined as 0.5- to 2-fold increase in circulating bacterial lipopolysaccharides (LPS) in the circulation.[17,18] Endotoxemia is a potent trigger of inflammation that can affect both the periphery and the CNS. LPS can induce neuroinflammation by compromising the function of the blood–brain barrier, a barrier that tightly regulates exchanges of molecules between the peripheral blood and the CNS.[19,20] In obesity, chronic inflammation and increased circulating levels of metabolites and proinflammatory molecules (*e.g.* fatty acids, glucose, cytokines and LPS) can affect the functioning of the barrier.[13,21–24] In fact, the blood–brain barrier at the hippocampus is disrupted in mice chronically fed a high fat diet (HFD), which is proposed to cause neuroinflammation and impaired memory.[13,24]

While consumption of HFD and obesity can have a detrimental impact on the brain, dietary components may be able to mitigate these harmful effects. Epidemiological evidence suggests that consumption of cocoa flavanols can improve cognitive function including hippocampal-dependent memory in humans.[25–27] (–)-Epicatechin (EC) is one of the most abundant flavanols found in cocoa products and one of the most widely consumed flavanols by humans.[28] EC consumption mainly derives from tea and cocoa products, and fruits such as grapes, berries, and apples. In humans, the effect of pure EC

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on cognition has not yet been investigated. In mice, EC improved cerebrovascular function, hippocampal angiogenesis, neuronal spine density, and spatial memory.[29] Thus, EC emerges as a dietary bioactive that may have beneficial effects on behavior and cognition.

Although there are studies implicating a relationship between obesity and cognitive impairment; and indicating beneficial neuroprotective effects of EC, there are no previous studies investigating the neuroprotective actions of EC in both obese humans and animal models of obesity. In humans, improvements in cognition in an elderly population was attributed in part to an improvement in insulin sensitivity.[30] We previously showed that dietary EC supplementation mitigates obesity- and high fructose/high fat diet-induced inflammation and insulin resistance in mice.[31–35] However, the anti-inflammatory capacity of EC in mitigating obesity-induced neuroinflammation in the hippocampus and in improving obesity-associated cognitive changes have not yet been characterized. This paper investigated if dietary supplementation with EC can mitigate hippocampal neuroinflammation and improve behavior in mice fed a HFD. EC supplementation prevented HFD-induced metabolic endotoxemia and increases in parameters of inflammation and oxidative stress, improving mouse performance on the novel object recognition task.

## Materials and methods

### 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 male C57BL/6J mice (20–25 g) (10 mice per group) were fed for 13 weeks either: (A) a diet containing approximately 10% total calories from fat (C) (TD.06416, Envigo, Indianapolis, IN), (B) a diet containing approximately 60% total calories from fat (lard) (HF) (TD.06414, Envigo, Indianapolis, IN), (C) the control diet supplemented with 20 mg EC per kg body weight (CE), and (D) the HFD supplemented with 20 mg EC per kg body weight (HFE). The composition of the control and the high fat diets is listed in ESI Table 1.† 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. The amount of EC supplemented has been found to improve insulin resistance in rats fed high fructose levels[34] and in mice fed a HFD.[31] In comparison to EC intake in human populations,[36] the amount of EC supplementation is relatively high. However, it can be reached by supplementation or consumption of select EC-rich fruits/vegetables and derivatives.[28]

Body weight and food intake were measured weekly throughout the study as previously described.[31] After 13 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 3000*g* for 10 min at room temperature. Tissues were dissected and flash frozen in liquid nitrogen and then stored at −80 °C for further analysis.

### Determination of plasma metabolic parameters

Plasma LPS levels were determined using a kit from Abbexa (Abbexa, Cambridge, UK) and following the manufacturer's protocol. Triglyceride concentrations were determined using kits purchased from Wiener Lab Group (Rosario, Argentina) and glucose concentrations using a kit from Sigma-Aldrich Co (St Louis, MO), following the manufacturer's protocols.

### RNA isolation and real-time PCR (RT-PCR)

For quantitative RT-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), Iba-1 (ionized calcium binding adaptor molecule 1), iNOS (inducible nitric oxide synthase), NOX (NADPH oxidase) 2 and 4, TLR4 (toll-like receptor 4), and TNFα (tumor necrosis factor alpha) were assessed by quantitative real-time PCR (iCycler, Bio-Rad, Hercules, CA) with the primers listed in Table 1.

### Cognitive function test

Behavioral tests were performed between week 10 and 12 of the dietary intervention. After being exposed to the diets for 10 weeks, each animal was habituated in a white, square arena (40 × 40 cm) where the animal was naïve to, for 15 minutes each day for two consecutive days. Next day, short-term object recognition memory was evaluated using the novel object recognition (NOR) task. On the following day, short-term spatial memory was evaluated with the object location memory (OLM) task. At week 11, animals started training for the Morris water maze (MWM) to be evaluated for spatial learning and reference memory. Animals were acclimated to a behavioral testing room separate from the housing room at least 1 hour prior to all handlings and behavioral tests. All objects and arena were cleaned with 70% ethanol after each trial. The pool used for the MWM was emptied and cleaned daily.

### Novel object recognition (NOR) and object location memory (OLM) tasks

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 NOR task, one of the objects was changed to a novel object during the test phase (*A*, *B*). For OLM task, location of one of the objects was changed to a novel location (*A*, *B*) and each arena had

**Table 1** Primers used in the study

| Gene | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|---|---|---|
| β-Actin | TCATGAAGTGTGACGTGGACATCCGC | CCTAGAAGCATTTGCGGTGCACGATG |
| BDNF | ATGGGACTCTGGAGAGCCTGAA | CGCCAGCCAATTCTCTTTTTGC |
| Iba-1 | GTCCTTGAAGCGAATGCTGG | CATTCTCAAGATGGCAGATC |
| iNOS | CGAAACGCTTCACTTCCAA | TGAGCCTATATTGCTGTGGCT |
| NOX2 | AACTGTATGCTGATCCTGCTGC | GTTCTCATTGTCACCGATGTCAG |
| NOX4 | TGAGGAGTCACTGAACTATGAAGTTAATC | TGACTGAGGTACAGCTGGATGTTCACA |
| TLR4 | GGAAGTTCACATAGCTGAATGAC | CAAGGCATGTCCAGAAATGAGA |
| TNFα | CCCCTCAGCAAACCACCAAGT | CTTGGGCAGATTGACCTCAGC |

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 ($A'/(A + A') \times 100\%$ or $B/(A + B) \times 100\%$ respectively).[37,38] 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 sample and the testing phases were excluded from analysis.

### Morris water maze (MWM)

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 (diameter 120 cm) described above and a plexiglass platform (10 cm top diameter). Each animal was placed on the platform, which was located 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 5 times daily with a 1 hour intertrial interval.

(4) Hidden platform task (MWM days 4–7): large and high-contrast geometrical patterns made with construction papers were mounted on the walls of the testing room and the pool 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 5 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 8): 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.

### 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 plasma endotoxin concentration and a-TLR4 mRNA and b-TNFα mRNA levels, and between TLR4 mRNA and TNFα mRNA levels. Tests for interaction were performed by two-way analysis of variance (ANOVA) and post-tested using Fisher's Least Significant Difference (LSD) to examine differences between group means. Within group performance of NOR and OLM was evaluated with two-tailed paired $t$-test. Differences were considered statistically significant at $p < 0.05$. Data are shown as mean ± SEM.

## Results

### Animal outcome

Daily food intake in the groups fed the HFD was significantly lower than in those fed the control diets (Fig. 1A) while the calorie intake was similar among groups (Fig. 1C). Compared to the control diet, consumption of the HFD caused a higher increase in body weight gain, which became significant after only one week on the diet (Fig. 1B). After 13 weeks on the HFD, the average body weight of the HF group was 24% higher than that of the control group. This was accompanied by a 2.3- and 2.6-fold increase in the visceral fat pad weight in mice fed the HFD or the HFD supplemented with EC, respectively (Fig. 1C). Supplementation with EC did not affect body weight gain neither in mice fed the control diet nor the HFD. Hippocampal tissue weights were similar among groups. Ratio of hippocampus weight to body weight was similar for the HF and HFE groups (0.50 ± 0.05 mg and 0.50 ± 0.03 mg respectively).

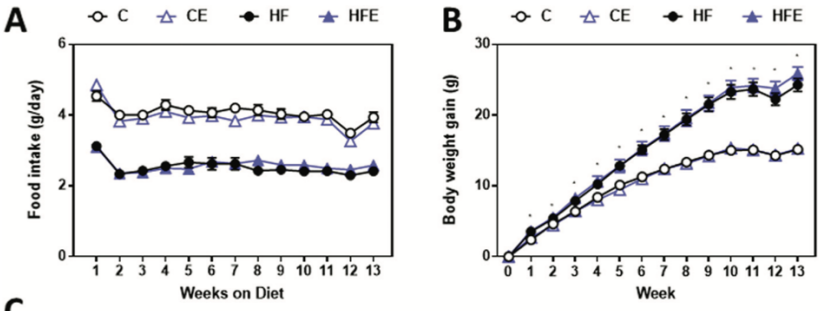
### EC supplementation attenuates parameters of neuroinflammation in the hippocampus from HFD-fed mice

To evaluate neuroinflammation, we measured the mRNA levels of TLR4, Iba-1, and TNFα in the hippocampus by RT-PCR (Fig. 2A). Consumption of the HFD did not affect hippocampal TNFα mRNA content. On the other hand, Iba-1 mRNA levels were 16% higher in HF mice than in controls, and EC supplementation prevented these increases. A significant interaction between EC supplementation and diet on TLR4 mRNA levels was observed (interaction from two-way ANOVA: $p < 0.04$). TLR4 mRNA levels were 52% higher in HF mice than in controls, and EC supplementation also fully prevented these increases.

We also measured enzymes involved in inflammation and reactive nitrogen/oxygen species (nitric oxide, superoxide anion, $H_2O_2$) production, *i.e.* NOX2, NOX4, and iNOS (Fig. 2B). NOX2 and iNOS mRNA levels were similar among groups, while 44% higher NOX4 mRNA levels were observed in the HF group compared to controls, which was prevented by EC supplementation.

### EC supplementation prevents HFD-induced metabolic endotoxemia

Consumption of the HFD caused metabolic endotoxemia in mice. LPS concentration in plasma was 32% higher in HF than in control mice, and EC supplementation prevented this increase (Fig. 3A). There was a strong positive correlation ($r$: 0.57, $p = 0.001$) between plasma endotoxin concentration and




C

| Parameter | C | CE | HF | HFE |
|---|---|---|---|---|
| Food intake (g/d) | 4.07 ± 0.07$^a$ | 3.95 ± 0.09$^a$ | 2.80 ± 0.08$^b$ | 2.87 ± 0.07$^b$ |
| Caloric intake | 15.1 ± 0.2 | 14.6 ± 0.3 | 14.4 ± 0.3 | 14.7 ± 0.3 |
| Body weight (g) | 37.0 ± 0.7$^a$ | 36.2 ± 0.8$^a$ | 46.0 ± 1.1$^b$ | 47.5 ± 1.0$^b$ |
| Brain (mg) | 432 ± 5 | 432 ± 6 | 421 ± 4 | 428 ± 6 |
| Hippocampus (mg) | 23.8 ± 1.0 | 25.8 ± 1.7 | 22.8 ± 2.5 | 24.2 ± 1.2 |
| Visceral Fat (g) | 0.66 ± 0.05$^a$ | 0.63 ± 0.04$^a$ | 1.51 ± 0.19$^b$ | 1.70 ± 0.06$^b$ |
| Epididymal Fat (g) | 1.65 ± 0.08$^a$ | 1.52 ± 0.07$^a$ | 2.15 ± 0.17$^b$ | 2.41 ± 0.16$^b$ |
| Retroperitoneal Fat (g) | 0.69 ± 0.05$^a$ | 0.66 ± 0.09$^a$ | 1.74 ± 0.14$^b$ | 1.78 ± 0.08$^b$ |
| Subcutaneous fat (g) | 2.3 ± 0.2$^a$ | 2.4 ± 0.2$^a$ | 4.9 ± 0.3$^b$ | 5.5 ± 0.3$^b$ |
| Brown Fat (mg) | 0.23 ± 0.02 | 0.24 ± 0.02 | 0.24 ± 0.03 | 0.28 ± 0.02 |
| Plasma glucose (mg/dl) | 190 ± 9$^a$ | 179 ± 5$^a$ | 247 ± 12$^b$ | 208 ± 9$^a$ |
| Plasma triglycerides (mg/dl) | 68 ± 5$^a$ | 81 ± 4$^a$ | 98 ± 5$^b$ | 101 ± 4$^b$ |

**Fig. 1** Effects of supplementation with EC on mice general outcome. (A) Food intake and (B) body weight gain. Mice were fed a control diet (empty circles), the control diet supplemented with 20 mg EC per kg body weight (empty triangles), a HFD (black circles), or the HFD supplemented with 20 mg EC per kg body weight (blue triangles). Body weight was measured weekly. *Differences between the HF and control body weight gain values are significant between week 1 and 13 on the diets ($p < 0.05$, two-way ANOVA with Fisher's LSD). (C) Mice general outcome parameters after 13 weeks on the diets. Results are shown as means ± SEM and are the average of 6–10 animals per group. Values having different superscripts are significantly different ($p < 0.05$, two-way ANOVA with Fisher's LSD).

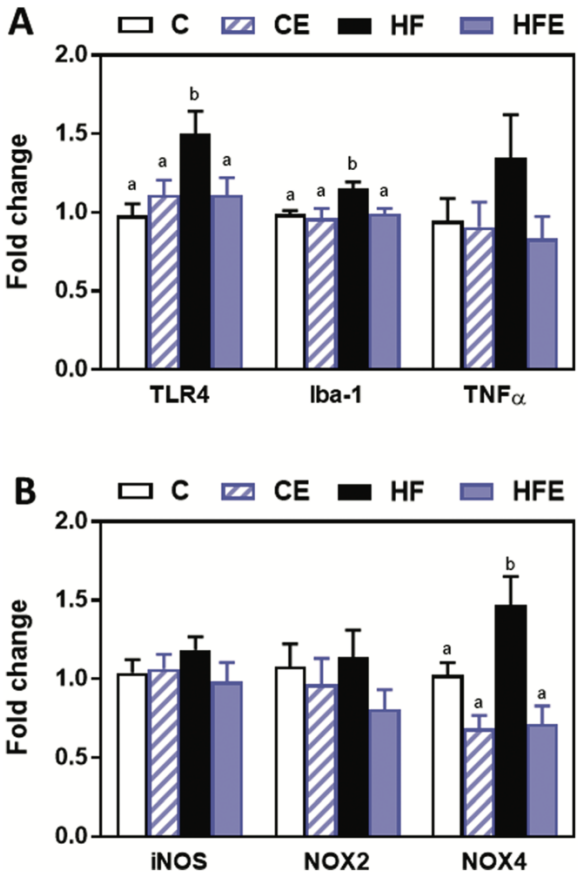



**Fig. 2** EC supplementation improves parameters of inflammation and oxidant production in the hippocampus. (A, B) TLR4, Iba-1, TNFα, iNOS, NOX2 and NOX4 mRNA levels in the hippocampus were determined by RT-PCR and the relative gene expression was normalized to β-actin as housekeeping gene. Determinations were done after 13 weeks on the respective diets. Results are shown as mean ± SEM of 6–10 animals per group. Data were normalized to control values. Values having different superscripts are significantly different ($p < 0.05$, two-way ANOVA with Fisher's LSD).

TLR4 mRNA levels in the hippocampus (Fig. 3B). Although hippocampal TNFα mRNA levels were similar among groups, a significant correlation was observed between TNFα mRNA levels and (i) plasma endotoxin ($r$: 0.41, $p = 0.03$) (Fig. 3C) and (ii) TLR4 ($r$: 0.58, $p = 0.0004$) (Fig. 3D) mRNA levels.

### EC supplementation improves novel object recognition memory in HFD-fed mice while did not affect spatial memory and 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 (Fig. 4A). Comparing within group preference index of the sample and test phase, all groups significantly preferred the novel object (ESI Fig. 1A†). During the test phase, a significant interaction between EC supplementation and diet on NOR performance was observed (interaction from two-way ANOVA: $p < 0.03$). EC supplemented HF group (HFE) exhibited greater novel object preference compared to the HF group as measured by the preference index (Fig. 4A). This demonstrates the greater ability of the EC supplemented group in recognizing the novel object from the familiar object.

EC supplementation did not affect spatial memory and learning. OLM task was conducted to assess short-term spatial memory of mice. All groups performed similarly in both sample and test phases as measured by the preference index (Fig. 4B). Interestingly, no significant within group changes in the preference index were observed in all C, CE, HF, HFE groups, indicating that there was no overall novel location preference (ESI Fig. 1B†). Spatial learning was also evaluated with Morris water maze with the hidden platform task. Comparing the first (MWM day 4) and the last day (MWM day 7) of the hidden platform task, all groups found the hidden platform more quickly (Fig. 5A). On the last day of the hidden platform task, all groups had similar escape latencies exhibiting comparable spatial learning (Fig. 5A). Similarly, all groups spent a comparable amount of time in the target southwest quadrant zone during the probe trial, indicating no differences in spatial reference memory among groups (Fig. 5B).

### 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,[39] in the hippocampus. Consumption of the HFD did not affect hippocampal BDNF mRNA content. However, BDNF mRNA levels were 90 and 76% higher in the CE and HFE groups compared to the control group (Fig. 5C).

## Discussion

This work showed that supplementation with EC improves parameters of neuroinflammation and impaired behavior in HFD-induced obese mice. Thus, consumption of the HFD caused metabolic endotoxemia and upregulation of hippocampal neuroinflammatory markers, *i.e.* TLR4, Iba-1, and NOX4, in association with impaired recognition memory. EC supplementation prevented all these changes, supporting a potential benefit of an EC-rich diet on obesity-induced inflammation and altered behavior.

The HFD induced significant increase in body weight and fat pads weight in mice after 13 weeks which was not prevented by EC supplementation. The increase in adiposity in the HF and HFE groups despite of the similar caloric intake among all four groups could be explained by the fact that different macronutrients exert differential effects on the thermic effects of food.[40] Fat is less thermogenic than carbohydrates and proteins and thus can prompt greater positive energy balance in the body compared to carbohydrates and proteins when consumed over time.[41–45]

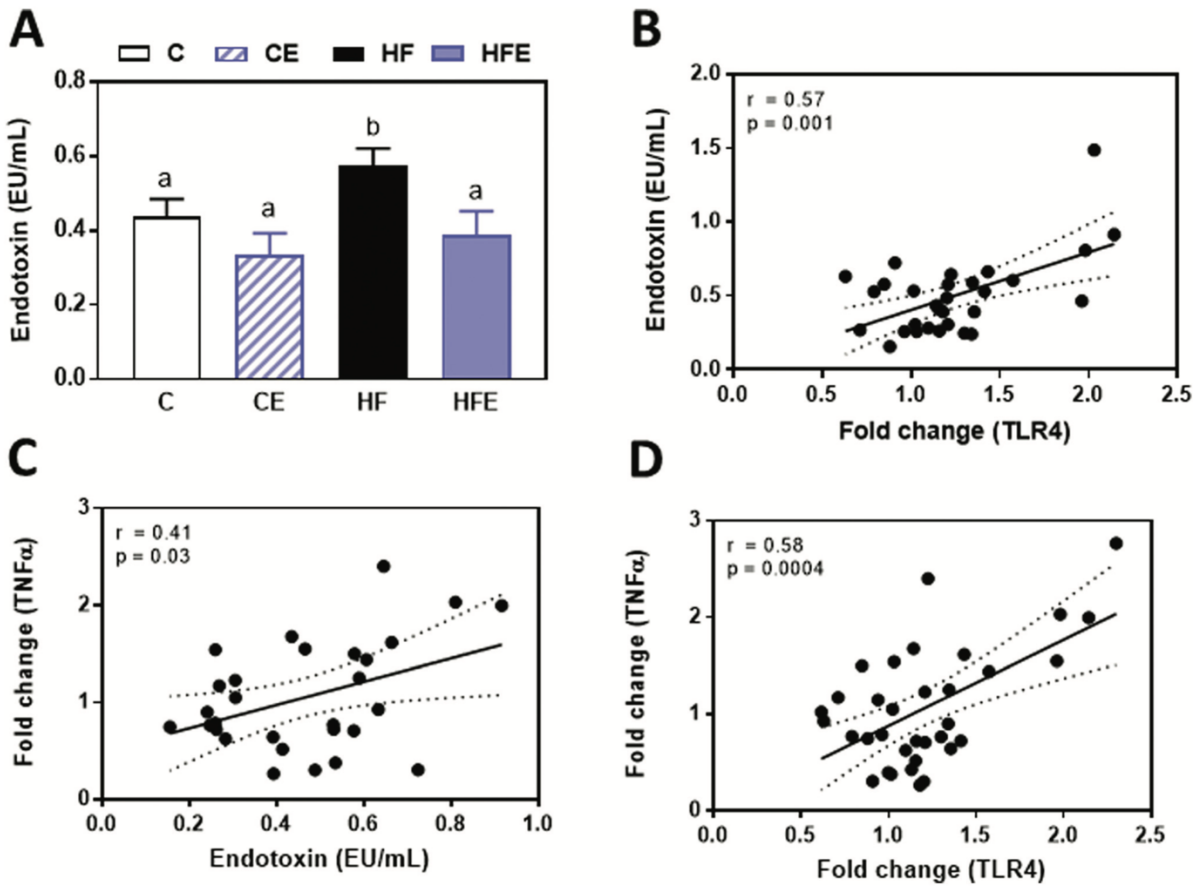



**Fig. 3** Effects of EC supplementation on HFD-induced endotoxemia. (A) Plasma endotoxin concentration on week 13 on the respective diets. Results are shown as mean ± SEM of 6–10 animals per group. Values having different superscripts are significantly different ($p < 0.05$, two-way ANOVA with Fisher's LSD). (B–D) Correlations between plasma endotoxin concentration and (B) TLR4 mRNA and (C) TNFα mRNA levels. (D) Correlation between TLR4 and TNFα mRNA levels. Solid line represents the regression line and dashed lines delineate the 95% confidence band.

EC supplementation prevented the metabolic endotoxemia associated with HFD consumption. This finding is in agreement with a similar observation in mice fed the HFD for 15 weeks,[33] in which the metabolic endotoxemia was associated with an increase in intestinal permeability. EC protects the intestinal barrier from permeabilization by inhibiting HFD-associated down regulation of tight junction proteins and by modulating signaling pathways that promote tight junction opening. In this regard, EC prevents both TNFα and bile acid-induced Caco-2 monolayer permeabilization by inhibiting NF-κB and ERK1/2.[33,46,47] On the other hand, other mechanisms could be involved in HFD-induced metabolic endotoxemia. In this regard, LPS is also transported through intestinal epithelial cells and into the lymph, upon incorporation and secretion into chylomicrons.[48] Once in the circulation, endotoxins can reach different organs and initiate pro-inflammatory responses. Endotoxins bind to the TLR4 to initiate a cascade of events that leads to the activation of, among other signals, transcription factors NF-κB and AP-1 that increase the expression of proinflammatory molecules.[49] The increased expression of TLR4 in the hippocampus of HFD-mice suggests the activation of this pathway. This is paralleled by a pro-inflammatory condition as evidenced by an increased expression of Iba-1, a protein participating in microglia endocytosis, and a trend for higher TNFα expression. This is further supported by positive correlations among plasma endotoxin levels, TLR4 and TNFα expression. Overall, the capacity of EC to prevent metabolic endotoxemia and suppress TLR4 and Iba-1 upregulation supports a link between metabolic endotoxemia and neuroinflammation. It also underscores the health relevance of the actions of EC at the gastrointestinal tract, where it prevents endotoxin transport into the circulation.[33]

While EC-mediated decrease in metabolic endotoxemia can be a relevant mechanism in EC's capacity to mitigate high fat diet-induced neuroinflammation, other potential contributing mechanism is a direct action of EC and/or EC metabolites at the level of the brain. In humans, 95% of EC is absorbed either as EC, structurally related EC metabolites (SREM) (glu-

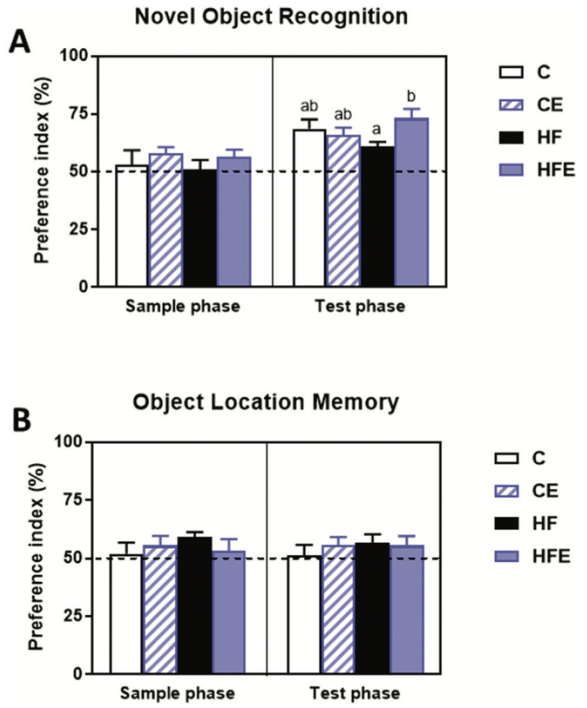



**Fig. 4** Effects of EC supplementation on short-term recognition memory and spatial memory. For both novel object recognition (NOR) and object location memory (OLM) tasks, animals explored two identical unfamiliar objects for 5 minutes (sample phase). After being placed in the home cage for 1 hour (retention phase), they were reintroduced to the arena for 5 minutes (test phase). (A) EC supplemented HF group (HFE) exhibited greater novel object preference compared to the HF group as measured by the preference index. (B) All groups performed similarly in both OLM sample and test phases as measured by the preference index. Neither HFD nor EC supplementation affected spatial memory in the OLM task. Dashed lines delineate 50% null preference. Results are shown as mean ± SEM of 8–10 animals per group. Values having different superscripts are significantly different ($p < 0.05$, two-way ANOVA with Fisher's LSD).

curonyl, methyl and/or sulphated EC derivatives) or as smaller metabolites generated after EC metabolism by the microbiota.[50] SREM, mainly catechin and EC glucuronidated derivatives, were measured in the brain of Tg2576 AD transgenic mice consuming a flavan-3-ol-rich grape powder.[51] When a synthetic SREM (3-*O*-Me-EC-5-*O*-β-glucuronide) was added to brain slices from Tg2576 AD mice, it improved basal synaptic transmission and long-term potentiation.[51] In mice fed EC for 13 days, both EC and 3′-*O*-methyl-(−) EC were detected in perfused brains.[52] This was associated to an improvement in the retention of spatial memory that was attributed to increased angiogenesis.[52] Recently, the main EC microbiota-derived metabolite 5-(hydroxyphenyl)-γ-valerolactone-sulfate was found in mouse brain after 5-(hydroxyphenyl)-γ-valerolactone i.p. injection, and in rat and pig brain upon consumption of EC-rich foods.[53] Overall, although evidence is limited, EC and

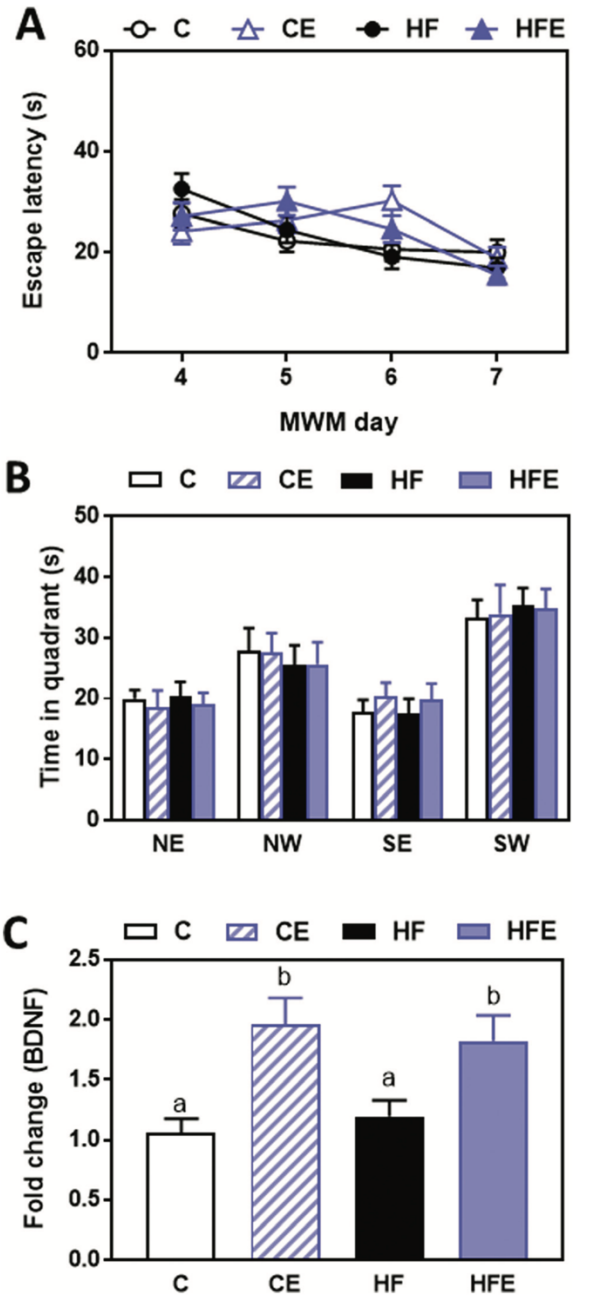



**Fig. 5** Effects of EC supplementation on spatial learning and memory and levels of BDNF in the hippocampus. (A) Learning curves of mice in the hidden platform task and (B) time spent in each quadrant during the probe trial. (C) BDNF mRNA levels were determined by RT-PCR and the relative gene expression was normalized to β-actin as housekeeping gene. Results are shown as mean ± SEM of 6–10 animals per group. Data were normalized to control values. Values having different superscripts are significantly different ($p < 0.05$, two-way ANOVA with Fisher's LSD).

SREM can reach the brain where they could have direct protective effects against neuroinflammation.

NADPH oxidase is one of the key producers of cell reactive oxygen species, in particular of superoxide anion and $H_2O_2$. Although adequate amounts of select oxidants are critical for normal brain function such as hippocampal long-term potentiation,[54] excessive generation of oxidants can result in oxidative stress.[55] Oxidative damage to cellular components may play a role in the development of cognitive impairment associated with CNS disorders.[56–58] For instance, postmortem brain tissue analysis of Alzheimer's disease patients showed an upregulation of the NOX cytosolic subunits p67phox, p47phox, and p40phox and an increase in NOX enzymatic activity.[56] In our study, EC mitigated HFD-induced increased expression of NOX4 in the hippocampus while neither HFD nor EC supplementation affected iNOS and NOX2 mRNA levels. Consumption of a HFD increases the expression of Iba-1 and NOX subunits and the activity of NOX in mouse brain compared to their age-matched controls.[59] Also, a HFD induces protein oxidation in the hippocampus of aged mice and impairs their cognitive performance in a T-maze.[58] The observed capacity of EC to regulate hippocampal regulation of NOX4 expression is in agreement with its capacity to modulate the expression and activity of several NOX isoforms and mitigate oxidative stress.[60] Collectively, current evidence suggests a relationship between oxidative stress and neuroinflammatory status in obesity that can be mitigated by consumption of EC.

Growing evidence indicates that diet-induced obesity contributes to neuroinflammation, as well as to cognitive dysfunction in rodent models.[13,16,24,61–63] Both NOR and OLM tasks are based on rodents' innate tendency to explore a novel stimulus. The increased time spent exploring the replaced or relocated object during the test phase suggests their ability to remember what type of object they were previously exposed to or where the object was previously located during the sample phase. All C, CE, HF, HFE groups had no significant novel location preference shown in OLM task, while novel object preference was observed in NOR task in all groups. It is possible that the spatial cues chosen for OLM were not recognizable or distinguishable by mice to spatially differentiate the two identical objects or the animals were simply not interested in the chosen objects.[38,64] It is also plausible that they needed a shorter retention phase or longer time to explore the objects in the arena.[64] In the current study, consumption of the HFD did not cause large effects on learning and memory. In terms of HFD-mediated cognitive impairment, it is possible that in our study the HFD did not induce cognitive impairment due to its duration. Indeed, it is suggested that specific effects of a HFD on cognition are dependent on the duration of dietary exposure.[22] For instance, while 5 weeks on a HFD (60% kcal from fat) did not impair object recognition memory in mice,[65] 21 weeks on a HFD (40% kcal from fat) impaired recognition memory.[62] In terms of the MWM, we observed that HFD-fed mice performed similarly to the control group in both the hidden platform task and the probe trial. It has been reported that mice fed a HFD (60% kcal from fat) display impaired spatial memory compared to mice fed control diets, but the duration of the HFD was longer than in our study. For instance, impaired spatial memory was observed in mice after consumption of HFD for 19 weeks,[61] 20 weeks,[13] and 5 months.[66] Therefore, future studies with longer durations of HFD consumption are warranted to investigate the effects of EC on HFD-induced impaired learning and memory in mice.

EC supplementation significantly improved recognition memory in HFD-fed mice. Several studies have characterized the effects of EC-rich cocoa on cognition in humans.[30,67] In elderly individuals with mild cognitive impairment, consumption of cocoa flavanols improved cognitive functions.[30,67] So far, most of the effects of EC on cognition have been attributed to improvements in blood flow.[68] However, EC and/or its metabolites could also act through the mitigation of HFD/obesity-induced neuroinflammation and through the observed increase in hippocampal BDNF. BDNF is a member of the neurotrophin family of growth factors crucial for the differentiation and survival of neurons. BDNF plays a critical role in the induction of hippocampal long-term potentiation, a form of synaptic plasticity considered to underlie learning and memory.[39] BDNF deletion within dorsal hippocampus of mouse impairs learning and memory in novel object recognition task and Morris water maze.[69] In humans, it has been proposed that circulating BDNF may be used as a biomarker for select psychiatric disorders.[70] Circulating and brain BDNF levels are found to be increased upon flavanol supplementation in rodents and humans. An effect of EC on BDNF metabolism is supported by findings in humans consuming EC-rich cocoa.[71] High serum levels of BDNF were found in a population of males and females (62–75 years of age) consuming 494 mg flavanols per d for 12 weeks. The increase in serum BDNF was correlated with an improvement in global cognitive performance. Adult male mice fed a control diet and administered with 4 mg EC $day^{-1}$ for 14 weeks show a decrease in anxiety assessed in open field and elevated plus maze tests, and an increase in BDNF hippocampal levels.[72] Although we also observed an increase in hippocampal BDNF mRNA levels in the EC supplemented groups, we did not observe significant changes in the open field behavior among groups (data not shown). On the other hand, the 7-times lower EC intake in our experimental model compared to this previous study,[72] can explain the differential response. Overall, given the relevant role of BDNF in neurogenesis and in supporting brain physiology, future studies investigating the mechanisms of brain BDNF increase by EC will be of outmost relevance. It is important to mention that other flavonoids can have neuroprotective actions. However, rather than on obesity-associated altered behavior, most studies were focused on ageing-related cognitive dysfunction.[73] In this regard, high consumption of green tea, which contains catechin, epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), was associated with a lower prevalence of cognitive impairment in Japanese subjects aged ≥70 years, as assessed by Mini-Mental State Examination.[74,75]

In summary, EC supplementation improved recognition memory and mitigated neuroinflammation in a model of diet (HFD)-induced obesity in mice. Among the underlying mechanisms, we provide evidence that EC can in part act by promoting BDNF upregulation and by preventing obesity-induced metabolic endotoxemia and the associated activation of pro-inflammatory responses and oxidative stress in the hippocampus. Further studies, particularly in obese humans, will be essential to support the concept that consumption of EC-rich foods could contribute to improve behavior and cognition in obesity.

## Abbreviations

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| CNS | Central nervous system |
| EC | (−)-Epicatechin |
| HFD | High fat diet |
| LPS | Lipopolysaccharides |
| iNOS | Inducible nitric oxide synthase |
| Iba-1 | Ionized calcium binding adaptor molecule 1 |
| NOX | NADPH oxidase |
| NOR | Novel object recognition |
| MWM | Morris water maze |
| OLM | Object location memory |
| TLR4 | Toll-like receptor 4 |
| TNFα | Tumor necrosis factor alpha |

## Author contributions

J. K. and Z. W. ran all experiments. P. I. O. designed the study. J. K. and P. I. O. wrote the manuscript. All authors revised the article, critically reviewed it for intellectual content, and approved the final version.

## Conflicts of interest

There are no conflicts to declare.

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# Chapter 3

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

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

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**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, ingunal 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**.

**Table 1. Primers used in the study.**

| 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 |

*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.

**Table 2. Body and tissue weights after 24 weeks on the diets.**

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

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).

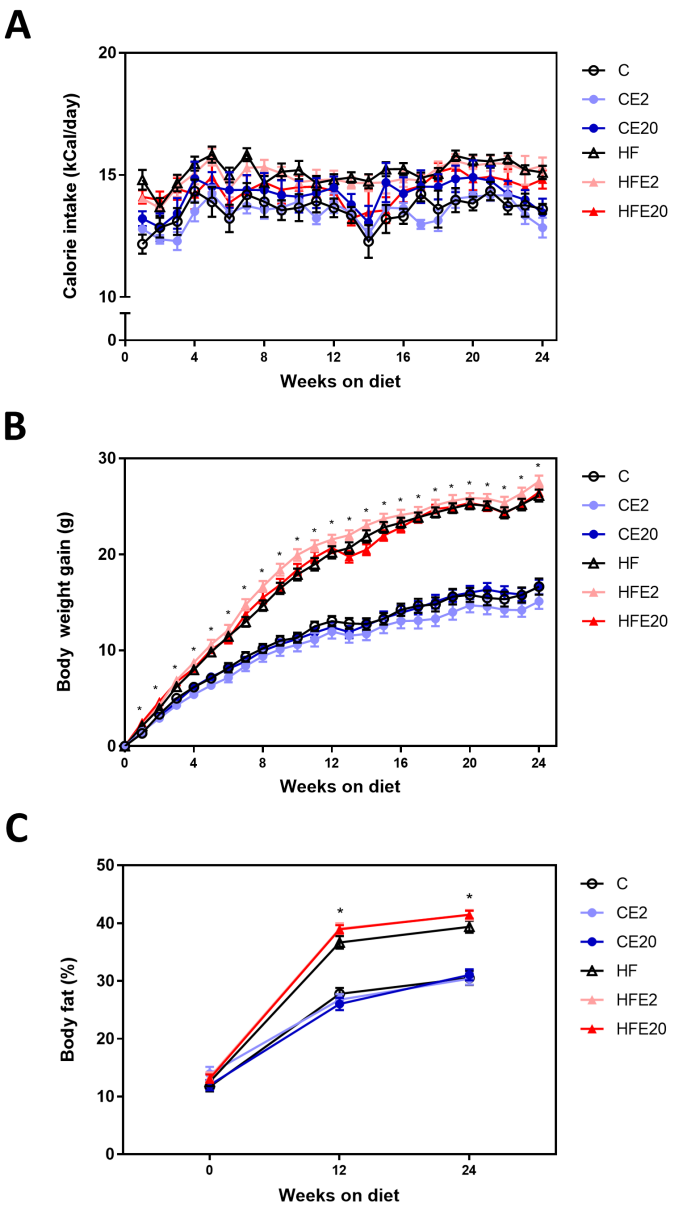



**Figure 1. Effects of supplementation with EC on body weight gain and body fat mass. A-** Calorie intake, **B-** body weight gain, and **C-** % fat mass. Mice were fed a control diet (empty circles), the control diet supplemented with 2 mg EC/kg (light blue circles) or 20 mg EC/kg (dark blue circles) 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).

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.

**Table 3. Blood parameters after 12 and 24 weeks on the diets.**

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

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).

*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.

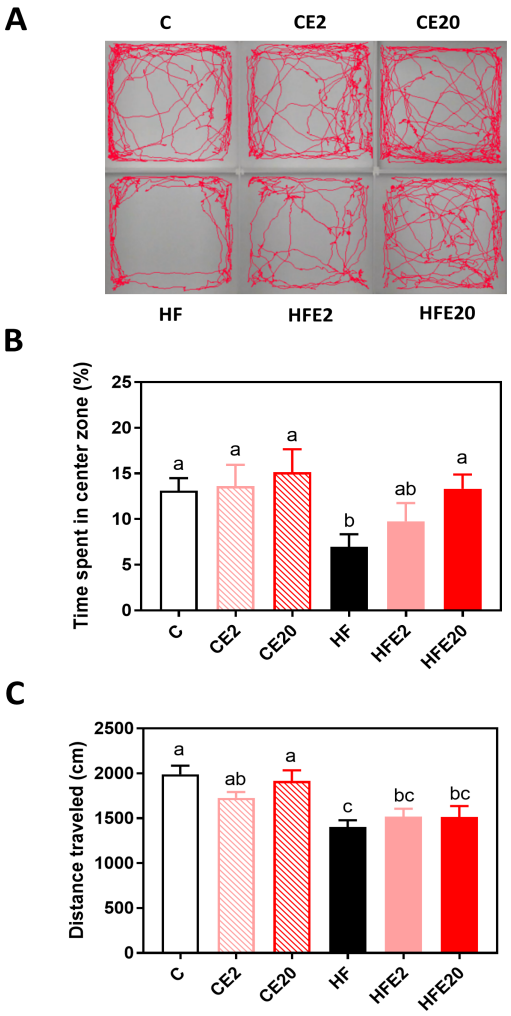



**Figure 2. EC supplementation mitigates anxiety-related behavior in mice in a dose-dependent manner.** **A-** Representative tracks of C, CE2, CE20, HF, HFE2, and HFE20 mice in the open field arena over 5 min. **B-** EC supplemented mice exhibit reduced anxiety-like behavior, spending significantly 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).

*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.

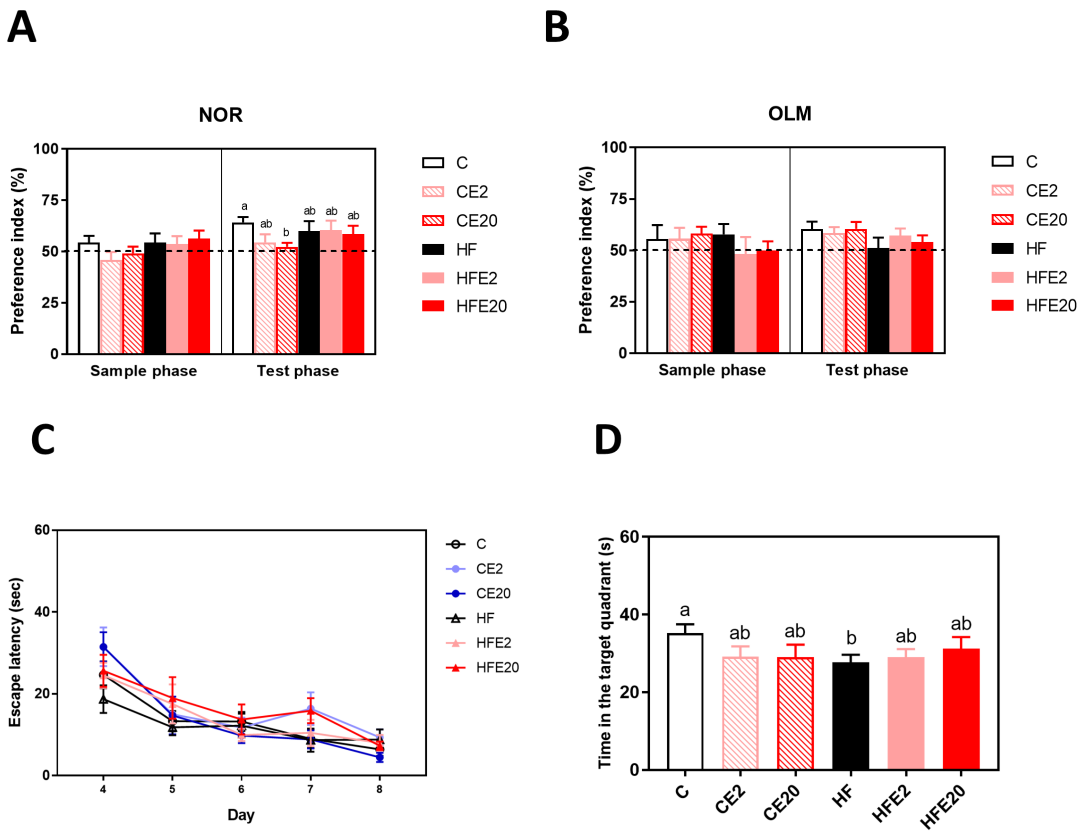



**Figure 3. Effects of EC supplementation on short-term recognition, spatial, and reference memory and spatial learning.** For both NOR and OLM tasks, animals explored two identical unfamiliar objects for 5 minutes (sample phase). After being placed in the home cage for 1 hour (retention phase), they 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).

*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**).

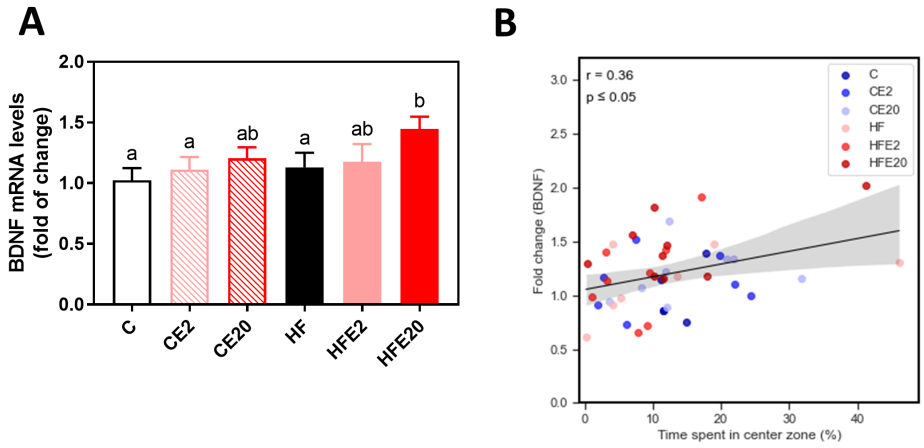



**Figure 4. Effects of EC supplementation on the anxiety-related behavior and its correlation with BDNF levels. A-** BDNF mRNA levels in the hippocampus were determined by q-PCR and the relative gene expression was normalized to β-actin as housekeeping gene. Determinations were done after 24 weeks on 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.

*3.5 EC supplementation increases the expression of the hippocampal glucocorticoid and mineralocorticoid receptors and decreases the expression of 11ß-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).

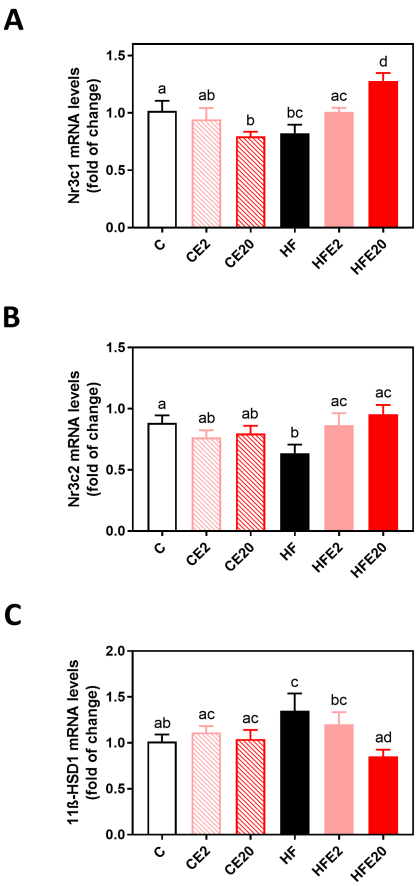



**Figure 5. Effects of EC consumption on the mRNA levels of the glucocorticoid receptors (GR; Nr3c1), the mineralocorticoids receptor (MR; Nr3c2), and 11β-HSD1.** The mRNA levels of the **A-** GR, **B-** MR, and **C-** 11β-HSD1 in the hippocampus were determined by q-PCR and the relative gene expression 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).

*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).

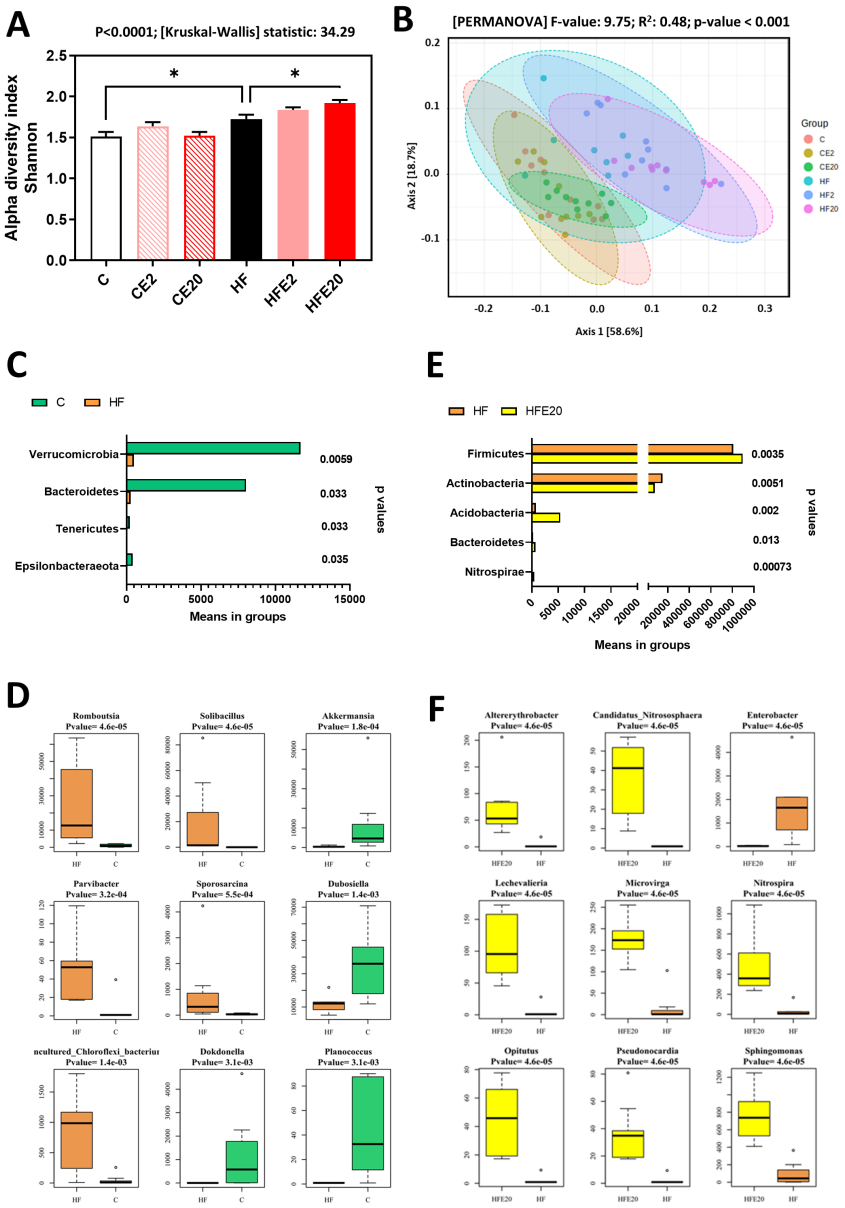
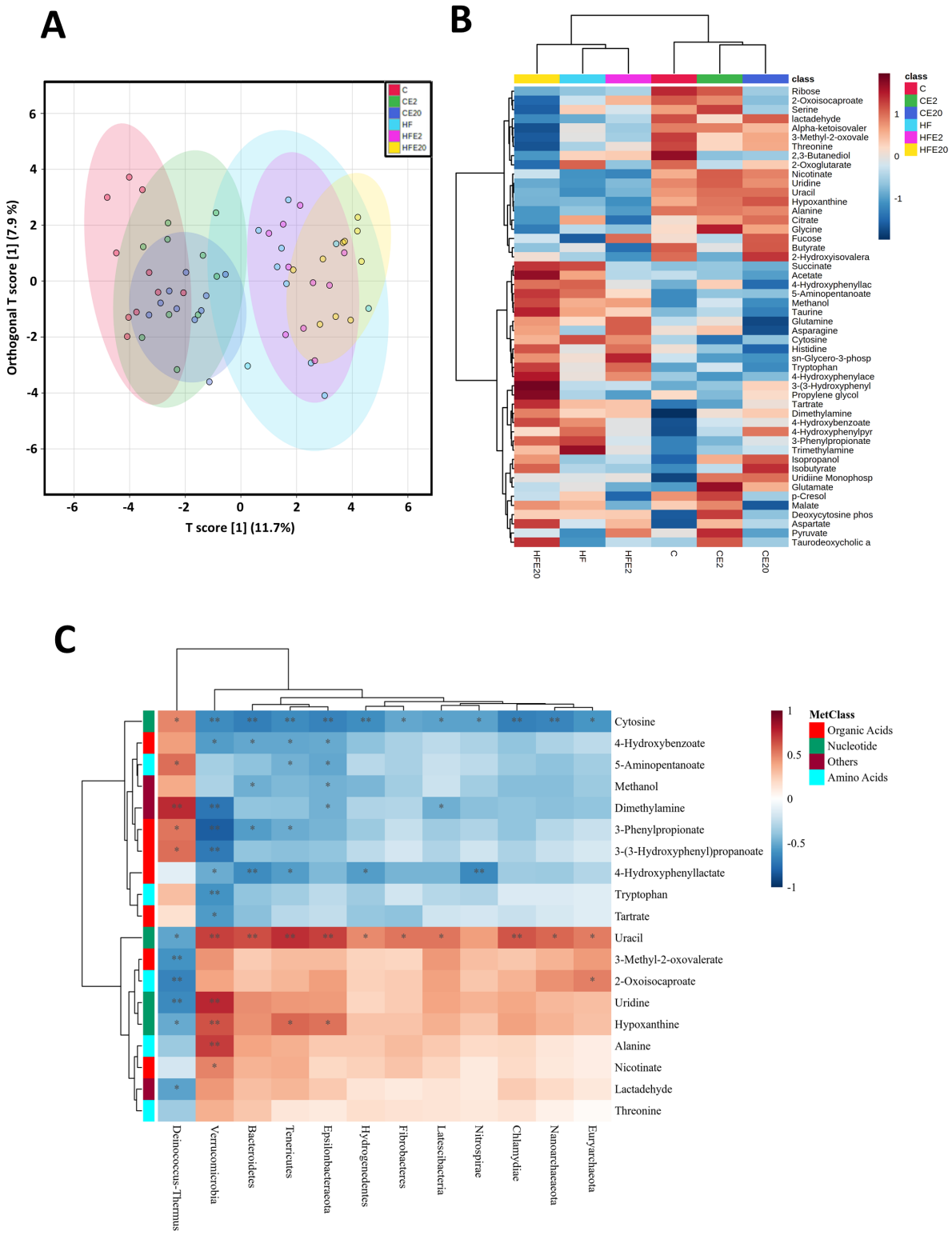



**Figure 6. Effect of EC supplementation on microbiota diversity. A-** α diversity as assessed by Shannon index showed a higher diversity in HF and HF treated with EC 20 mg/kg B.W.. **B-** β diversity, assessed using weighted Unifrac distance and PERMANOVA analyses showed a robust separation of control 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.


A
C
CE2
CE20
HF
HFE2
HFE20
Orthogonal T score [1] (7.9 %)
T score [1] (11.7%)
B
class
Ribose
2-Oxoisocaproate
Serine
lactadehyde
Alpha-ketoisovaler
3-Methyl-2-oxovale
Threonine
2,3-Butanediol
2-Oxoglutarate
Nicotinate
Uridine
Uracil
Hypoxanthine
Alanine
Citrate
Glycine
Fucose
Butyrate
2-Hydroxyisovalera
Succinate
Acetate
4-Hydroxyphenyllac
5-Aminopentanoate
Methanol
Taurine
Glutamine
Asparagine
Cytosine
Histidine
sn-Glycero-3-phosp
Tryptophan
4-Hydroxyphenylace
3-(3-Hydroxyphenyl
Propylene glycol
Tartrate
Dimethylamine
4-Hydroxybenzoate
4-Hydroxyphenylpyr
3-Phenylpropionate
Trimethylamine
Isopropanol
Isobutyrate
Uridine Monophosp
Glutamate
p-Cresol
Malate
Deoxycytosine phos
Aspartate
Pyruvate
Taurodeoxycholic a
HFE20
HF
HFE2
C
CE2
CE20
C
CE2
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1
0
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C
Cytosine
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5-Aminopentanoate
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3-Phenylpropionate
3-(3-Hydroxyphenyl)propanoate
4-Hydroxyphenyllactate
Tryptophan
Tartrate
Uracil
3-Methyl-2-oxovalerate
2-Oxoisocaproate
Uridine
Hypoxanthine
Alanine
Nicotinate
Lactadehyde
Threonine
Deinococcus-Thermus
Verrucomicrobia
Bacteroidetes
Tenericutes
Epsilonbacteraeota
Hydrogenedentes
Fibrobacteres
Latescibacteria
Nitrospirae
Chlamydiae
Nanoarchaeaeota
Euryarchaeota
1
0.5
0
-0.5
-1
MetClass
Organic Acids
Nucleotide
Others
Amino Acids

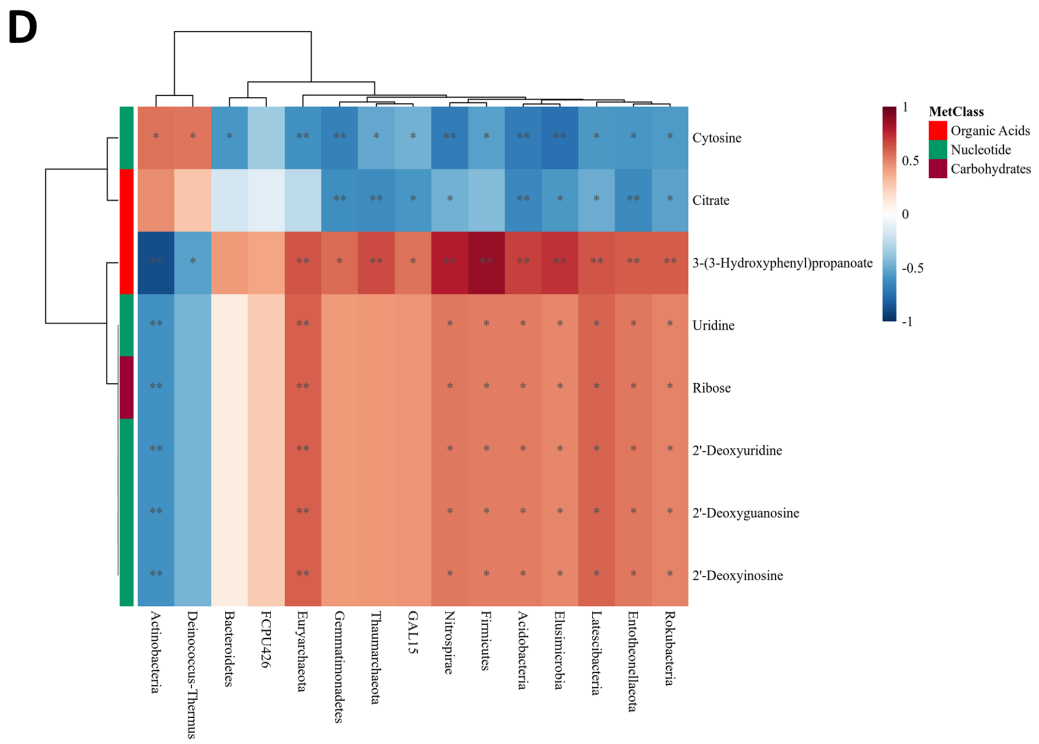


**Figure 7. Effect of EC supplementation on the fecal metabolome. A-** Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot of all metabolite features showed a clear separation of the fecal metabolites in the different treatment groups. **B-** Clustering result shown 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.

## 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 [92, 93], and indirectly, by improving cerebral blood flow [31, 32] and/or affecting select receptors present at the blood-brain barrier (BBB) [33, 93, 94]. Ingested EC is highly bioavailable and extensively metabolized into a wide range of metabolites [95], and some metabolites were shown to cross the BBB and detected in the brain [92, 93, 96]. However, the primary route by which EC metabolites cross the BBB and their further metabolism in the brain is not yet completely understood [97]. 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 a specific receptor expressed in the brain, similar to the one described in arterial endothelial cell membrane [93, 94]. 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.

**Conflicts of interest**

There are no conflicts to declare.

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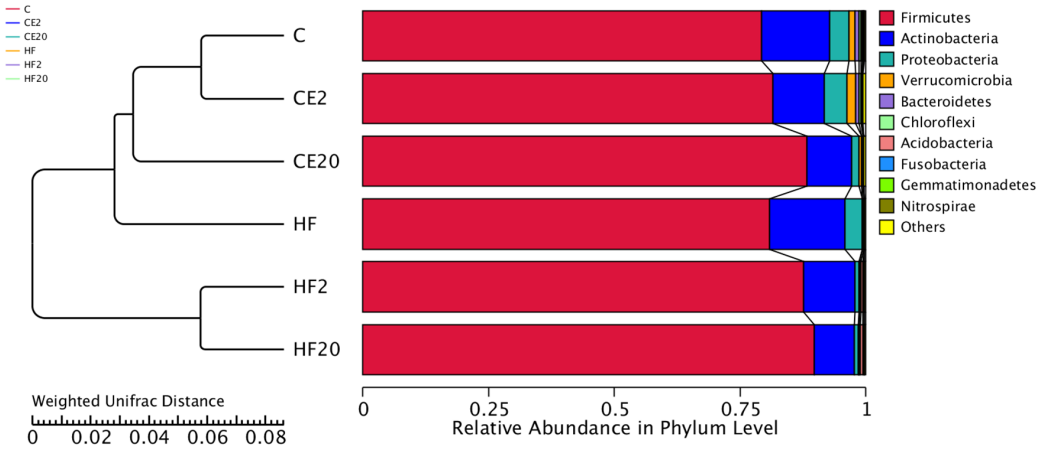
**Supplementary materials**

**Supplementary Figure S1.** Relative abundance at the Phylum level using weighted Unifrac distances of all the treatment groups



**Supplementary Table S1.** Composition of control and high fat diet. The original recipe is from Envigo (Envigo, Indianapolis, IN). The product codes are TD.06415 (high fat diet) and TD.06416 (control diet). The original documents containing nutrient information and ingredients are found in Envigo (www.envigo.com). All ingredients are purchased from Dyets, Inc. (Dyets, Inc., Bethlehem, PA).

| | **High Fat Diet (4.6kcal/g)** | **Control Diet (3.7kcal/g)** |
|---|---|---|
| **Nutrient Information** | **% kcal from** | **% kcal from** |
| Protein | 19.0 | 20.1 |
| Carbohydrate | 36.2 | 69.8 |
| Fat | 44.8 | 10.2 |
| **Ingredient** | **g/kg** | **g/kg** |
| Casein | 245.0 | 210.0 |
| L-Cystine | 3.5 | 3.0 |
| Corn Starch | 85.0 | 280.0 |
| Dextrose | 115.0 | 50.0 |
| Sucrose | 200.0 | 325.0 |
| Lard | 195.0 | 20.0 |
| Soybean Oil | 30.0 | 20.0 |
| Cellulose | 58.0 | 37.15 |
| Mineral Mix | 43.0 | 35.0 |
| Calcium Phosphate, dibasic | 3.4 | 2.0 |
| Vitamin Mix | 19.0 | 15.0 |
| Choline Bitartrate | 3.0 | 2.75 |
| Yellow Food Color | | 0.1 |
| Pink Food Color | 0.1 | |

**Supplementary Table S2.** Univariate analysis (Mann–Whitney U test) of the control and high fat taxa at the Phylum level and corrected for multiple testing with FDR (q<0.1)

| Taxa | HF.MeanSD | C.MeanSD | HF.MedianIQR | C.MedianIQR | FC | P | FDR.P |
|---|---|---|---|---|---|---|---|
| Verrucomicrobia | 483.6 (368.3) | 11697.98 (16550.77) | 371.49 [277.862,568.847] | 5290.58 [3113.723,11683.308] | 24.19 | 0.00018 | 0.0059 |
| Bacteroidetes | 272.58 (156.69) | 8039.57 (7240.54) | 261.81 [178.581,372.011] | 8361.05 [682.396,13258.633] | 29.49 | 0.0021 | 0.033 |
| Tenericutes | 0.9 (0.05) | 232.38 (195.89) | 0.89 [0.871,0.928] | 205.94 [43.396,418.557] | 257.29 | 0.0031 | 0.033 |
| Epsilonbacteraeota | 0.9 (0.05) | 415.27 (426.53) | 0.89 [0.871,0.928] | 314.46 [18.049,791.888] | 459.77 | 0.0044 | 0.035 |
| Chlamydiae | 0.9 (0.05) | 30.65 (37.07) | 0.89 [0.871,0.928] | 13.68 [1.03,59.482] | 33.94 | 0.0085 | 0.055 |
| Deinococcus-Thermus | 648.93 (528.89) | 33.71 (66.56) | 643.73 [221.465,1076.613] | 1.15 [0.967,9.51] | 0.05 | 0.012 | 0.062 |
| Nanoarchaeaeota | 4.29 (9.58) | 75.74 (124.57) | 0.89 [0.871,0.944] | 26.8 [1.147,67.104] | 17.67 | 0.016 | 0.071 |
| Latescibacteria | 19.45 (52.47) | 203.71 (318.75) | 0.89 [0.871,0.944] | 53.35 [13.732,176.802] | 10.47 | 0.021 | 0.082 |
| Hydrogenedentes | 2.88 (3.65) | 36.38 (72.77) | 0.93 [0.892,2.933] | 13.52 [1.147,18.748] | 12.63 | 0.027 | 0.085 |
| Nitrospirae | 31.29 (56.16) | 1068.9 (1436.42) | 8.93 [6.7,26.626] | 441.52 [188.302,1464.634] | 34.16 | 0.027 | 0.085 |
| Euryarchaeota | 7.79 (19.48) | 45.46 (73.02) | 0.89 [0.871,0.944] | 31.46 [1.147,45.222] | 5.84 | 0.034 | 0.091 |
| Fibrobacteres | 3.94 (6.03) | 41.93 (66.75) | 0.93 [0.896,2.956] | 13.52 [1.147,38.09] | 10.64 | 0.034 | 0.091 |
| Elusimicrobia | 0.9 (0.05) | 6.89 (10.72) | 0.89 [0.871,0.928] | 0.95 [0.934,7.479] | 7.63 | 0.068 | 0.166 |
| Acidobacteria | 753.08 (1450.33) | 3509.54 (3980.52) | 303.55 [183.421,362.775] | 2093.88 [810.714,5189.124] | 4.66 | 0.083 | 0.166 |
| Armatimonadetes | 320.19 (199.75) | 146.95 (162.04) | 356.2 [243.91,471.592] | 105.17 [3.341,254.362] | 0.46 | 0.083 | 0.166 |
| Planctomycetes | 418.32 (342.58) | 155.86 (206.58) | 374.23 [166.451,673.632] | 48.09 [9.599,306.805] | 0.37 | 0.083 | 0.166 |
| Gemmatimonadetes | 423.96 (779.98) | 1620.39 (1957.04) | 162.76 [97.945,243.449] | 791.17 [544.646,1885.517] | 3.82 | 0.101 | 0.19 |
| Thaumarchaeota | 5.45 (12.88) | 10.49 (20.07) | 0.89 [0.871,0.944] | 0.96 [0.934,1.149] | 1.92 | 0.173 | 0.307 |
| Entotheonellaeota | 14.78 (39.27) | 14.62 (20.99) | 0.89 [0.871,0.944] | 1.05 [0.939,28.128] | 0.99 | 0.203 | 0.325 |
| Rokubacteria | 78.94 (220.73) | 70.3 (129.23) | 0.89 [0.871,0.944] | 1.05 [0.939,52.258] | 0.89 | 0.203 | 0.325 |
| Cyanobacteria | 919.63 (1052.17) | 324.39 (369.16) | 607.99 [67.233,1361.624] | 195.76 [27.487,488.336] | 0.35 | 0.237 | 0.361 |
| Dependentiae | 5 (6.34) | 29.63 (45.97) | 0.96 [0.916,8.638] | 1.15 [0.966,44.694] | 5.93 | 0.274 | 0.399 |
| FCPU426 | 1.95 (2.98) | 0.97 (0.1) | 0.89 [0.871,0.944] | 0.95 [0.934,0.983] | 0.5 | 0.315 | 0.421 |
| GAL15 | 6.62 (16.18) | 0.97 (0.1) | 0.89 [0.871,0.944] | 0.95 [0.934,0.983] | 0.15 | 0.315 | 0.421 |
| Actinobacteria | 149670.2 (47585.99) | 134971.52 (85882.47) | 155996.77 [108531.2,180592.962] | 124357.51 [71457.1,166232.271] | 0.9 | 0.46 | 0.566 |
| Chloroflexi | 2062.64 (1201.91) | 4096.78 (6602.92) | 2202.27 [1398.875,2794.068] | 958.3 [766.021,2418.193] | 1.99 | 0.46 | 0.566 |
| Others | 172.12 (237.98) | 183.25 (185.6) | 56.98 [41.101,180.414] | 136.91 [56.215,197.001] | 1.06 | 0.515 | 0.61 |
| BRC1 | 6.31 (8.12) | 17.79 (41.2) | 0.93 [0.898,10.824] | 1.07 [0.939,14.373] | 2.82 | 0.573 | 0.611 |
| Proteobacteria | 34467.25 (65993.71) | 38662.15 (43696.24) | 13723.96 [6951.071,18457.478] | 16373.41 [11574.367,75546.399] | 1.12 | 0.573 | 0.611 |
| WPS-2 | 48.79 (29.57) | 96.38 (110.17) | 52.11 [34.528,64.461] | 70.8 [1.147,165.671] | 1.98 | 0.573 | 0.611 |
| Firmicutes | 808874.56 (89958.47) | 793693.59 (82777.57) | 834916.03 [768297.736,877981.873] | 783804.75 [744101.431,840650.144] | 0.98 | 0.633 | 0.654 |
| Patescibacteria | 271.84 (269.93) | 465.86 (490.72) | 258.38 [13.136,498.383] | 298.58 [43.83,949.078] | 1.71 | 0.696 | 0.696 |

**Supplementary Table S3.** Univariate analysis (Mann–Whitney U test) of the control and high fat taxa at the Genus level and corrected for multiple testing with FDR (q<0.1)

| Taxa | HF.MeanSD | C.MeanSD | HF.MedianIQR | C.MedianIQR | FC | P | FDR.P |
|---|---|---|---|---|---|---|---|
| Romboutsia | 24104.04 (24720.13) | 1012.1 (802.81) | 12688.29 [6969.59,38754.615] | 866.72 [291.669,1775.761] | 0.04 | 4.60E-05 | 6.60E-03 |
| Solibacillus | 18285.3 (32028.06) | 29.96 (30.2) | 1518.05 [1254.128,15605.079] | 19.75 [8.67,44.641] | 1.64E-03 | 4.60E-05 | 6.60E-03 |
| Akkermansia | 444.14 (378.81) | 11323.67 (16589.83) | 342.78 [176.248,546.405] | 4583.31 [2725.737,11753.886] | 25.5 | 1.80E-04 | 1.80E-02 |
| Parvibacter | 49.6 (34.27) | 4.83 (12.12) | 52.75 [18.299,56.851] | 0.99 [0.954,1.117] | 0.1 | 3.20E-04 | 2.30E-02 |
| Sporosarcina | 855.78 (1414.31) | 34.81 (29.64) | 323.75 [109.209,705.058] | 29.39 [9.113,56.175] | 0.04 | 5.50E-04 | 3.20E-02 |
| Dubosiella | 11719.9 (4968.96) | 36856.1 (19116.61) | 12075.3 [8825.407,12970.226] | 36001.28 [20821.286,45175.963] | 3.14 | 1.40E-03 | 4.00E-02 |
| uncultured_Chloroflexi_bacterium | 825.04 (613.95) | 41.28 (79.73) | 986.07 [288.893,1157.725] | 9.06 [1.039,30.953] | 0.05 | 1.40E-03 | 4.00E-02 |
| Dokdonella | 0.9 (0.05) | 1065.28 (1487.22) | 0.89 [0.871,0.928] | 575.7 [16.102,1519.139] | 1179.5 | 3.10E-03 | 4.00E-02 |
| Planococcus | 0.9 (0.05) | 42.3 (35.46) | 0.89 [0.871,0.928] | 32.57 [15.773,77.921] | 46.83 | 3.10E-03 | 4.00E-02 |
| SWB02 | 0.9 (0.05) | 128.84 (182.08) | 0.89 [0.871,0.928] | 19.39 [5.292,272.715] | 142.65 | 3.10E-03 | 4.00E-02 |
| Sphaerotilus | 0.9 (0.05) | 632.72 (938.3) | 0.89 [0.871,0.928] | 292.29 [3.346,970.176] | 700.56 | 3.10E-03 | 4.00E-02 |
| Acinetobacter | 0.9 (0.05) | 3702.06 (6461.11) | 0.89 [0.871,0.928] | 1455.71 [18.198,4527.792] | 4099 | 4.40E-03 | 4.00E-02 |
| CL500-29_marine_group | 6.62 (16.18) | 142.91 (114.59) | 0.89 [0.871,0.944] | 126.49 [68.744,230.908] | 21.59 | 4.40E-03 | 4.00E-02 |
| Candidatus_Berkiella | 0.9 (0.05) | 54.47 (64.62) | 0.89 [0.871,0.928] | 23.07 [1.156,106.146] | 60.32 | 4.40E-03 | 4.00E-02 |
| Christensenellaceae_R-7_group | 1.89 (2.77) | 75.3 (69.24) | 0.91 [0.878,0.949] | 64.04 [14.074,121.378] | 39.9 | 4.40E-03 | 4.00E-02 |
| Chthoniobacter | 0.9 (0.05) | 19.5 (21.26) | 0.89 [0.871,0.928] | 14.64 [3.08,27.462] | 21.59 | 4.40E-03 | 4.00E-02 |
| Defluviicoccus | 0.9 (0.05) | 101.09 (91.08) | 0.89 [0.871,0.928] | 92.86 [17.452,147.309] | 111.92 | 4.40E-03 | 4.00E-02 |
| Denitratisoma | 153.68 (111.78) | 4.83 (12.12) | 198.38 [53.725,225.191] | 0.99 [0.954,1.117] | 0.03 | 4.40E-03 | 4.00E-02 |
| Desulfotomaculum | 0.9 (0.05) | 14.89 (14.21) | 0.89 [0.871,0.928] | 14.46 [1.156,24.829] | 16.49 | 4.40E-03 | 4.00E-02 |
| Desulfovibrio | 0.9 (0.05) | 273.79 (325.24) | 0.89 [0.871,0.928] | 236.67 [3.756,367.811] | 303.15 | 4.40E-03 | 4.00E-02 |
| Flavobacterium | 0.9 (0.05) | 32.66 (43.94) | 0.89 [0.871,0.928] | 14.34 [1.156,54.757] | 36.16 | 4.40E-03 | 4.00E-02 |
| Geobacter | 0.9 (0.05) | 63.52 (88.31) | 0.89 [0.871,0.928] | 14.74 [1.156,92.833] | 70.33 | 4.40E-03 | 4.00E-02 |
| Geothrix | 0.9 (0.05) | 57.81 (102.63) | 0.89 [0.871,0.928] | 9.6 [1.156,63.846] | 64.01 | 4.40E-03 | 4.00E-02 |
| Hirschia | 0.9 (0.05) | 25.04 (30.4) | 0.89 [0.871,0.928] | 15.25 [3.08,27.241] | 27.72 | 4.40E-03 | 4.00E-02 |
| Ignavibacterium | 0.9 (0.05) | 85.11 (113) | 0.89 [0.871,0.928] | 36.55 [1.156,102.493] | 94.24 | 4.40E-03 | 4.00E-02 |
| Parabacteroides | 0.9 (0.05) | 124.63 (136.36) | 0.89 [0.871,0.928] | 68.83 [6.644,203.157] | 138 | 4.40E-03 | 4.00E-02 |
| Rhizobacter | 1.95 (2.98) | 94.42 (111.5) | 0.89 [0.871,0.944] | 45.23 [31.353,140.252] | 48.35 | 4.40E-03 | 4.00E-02 |
| Tropicimonas | 0.9 (0.05) | 83.96 (91.07) | 0.89 [0.871,0.928] | 58.05 [7.975,119.668] | 92.97 | 4.40E-03 | 4.00E-02 |
| [Eubacterium]_xylanophilum_group | 0.9 (0.05) | 276.61 (372.01) | 0.89 [0.871,0.928] | 172.54 [3.756,257.408] | 306.27 | 4.40E-03 | 4.00E-02 |
| uncultured_Bacteroidetes_bacterium | 0.9 (0.05) | 72.38 (114.42) | 0.89 [0.871,0.928] | 37.58 [1.156,54.939] | 80.14 | 4.40E-03 | 4.00E-02 |
| uncultured_gamma_proteobacterium | 0.9 (0.05) | 260.44 (484.22) | 0.89 [0.871,0.928] | 32.23 [1.156,167.081] | 288.37 | 4.40E-03 | 4.00E-02 |
| uncultured_prokaryote | 0.9 (0.05) | 167.09 (299.39) | 0.89 [0.871,0.928] | 18.64 [1.156,107.266] | 185.01 | 4.40E-03 | 4.00E-02 |
| Anaerolinea | 0.9 (0.05) | 42.8 (82.01) | 0.89 [0.871,0.928] | 5 [1.033,9.873] | 47.39 | 6.20E-03 | 4.00E-02 |
| Brevundimonas | 5.07 (8.99) | 98.72 (103.25) | 0.93 [0.892,2.956] | 41.96 [30.541,199.266] | 19.48 | 6.20E-03 | 4.00E-02 |
| Enterobacter | 14938.86 (40682.95) | 761.3 (2288.46) | 697.38 [453.94,835.793] | 24.6 [9.007,84.071] | 0.05 | 6.20E-03 | 4.00E-02 |
| Iamia | 13.21 (21.95) | 166.87 (179.07) | 5.08 [0.871,11.884] | 77.93 [61.967,274.223] | 12.63 | 6.20E-03 | 4.00E-02 |
| Lachnoclostridium | 396.51 (279) | 904.4 (432.62) | 352.79 [259.908,426.691] | 869.29 [564.87,1271.316] | 2.28 | 6.20E-03 | 4.00E-02 |
| Marinobacterium | 0.9 (0.05) | 32.05 (47.19) | 0.89 [0.871,0.928] | 5 [1.033,62.396] | 35.49 | 6.20E-03 | 4.00E-02 |
| Mycobacterium | 4.11 (6.5) | 137.14 (154.88) | 0.91 [0.871,2.956] | 98.19 [41.706,163.71] | 33.34 | 6.20E-03 | 4.00E-02 |
| Paenarthrobacter | 0.9 (0.05) | 25.09 (33.95) | 0.89 [0.871,0.928] | 5 [1.033,37.305] | 27.78 | 6.20E-03 | 4.00E-02 |
| Truepera | 648.9 (528.89) | 29.42 (60.37) | 643.68 [221.433,1076.517] | 1 [0.954,1.156] | 0.05 | 6.20E-03 | 4.00E-02 |
| uncultured_Acidobacteriaceae_bacterium | 0.9 (0.05) | 84.74 (169.65) | 0.89 [0.871,0.928] | 5 [0.979,27.077] | 93.83 | 6.20E-03 | 4.00E-02 |
| uncultured_Actinomycetales_bacterium | 0.9 (0.05) | 156.75 (355.14) | 0.89 [0.871,0.928] | 9.34 [1.033,27.371] | 173.55 | 6.20E-03 | 4.00E-02 |
| uncultured_Caldilineaceae_bacterium | 0.9 (0.05) | 14.19 (21.81) | 0.89 [0.871,0.928] | 5.32 [1.033,15.733] | 15.71 | 6.20E-03 | 4.00E-02 |
| wastewater_metagenome | 2.99 (3.9) | 189.12 (215.57) | 0.91 [0.881,2.85] | 86.27 [41.301,321.164] | 63.24 | 6.20E-03 | 4.00E-02 |
| Anaerovorax | 1.89 (2.77) | 60.8 (68.26) | 0.91 [0.878,0.949] | 24.73 [3.368,117.682] | 32.22 | 8.50E-03 | 4.30E-02 |
| Bacteroides | 1.86 (2.68) | 407.76 (418.05) | 0.91 [0.881,0.949] | 305.82 [3.756,756.933] | 219.45 | 8.50E-03 | 4.30E-02 |
| Crassaminicella | 2.02 (3.2) | 235.74 (256.54) | 0.89 [0.871,0.928] | 227.72 [1.156,321.598] | 116.53 | 8.50E-03 | 4.30E-02 |
| Cyanobacteria/Melainabacteria_group_bacterium_S15B-MN24_CBMW_12 | 0.9 (0.05) | 9.34 (13.07) | 0.89 [0.871,0.928] | 1.16 [0.977,15.733] | 10.34 | 8.50E-03 | 4.30E-02 |
| Prosthecobacter | 0.9 (0.05) | 9.43 (14) | 0.89 [0.871,0.928] | 1.16 [0.977,9.64] | 10.44 | 8.50E-03 | 4.30E-02 |
| Pseudomonas | 5.07 (6.32) | 866.19 (1044.29) | 0.96 [0.871,9.106] | 499.57 [33.864,1683.539] | 170.92 | 8.50E-03 | 4.30E-02 |
| Reyranella | 7.79 (19.48) | 100.03 (83.29) | 0.89 [0.871,0.944] | 121.4 [20.567,146.43] | 12.85 | 8.50E-03 | 4.30E-02 |
| Rhodobacter | 3.1 (6.23) | 430.44 (551.11) | 0.89 [0.871,0.949] | 188.01 [9.539,892.661] | 138.69 | 8.50E-03 | 4.30E-02 |
| Rhodococcus | 1.87 (2.72) | 126.28 (176.15) | 0.91 [0.881,0.949] | 55.29 [6.644,141.718] | 67.5 | 8.50E-03 | 4.30E-02 |

**Supplementary Table S3.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ruminococcaceae_UCG-014 | 40.35 (43.69) | 872 (780.09) | 30.87 [6.684,51.953] | 671.58 [233.948,1408.221] | 21.61 | 8.50E-03 | 4.30E-02 |
| Sandaracinus | 0.9 (0.05) | 6.35 (7.54) | 0.89 [0.871,0.928] | 1.16 [0.977,9.886] | 7.03 | 8.50E-03 | 4.30E-02 |
| Shewanella | 2.02 (3.2) | 60.84 (69.63) | 0.89 [0.871,0.928] | 35.26 [3.297,101.616] | 30.07 | 8.50E-03 | 4.30E-02 |
| Aquicella | 0.9 (0.05) | 2.68 (3.54) | 0.89 [0.871,0.928] | 1 [0.975,1.156] | 2.97 | 1.20E-02 | 0.05 |
| Clostridium_sensu_stricto_13 | 3 (5.93) | 74.06 (74.5) | 0.91 [0.871,0.949] | 72.06 [1.156,114.377] | 24.67 | 1.20E-02 | 0.05 |
| Conexibacter | 9.2 (17.52) | 180.07 (193.81) | 0.89 [0.871,5.332] | 127.27 [21.94,281.021] | 19.57 | 1.20E-02 | 0.05 |
| Dechloromonas | 1.9 (2.8) | 917.17 (1294.26) | 0.91 [0.871,0.949] | 520.71 [1.156,1249.856] | 483.4 | 1.20E-02 | 0.05 |
| Marmoricola | 0.9 (0.05) | 5.6 (7.69) | 0.89 [0.871,0.928] | 1 [0.975,8.954] | 6.2 | 1.20E-02 | 0.05 |
| Microlunatus | 5.45 (12.88) | 74.62 (100.37) | 0.89 [0.871,0.944] | 37.71 [11.969,88.607] | 13.69 | 1.20E-02 | 0.05 |
| Phaselicystis | 3.12 (6.28) | 58.92 (79.58) | 0.89 [0.871,0.944] | 21.28 [3.08,97.477] | 18.89 | 1.20E-02 | 0.05 |
| Ruminococcaceae_NK4A214_group | 1.9 (2.8) | 63.28 (69.96) | 0.91 [0.871,0.949] | 42.46 [1.156,112.165] | 33.35 | 1.20E-02 | 0.05 |
| Stenotrophomonas | 1.89 (2.77) | 19.78 (21.78) | 0.91 [0.878,0.949] | 19.2 [1.156,24.864] | 10.48 | 1.20E-02 | 0.05 |
| Terrimonas | 1.95 (2.98) | 109.99 (131.76) | 0.89 [0.871,0.944] | 74.29 [1.156,141.034] | 56.32 | 1.20E-02 | 0.05 |
| Bauldia | 3.12 (6.28) | 52.19 (99.49) | 0.89 [0.871,0.944] | 19.54 [3.08,33.371] | 16.73 | 1.60E-02 | 0.06 |
| Coxiella | 3.12 (6.28) | 51.67 (72.24) | 0.89 [0.871,0.944] | 13.9 [1.156,92.259] | 16.56 | 1.60E-02 | 0.06 |
| Faecalibacterium | 1.95 (2.98) | 10.16 (10.03) | 0.89 [0.871,0.944] | 9.16 [1.156,17.181] | 5.2 | 1.60E-02 | 0.06 |
| Nannocystis | 1.95 (2.98) | 35.94 (50.83) | 0.89 [0.871,0.944] | 9.16 [1.156,76.182] | 18.4 | 1.60E-02 | 0.06 |
| Ruminococcaceae_UCG-010 | 4.26 (9.51) | 91.11 (111.65) | 0.89 [0.871,0.949] | 44.78 [1.156,164.666] | 21.38 | 1.60E-02 | 0.06 |
| Staphylococcus | 108226.02 (69256.07) | 31045.86 (29423.34) | 120869.7 [49826.976,152502.513] | 24167.39 [8943.856,38809.378] | 0.29 | 1.60E-02 | 0.06 |
| Trichococcus | 90.21 (62.69) | 7.39 (9.9) | 116.14 [34.374,141.397] | 1.16 [0.985,9.716] | 0.08 | 1.60E-02 | 0.06 |
| uncultured_delta_proteobacterium | 362.08 (249.14) | 96.81 (212.52) | 365.79 [202.842,587.571] | 14.63 [1.039,19.323] | 0.27 | 1.60E-02 | 0.06 |
| Actinoplanes | 0.9 (0.05) | 5.64 (9.23) | 0.89 [0.871,0.928] | 1.08 [0.954,7.664] | 6.25 | 2.10E-02 | 0.073 |
| Arenimonas | 1005.34 (733.48) | 84.15 (110.43) | 1342.71 [331.3,1585.345] | 38.4 [7.505,113.168] | 0.08 | 2.10E-02 | 0.073 |
| Escherichia-Shigella | 3.27 (6.72) | 63.33 (123.91) | 0.89 [0.871,0.928] | 29.09 [1.039,45.803] | 19.38 | 2.10E-02 | 0.073 |
| Ideonella | 0.9 (0.05) | 25.28 (42.69) | 0.89 [0.871,0.928] | 1.16 [0.977,19.594] | 27.99 | 2.10E-02 | 0.073 |
| Nitrospira | 30.32 (56.68) | 881.84 (1027.51) | 8.93 [0.962,26.627] | 445.77 [190.268,1523.552] | 29.08 | 2.10E-02 | 0.073 |
| Sphingobium | 0.9 (0.05) | 22.64 (33.13) | 0.89 [0.871,0.928] | 5.44 [0.959,28.06] | 25.07 | 2.10E-02 | 0.073 |
| ADurb.Bin063-1 | 0.9 (0.05) | 15.97 (28.63) | 0.89 [0.871,0.928] | 1 [0.954,22.6] | 17.68 | 2.70E-02 | 0.08 |
| Caenimonas | 0.9 (0.05) | 21.56 (40.69) | 0.89 [0.871,0.928] | 1.07 [0.954,15.144] | 23.88 | 2.70E-02 | 0.08 |
| Candidatus_Nitrocosmicus | 0.9 (0.05) | 5.75 (10.29) | 0.89 [0.871,0.928] | 0.99 [0.954,1.156] | 6.36 | 2.70E-02 | 0.08 |
| Desulfosporosinus | 2.93 (3.75) | 36.22 (48.27) | 0.92 [0.878,2.933] | 24.75 [1.156,49.292] | 12.36 | 2.70E-02 | 0.08 |
| Ellin6067 | 21.49 (51.78) | 244.6 (283.54) | 0.95 [0.871,8.944] | 105.09 [76.366,450.917] | 11.38 | 2.70E-02 | 0.08 |
| Exiguobacterium | 8.33 (10.58) | 773.59 (691.09) | 0.96 [0.91,17.634] | 890.77 [29.775,1389.81] | 92.89 | 2.70E-02 | 0.08 |
| Lactobacillus | 6450.17 (9861.64) | 23937.3 (26784.15) | 3235.28 [2030.836,4361.013] | 9840.22 [4284.767,47299.484] | 3.71 | 2.70E-02 | 0.08 |
| Mesorhizobium | 15.26 (15.58) | 258.7 (364.97) | 13.7 [0.878,21.362] | 129.23 [37.981,355.713] | 16.95 | 2.70E-02 | 0.08 |
| Nocardioides | 6.29 (9.49) | 147.95 (379.61) | 0.96 [0.878,8.817] | 15.69 [9.539,55.313] | 23.54 | 2.70E-02 | 0.08 |
| Pontibacter | 0.9 (0.05) | 3.67 (4.31) | 0.89 [0.871,0.928] | 1.07 [0.954,7.664] | 4.06 | 2.70E-02 | 0.08 |
| Roseomonas | 0.9 (0.05) | 64.93 (121.07) | 0.89 [0.871,0.928] | 1.07 [0.954,47.908] | 71.89 | 2.70E-02 | 0.08 |
| Tumebacillus | 0.9 (0.05) | 3.75 (6.25) | 0.89 [0.871,0.928] | 1 [0.954,1.156] | 4.15 | 2.70E-02 | 0.08 |
| UTCFX1 | 2.94 (3.77) | 22.87 (33.85) | 0.91 [0.878,2.933] | 9.74 [1.156,26.736] | 7.79 | 2.70E-02 | 0.08 |
| metagenome | 129.5 (78.92) | 1371.59 (1511.76) | 141.92 [76.85,155.518] | 745.38 [370.484,2652.398] | 10.59 | 2.70E-02 | 0.08 |
| uncultured_Verrucomicrobia_bacterium | 0.9 (0.05) | 12.67 (27.41) | 0.89 [0.871,0.928] | 1 [0.954,8.954] | 14.02 | 2.70E-02 | 0.08 |
| Adhaeribacter | 0.9 (0.05) | 2.92 (4.14) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 3.24 | 3.40E-02 | 0.088 |
| Cellulomonas | 0.9 (0.05) | 1.88 (2.82) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 2.09 | 3.40E-02 | 0.088 |
| Erythrobacter | 1.9 (2.8) | 175.24 (309.19) | 0.91 [0.871,0.949] | 45.76 [1.033,79.472] | 92.36 | 3.40E-02 | 0.088 |
| GCA-900066575 | 12.51 (20.09) | 130.23 (157.49) | 8.76 [0.967,9.282] | 86.7 [16.168,164.244] | 10.41 | 3.40E-02 | 0.088 |
| Gordonia | 1.95 (2.96) | 31.15 (43.16) | 0.89 [0.871,0.949] | 5.44 [1.033,63.74] | 16.01 | 3.40E-02 | 0.088 |
| Luteimonas | 308.03 (240.28) | 28.19 (25.49) | 357.46 [81.648,501.387] | 19.75 [11.376,38.376] | 0.09 | 3.40E-02 | 0.088 |
| Luteolibacter | 1.95 (2.98) | 61.43 (119.48) | 0.89 [0.871,0.944] | 5.44 [0.961,53.242] | 31.45 | 3.40E-02 | 0.088 |
| Microbacterium | 6.18 (6.36) | 86.97 (118.49) | 4.77 [0.881,9.437] | 23.72 [9.609,144.316] | 14.08 | 3.40E-02 | 0.088 |
| Ohtaekwangia | 0.9 (0.05) | 6.06 (10.85) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 6.71 | 3.40E-02 | 0.088 |
| Phenylobacterium | 4.29 (9.58) | 40.01 (39.2) | 0.89 [0.871,0.944] | 39.17 [1,74.351] | 9.34 | 3.40E-02 | 0.088 |
| Ramlibacter | 7.42 (15.49) | 56.83 (65.74) | 0.93 [0.878,2.933] | 40.7 [1.156,77.955] | 7.66 | 3.40E-02 | 0.088 |
| Shinella | 35.67 (32.04) | 6.51 (9.89) | 26.9 [15.234,47.701] | 1.08 [0.977,7.664] | 0.18 | 3.40E-02 | 0.088 |
| Stenotrophobacter | 0.9 (0.05) | 3.91 (6.5) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 4.33 | 3.40E-02 | 0.088 |

**Supplementary Table S3.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| [Eubacterium]_brachy_group | 10.7 (15.09) | 48.35 (43.53) | 8.54 [0.898,9.437] | 36.97 [11.556,89.813] | 4.52 | 3.40E-02 | 0.088 |
| [Eubacterium]_nodatum_group | 25.11 (68.47) | 84.91 (85.56) | 0.89 [0.871,0.949] | 61.21 [13.159,119.556] | 3.38 | 3.40E-02 | 0.088 |
| uncultured_Armatimonadetes_bacterium | 0.9 (0.05) | 14.76 (43.53) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 16.34 | 3.40E-02 | 0.088 |
| uncultured_Holophaga_sp. | 0.9 (0.05) | 4.9 (9.33) | 0.89 [0.871,0.928] | 0.99 [0.954,1.117] | 5.43 | 3.40E-02 | 0.088 |
| Bdellovibrio | 5.09 (6.32) | 158.14 (192.54) | 0.93 [0.892,9.116] | 90.69 [1.156,289.73] | 31.04 | 4.30E-02 | 0.098 |
| Bradyrhizobium | 19.28 (45.18) | 113.5 (119.99) | 0.89 [0.871,9.437] | 73.59 [5.292,226.128] | 5.89 | 4.30E-02 | 0.098 |
| Candidatus_Kuenenia | 411.42 (351.34) | 0.99 (0.1) | 374.19 [152.671,673.588] | 0.99 [0.954,0.998] | 2.41E-03 | 4.30E-02 | 0.098 |
| Candidatus_Nitrososphaera | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| KF-JG30-B11 | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| Kibdelosporangium | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| Leucobacter | 39.41 (32.47) | 0.99 (0.1) | 40.39 [19.584,48.942] | 0.99 [0.954,0.998] | 0.03 | 4.30E-02 | 0.098 |
| Massilia | 12.67 (21.56) | 125.57 (152.03) | 0.96 [0.91,13.401] | 68.56 [3.346,201.636] | 9.91 | 4.30E-02 | 0.098 |
| Nitrolancea | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| Nitrosospira | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| Nonomuraea | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| Sorangium | 0.9 (0.05) | 5.5 (14.31) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 6.09 | 4.30E-02 | 0.098 |
| Vicinamibacter | 0.9 (0.05) | 0.99 (0.1) | 0.89 [0.871,0.928] | 0.99 [0.954,0.998] | 1.1 | 4.30E-02 | 0.098 |
| uncultured_Rhodospirillaceae_bacterium | 52.49 (39.92) | 0.99 (0.1) | 66.44 [13.134,84.401] | 0.99 [0.954,0.998] | 0.02 | 4.30E-02 | 0.098 |
| uncultured_anaerobic_ammonium-oxidizing_bacterium | 283.75 (186.82) | 0.99 (0.1) | 335.63 [201.739,431.875] | 0.99 [0.954,0.998] | 3.50E-03 | 4.30E-02 | 0.098 |
| Agromyces | 5.45 (12.88) | 57.37 (118.17) | 0.89 [0.871,0.944] | 5.32 [0.979,44.56] | 10.52 | 0.055 | 0.113 |
| Alistipes | 10.46 (17.8) | 208.14 (212.01) | 0.96 [0.896,11.294] | 232.71 [1.156,309.436] | 19.91 | 0.055 | 0.113 |
| Blautia | 266.13 (190.25) | 187.2 (280.3) | 213.42 [176.512,243.669] | 77.63 [57.947,182.147] | 0.7 | 0.055 | 0.113 |
| Bosea | 4.22 (6.59) | 19.69 (31.5) | 0.89 [0.871,3.188] | 1.07 [0.975,28.92] | 4.66 | 0.055 | 0.113 |
| Family_XIII_AD3011_group | 11.21 (29.16) | 19.75 (25.49) | 0.89 [0.871,0.949] | 9.43 [1.156,27.2] | 1.76 | 0.055 | 0.113 |
| Fictibacillus | 1.95 (2.96) | 6.65 (10.01) | 0.89 [0.871,0.949] | 1 [0.975,7.717] | 3.42 | 0.055 | 0.113 |
| Methyloceanibacter | 1.95 (2.98) | 22.69 (48.85) | 0.89 [0.871,0.944] | 1.08 [0.954,19.789] | 11.62 | 0.055 | 0.113 |
| Nitrosomonas | 925.85 (685.87) | 87.19 (120.29) | 1011.12 [529.16,1310.69] | 18.57 [1.156,164.088] | 0.09 | 0.055 | 0.113 |
| Pseudonocardia | 1.95 (2.98) | 23.36 (43.73) | 0.89 [0.871,0.944] | 1.08 [0.954,24.741] | 11.96 | 0.055 | 0.113 |
| Ruminiclostridium_5 | 16.08 (26.93) | 67.15 (69.6) | 4.77 [0.898,14.414] | 72.5 [2.968,79.543] | 4.18 | 0.055 | 0.113 |
| Skermanella | 3.12 (6.28) | 26.05 (30.84) | 0.89 [0.871,0.944] | 5.32 [0.977,58.693] | 8.35 | 0.055 | 0.113 |
| uncultured_proteobacterium | 1.95 (2.98) | 33.69 (45.02) | 0.89 [0.871,0.944] | 1.08 [0.954,59.477] | 17.25 | 0.055 | 0.113 |
| Acetobacter | 2386.53 (2059.74) | 10.18 (12.62) | 3067.72 [21.079,3860.213] | 5.44 [1.033,15.776] | 4.27E-03 | 0.068 | 0.134 |
| Altererythrobacter | 3.12 (6.28) | 27.53 (55.13) | 0.89 [0.871,0.944] | 5.54 [0.954,19.831] | 8.82 | 0.068 | 0.134 |
| Brooklawnia | 4.28 (6.93) | 10.16 (10.68) | 0.91 [0.871,2.958] | 5.49 [1.033,19.28] | 2.37 | 0.068 | 0.134 |
| Enterorhabdus | 1194.84 (591.28) | 2100.85 (1180.95) | 1147.09 [765.046,1493.608] | 1908.72 [1416.282,2551.422] | 1.76 | 0.068 | 0.134 |
| Ileibacterium | 535462.35 (66167.84) | 584846.15 (56346.52) | 541242.48 [480362.459,552503.726] | 582321.34 [566061.17,598533.746] | 1.09 | 0.068 | 0.134 |
| uncultured_Myxococcales_bacterium | 3.12 (6.28) | 19.39 (31.02) | 0.89 [0.871,0.944] | 1.16 [0.959,24.714] | 6.22 | 0.068 | 0.134 |
| Gemmatirosa | 5.45 (12.88) | 14.54 (25.56) | 0.89 [0.871,0.944] | 1.08 [0.959,11.096] | 2.67 | 0.083 | 0.153 |
| Haliangium | 58.18 (116.49) | 472.31 (615.04) | 22 [8.923,28.311] | 149.47 [81.53,931.574] | 8.12 | 0.083 | 0.153 |
| Hyphomicrobium | 2802.06 (2650.28) | 483.82 (678.27) | 2785.24 [113.61,4891.32] | 168.03 [16.102,796.935] | 0.17 | 0.083 | 0.153 |
| Janthinobacterium | 3.95 (4.21) | 32.93 (42.25) | 0.96 [0.878,8.817] | 9.38 [1.156,62.246] | 8.33 | 0.083 | 0.153 |
| Opitutus | 1.95 (2.98) | 7.27 (14.17) | 0.89 [0.871,0.944] | 1 [0.954,7.664] | 3.72 | 0.083 | 0.153 |
| Oscillibacter | 7.41 (9.3) | 307.9 (402.69) | 4.84 [0.878,9.261] | 217.21 [1.156,410.453] | 41.58 | 0.083 | 0.153 |
| Ottowia | 374.41 (367.16) | 59.23 (78.4) | 332.57 [116.339,548.667] | 26.99 [1.156,100.348] | 0.16 | 0.083 | 0.153 |
| Paeniclostridium | 11.92 (12.56) | 60.47 (75.52) | 8.79 [6.684,11.405] | 17.34 [9.007,83.137] | 5.07 | 0.083 | 0.153 |
| Paracoccus | 8.58 (8.84) | 223.96 (282.43) | 4.98 [0.871,17.888] | 137.55 [1.156,299.733] | 26.1 | 0.083 | 0.153 |
| RB41 | 134.94 (379.13) | 56.94 (96.06) | 0.89 [0.871,0.944] | 5.58 [0.975,78.701] | 0.42 | 0.083 | 0.153 |
| uncultured_Gemmatimonadetes_bacterium | 3.12 (6.28) | 15.85 (33.8) | 0.89 [0.871,0.944] | 1 [0.954,15.039] | 5.08 | 0.083 | 0.153 |
| Acidibacter | 10.12 (26.08) | 22.95 (36.84) | 0.89 [0.871,0.944] | 10.41 [0.954,19.958] | 2.27 | 0.101 | 0.175 |
| Candidatus_Solibacter | 11.29 (29.38) | 58.58 (120.7) | 0.89 [0.871,0.944] | 1.07 [0.954,19.958] | 5.19 | 0.101 | 0.175 |
| Gaiella_sp._EBR4-RS1 | 72.23 (76.42) | 9.58 (16.63) | 35.26 [20.116,131.207] | 1.07 [0.954,7.792] | 0.13 | 0.101 | 0.175 |
| Marvinbryantia | 4.04 (6.31) | 5.34 (6.31) | 0.93 [0.871,2.956] | 1.16 [0.993,9.208] | 1.32 | 0.101 | 0.175 |
| Paludibaculum | 4.11 (6.5) | 45.86 (62.14) | 0.91 [0.871,2.956] | 5.44 [1.033,100.348] | 11.15 | 0.101 | 0.175 |
| Pedomicrobium | 14.28 (21.51) | 93.2 (114) | 8.93 [0.871,12.098] | 44.24 [7.505,155.278] | 6.53 | 0.101 | 0.175 |
| Rhodoplanes | 6.62 (16.18) | 16.92 (26.46) | 0.89 [0.871,0.944] | 1.08 [0.954,28.454] | 2.56 | 0.101 | 0.175 |

**Supplementary Table S3.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Streptomyces | 5.45 (12.88) | 13.02 (16.39) | 0.89 [0.871,0.944] | 5.32 [0.975,24.762] | 2.39 | 0.101 | 0.175 |
| uncultured_Desulfuromonadales_bacterium | 3.12 (6.28) | 14.61 (28.71) | 0.89 [0.871,0.944] | 1 [0.954,1.156] | 4.68 | 0.101 | 0.175 |
| uncultured_actinobacterium | 25.28 (68.97) | 76.07 (128.19) | 0.89 [0.871,0.944] | 1.08 [0.954,95.339] | 3.01 | 0.101 | 0.175 |
| Anaeromyxobacter | 2.96 (3.82) | 14.4 (22.47) | 0.91 [0.871,3.004] | 1.16 [0.977,24.652] | 4.86 | 0.122 | 0.195 |
| Candidatus_Entotheonella | 5.45 (12.88) | 7.72 (18.38) | 0.89 [0.871,0.944] | 1 [0.954,1.156] | 1.42 | 0.122 | 0.195 |
| Candidatus_Udaeobacter | 4.29 (9.58) | 74.45 (158.18) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 17.37 | 0.122 | 0.195 |
| Demequina | 4.02 (8.8) | 43.48 (86.72) | 0.91 [0.881,0.949] | 1 [0.954,17.619] | 10.81 | 0.122 | 0.195 |
| Dongia | 21.78 (59.07) | 46.25 (48.49) | 0.89 [0.871,0.944] | 40.24 [0.954,86.595] | 2.12 | 0.122 | 0.195 |
| GCA-900066225 | 2.98 (5.86) | 5.61 (7.87) | 0.91 [0.878,0.949] | 1.07 [0.958,7.792] | 1.88 | 0.122 | 0.195 |
| IS-44 | 6.62 (16.18) | 11.91 (24.75) | 0.89 [0.871,0.944] | 1 [0.954,7.664] | 1.8 | 0.122 | 0.195 |
| Kribbella | 3.12 (6.28) | 7.22 (13.22) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 2.31 | 0.122 | 0.195 |
| Lysobacter | 28.78 (78.87) | 5.61 (9.22) | 0.89 [0.871,0.944] | 1.08 [0.975,7.397] | 0.19 | 0.122 | 0.195 |
| [Eubacterium]_coprostanoligenes_group | 88.15 (178.85) | 866.08 (1260.46) | 26.37 [15.164,46.882] | 462.17 [20.674,1008.461] | 9.82 | 0.122 | 0.195 |
| uncultured_Acidobacteria_bacterium | 28.78 (78.87) | 37.86 (51.99) | 0.89 [0.871,0.944] | 1.08 [0.954,71.713] | 1.32 | 0.122 | 0.195 |
| uncultured_Syntrophobacterales_bacterium | 3.12 (6.28) | 6.74 (15.29) | 0.89 [0.871,0.944] | 0.99 [0.954,1.156] | 2.16 | 0.122 | 0.195 |
| uncultured_beta_proteobacterium | 24.12 (65.67) | 38.5 (59.71) | 0.89 [0.871,0.944] | 1.08 [0.954,63.953] | 1.6 | 0.122 | 0.195 |
| uncultured_candidate_division_SPAM_bacterium | 6.62 (16.18) | 16.04 (28.83) | 0.89 [0.871,0.944] | 0.99 [0.954,22.572] | 2.42 | 0.122 | 0.195 |
| BD1-7_clade | 14.03 (13.28) | 148.62 (241.28) | 13.26 [0.915,21.879] | 43.14 [1.156,173.057] | 10.6 | 0.146 | 0.219 |
| Chryseolinea | 34.92 (24.77) | 22.15 (43.96) | 32.13 [23.179,46.984] | 0.99 [0.954,22.572] | 0.63 | 0.146 | 0.219 |
| Dactylosporangium | 3.12 (6.28) | 4.08 (7.25) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 1.31 | 0.146 | 0.219 |
| Devosia | 4.12 (6.33) | 22.88 (40.42) | 0.91 [0.871,3.188] | 1 [0.954,26.284] | 5.55 | 0.146 | 0.219 |
| Faecalibaculum | 2193.4 (1252.13) | 6325.28 (5771.48) | 2266.45 [1267.062,2794.151] | 4171.23 [2236.488,10870.588] | 2.88 | 0.146 | 0.219 |
| Pseudoduganella | 1.95 (2.98) | 1.88 (2.82) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.96 | 0.146 | 0.219 |
| Psychrobacillus | 10.01 (22.38) | 18.53 (28.2) | 0.89 [0.871,3.188] | 5.41 [0.958,17.895] | 1.85 | 0.146 | 0.219 |
| Steroidobacter | 8.95 (22.78) | 7.94 (11.48) | 0.89 [0.871,0.944] | 0.99 [0.954,15.163] | 0.89 | 0.146 | 0.219 |
| uncultured_Acidimicrobidae_bacterium | 1.95 (2.98) | 37.73 (108.75) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 19.32 | 0.146 | 0.219 |
| uncultured_Acidobacteriales_bacterium | 4.29 (9.58) | 6.88 (15.34) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 1.61 | 0.146 | 0.219 |
| uncultured_Rubrobacterales_bacterium | 4.29 (9.58) | 8.05 (16.17) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 1.88 | 0.146 | 0.219 |
| Flavitalea | 3.12 (6.28) | 1.88 (2.82) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.6 | 0.173 | 0.246 |
| Geodermatophilus | 5.45 (12.88) | 6.06 (10.84) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 1.11 | 0.173 | 0.246 |
| MND1 | 41.42 (111.51) | 171.36 (343.4) | 0.91 [0.881,2.897] | 16.57 [0.954,159.861] | 4.14 | 0.173 | 0.246 |
| Polycyclovorans | 4.29 (9.58) | 1.85 (2.68) | 0.89 [0.871,0.944] | 0.99 [0.975,1.117] | 0.43 | 0.173 | 0.246 |
| Rhodomicrobium | 3.12 (6.28) | 1.88 (2.82) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.6 | 0.173 | 0.246 |
| Rubrobacter | 42.78 (118.47) | 16.8 (33.37) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.39 | 0.173 | 0.246 |
| Ruminiclostridium | 33.49 (34.99) | 413.71 (585.34) | 26.47 [15.221,36.424] | 101.35 [20.656,587.2] | 12.35 | 0.173 | 0.246 |
| Ruminococcaceae_UCG-005 | 16.06 (14.35) | 91.65 (163.48) | 17.75 [0.926,23.676] | 29.86 [11.638,76.037] | 5.71 | 0.173 | 0.246 |
| Solirubrobacter | 36.95 (101.97) | 22.91 (47.04) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.62 | 0.173 | 0.246 |
| Sphingoaurantiacus | 8.95 (22.78) | 2.89 (4.05) | 0.89 [0.871,0.944] | 0.99 [0.975,1.117] | 0.32 | 0.173 | 0.246 |
| uncultured_Acidiferrimonas_sp. | 40.45 (111.87) | 1.88 (2.82) | 0.89 [0.871,0.944] | 0.99 [0.954,1.117] | 0.05 | 0.173 | 0.246 |
| Actinophytocola | 1.95 (2.98) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.51 | 0.203 | 0.262 |
| Adlercreutzia | 1619.91 (886.41) | 959.72 (846.6) | 1823.84 [1198.791,2385.269] | 543.25 [342.098,1342.873] | 0.59 | 0.203 | 0.262 |
| Crossiella | 3.12 (6.28) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.32 | 0.203 | 0.262 |
| Dietzia | 3.04 (3.99) | 19.09 (54.26) | 0.91 [0.871,2.958] | 0.99 [0.954,1.117] | 6.28 | 0.203 | 0.262 |
| Herpetosiphon | 7.79 (19.48) | 2.03 (3.35) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.26 | 0.203 | 0.262 |
| Lechevalieria | 4.29 (9.58) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.23 | 0.203 | 0.262 |
| Limnobacter | 136.86 (94.87) | 71.63 (172.06) | 152.52 [88.281,202.659] | 1.07 [0.954,7.792] | 0.52 | 0.203 | 0.262 |
| Lysinibacillus | 68.62 (134.22) | 3.82 (4.61) | 13.23 [0.972,54.88] | 0.99 [0.954,7.717] | 0.06 | 0.203 | 0.262 |
| Microvirga | 15.76 (35.62) | 14.31 (20.88) | 0.91 [0.871,5.262] | 9.38 [1.027,17.104] | 0.91 | 0.203 | 0.262 |
| Myxococcus | 1.95 (2.98) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.51 | 0.203 | 0.262 |
| Nordella | 15.95 (42.58) | 4.34 (10.65) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.27 | 0.203 | 0.262 |
| Novosphingobium | 11.57 (14.28) | 130.12 (248.55) | 9.3 [0.892,11.884] | 19.64 [3.237,80.81] | 11.25 | 0.203 | 0.262 |
| Parviterribacter | 1.95 (2.98) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.51 | 0.203 | 0.262 |
| Psychroglaciecola | 3.12 (6.28) | 1.89 (2.85) | 0.89 [0.871,0.944] | 0.99 [0.954,1.114] | 0.61 | 0.203 | 0.262 |
| Streptococcus | 30.88 (15.96) | 290.31 (775.37) | 35.26 [22.07,37.951] | 44.14 [26.822,74.592] | 9.4 | 0.203 | 0.262 |
| Tyzzerella | 11.33 (17.08) | 67.58 (92.71) | 8.54 [0.98,9.089] | 44.42 [1.156,86.181] | 5.96 | 0.203 | 0.262 |

**Supplementary Table S3.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| uncultured_Acidimicrobiales_bacterium | 3.12 (6.28) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.32 | 0.203 | 0.262 |
| uncultured_Nitrosomonadales_bacterium | 3.12 (6.28) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.32 | 0.203 | 0.262 |
| uncultured_Stella_sp. | 3.12 (6.28) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.32 | 0.203 | 0.262 |
| uncultured_archaeon | 4.29 (9.58) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.23 | 0.203 | 0.262 |
| wb1-P19 | 12.45 (32.68) | 0.99 (0.1) | 0.89 [0.871,0.944] | 0.99 [0.954,0.998] | 0.08 | 0.203 | 0.262 |
| Blastococcus | 22.9 (58.71) | 10.69 (22.5) | 0.89 [0.871,3.183] | 1 [0.954,1.156] | 0.47 | 0.237 | 0.296 |
| Chlorobi_bacterium_OLB6 | 11.61 (10.95) | 0.99 (0.1) | 9.49 [0.915,20.456] | 0.99 [0.954,0.998] | 0.09 | 0.237 | 0.296 |
| Erysipelatoclostridium | 316.07 (219.73) | 171.59 (174.44) | 298.25 [198.452,496.227] | 123.62 [90.958,178.617] | 0.54 | 0.237 | 0.296 |
| Ilumatobacter | 29.15 (25.69) | 100.42 (137.85) | 26.02 [13.779,38.092] | 43.26 [21.511,111.985] | 3.45 | 0.237 | 0.296 |
| Luteitalea | 28.05 (33.52) | 6.93 (10.69) | 13.35 [6.684,38.337] | 1 [0.954,7.726] | 0.25 | 0.237 | 0.296 |
| PAUC26f | 4.14 (6.52) | 22.56 (42.9) | 0.91 [0.871,3.004] | 1 [0.954,8.954] | 5.45 | 0.237 | 0.296 |
| uncultured_Acidobacterium_sp. | 4.16 (6.55) | 10.92 (21.77) | 0.89 [0.871,3.063] | 0.99 [0.954,7.717] | 2.62 | 0.237 | 0.296 |
| Alkaliphilus | 30.98 (40.11) | 3.19 (7) | 22.48 [0.915,38.726] | 0.99 [0.954,0.998] | 0.1 | 0.274 | 0.332 |
| IMCC26207 | 126.53 (103.27) | 54.83 (60.6) | 167.76 [13.134,202.793] | 27.26 [18.136,98.608] | 0.43 | 0.274 | 0.332 |
| Jeotgalicoccus | 114.31 (84.61) | 268.05 (317.37) | 94.29 [56.457,137.199] | 188.64 [102.03,257.061] | 2.34 | 0.274 | 0.332 |
| Kineosporia | 4.1 (6.49) | 4.65 (6.49) | 0.91 [0.878,2.933] | 1.08 [0.954,7.664] | 1.13 | 0.274 | 0.332 |
| Others | 17939.84 (22865.18) | 35657.64 (36988.55) | 10801.28 [8019.006,16412.581] | 21953.89 [9968.934,50709.745] | 1.99 | 0.274 | 0.332 |
| Parasutterella | 59.69 (60.37) | 827.19 (1000.96) | 47.81 [15.668,82.045] | 547.52 [10.325,1293.762] | 13.86 | 0.274 | 0.332 |
| Rothia | 3.96 (6.02) | 17.4 (39.09) | 0.92 [0.881,3.063] | 0.99 [0.954,11.174] | 4.39 | 0.274 | 0.332 |
| Sulfurifustis | 4.07 (6.46) | 57.25 (154.93) | 0.91 [0.881,2.869] | 1 [0.954,8.954] | 14.05 | 0.274 | 0.332 |
| Bacillus | 111.69 (93.18) | 716.4 (699.11) | 101.09 [48.066,169.802] | 723.6 [9.532,1161.625] | 6.41 | 0.315 | 0.377 |
| Thauera | 7.35 (10.22) | 57.1 (88.41) | 0.96 [0.878,11.146] | 1.16 [0.959,108.848] | 7.76 | 0.315 | 0.377 |
| [Eubacterium]_ventriosum_group | 2.9 (3.69) | 2.74 (3.67) | 0.92 [0.881,2.869] | 1 [0.975,1.156] | 0.94 | 0.315 | 0.377 |
| Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium | 22.52 (17.92) | 69.09 (81.71) | 17.93 [9.723,31.892] | 38.4 [11.862,107.37] | 3.07 | 0.36 | 0.411 |
| Blastocatella | 27.36 (20.57) | 14.29 (18.91) | 30.39 [14.13,37.045] | 1.16 [0.977,26.55] | 0.52 | 0.36 | 0.411 |
| Clostridium_sensu_stricto_1 | 7450.72 (14501.08) | 5435.62 (8824.68) | 1546.53 [1168.495,3758.959] | 1206.01 [216.754,4780.086] | 0.73 | 0.36 | 0.411 |
| Defluviimonas | 46.68 (52.25) | 257.03 (321.65) | 27.44 [19.584,59.445] | 126.02 [6.644,535.559] | 5.51 | 0.36 | 0.411 |
| Frigoribacterium | 12.63 (21.04) | 16.5 (14.6) | 0.96 [0.916,17.416] | 15.26 [3.174,22.332] | 1.31 | 0.36 | 0.411 |
| Membranicola | 9.38 (12.11) | 0.99 (0.1) | 4.92 [0.915,11.405] | 0.99 [0.954,0.998] | 0.11 | 0.36 | 0.411 |
| Ornithinimicrobium | 6.59 (11.05) | 0.99 (0.1) | 0.91 [0.878,5.073] | 0.99 [0.954,0.998] | 0.15 | 0.36 | 0.411 |
| Pseudorhodoplanes | 3.92 (4.16) | 7.68 (12.09) | 0.96 [0.892,8.769] | 1.07 [0.954,9.962] | 1.96 | 0.36 | 0.411 |
| Rubellimicrobium | 5.09 (6.35) | 15.37 (25.6) | 0.93 [0.896,8.939] | 5.32 [1.033,9.806] | 3.02 | 0.36 | 0.411 |
| Ruminiclostridium_9 | 61.73 (89.67) | 492.18 (513.51) | 39.28 [16.487,47.643] | 441.15 [9.532,834.494] | 7.97 | 0.36 | 0.411 |
| Thermomonas | 168.25 (395.23) | 446.72 (554.01) | 27.45 [7.006,60.612] | 221.18 [3.756,882.261] | 2.66 | 0.36 | 0.411 |
| Acetatifactor | 64.08 (67.04) | 37.74 (44) | 40.41 [23.979,76.913] | 19.48 [3.756,59.895] | 0.59 | 0.408 | 0.459 |
| Lactococcus | 74.38 (45.4) | 57.53 (49.37) | 62.2 [42.462,117.104] | 48.12 [12.889,92.728] | 0.77 | 0.408 | 0.459 |
| mle1-7 | 6.26 (9.48) | 21.45 (41.87) | 0.96 [0.892,8.769] | 1.16 [0.954,9.895] | 3.42 | 0.408 | 0.459 |
| uncultured_soil_bacterium | 33.81 (66.26) | 117.84 (237.51) | 12.97 [0.967,20.494] | 19.22 [3.298,66.865] | 3.49 | 0.408 | 0.459 |
| Corynebacterium_1 | 50.2 (75.51) | 92.49 (142.32) | 22 [15.755,38.343] | 31.23 [19.857,60.257] | 1.84 | 0.46 | 0.505 |
| JTB255_marine_benthic_group | 10.75 (12.22) | 54.87 (90.32) | 4.77 [0.921,19.815] | 1.08 [0.954,61.907] | 5.11 | 0.46 | 0.505 |
| Lachnospiraceae_NK4A136_group | 121.44 (114) | 1928.28 (1870.48) | 104.35 [53.164,130.967] | 2091.14 [3.757,3210.374] | 15.88 | 0.46 | 0.505 |
| OM27_clade | 27.08 (22.85) | 80.8 (118.45) | 26.37 [13.761,32.511] | 37.98 [3.08,89.617] | 2.98 | 0.46 | 0.505 |
| Roseburia | 28.24 (25.14) | 73.5 (90.8) | 22.88 [6.615,53.663] | 43.26 [6.644,77.358] | 2.6 | 0.46 | 0.505 |
| Turicibacter | 155.97 (240.52) | 282.42 (270.77) | 60.32 [23.866,134.382] | 285.97 [49.994,382.267] | 1.81 | 0.46 | 0.505 |
| Candidatus_Alysiosphaera | 109.13 (76.12) | 95.83 (117.4) | 111.62 [62.719,142.788] | 36.65 [13.623,179.268] | 0.88 | 0.515 | 0.559 |
| Cutibacterium | 7.01 (8.55) | 1.88 (2.82) | 4.87 [0.932,8.885] | 0.99 [0.954,1.117] | 0.27 | 0.515 | 0.559 |
| Lautropia | 51.04 (41.21) | 35.16 (45.51) | 42 [26.035,86.434] | 13.59 [1.033,72.153] | 0.69 | 0.515 | 0.559 |
| Bryobacter | 45.45 (84.63) | 56.01 (58.18) | 18.01 [6.843,26.219] | 52.14 [1.018,91.578] | 1.23 | 0.573 | 0.615 |
| Coriobacteriaceae_UCG-002 | 103464.05 (30339.31) | 97777.55 (88162.11) | 97390.96 [79104.451,119979.954] | 80790.19 [28098.049,142586.205] | 0.95 | 0.573 | 0.615 |
| Gemmatimonas | 36.49 (30.15) | 57.18 (46.93) | 35.64 [16.047,47.439] | 39.45 [34.845,88.431] | 1.57 | 0.573 | 0.615 |
| Pajaroellobacter | 21.68 (13.53) | 47.59 (54.08) | 18.36 [17.5,23.676] | 29.55 [1,100.685] | 2.19 | 0.633 | 0.676 |
| Sphingomonas | 93.07 (127.59) | 128.67 (155.28) | 44.08 [7.697,109.939] | 68.19 [32.939,180.785] | 1.38 | 0.633 | 0.676 |
| Facklamia | 20.16 (15.92) | 20.52 (29.59) | 18.3 [13.352,25.541] | 10.64 [2.851,19.594] | 1.02 | 0.696 | 0.729 |
| Hymenobacter | 2.88 (3.65) | 0.99 (0.1) | 0.93 [0.892,2.933] | 0.99 [0.954,0.998] | 0.34 | 0.696 | 0.729 |
| Litorilinea | 8.31 (9.55) | 1.85 (2.68) | 4.77 [0.916,11.98] | 0.99 [0.975,1.117] | 0.22 | 0.696 | 0.729 |

**Supplementary Table S3.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Paenibacillus | 20.58 (29.74) | 22.73 (23.34) | 8.66 [0.921,21.464] | 14.43 [1.156,46.364] | 1.1 | 0.696 | 0.729 |
| Subgroup_10 | 137.82 (86.71) | 581.69 (761.49) | 137.63 [80.307,191.409] | 184.21 [26.018,1267.956] | 4.22 | 0.696 | 0.729 |
| Aeromicrobium | 10.87 (11.15) | 9.23 (12.95) | 9.21 [0.878,18.523] | 1.08 [0.977,11.151] | 0.85 | 0.762 | 0.781 |
| Bifidobacterium | 40062.33 (33655.34) | 29469.07 (16088.93) | 29885.88 [15838.17,52463.921] | 25955.79 [17495.655,36777.113] | 0.74 | 0.762 | 0.781 |
| Ellin6055 | 83.4 (127.71) | 51.11 (52.58) | 18.56 [0.91,118.889] | 38.84 [2.968,89.841] | 0.61 | 0.762 | 0.781 |
| Gluconobacter | 39.09 (77.26) | 0.99 (0.1) | 0.96 [0.921,30.393] | 0.99 [0.954,0.998] | 0.03 | 0.762 | 0.781 |
| Methylobacterium | 7.06 (9.68) | 3.58 (4.16) | 0.96 [0.921,10.694] | 1.07 [0.975,6.926] | 0.51 | 0.762 | 0.781 |
| Tissierella | 7.73 (10.97) | 0.99 (0.1) | 0.91 [0.871,11.405] | 0.99 [0.954,0.998] | 0.13 | 0.762 | 0.781 |
| Enterococcus | 89153.33 (44544.21) | 98702.39 (66772.2) | 101890.9 [69635.87,125285.442] | 79531.53 [42039.398,157561.055] | 1.11 | 0.829 | 0.843 |
| Gaiella | 99.71 (149.38) | 138.9 (214.69) | 39.78 [20.098,106.811] | 58.29 [3.237,180.565] | 1.39 | 0.829 | 0.843 |
| Aerococcus | 465.8 (692.55) | 478.98 (928.6) | 146.1 [53.628,509.256] | 140.12 [112.172,356.906] | 1.03 | 0.897 | 0.903 |
| Lachnospiraceae_UCG-006 | 17.42 (12.22) | 19.63 (23.56) | 22.06 [6.78,27.267] | 9.7 [3.08,27.144] | 1.13 | 0.897 | 0.903 |
| OLB13 | 16.24 (21.23) | 5.63 (7.93) | 9.07 [0.916,21.011] | 1.07 [0.958,7.664] | 0.35 | 0.897 | 0.903 |
| Acidobacteria_bacterium_enrichment_culture_clone_CV-04 | 7.21 (7.42) | 5.62 (12.1) | 4.98 [0.91,11.838] | 1 [0.977,1.156] | 0.78 | 1 | 1 |
| Propioniciclava | 7.4 (12.5) | 2.87 (5.95) | 0.96 [0.892,8.769] | 0.99 [0.954,1.117] | 0.39 | 1 | 1 |

**Supplementary Table S4.** Univariate analysis (Mann–Whitney U test) of the high fat and high fat containing EC 20mg/Kg taxa at the Phylum level and corrected for multiple testing with FDR (q<0.1)

| Taxa | HFE20.MeanSD | HF.MeanSD | HFE20.MedianIQR | HF.MedianIQR | FC | P | FDR.P |
|---|---|---|---|---|---|---|---|
| Nitrospirae | 487.47 (274.89) | 31.29 (56.16) | 357.71 [294.447,592.58] | 8.93 [6.7,26.626] | 0.06 | 4.60E-05 | 7.30E-04 |
| Thaumarchaeota | 180.67 (92.26) | 5.45 (12.88) | 181.21 [101.618,230.197] | 0.89 [0.871,0.944] | 0.03 | 4.60E-05 | 7.30E-04 |
| Acidobacteria | 5405.54 (1752.33) | 753.08 (1450.33) | 5519.67 [4846.803,6204.249] | 303.55 [183.421,362.775] | 0.14 | 1.80E-04 | 2.00E-03 |
| Entotheonellaeota | 154.21 (132.74) | 14.78 (39.27) | 138.03 [37.675,206.652] | 0.89 [0.871,0.944] | 0.1 | 5.50E-04 | 3.50E-03 |
| Elusimicrobia | 57.67 (61.15) | 0.9 (0.05) | 41.17 [18.232,77.612] | 0.89 [0.871,0.928] | 0.02 | 8.70E-04 | 3.50E-03 |
| Firmicutes | 897225.3 (41267.08) | 808874.56 (89958.47) | 910837.85 [900273.345,911971.643] | 834916.03 [768297.736,877981.873] | 0.9 | 8.70E-04 | 3.50E-03 |
| GAL15 | 68.17 (63.72) | 6.62 (16.18) | 44.14 [20.24,106.931] | 0.89 [0.871,0.944] | 0.1 | 8.70E-04 | 3.50E-03 |
| Gemmatimonadetes | 2687.91 (863.7) | 423.96 (779.98) | 2346.58 [2199.856,2899.898] | 162.76 [97.945,243.449] | 0.16 | 8.70E-04 | 3.50E-03 |
| Actinobacteria | 79601.55 (43104.02) | 149670.2 (47585.99) | 64521.14 [62162.309,78153.004] | 155996.77 [108531.2,180592.962] | 1.88 | 2.10E-03 | 5.10E-03 |
| Deinococcus-Thermus | 2.54 (3.43) | 648.93 (528.89) | 0.93 [0.895,0.959] | 643.73 [221.465,1076.613] | 255.83 | 2.10E-03 | 5.10E-03 |
| Euryarchaeota | 45.78 (52.53) | 7.79 (19.48) | 32.48 [11.548,54.485] | 0.89 [0.871,0.944] | 0.17 | 2.10E-03 | 5.10E-03 |
| Others | 837.61 (570.34) | 172.12 (237.98) | 761.26 [469.485,1087.523] | 56.98 [41.101,180.414] | 0.21 | 2.10E-03 | 5.10E-03 |
| Rokubacteria | 419.65 (312.12) | 78.94 (220.73) | 283.81 [184.923,606.241] | 0.89 [0.871,0.944] | 0.19 | 2.10E-03 | 5.10E-03 |
| Bacteroidetes | 655.68 (313.67) | 272.58 (156.69) | 618.22 [388.322,855.815] | 261.81 [178.581,372.011] | 0.42 | 6.20E-03 | 1.30E-02 |
| Latescibacteria | 98.06 (60.32) | 19.45 (52.47) | 91.22 [68.163,127.623] | 0.89 [0.871,0.944] | 0.2 | 6.20E-03 | 1.30E-02 |
| FCPU426 | 13.16 (16.01) | 1.95 (2.98) | 8.86 [0.932,15.57] | 0.89 [0.871,0.944] | 0.15 | 3.40E-02 | 0.069 |
| Planctomycetes | 101.97 (56.98) | 418.32 (342.58) | 87.2 [75.129,128.938] | 374.23 [166.451,673.632] | 4.1 | 0.055 | 0.103 |
| WPS-2 | 19.33 (18.56) | 48.79 (29.57) | 14.17 [2.84,28.705] | 52.11 [34.528,64.461] | 2.52 | 0.068 | 0.12 |
| Armatimonadetes | 166.83 (115.77) | 320.19 (199.75) | 203.34 [82.976,232.492] | 356.2 [243.91,471.592] | 1.92 | 0.083 | 0.14 |
| Verrucomicrobia | 663.59 (281.33) | 483.6 (368.3) | 582.68 [469.088,777.084] | 371.49 [277.862,568.847] | 0.73 | 0.122 | 0.195 |
| Chlamydiae | 7.98 (14.62) | 0.9 (0.05) | 0.93 [0.895,7.315] | 0.89 [0.871,0.928] | 0.11 | 0.203 | 0.295 |
| Tenericutes | 2.52 (3.39) | 0.9 (0.05) | 0.93 [0.895,0.959] | 0.89 [0.871,0.928] | 0.36 | 0.203 | 0.295 |
| Proteobacteria | 8457.15 (1930.34) | 34467.25 (65993.71) | 7844 [7200.244,10049.419] | 13723.96 [6951.071,18457.478] | 4.08 | 0.274 | 0.382 |
| Cyanobacteria | 321.91 (417.57) | 919.63 (1052.17) | 118.31 [47.826,425.645] | 607.99 [67.233,1361.624] | 2.86 | 0.46 | 0.61 |
| Dependentiae | 3.54 (5.95) | 5 (6.34) | 0.9 [0.883,0.95] | 0.96 [0.916,8.638] | 1.41 | 0.515 | 0.61 |
| Fibrobacteres | 13.75 (32.54) | 3.94 (6.03) | 0.95 [0.914,8.629] | 0.93 [0.896,2.956] | 0.29 | 0.515 | 0.61 |
| Hydrogenedentes | 0.91 (0.04) | 2.88 (3.65) | 0.9 [0.883,0.94] | 0.93 [0.892,2.933] | 3.17 | 0.515 | 0.61 |
| BRC1 | 9.71 (11.23) | 6.31 (8.12) | 4.78 [0.901,16.606] | 0.93 [0.898,10.824] | 0.65 | 0.573 | 0.632 |
| Epsilonbacteraeota | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.883,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.632 |
| Nanoarchaeaeota | 2.64 (5.47) | 4.29 (9.58) | 0.91 [0.883,0.95] | 0.89 [0.871,0.944] | 1.62 | 0.633 | 0.654 |
| Patescibacteria | 147.11 (126.31) | 271.84 (269.93) | 118.38 [49.658,199.579] | 258.38 [13.136,498.383] | 1.85 | 0.633 | 0.654 |
| Chloroflexi | 2139.17 (436.84) | 2062.64 (1201.91) | 2225.36 [1782.003,2454.914] | 2202.27 [1398.875,2794.068] | 0.96 | 1 | 1 |

**Supplementary Table S5.** Univariate analysis (Mann–Whitney U test) of the high fat and high fat containing EC 20mg/Kg taxa at the Genus level and corrected for multiple testing with FDR (q<0.1)

| Taxa | HFE20.MeanSD | HF.MeanSD | HFE20.MedianIQR | HF.MedianIQR | FC | P | FDR.P |
|---|---|---|---|---|---|---|---|
| Altererythrobacter | 69.12 (51.52) | 3.12 (6.28) | 53.24 [43.364,77.146] | 0.89 [0.871,0.944] | 0.05 | 4.60E-05 | 0.001 |
| Candidatus_Nitrososphaera | 35.77 (19.06) | 0.9 (0.05) | 41.19 [19.897,50.876] | 0.89 [0.871,0.928] | 0.03 | 4.60E-05 | 0.001 |
| Enterobacter | 14.63 (11.8) | 14938.86 (40682.95) | 9.27 [8.713,24.453] | 697.38 [453.94,835.793] | 1020.77 | 4.60E-05 | 0.001 |
| Lechevalieria | 108.39 (49.7) | 4.29 (9.58) | 95.56 [68.769,156.643] | 0.89 [0.871,0.944] | 0.04 | 4.60E-05 | 0.001 |
| Microvirga | 177.2 (46.85) | 15.76 (35.62) | 173.4 [154.626,193.828] | 0.91 [0.871,5.262] | 0.09 | 4.60E-05 | 0.001 |
| Nitrospira | 487.77 (275.08) | 30.32 (56.68) | 357.98 [294.51,592.779] | 8.93 [0.962,26.627] | 0.06 | 4.60E-05 | 0.001 |
| Opitutus | 45.33 (23.72) | 1.95 (2.98) | 45.82 [21.397,65.194] | 0.89 [0.871,0.944] | 0.04 | 4.60E-05 | 0.001 |
| Pseudonocardia | 36.29 (19.41) | 1.95 (2.98) | 34.87 [20.76,38.291] | 0.89 [0.871,0.944] | 0.05 | 4.60E-05 | 0.001 |
| Sphingomonas | 754.34 (290.84) | 93.07 (127.59) | 737.18 [536.644,905.396] | 44.08 [7.697,109.939] | 0.12 | 4.60E-05 | 0.001 |
| Terrimonas | 32.6 (22.97) | 1.95 (2.98) | 23.91 [18.031,35.039] | 0.89 [0.871,0.944] | 0.06 | 4.60E-05 | 0.001 |
| UTCFX1 | 58.52 (41.01) | 2.94 (3.77) | 41.25 [36.632,89.458] | 0.91 [0.878,2.933] | 0.05 | 4.60E-05 | 0.001 |
| uncultured_Acidimicrobiales_bacterium | 121.51 (89.38) | 3.12 (6.28) | 92.4 [59.953,199.903] | 0.89 [0.871,0.944] | 0.03 | 4.60E-05 | 0.001 |
| uncultured_Acidobacterium_sp. | 141.72 (83.19) | 4.16 (6.55) | 109.09 [73.134,212.904] | 0.89 [0.871,3.063] | 0.03 | 4.60E-05 | 0.001 |
| Chthoniobacter | 42.36 (35.66) | 0.9 (0.05) | 32.81 [26.544,49.893] | 0.89 [0.871,0.928] | 0.02 | 9.10E-05 | 0.0015 |
| Hirschia | 30.09 (18.17) | 0.9 (0.05) | 28.09 [26.163,35.039] | 0.89 [0.871,0.928] | 0.03 | 9.10E-05 | 0.0015 |
| Nonomuraea | 23.54 (11) | 0.9 (0.05) | 22.82 [18.142,32.857] | 0.89 [0.871,0.928] | 0.04 | 9.10E-05 | 0.0015 |
| Skermanella | 101.03 (63.32) | 3.12 (6.28) | 105.96 [52.58,155.648] | 0.89 [0.871,0.944] | 0.03 | 9.10E-05 | 0.0015 |
| uncultured_soil_bacterium | 313.2 (78.8) | 33.81 (66.26) | 299.62 [262.488,359.005] | 12.97 [0.967,20.494] | 0.11 | 9.10E-05 | 0.0015 |
| Acidibacter | 112.86 (56.32) | 10.12 (26.08) | 89.91 [77.362,135.257] | 0.89 [0.871,0.944] | 0.09 | 1.80E-04 | 0.0019 |
| Bdellovibrio | 72.63 (64.87) | 5.09 (6.32) | 47.28 [35.427,100.039] | 0.93 [0.892,9.116] | 0.07 | 1.80E-04 | 0.0019 |
| Bryobacter | 419.05 (187.12) | 45.45 (84.63) | 367.89 [316.797,525.954] | 18.01 [6.843,26.219] | 0.11 | 1.80E-04 | 0.0019 |
| Dongia | 231.44 (104.45) | 21.78 (59.07) | 255.38 [194.121,273.147] | 0.89 [0.871,0.944] | 0.09 | 1.80E-04 | 0.0019 |
| Kribbella | 51.28 (25.47) | 3.12 (6.28) | 54.71 [29.535,70.532] | 0.89 [0.871,0.944] | 0.06 | 1.80E-04 | 0.0019 |
| MND1 | 476.69 (189.14) | 41.42 (111.51) | 457.23 [368.857,535.667] | 0.91 [0.881,2.897] | 0.09 | 1.80E-04 | 0.0019 |
| Phenylobacterium | 58.76 (36.74) | 4.29 (9.58) | 58.08 [34.636,72.556] | 0.89 [0.871,0.944] | 0.07 | 1.80E-04 | 0.0019 |
| Steroidobacter | 155.3 (124.02) | 8.95 (22.78) | 138.76 [82.213,179.64] | 0.89 [0.871,0.944] | 0.06 | 1.80E-04 | 0.0019 |
| Streptomyces | 72.72 (42.73) | 5.45 (12.88) | 53.26 [47.286,101.519] | 0.89 [0.871,0.944] | 0.07 | 1.80E-04 | 0.0019 |
| Vicinamibacter | 22.1 (21.29) | 0.9 (0.05) | 17.26 [9.015,26.241] | 0.89 [0.871,0.928] | 0.04 | 1.80E-04 | 0.0019 |
| Candidatus_Udaeobacter | 69.91 (37.93) | 4.29 (9.58) | 68.45 [45.963,97.423] | 0.89 [0.871,0.944] | 0.06 | 3.20E-04 | 0.0025 |
| Denitratisoma | 0.91 (0.04) | 153.68 (111.78) | 0.9 [0.884,0.94] | 198.38 [53.725,225.191] | 168.91 | 3.20E-04 | 0.0025 |
| Hyphomicrobium | 20.58 (32.67) | 2802.06 (2650.28) | 8.96 [0.946,17.74] | 2785.24 [113.61,4891.32] | 136.17 | 3.20E-04 | 0.0025 |
| Parviterribacter | 39.04 (40.95) | 1.95 (2.98) | 18.57 [17.352,56.976] | 0.89 [0.871,0.944] | 0.05 | 3.20E-04 | 0.0025 |
| Sorangium | 12.87 (11.27) | 0.9 (0.05) | 8.97 [8.631,16.809] | 0.89 [0.871,0.928] | 0.07 | 3.20E-04 | 0.0025 |
| Trichococcus | 0.91 (0.04) | 90.21 (62.69) | 0.9 [0.884,0.94] | 116.14 [34.374,141.397] | 99.15 | 3.20E-04 | 0.0025 |
| uncultured_Acidobacteria_bacterium | 304.23 (137.93) | 28.78 (78.87) | 281.34 [210.427,321.887] | 0.89 [0.871,0.944] | 0.09 | 3.20E-04 | 0.0025 |
| uncultured_Nitrosomonadales_bacterium | 40.37 (26.68) | 3.12 (6.28) | 35.87 [29.309,54.763] | 0.89 [0.871,0.944] | 0.08 | 3.20E-04 | 0.0025 |
| uncultured_Syntrophobacterales_bacterium | 40.82 (22.02) | 3.12 (6.28) | 41.49 [21.151,58.778] | 0.89 [0.871,0.944] | 0.08 | 3.20E-04 | 0.0025 |
| Ellin6055 | 497.94 (241.45) | 83.4 (127.71) | 482.37 [296.366,732.118] | 18.56 [0.91,118.889] | 0.17 | 5.50E-04 | 0.0038 |
| Lactobacillus | 39878.79 (21822) | 6450.17 (9861.64) | 36342.81 [25916.147,43840.689] | 3235.28 [2030.836,4361.013] | 0.16 | 5.50E-04 | 0.0038 |
| Nordella | 144.1 (77.72) | 15.95 (42.58) | 157.86 [77.757,203.132] | 0.89 [0.871,0.944] | 0.11 | 5.50E-04 | 0.0038 |
| Paenarthrobacter | 80.85 (121.86) | 0.9 (0.05) | 36.95 [27.021,62.932] | 0.89 [0.871,0.928] | 0.01 | 5.50E-04 | 0.0038 |
| metagenome | 382.33 (139.86) | 129.5 (78.92) | 388.81 [294.785,455.549] | 141.92 [76.85,155.518] | 0.34 | 5.50E-04 | 0.0038 |
| Bacillus | 334.25 (193.92) | 111.69 (93.18) | 281.52 [218.209,357.719] | 101.09 [48.066,169.802] | 0.33 | 8.70E-04 | 0.005 |
| Lachnoclostridium | 97.48 (70.84) | 396.51 (279) | 70.32 [51.64,130.218] | 352.79 [259.908,426.691] | 4.07 | 8.70E-04 | 0.005 |
| Paludibaculum | 34.3 (18.19) | 4.11 (6.5) | 36.51 [20.746,46.072] | 0.91 [0.871,2.956] | 0.12 | 8.70E-04 | 0.005 |
| RB41 | 1067.41 (633.73) | 134.94 (379.13) | 1063.58 [607.714,1444.277] | 0.89 [0.871,0.944] | 0.13 | 8.70E-04 | 0.005 |
| Rubrobacter | 368.19 (229.47) | 42.78 (118.47) | 342.57 [158.312,584.574] | 0.89 [0.871,0.944] | 0.12 | 8.70E-04 | 0.005 |
| Sphingoaurantiacus | 56.37 (25.72) | 8.95 (22.78) | 61.7 [31.693,78.291] | 0.89 [0.871,0.944] | 0.16 | 8.70E-04 | 0.005 |
| uncultured_Acidobacteriales_bacterium | 70.44 (57.66) | 4.29 (9.58) | 54.74 [24.392,109.47] | 0.89 [0.871,0.944] | 0.06 | 8.70E-04 | 0.005 |
| uncultured_actinobacterium | 176.61 (87.38) | 25.28 (68.97) | 175.62 [107.103,223.131] | 0.89 [0.871,0.944] | 0.14 | 8.70E-04 | 0.005 |
| Blastococcus | 186.17 (103.64) | 22.9 (58.71) | 142.99 [121.549,198.498] | 0.89 [0.871,3.183] | 0.12 | 1.40E-03 | 0.0065 |
| Caenimonas | 14.64 (16.59) | 0.9 (0.05) | 9.05 [2.873,21.739] | 0.89 [0.871,0.928] | 0.06 | 1.40E-03 | 0.0065 |
| Candidatus_Solibacter | 65.82 (40.42) | 11.29 (29.38) | 74.76 [28.941,94.816] | 0.89 [0.871,0.944] | 0.17 | 1.40E-03 | 0.0065 |
| Ellin6067 | 153.37 (91.88) | 21.49 (51.78) | 132.58 [83.096,202.715] | 0.95 [0.871,8.944] | 0.14 | 1.40E-03 | 0.0065 |
| Gaiella | 409.52 (196.85) | 99.71 (149.38) | 479.1 [249.351,544.784] | 39.78 [20.098,106.811] | 0.24 | 1.40E-03 | 0.0065 |
| Haliangium | 304.63 (148.34) | 58.18 (116.49) | 291.78 [196.304,424.304] | 22 [8.923,28.311] | 0.19 | 1.40E-03 | 0.0065 |

**Supplementary Table S5.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Microlunatus | 68.96 (44.53) | 5.45 (12.88) | 68.32 [41.511,95.335] | 0.89 [0.871,0.944] | 0.08 | 1.40E-03 | 0.0065 |
| Nocardioides | 63.94 (77.75) | 6.29 (9.49) | 35.33 [18.914,59.242] | 0.96 [0.878,8.817] | 0.1 | 1.40E-03 | 0.0065 |
| Solirubrobacter | 312.72 (153.73) | 36.95 (101.97) | 258.81 [211.142,396.951] | 0.89 [0.871,0.944] | 0.12 | 1.40E-03 | 0.0065 |
| uncultured_Aciditerrimonas_sp. | 282.9 (133.49) | 40.45 (111.87) | 254.94 [208.987,378.437] | 0.89 [0.871,0.944] | 0.14 | 1.40E-03 | 0.0065 |
| uncultured_Gemmatimonadetes_bacterium | 41.49 (30.64) | 3.12 (6.28) | 46 [11.86,64.633] | 0.89 [0.871,0.944] | 0.08 | 1.40E-03 | 0.0065 |
| Candidatus_Nitrocosmicus | 44.15 (51.55) | 0.9 (0.05) | 23 [11.616,56.796] | 0.89 [0.871,0.928] | 0.02 | 2.10E-03 | 0.0085 |
| Conexibacter | 41.79 (19.89) | 9.2 (17.52) | 49.12 [28.372,53.962] | 0.89 [0.871,5.332] | 0.22 | 2.10E-03 | 0.0085 |
| Iamia | 106.79 (66.86) | 13.21 (21.95) | 90.96 [58.118,156.479] | 5.08 [0.871,11.884] | 0.12 | 2.10E-03 | 0.0085 |
| Lysobacter | 163.19 (84.18) | 28.78 (78.87) | 146.35 [117.464,223.466] | 0.89 [0.871,0.944] | 0.18 | 2.10E-03 | 0.0085 |
| Mycobacterium | 40.24 (29.04) | 4.11 (6.5) | 32.25 [20.746,51.229] | 0.91 [0.871,2.956] | 0.1 | 2.10E-03 | 0.0085 |
| Nannocystis | 19.86 (17.91) | 1.95 (2.98) | 17.58 [9.481,25.668] | 0.89 [0.871,0.944] | 0.1 | 2.10E-03 | 0.0085 |
| Reyranella | 53.71 (41.85) | 7.79 (19.48) | 40.6 [30.21,71.223] | 0.89 [0.871,0.944] | 0.14 | 2.10E-03 | 0.0085 |
| Sulfurifustis | 30.56 (31.76) | 4.07 (6.46) | 17.58 [11.459,25.719] | 0.91 [0.881,2.869] | 0.13 | 2.10E-03 | 0.0085 |
| Truepera | 2.54 (3.43) | 648.9 (528.89) | 0.93 [0.896,0.96] | 643.68 [221.433,1076.517] | 255.64 | 2.10E-03 | 0.0085 |
| Polycyclovorans | 47.88 (37.42) | 4.29 (9.58) | 41 [26.261,77.146] | 0.89 [0.871,0.944] | 0.09 | 3.10E-03 | 0.012 |
| Ramlibacter | 40.51 (30.68) | 7.42 (15.49) | 32.01 [18.237,69.04] | 0.93 [0.878,2.933] | 0.18 | 3.10E-03 | 0.012 |
| uncultured_Acidimicrobidae_bacterium | 35.41 (32.72) | 1.95 (2.98) | 36.19 [8.754,46.567] | 0.89 [0.871,0.944] | 0.06 | 3.10E-03 | 0.012 |
| uncultured_Holophaga_sp. | 23.24 (20.91) | 0.9 (0.05) | 17.93 [11.283,30.619] | 0.89 [0.871,0.928] | 0.04 | 3.10E-03 | 0.012 |
| uncultured_Verrucomicrobia_bacterium | 12.7 (12.76) | 0.9 (0.05) | 9.27 [8.958,9.595] | 0.89 [0.871,0.928] | 0.07 | 3.10E-03 | 0.012 |
| uncultured_beta_proteobacterium | 155.48 (46.95) | 24.12 (65.67) | 153.56 [132.748,178.88] | 0.89 [0.871,0.944] | 0.16 | 3.10E-03 | 0.012 |
| CL500-29_marine_group | 37.53 (30.54) | 6.62 (16.18) | 31.53 [17.916,54.288] | 0.89 [0.871,0.944] | 0.18 | 4.40E-03 | 0.016 |
| Clostridium_sensu_stricto_1 | 27501.66 (17440.69) | 7450.72 (14501.08) | 27509.74 [13554.328,40012.297] | 1546.53 [1168.495,3758.959] | 0.27 | 4.40E-03 | 0.016 |
| IS-44 | 43.04 (33.55) | 6.62 (16.18) | 37.2 [26.502,46.031] | 0.89 [0.871,0.944] | 0.15 | 4.40E-03 | 0.016 |
| Myxococcus | 30.92 (29.89) | 1.95 (2.98) | 23.86 [11.358,42.488] | 0.89 [0.871,0.944] | 0.06 | 4.40E-03 | 0.016 |
| Planococcus | 46.02 (37.45) | 0.9 (0.05) | 57.34 [5.098,75.081] | 0.89 [0.871,0.928] | 0.02 | 4.40E-03 | 0.016 |
| Coriobacteriaceae_UCG-002 | 59986.86 (44261.73) | 103464.05 (30339.31) | 47384.04 [33221.453,65108.691] | 97390.96 [79104.451,119979.954] | 1.72 | 6.20E-03 | 0.02 |
| Facklamia | 0.91 (0.04) | 20.16 (15.92) | 0.9 [0.884,0.94] | 18.3 [13.352,25.541] | 22.15 | 6.20E-03 | 0.02 |
| Jeotgalicoccus | 34.53 (57.61) | 114.31 (84.61) | 9.03 [0.955,43.646] | 94.29 [56.457,137.199] | 3.31 | 6.20E-03 | 0.02 |
| Kibdelosporangium | 10.21 (7.45) | 0.9 (0.05) | 9.36 [3,17.266] | 0.89 [0.871,0.928] | 0.09 | 6.20E-03 | 0.02 |
| Luteimonas | 17.47 (35.88) | 308.03 (240.28) | 0.96 [0.919,8.87] | 357.46 [81.648,501.387] | 17.63 | 6.20E-03 | 0.02 |
| Marmoricola | 21.28 (17.57) | 0.9 (0.05) | 18.42 [8.925,33.102] | 0.89 [0.871,0.928] | 0.04 | 6.20E-03 | 0.02 |
| uncultured_Stella_sp. | 31.39 (32.04) | 3.12 (6.28) | 27.04 [9.24,36.857] | 0.89 [0.871,0.944] | 0.1 | 6.20E-03 | 0.02 |
| uncultured_archaeon | 33.61 (44.39) | 4.29 (9.58) | 21.93 [8.834,34.485] | 0.89 [0.871,0.944] | 0.13 | 6.20E-03 | 0.02 |
| Aeromicrobium | 83.06 (71.16) | 10.87 (11.15) | 63.83 [23.093,126.904] | 9.21 [0.878,18.523] | 0.13 | 8.50E-03 | 0.025 |
| Bifidobacterium | 8871.55 (6417.46) | 40062.33 (33655.34) | 6160.11 [3647.824,14897.749] | 29885.88 [15838.17,52463.921] | 4.52 | 8.50E-03 | 0.025 |
| Novosphingobium | 39.82 (28.86) | 11.57 (14.28) | 36.01 [20.814,46.567] | 9.3 [0.892,11.884] | 0.29 | 8.50E-03 | 0.025 |
| Paenibacillus | 76.94 (55.78) | 20.58 (29.74) | 75.9 [28.718,117.161] | 8.66 [0.921,21.464] | 0.27 | 8.50E-03 | 0.025 |
| Pseudoduganella | 25.23 (26.94) | 1.95 (2.98) | 13.28 [9.015,33.452] | 0.89 [0.871,0.944] | 0.08 | 8.50E-03 | 0.025 |
| Rhizobacter | 27.98 (45.98) | 1.95 (2.98) | 9.57 [8.925,18.643] | 0.89 [0.871,0.944] | 0.07 | 8.50E-03 | 0.025 |
| SWB02 | 14.73 (19.97) | 0.9 (0.05) | 8.81 [0.946,16.342] | 0.89 [0.871,0.928] | 0.06 | 8.50E-03 | 0.025 |
| Tumebacillus | 18.23 (18.55) | 0.9 (0.05) | 13.57 [3.055,26.924] | 0.89 [0.871,0.928] | 0.05 | 8.50E-03 | 0.025 |
| mle1-7 | 21.88 (16.08) | 6.26 (9.48) | 22.48 [9.096,28.244] | 0.96 [0.892,8.769] | 0.29 | 8.50E-03 | 0.025 |
| uncultured_delta_proteobacterium | 29.37 (19.35) | 362.08 (249.14) | 26.16 [19.897,40.654] | 365.79 [202.842,587.571] | 12.33 | 8.50E-03 | 0.025 |
| wb1-P19 | 70.64 (84.67) | 12.45 (32.68) | 37.58 [8.71,104.484] | 0.89 [0.871,0.944] | 0.18 | 8.50E-03 | 0.025 |
| Adhaeribacter | 28 (37.07) | 0.9 (0.05) | 9.41 [0.948,49.779] | 0.89 [0.871,0.928] | 0.03 | 1.20E-02 | 0.03 |
| Aquicella | 13 (12.39) | 0.9 (0.05) | 13.43 [0.955,18.798] | 0.89 [0.871,0.928] | 0.07 | 1.20E-02 | 0.03 |
| Bradyrhizobium | 69.53 (42.17) | 19.28 (45.18) | 59.06 [46.75,109.887] | 0.89 [0.871,9.437] | 0.28 | 1.20E-02 | 0.03 |
| Nitrosospira | 188.43 (228.24) | 0.9 (0.05) | 36.61 [0.955,438.752] | 0.89 [0.871,0.928] | 4.79E-03 | 1.20E-02 | 0.03 |
| Parasutterella | 6.87 (7.13) | 59.69 (60.37) | 4.95 [0.916,9.46] | 47.81 [15.668,82.045] | 8.69 | 1.20E-02 | 0.03 |
| Rhodomicrobium | 11.91 (9.02) | 3.12 (6.28) | 13.13 [2.922,18.412] | 0.89 [0.871,0.944] | 0.26 | 1.20E-02 | 0.03 |
| Staphylococcus | 254366 (120417.94) | 108226.02 (69256.07) | 249456.02 [156496.464,359072.808] | 120869.7 [49826.976,152502.513] | 0.43 | 1.20E-02 | 0.03 |
| uncultured_Chloroflexi_bacterium | 105.19 (73.87) | 825.04 (613.95) | 88.93 [54.948,131.423] | 986.07 [288.893,1157.725] | 7.84 | 1.20E-02 | 0.03 |
| uncultured_Myxococcales_bacterium | 11.98 (10.14) | 3.12 (6.28) | 9.16 [2.879,17.949] | 0.89 [0.871,0.944] | 0.26 | 1.20E-02 | 0.03 |
| uncultured_Rubrobacterales_bacterium | 22.96 (23.48) | 4.29 (9.58) | 17.75 [8.853,24.617] | 0.89 [0.871,0.944] | 0.19 | 1.20E-02 | 0.03 |
| uncultured_candidate_division_SPAM_bacterium | 33.63 (39.73) | 6.62 (16.18) | 27.41 [8.673,42.523] | 0.89 [0.871,0.944] | 0.2 | 1.20E-02 | 0.03 |
| Acetobacter | 0.91 (0.04) | 2386.53 (2059.74) | 0.9 [0.884,0.94] | 3067.72 [21.079,3860.213] | 2623.07 | 1.60E-02 | 0.034 |
| Candidatus_Alysiosphaera | 21.99 (14.62) | 109.13 (76.12) | 23.72 [11.032,35.039] | 111.62 [62.719,142.788] | 4.96 | 1.60E-02 | 0.034 |
| Candidatus_Entotheonella | 31.05 (29.07) | 5.45 (12.88) | 27.19 [8.984,45.505] | 0.89 [0.871,0.944] | 0.18 | 1.60E-02 | 0.034 |
| Candidatus_Kuenenia | 0.91 (0.04) | 411.42 (351.34) | 0.9 [0.884,0.94] | 374.19 [152.671,673.588] | 452.2 | 1.60E-02 | 0.034 |

**Supplementary Table S5.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cellulomonas | 16.66 (27.76) | 0.9 (0.05) | 5.13 [0.946,17.74] | 0.89 [0.871,0.928] | 0.05 | 1.60E-02 | 0.034 |
| Chryseolinea | 7.85 (9.46) | 34.92 (24.77) | 4.79 [0.916,8.896] | 32.13 [23.179,46.984] | 4.45 | 1.60E-02 | 0.034 |
| Defluviimonas | 0.91 (0.04) | 46.68 (52.25) | 0.9 [0.884,0.94] | 27.44 [19.584,59.445] | 51.31 | 1.60E-02 | 0.034 |
| Gaiella_sp._EBR4-RS1 | 0.91 (0.04) | 72.23 (76.42) | 0.9 [0.884,0.94] | 35.26 [20.116,131.207] | 79.39 | 1.60E-02 | 0.034 |
| Geodermatophilus | 33.78 (36.39) | 5.45 (12.88) | 23.09 [9.071,41.18] | 0.89 [0.871,0.944] | 0.16 | 1.60E-02 | 0.034 |
| Herpetosiphon | 25.99 (21.25) | 7.79 (19.48) | 22.95 [11.052,34.309] | 0.89 [0.871,0.944] | 0.3 | 1.60E-02 | 0.034 |
| IMCC26207 | 0.91 (0.04) | 126.53 (103.27) | 0.9 [0.884,0.94] | 167.76 [13.134,202.793] | 139.07 | 1.60E-02 | 0.034 |
| Ileibacterium | 428572.22 (93960.71) | 535462.35 (66167.84) | 451646.27 [362547.363,480497.24] | 541242.48 [480362.459,552503.726] | 1.25 | 1.60E-02 | 0.034 |
| Ilumatobacter | 0.91 (0.04) | 29.15 (25.69) | 0.9 [0.884,0.94] | 26.02 [13.779,38.092] | 32.04 | 1.60E-02 | 0.034 |
| Nitrolancea | 17.34 (22.52) | 0.9 (0.05) | 9.43 [0.946,25.151] | 0.89 [0.871,0.928] | 0.05 | 1.60E-02 | 0.034 |
| Ottowia | 0.91 (0.04) | 374.41 (367.16) | 0.9 [0.884,0.94] | 332.57 [116.339,548.667] | 411.51 | 1.60E-02 | 0.034 |
| Phaselicystis | 15.67 (11.67) | 3.12 (6.28) | 17.93 [5.029,19.145] | 0.89 [0.871,0.944] | 0.2 | 1.60E-02 | 0.034 |
| Psychrobacillus | 170.73 (323.21) | 10.01 (22.38) | 72.38 [14.22,87.63] | 0.89 [0.871,3.188] | 0.06 | 1.60E-02 | 0.034 |
| Rubellimicrobium | 46.24 (79.81) | 5.09 (6.35) | 18.48 [8.798,43.642] | 0.93 [0.896,8.939] | 0.11 | 1.60E-02 | 0.034 |
| Stenotrophobacter | 57.98 (72.36) | 0.9 (0.05) | 17.74 [0.946,99.03] | 0.89 [0.871,0.928] | 0.02 | 1.60E-02 | 0.034 |
| uncultured_anaerobic_ammonium-oxidizing_bacterium | 0.91 (0.04) | 283.75 (186.82) | 0.9 [0.884,0.94] | 335.63 [201.739,431.875] | 311.87 | 1.60E-02 | 0.034 |
| Actinoplanes | 21.16 (24.12) | 0.9 (0.05) | 9.14 [0.919,42.933] | 0.89 [0.871,0.928] | 0.04 | 2.10E-02 | 0.043 |
| Agromyces | 21.99 (20.98) | 5.45 (12.88) | 18.51 [8.795,24.947] | 0.89 [0.871,0.944] | 0.25 | 2.10E-02 | 0.043 |
| Arenimonas | 33.66 (30.57) | 1005.34 (733.48) | 27.1 [5.493,53.718] | 1342.71 [331.3,1585.345] | 29.87 | 2.10E-02 | 0.043 |
| KF-JG30-B11 | 4.96 (4.26) | 0.9 (0.05) | 4.8 [0.946,8.975] | 0.89 [0.871,0.928] | 0.18 | 2.10E-02 | 0.043 |
| Leucobacter | 1.7 (2.5) | 39.41 (32.47) | 0.92 [0.896,0.95] | 40.39 [19.584,48.942] | 23.16 | 2.10E-02 | 0.043 |
| Massilia | 52.13 (48.99) | 12.67 (21.56) | 37.63 [9.142,97.808] | 0.96 [0.91,13.401] | 0.24 | 2.10E-02 | 0.043 |
| Ohtaekwangia | 26.61 (44.38) | 0.9 (0.05) | 9.36 [0.912,28.117] | 0.89 [0.871,0.928] | 0.03 | 2.10E-02 | 0.043 |
| Turicibacter | 489.73 (447.58) | 155.97 (240.52) | 322.77 [162.485,620.985] | 60.32 [23.866,134.382] | 0.32 | 2.10E-02 | 0.043 |
| Akkermansia | 138.83 (104.06) | 444.14 (378.81) | 118.03 [61.222,190.187] | 342.78 [176.248,546.405] | 3.2 | 2.70E-02 | 0.052 |
| Corynebacterium_1 | 3.7 (8.84) | 50.2 (75.51) | 0.9 [0.884,0.94] | 22 [15.755,38.343] | 13.57 | 2.70E-02 | 0.052 |
| Lautropia | 4.39 (8.48) | 51.04 (41.21) | 0.92 [0.884,0.96] | 42 [26.035,86.434] | 11.63 | 2.70E-02 | 0.052 |
| Limnobacter | 4.33 (8.44) | 136.86 (94.87) | 0.94 [0.896,0.96] | 152.52 [88.281,202.659] | 31.61 | 2.70E-02 | 0.052 |
| Luteolibacter | 15.76 (14.25) | 1.95 (2.98) | 13.57 [2.952,25.623] | 0.89 [0.871,0.944] | 0.12 | 2.70E-02 | 0.052 |
| Pedomicrobium | 41.12 (24.19) | 14.28 (21.51) | 47.04 [20.142,56.489] | 8.93 [0.871,12.098] | 0.35 | 2.70E-02 | 0.052 |
| Rhodoplanes | 14.15 (16.17) | 6.62 (16.18) | 8.88 [2.872,15.274] | 0.89 [0.871,0.944] | 0.47 | 2.70E-02 | 0.052 |
| uncultured_Acidobacteriaceae_bacterium | 12.04 (24.29) | 0.9 (0.05) | 0.96 [0.933,8.993] | 0.89 [0.871,0.928] | 0.08 | 2.70E-02 | 0.052 |
| Bauldia | 14.17 (12.57) | 3.12 (6.28) | 13.28 [2.841,18.779] | 0.89 [0.871,0.944] | 0.22 | 3.40E-02 | 0.066 |
| Janthinobacterium | 35.9 (49.14) | 3.95 (4.21) | 13.88 [3.044,54.739] | 0.96 [0.878,8.817] | 0.11 | 3.40E-02 | 0.066 |
| Crossiella | 39.42 (42.37) | 3.12 (6.28) | 30.38 [0.916,65.727] | 0.89 [0.871,0.944] | 0.08 | 4.30E-02 | 0.081 |
| Gemmatirosa | 31.36 (31.79) | 5.45 (12.88) | 27.18 [0.935,61.211] | 0.89 [0.871,0.944] | 0.17 | 4.30E-02 | 0.081 |
| Luteitalea | 2.68 (5.59) | 28.05 (33.52) | 0.9 [0.884,0.95] | 13.35 [6.684,38.337] | 10.49 | 4.30E-02 | 0.081 |
| Nitrosomonas | 13.11 (19.15) | 925.85 (685.87) | 0.96 [0.919,21.617] | 1011.12 [529.16,1310.69] | 70.63 | 4.30E-02 | 0.081 |
| uncultured_Desulfuromonadales_bacterium | 13.9 (15.32) | 3.12 (6.28) | 9.21 [0.916,19.005] | 0.89 [0.871,0.944] | 0.22 | 4.30E-02 | 0.081 |
| Dactylosporangium | 9.5 (9.14) | 3.12 (6.28) | 8.89 [0.916,16.646] | 0.89 [0.871,0.944] | 0.33 | 0.055 | 0.097 |
| Flavitalea | 16.35 (20.94) | 3.12 (6.28) | 9.36 [0.924,22.189] | 0.89 [0.871,0.944] | 0.19 | 0.055 | 0.097 |
| Ideonella | 5.89 (5.77) | 0.9 (0.05) | 4.88 [0.902,9.399] | 0.89 [0.871,0.928] | 0.15 | 0.055 | 0.097 |
| Others | 18242.32 (3933.75) | 17939.84 (22865.18) | 17262.94 [15948.997,19378.181] | 10801.28 [8019.006,16412.581] | 0.98 | 0.055 | 0.097 |
| Pontibacter | 14.11 (22.84) | 0.9 (0.05) | 0.96 [0.91,18.252] | 0.89 [0.871,0.928] | 0.06 | 0.055 | 0.097 |
| Psychroglaciecola | 20.86 (29.65) | 3.12 (6.28) | 8.97 [0.916,30.619] | 0.89 [0.871,0.944] | 0.15 | 0.055 | 0.097 |
| Thermomonas | 5.05 (8.19) | 168.25 (395.23) | 0.95 [0.916,6.713] | 27.45 [7.006,60.612] | 33.33 | 0.055 | 0.097 |
| uncultured_Rhodospirillaceae_bacterium | 8.55 (13.53) | 52.49 (39.92) | 0.96 [0.902,9.277] | 66.44 [13.134,84.401] | 6.14 | 0.055 | 0.097 |
| Defluviicoccus | 4.21 (5.9) | 0.9 (0.05) | 0.95 [0.906,6.713] | 0.89 [0.871,0.928] | 0.21 | 0.068 | 0.117 |
| Fictibacillus | 9.62 (12.13) | 1.95 (2.96) | 4.8 [0.906,15.303] | 0.89 [0.871,0.949] | 0.2 | 0.068 | 0.117 |
| Prosthecobacter | 7.71 (11.93) | 0.9 (0.05) | 0.95 [0.906,13.185] | 0.89 [0.871,0.928] | 0.12 | 0.068 | 0.117 |
| Ruminiclostridium_5 | 38 (32.45) | 16.08 (26.93) | 30.21 [9.481,68.106] | 4.77 [0.898,14.414] | 0.42 | 0.068 | 0.117 |
| Sandaracinus | 5.06 (7.06) | 0.9 (0.05) | 0.95 [0.916,6.977] | 0.89 [0.871,0.928] | 0.18 | 0.068 | 0.117 |
| Actinophytocola | 9.59 (10.01) | 1.95 (2.98) | 4.96 [0.904,18.845] | 0.89 [0.871,0.944] | 0.2 | 0.083 | 0.139 |
| Aerococcus | 2989.29 (7884.91) | 465.8 (692.55) | 540.06 [385.035,661.416] | 146.1 [53.628,509.256] | 0.16 | 0.083 | 0.139 |
| Frigoribacterium | 51.86 (79.53) | 12.63 (21.04) | 27.71 [5.439,47.466] | 0.96 [0.916,17.416] | 0.24 | 0.083 | 0.139 |
| Pseudomonas | 20.36 (30.61) | 5.07 (6.32) | 9.57 [2.986,17.789] | 0.96 [0.871,9.106] | 0.25 | 0.083 | 0.139 |
| uncultured_Actinomycetales_bacterium | 8.72 (14.69) | 0.9 (0.05) | 0.95 [0.906,13.185] | 0.89 [0.871,0.928] | 0.1 | 0.083 | 0.139 |
| uncultured_proteobacterium | 9.56 (9.44) | 1.95 (2.98) | 9.05 [0.912,15.267] | 0.89 [0.871,0.944] | 0.2 | 0.083 | 0.139 |
| Mesorhizobium | 32.94 (27.03) | 15.26 (15.58) | 35.49 [7.676,41.902] | 13.7 [0.878,21.362] | 0.46 | 0.101 | 0.167 |

**Supplementary Table S5.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Methyloceanibacter | 9.68 (12.09) | 1.95 (2.98) | 4.88 [0.904,15.855] | 0.89 [0.871,0.944] | 0.2 | 0.101 | 0.167 |
| Blautia | 189.19 (93.36) | 266.13 (190.25) | 154.53 [137.69,196.703] | 213.42 [176.512,243.669] | 1.41 | 0.122 | 0.191 |
| Cutibacterium | 2.68 (5.59) | 7.01 (8.55) | 0.9 [0.884,0.95] | 4.87 [0.932,8.885] | 2.62 | 0.122 | 0.191 |
| Gluconobacter | 0.91 (0.04) | 39.09 (77.26) | 0.9 [0.884,0.94] | 0.96 [0.921,30.393] | 42.96 | 0.122 | 0.191 |
| JTB255_marine_benthic_group | 0.91 (0.04) | 10.75 (12.22) | 0.9 [0.884,0.94] | 4.77 [0.921,19.815] | 11.81 | 0.122 | 0.191 |
| Kineosporia | 12.28 (10.33) | 4.1 (6.49) | 9.48 [2.898,18.985] | 0.91 [0.878,2.933] | 0.33 | 0.122 | 0.191 |
| Solibacillus | 2499.51 (3770.32) | 18285.3 (32028.06) | 402.53 [174.872,2719.456] | 1518.05 [1254.128,15605.079] | 7.32 | 0.122 | 0.191 |
| Sphingobium | 5.97 (10.64) | 0.9 (0.05) | 0.94 [0.902,0.96] | 0.89 [0.871,0.928] | 0.15 | 0.122 | 0.191 |
| Tyzzerella | 4.26 (8.06) | 11.33 (17.08) | 0.92 [0.896,0.96] | 8.54 [0.98,9.089] | 2.66 | 0.122 | 0.191 |
| [Eubacterium]_coprostanoligenes_group | 13.7 (13.97) | 88.15 (178.85) | 13.44 [0.935,18.643] | 26.37 [15.164,46.882] | 6.44 | 0.122 | 0.191 |
| uncultured_Armatimonadetes_bacterium | 8.65 (14.83) | 0.9 (0.05) | 0.95 [0.896,7.076] | 0.89 [0.871,0.928] | 0.1 | 0.122 | 0.191 |
| ADurb.Bin063-1 | 6.95 (9.2) | 0.9 (0.05) | 0.95 [0.885,9.422] | 0.89 [0.871,0.928] | 0.13 | 0.146 | 0.218 |
| Alkaliphilus | 0.91 (0.04) | 30.98 (40.11) | 0.9 [0.884,0.94] | 22.48 [0.915,38.726] | 34.05 | 0.146 | 0.218 |
| Brevundimonas | 14.06 (12.75) | 5.07 (8.99) | 9.32 [2.831,25.715] | 0.93 [0.892,2.956] | 0.36 | 0.146 | 0.218 |
| Chlorobi_bacterium_OLB6 | 0.91 (0.04) | 11.61 (10.95) | 0.9 [0.884,0.94] | 9.49 [0.915,20.456] | 12.76 | 0.146 | 0.218 |
| Lactococcus | 45.31 (44.63) | 74.38 (45.4) | 28.58 [20.162,57.371] | 62.2 [42.462,117.104] | 1.64 | 0.146 | 0.218 |
| Membranicola | 0.91 (0.04) | 9.38 (12.11) | 0.9 [0.884,0.94] | 4.92 [0.915,11.405] | 10.31 | 0.146 | 0.218 |
| PAUC26f | 16.11 (20.2) | 4.14 (6.52) | 5.18 [0.899,30.664] | 0.91 [0.871,3.004] | 0.26 | 0.146 | 0.218 |
| Shinella | 14.07 (15.62) | 35.67 (32.04) | 8.88 [2.873,16.475] | 26.9 [15.234,47.701] | 2.54 | 0.146 | 0.218 |
| Cyanobacteria/Melainabacteria_group_bacterium_S15B-MN24_CBMW_12 | 2.51 (3.36) | 0.9 (0.05) | 0.94 [0.896,0.96] | 0.89 [0.871,0.928] | 0.36 | 0.173 | 0.252 |
| Desulfotomaculum | 2.51 (3.36) | 0.9 (0.05) | 0.94 [0.896,0.96] | 0.89 [0.871,0.928] | 0.36 | 0.173 | 0.252 |
| Devosia | 9.54 (11.52) | 4.12 (6.33) | 0.96 [0.906,18.467] | 0.91 [0.871,3.188] | 0.43 | 0.173 | 0.252 |
| Lachnospiraceae_UCG-006 | 6.9 (9.29) | 17.42 (12.22) | 0.96 [0.91,8.87] | 22.06 [6.78,27.267] | 2.52 | 0.173 | 0.252 |
| Subgroup_10 | 278.99 (222.89) | 137.82 (86.71) | 224.54 [168.299,345.028] | 137.63 [80.307,191.409] | 0.49 | 0.173 | 0.252 |
| Coxiella | 5.07 (8.09) | 3.12 (6.28) | 0.94 [0.902,6.841] | 0.89 [0.871,0.944] | 0.62 | 0.203 | 0.291 |
| Dubosiella | 9837.5 (6603.88) | 11719.9 (4968.96) | 8655.59 [5829.408,10226.707] | 12075.3 [8825.407,12970.226] | 1.19 | 0.203 | 0.291 |
| Romboutsia | 37234.51 (16427.93) | 24104.04 (24720.13) | 39798.68 [29257.473,47889.351] | 12688.29 [6969.59,38754.615] | 0.65 | 0.203 | 0.291 |
| Ruminococcaceae_UCG-010 | 5.93 (7.08) | 4.26 (9.51) | 0.96 [0.907,9.214] | 0.89 [0.871,0.949] | 0.72 | 0.203 | 0.291 |
| Candidatus_Berkiella | 2.55 (3.47) | 0.9 (0.05) | 0.92 [0.896,0.95] | 0.89 [0.871,0.928] | 0.35 | 0.237 | 0.334 |
| Rothia | 14.81 (21.73) | 3.96 (6.02) | 4.96 [0.946,16.183] | 0.92 [0.881,3.063] | 0.27 | 0.237 | 0.334 |
| uncultured_Caldilineaceae_bacterium | 4.26 (8.06) | 0.9 (0.05) | 0.92 [0.896,0.96] | 0.89 [0.871,0.928] | 0.21 | 0.237 | 0.334 |
| Acidobacteria_bacterium_enrichment_culture_clone_CV-04 | 0.91 (0.04) | 7.21 (7.42) | 0.9 [0.884,0.94] | 4.98 [0.91,11.838] | 7.93 | 0.274 | 0.372 |
| Alistipes | 0.91 (0.04) | 10.46 (17.8) | 0.9 [0.884,0.94] | 0.96 [0.896,11.294] | 11.49 | 0.274 | 0.372 |
| BD1-7_clade | 3.46 (8.06) | 14.03 (13.28) | 0.92 [0.896,0.95] | 13.26 [0.915,21.879] | 4.05 | 0.274 | 0.372 |
| Blastocatella | 13.77 (14.36) | 27.36 (20.57) | 9.36 [2.993,21.821] | 30.39 [14.13,37.045] | 1.99 | 0.274 | 0.372 |
| Exiguobacterium | 0.91 (0.04) | 8.33 (10.58) | 0.9 [0.884,0.94] | 0.96 [0.91,17.634] | 9.15 | 0.274 | 0.372 |
| Gemmatimonas | 53.03 (26) | 36.49 (30.15) | 47.11 [37.829,73.684] | 35.64 [16.047,47.439] | 0.69 | 0.274 | 0.372 |
| Microbacterium | 27.33 (39.31) | 6.18 (6.36) | 4.96 [0.946,45.801] | 4.77 [0.881,9.437] | 0.23 | 0.274 | 0.372 |
| Parvibacter | 70.06 (37.24) | 49.6 (34.27) | 62.25 [40.599,84.858] | 52.75 [18.299,56.851] | 0.71 | 0.274 | 0.372 |
| Propioniciclava | 0.91 (0.04) | 7.4 (12.5) | 0.9 [0.884,0.94] | 0.96 [0.892,8.769] | 8.13 | 0.315 | 0.426 |
| Demequina | 4.16 (5.69) | 4.02 (8.8) | 0.95 [0.906,6.843] | 0.91 [0.881,0.949] | 0.97 | 0.36 | 0.475 |
| Rhodobacter | 4.27 (5.96) | 3.1 (6.23) | 0.95 [0.896,6.843] | 0.89 [0.871,0.949] | 0.73 | 0.36 | 0.475 |
| Ruminococcaceae_UCG-014 | 18.23 (20.46) | 40.35 (43.69) | 13.44 [8.961,18.494] | 30.87 [6.684,51.953] | 2.21 | 0.36 | 0.475 |
| Sphaerotilus | 1.72 (2.54) | 0.9 (0.05) | 0.92 [0.885,0.95] | 0.89 [0.871,0.928] | 0.53 | 0.36 | 0.475 |
| Streptococcus | 22.84 (18.24) | 30.88 (15.96) | 21.84 [9.158,28.144] | 35.26 [22.07,37.951] | 1.35 | 0.36 | 0.475 |
| Faecalibacterium | 4.38 (6.24) | 1.95 (2.98) | 0.93 [0.885,6.951] | 0.89 [0.871,0.944] | 0.45 | 0.408 | 0.522 |
| Flavobacterium | 1.73 (2.59) | 0.9 (0.05) | 0.91 [0.884,0.95] | 0.89 [0.871,0.928] | 0.52 | 0.408 | 0.522 |
| Paeniclostridium | 12.95 (10.24) | 11.92 (12.56) | 9.41 [8.75,18.627] | 8.79 [6.684,11.405] | 0.92 | 0.408 | 0.522 |
| Roseomonas | 2.64 (5.48) | 0.9 (0.05) | 0.91 [0.884,0.95] | 0.89 [0.871,0.928] | 0.34 | 0.408 | 0.522 |
| Thauera | 0.91 (0.04) | 7.35 (10.22) | 0.9 [0.884,0.94] | 0.96 [0.878,11.146] | 8.08 | 0.408 | 0.522 |
| [Eubacterium]_brachy_group | 5.31 (8.86) | 10.7 (15.09) | 0.93 [0.885,6.953] | 8.54 [0.898,9.437] | 2.01 | 0.408 | 0.522 |
| uncultured_gamma_proteobacterium | 4.46 (11.24) | 0.9 (0.05) | 0.91 [0.884,0.95] | 0.89 [0.871,0.928] | 0.2 | 0.408 | 0.522 |
| Parabacteroides | 3.59 (6.09) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.25 | 0.46 | 0.58 |
| Paracoccus | 0.91 (0.04) | 8.58 (8.84) | 0.9 [0.884,0.94] | 4.98 [0.871,17.888] | 9.43 | 0.46 | 0.58 |
| Ruminococcaceae_UCG-005 | 7.62 (8.86) | 16.06 (14.35) | 4.8 [0.946,9.169] | 17.75 [0.926,23.676] | 2.11 | 0.46 | 0.58 |
| OM27_clade | 22.44 (21.44) | 27.08 (22.85) | 18.06 [11.391,24.236] | 26.37 [13.761,32.511] | 1.21 | 0.515 | 0.636 |
| Ruminococcaceae_NK4A214_group | 6.25 (11.59) | 1.9 (2.8) | 0.92 [0.896,6.841] | 0.91 [0.871,0.949] | 0.3 | 0.515 | 0.636 |
| [Eubacterium]_xylanophilum_group | 1.77 (2.73) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.51 | 0.515 | 0.636 |
| uncultured_Bacteroidetes_bacterium | 2.74 (5.8) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.33 | 0.515 | 0.636 |

**Supplementary Table S5.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| wastewater_metagenome | 4.25 (5.84) | 2.99 (3.9) | 0.94 [0.902,6.951] | 0.91 [0.881,2.85] | 0.7 | 0.515 | 0.636 |
| Acinetobacter | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Anaerolinea | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Crassaminicella | 0.91 (0.04) | 2.02 (3.2) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 2.22 | 0.573 | 0.662 |
| Desulfovibrio | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Dokdonella | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Enterococcus | 88632.03 (28126.66) | 89153.33 (44544.21) | 92527.85 [64682.017,113651.741] | 101890.9 [69635.87,125285.442] | 1.01 | 0.573 | 0.662 |
| Escherichia-Shigella | 0.91 (0.04) | 3.27 (6.72) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 3.59 | 0.573 | 0.662 |
| Geobacter | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Geothrix | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Hymenobacter | 30.02 (62.62) | 2.88 (3.65) | 0.95 [0.906,6.713] | 0.93 [0.892,2.933] | 0.1 | 0.573 | 0.662 |
| Ignavibacterium | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Marinobacterium | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| Ornithinimicrobium | 6.06 (9.4) | 6.59 (11.05) | 0.95 [0.896,7.076] | 0.91 [0.878,5.073] | 1.09 | 0.573 | 0.662 |
| Shewanella | 0.91 (0.04) | 2.02 (3.2) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 2.22 | 0.573 | 0.662 |
| Tropicimonas | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| uncultured_prokaryote | 0.91 (0.04) | 0.9 (0.05) | 0.9 [0.884,0.94] | 0.89 [0.871,0.928] | 0.99 | 0.573 | 0.662 |
| GCA-900066575 | 17.37 (23.75) | 12.51 (20.09) | 8.96 [0.946,21.831] | 8.76 [0.967,9.282] | 0.72 | 0.633 | 0.724 |
| Pseudorhodoplanes | 2.55 (5.17) | 3.92 (4.16) | 0.92 [0.896,0.95] | 0.96 [0.892,8.769] | 1.54 | 0.633 | 0.724 |
| Roseburia | 18.43 (19.57) | 28.24 (25.14) | 14.08 [2.873,24.617] | 22.88 [6.615,53.663] | 1.53 | 0.633 | 0.724 |
| Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium | 27.79 (21.5) | 22.52 (17.92) | 17.93 [11.286,41.852] | 17.93 [9.723,31.892] | 0.81 | 0.696 | 0.783 |
| Bosea | 3.49 (5.83) | 4.22 (6.59) | 0.91 [0.884,0.96] | 0.89 [0.871,3.188] | 1.21 | 0.696 | 0.783 |
| Desulfosporosinus | 0.91 (0.04) | 2.93 (3.75) | 0.9 [0.884,0.94] | 0.92 [0.878,2.933] | 3.22 | 0.696 | 0.783 |
| Methylobacterium | 3.3 (3.83) | 7.06 (9.68) | 0.95 [0.906,6.713] | 0.96 [0.921,10.694] | 2.14 | 0.696 | 0.783 |
| Acetatifactor | 40.97 (29.79) | 64.08 (67.04) | 39.97 [19.097,51.667] | 40.41 [23.979,76.913] | 1.56 | 0.762 | 0.84 |
| Lachnospiraceae_NK4A136_group | 95.45 (68.69) | 121.44 (114) | 79.99 [49.317,131.652] | 104.35 [53.164,130.967] | 1.27 | 0.762 | 0.84 |
| OLB13 | 6.87 (11.06) | 16.24 (21.23) | 0.96 [0.91,8.76] | 9.07 [0.916,21.011] | 2.36 | 0.762 | 0.84 |
| Ruminiclostridium | 28.05 (28.31) | 33.49 (34.99) | 18.48 [8.877,41.982] | 26.47 [15.221,36.424] | 1.19 | 0.762 | 0.84 |
| Ruminiclostridium_9 | 36.61 (27.65) | 61.73 (89.67) | 31.56 [20.409,44.998] | 39.28 [16.487,47.643] | 1.69 | 0.762 | 0.84 |
| Enterorhabdus | 1147.54 (678.95) | 1194.84 (591.28) | 842.27 [700.353,1460.02] | 1147.09 [765.046,1493.608] | 1.04 | 0.829 | 0.89 |
| Family_XIII_AD3011_group | 0.91 (0.04) | 11.21 (29.16) | 0.9 [0.884,0.94] | 0.89 [0.871,0.949] | 12.32 | 0.829 | 0.89 |
| GCA-900066225 | 1.72 (2.54) | 2.98 (5.86) | 0.92 [0.885,0.95] | 0.91 [0.878,0.949] | 1.74 | 0.829 | 0.89 |
| Gordonia | 0.91 (0.04) | 1.95 (2.96) | 0.9 [0.884,0.94] | 0.89 [0.871,0.949] | 2.14 | 0.829 | 0.89 |
| Rhodococcus | 0.91 (0.04) | 1.87 (2.72) | 0.9 [0.884,0.94] | 0.91 [0.881,0.949] | 2.06 | 0.829 | 0.89 |
| Sporosarcina | 409.4 (571.41) | 855.78 (1414.31) | 165.27 [122.368,288.996] | 323.75 [109.209,705.058] | 2.09 | 0.829 | 0.89 |
| [Eubacterium]_nodatum_group | 0.91 (0.04) | 25.11 (68.47) | 0.9 [0.884,0.94] | 0.89 [0.871,0.949] | 27.6 | 0.829 | 0.89 |
| Anaeromyxobacter | 1.69 (2.44) | 2.96 (3.82) | 0.92 [0.896,0.95] | 0.91 [0.871,3.004] | 1.76 | 0.897 | 0.946 |
| Brooklawnia | 0.91 (0.04) | 4.28 (6.93) | 0.9 [0.884,0.94] | 0.91 [0.871,2.958] | 4.71 | 0.897 | 0.946 |
| Dietzia | 0.91 (0.04) | 3.04 (3.99) | 0.9 [0.884,0.94] | 0.91 [0.871,2.958] | 3.34 | 0.897 | 0.946 |
| Faecalibaculum | 2408.48 (1511.34) | 2193.4 (1252.13) | 2797.14 [801.873,3691.143] | 2266.45 [1267.062,2794.151] | 0.91 | 0.897 | 0.946 |
| Lysinibacillus | 82.74 (146.05) | 68.62 (134.22) | 27.1 [0.899,92.133] | 13.23 [0.972,54.88] | 0.83 | 0.897 | 0.946 |
| Marvinbryantia | 3.46 (5.74) | 4.04 (6.31) | 0.92 [0.885,0.96] | 0.93 [0.871,2.956] | 1.17 | 0.965 | 0.979 |
| Pajaroellobacter | 31.82 (30.51) | 21.68 (13.53) | 22.3 [9.373,41.641] | 18.36 [17.5,23.676] | 0.68 | 0.965 | 0.979 |
| Tissierella | 1.69 (2.44) | 7.73 (10.97) | 0.92 [0.896,0.95] | 0.91 [0.871,11.405] | 4.58 | 0.965 | 0.979 |
| Adlercreutzia | 1779.88 (536) | 1619.91 (886.41) | 1880.89 [1298.183,2132.106] | 1823.84 [1198.791,2385.269] | 0.91 | 0.965 | 0.979 |
| Anaerovorax | 0.91 (0.04) | 1.89 (2.77) | 0.9 [0.884,0.94] | 0.91 [0.878,0.949] | 2.07 | 0.965 | 0.979 |
| Bacteroides | 6.58 (17.96) | 1.86 (2.68) | 0.9 [0.884,0.94] | 0.91 [0.881,0.949] | 0.28 | 0.965 | 0.979 |
| Christensenellaceae_R-7_group | 0.91 (0.04) | 1.89 (2.77) | 0.9 [0.884,0.94] | 0.91 [0.878,0.949] | 2.07 | 0.965 | 0.979 |
| Litorilinea | 6.76 (7.87) | 8.31 (9.55) | 0.95 [0.916,15.349] | 4.77 [0.916,11.98] | 1.23 | 0.965 | 0.979 |
| Oscillibacter | 7.7 (18.71) | 7.41 (9.3) | 0.93 [0.896,0.96] | 4.84 [0.878,9.261] | 0.96 | 0.965 | 0.979 |
| Stenotrophomonas | 0.91 (0.04) | 1.89 (2.77) | 0.9 [0.884,0.94] | 0.91 [0.878,0.949] | 2.07 | 0.965 | 0.979 |
| [Eubacterium]_ventriosum_group | 1.69 (2.44) | 2.9 (3.69) | 0.92 [0.896,0.95] | 0.92 [0.881,2.869] | 1.72 | 0.965 | 0.979 |
| Clostridium_sensu_stricto_13 | 0.91 (0.04) | 3 (5.93) | 0.9 [0.884,0.94] | 0.91 [0.871,0.949] | 3.3 | 1 | 1 |
| Dechloromonas | 0.91 (0.04) | 1.9 (2.8) | 0.9 [0.884,0.94] | 0.91 [0.871,0.949] | 2.09 | 1 | 1 |
| Erysipelatoclostridium | 369.07 (296.12) | 316.07 (219.73) | 226.15 [180.308,485.651] | 298.25 [198.452,496.227] | 0.86 | 1 | 1 |
| Erythrobacter | 0.91 (0.04) | 1.9 (2.8) | 0.9 [0.884,0.94] | 0.91 [0.871,0.949] | 2.09 | 1 | 1 |

**Supplementary Table S6.** Univariate analysis (Mann–Whitney U test) of the control and high-fat metabolomic data

| Name | Class | HMDBID | KEGGID | HF.MeanSD | C.MeanSD | HF.MedianIQR | C.MedianIQR | FC | P | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5-Aminopentanoate | Amino Acids | HMDB0003355 | C00431 | 3375.98 (2853.47) | 1468.2 (855.29) | 2281.54 [2045.001,3021.252] | 1188.06 [989.483,1656.858] | 0.52 | 3.10E-03 | up |
| Alanine | Amino Acids | HMDB0000161 | C00041 | 66715.08 (10520.3) | 98077.74 (24718.86) | 67157.37 [58032.744,72263.212] | 95823.54 [75692.254,121573.226] | 1.43 | 3.10E-03 | down |
| Tryptophan | Amino Acids | HMDB0000929 | C00078 | 2293.93 (539.92) | 1639.32 (382.45) | 2471.53 [2073.401,2631.786] | 1726.91 [1258.16,1934.598] | 0.7 | 8.50E-03 | |
| Threonine | Amino Acids | HMDB0000167 | C00188 | 8037.66 (4426.64) | 12656.29 (2818.89) | 7791.96 [5523.154,10163.833] | 12111.21 [11226.939,13757.32] | 1.55 | 3.40E-02 | |
| 2-Oxoisocaproate | Amino Acids | HMDB0000695 | C00233 | 614.47 (1723.61) | 601.62 (731.94) | 5.2 [4.436,6.106] | 401.13 [165.057,630.38] | 77.22 | 4.30E-02 | |
| Ornithine | Amino Acids | HMDB0000214 | C00077 | 1958.47 (936.04) | 1083.73 (768.56) | 2340.49 [1884.123,2412.399] | 964.9 [801.995,1239.378] | 0.41 | 0.055 | |
| Histidine | Amino Acids | HMDB0000177 | C00135 | 3687.99 (1180.54) | 2881.57 (720.65) | 3833.03 [3062.453,4274.154] | 2549.47 [2386.004,3432.081] | 0.67 | 0.083 | |
| Cytosine | Nucleotide | HMDB0000630 | C00380 | 1302.59 (415.23) | 264.08 (221.77) | 1318.93 [1138.844,1518.962] | 257.32 [43.618,459.353] | 0.2 | 4.60E-05 | |
| Uracil | Nucleotide | HMDB0000300 | C00106 | 7131.52 (2313.69) | 12610.48 (1026.68) | 6806.46 [5842.153,8524.463] | 12446.64 [12197.951,12965.754] | 1.83 | 4.60E-05 | |
| Uridine | Nucleotide | HMDB0000296 | C00299 | 5.18 (0.91) | 369.78 (175.02) | 5.2 [4.436,5.902] | 319.91 [254.806,384.398] | 61.58 | 4.60E-05 | |
| Hypoxanthine | Nucleotide | HMDB0000157 | C00262 | 5336.82 (1441.03) | 8199.72 (1030.74) | 5119.53 [4213.796,6062.723] | 8355.57 [7284.495,8803.97] | 1.63 | 5.50E-04 | |
| 3-Phenylpropionate | Organic Acids | HMDB0000764 | C05629 | 659.42 (613.1) | 3.27 (0.79) | 448.72 [238.39,1176.487] | 3.15 [2.982,3.837] | 7.03E-03 | 4.60E-05 | |
| 3-(3-Hydroxyphenyl)propanoate | Organic Acids | HMDB0000375 | C11457 | 5.18 (0.91) | 3.27 (0.79) | 5.2 [4.436,5.902] | 3.15 [2.982,3.837] | 0.61 | 5.50E-04 | |
| 4-Hydroxybenzoate | Organic Acids | HMDB0000500 | C00156 | 263.55 (185.24) | 55.55 (88.23) | 240.29 [186.333,287.836] | 3.69 [3.13,99.913] | 0.02 | 2.10E-03 | |
| Nicotinate | Organic Acids | HMDB0001488 | C00253 | 806.72 (245.11) | 1243.41 (354.31) | 838.73 [670.998,963.357] | 1300.36 [960.555,1529.529] | 1.55 | 2.70E-02 | |
| Tartrate | Organic Acids | HMDB0000956 | C00898 | 2417.4 (2316.75) | 860.37 (384.6) | 1315.5 [1170.24,2690.917] | 804.66 [583.754,1204.693] | 0.61 | 2.70E-02 | |
| 3-Methyl-2-oxovalerate | Organic Acids | HMDB0000491 | C03465 | 550.99 (1305.62) | 1004.56 (1277.55) | 6.24 [5.242,273.416] | 341.21 [267.38,1066.991] | 54.65 | 3.40E-02 | |
| 4-Hydroxyphenyllactate | Organic Acids | HMDB0000755 | C03672 | 246.12 (84.77) | 149.45 (77.14) | 251.26 [186.333,316.082] | 147.2 [116.463,195.392] | 0.59 | 4.30E-02 | |
| Dimethylamine | Others | HMDB0000087 | C00543 | 569.17 (179.45) | 187.56 (114.59) | 554.1 [447.359,728.525] | 159.27 [126.268,205.195] | 0.29 | 3.20E-04 | |
| Methanol | Others | HMDB0001875 | C00132 | 684.25 (213.05) | 439.23 (98.12) | 657.52 [558.749,727.481] | 438.85 [390.038,472.955] | 0.67 | 3.10E-03 | |
| Lactadehyde | Others | HMDB0003052 | C00424 | 1318.12 (951.39) | 3105.65 (1542.2) | 973.07 [632.609,1592.833] | 2841 [1982.899,3637.325] | 2.92 | 6.20E-03 | |
| Acetate | Others | HMDB0000042 | C00033 | 363282.66 (54107.73) | 307755.13 (45548.53) | 382046.7 [309920.974,402420.453] | 312151.07 [263681.191,333535.729] | 0.82 | 0.055 | |
| Trimethylamine | Others | HMDB0000906 | C00565 | 1139.16 (627.02) | 617.52 (235.67) | 906.2 [665.986,1789.229] | 676.21 [472.514,763.262] | 0.75 | 0.083 | |
| 2,3-Butanediol | Alcohols | HMDB0003156 | C00265 | 4204.41 (7032.86) | 2508.42 (1310.21) | 1419.77 [6.381,4565.103] | 2365.31 [2026.714,2611.779] | 1.67 | 0.573 | |
| Glycerol | Alcohols | HMDB0000131 | C00116 | 934.84 (536.3) | 958.77 (815.16) | 933.55 [466.892,1285.725] | 527.3 [377.806,1616.16] | 0.56 | 0.829 | |
| Propylene glycol | Alcohols | HMDB0001881 | C00583 | 746.2 (1038.36) | 567.43 (466.08) | 349.43 [6.381,984.072] | 448.89 [273.325,916.507] | 1.28 | 1 | |
| Lysine | Amino Acids | HMDB0000182 | C00047 | 22643.96 (7257.37) | 27355 (3309.27) | 22933.45 [19886.164,26761.348] | 26779.04 [25283.882,28931.644] | 1.17 | 0.146 | |
| Alpha-ketoisovalerate | Amino Acids | HMDB0000019 | C00141 | 379.54 (641.57) | 630.56 (824.28) | 140.55 [87.488,290.373] | 197 [173.333,669.593] | 1.4 | 0.203 | |
| Glycine | Amino Acids | HMDB0000123 | C00037 | 41451.22 (10705.98) | 47727.94 (5422.93) | 40302.59 [38203.044,46353.356] | 48547.19 [43965.683,51356.995] | 1.2 | 0.203 | |
| 4-Aminobutyrate | Amino Acids | HMDB0000112 | C00334 | 1269.08 (308.44) | 971.13 (512.15) | 1271.38 [1060.047,1473.4] | 1140.72 [693.57,1193.847] | 0.9 | 0.237 | |
| Creatine | Amino Acids | HMDB0000064 | C00300 | 582.19 (296.98) | 407.04 (291) | 657.52 [458.28,833.929] | 414.54 [149.812,605.226] | 0.63 | 0.237 | |
| Tyramine | Amino Acids | HMDB0000306 | C00483 | 3522.67 (3306.38) | 5199.19 (2220.81) | 2240.47 [1250.525,5791.971] | 5860.89 [4325.025,6703.1] | 2.62 | 0.237 | |
| Glutamine | Amino Acids | HMDB0000641 | C00064 | 3440.03 (1856.99) | 2516.89 (1111.73) | 2511.14 [2358.09,4162.815] | 2234.32 [1825.283,2902.057] | 0.89 | 0.274 | |
| Asparagine | Amino Acids | HMDB0000168 | C00152 | 1673.33 (533.85) | 1432 (376.09) | 1666.13 [1262.001,2090.714] | 1403.88 [1229.569,1707.749] | 0.84 | 0.36 | |
| Valine | Amino Acids | HMDB0000883 | C00183 | 47408.84 (11605.23) | 43374.38 (6508.34) | 49507.01 [40847.387,53385.78] | 43651.29 [39693.672,47643.389] | 0.88 | 0.36 | |
| Aspartate | Amino Acids | HMDB0000191 | C00049 | 47524.93 (19698.89) | 40778.09 (21951.05) | 43454.61 [40132.916,55800.01] | 38761.98 [28728.234,58410.115] | 0.89 | 0.408 | |
| Leucine | Amino Acids | HMDB0000687 | C00123 | 31058.9 (8594.68) | 33215.41 (4343.2) | 31142.54 [25089.717,35511.207] | 33104.9 [30167.441,37133.742] | 1.06 | 0.515 | |
| Methionine | Amino Acids | HMDB0000696 | C00073 | 11885.34 (3844.34) | 13537.09 (2237.44) | 13122.2 [8561.484,14375.295] | 13599.42 [11589.441,15478.419] | 1.04 | 0.515 | |
| Tyrosine | Amino Acids | HMDB0000158 | C00082 | 9432.5 (5075.36) | 7390.92 (2595.31) | 10431.93 [4861.76,12643.906] | 6658.51 [5534.299,7350.926] | 0.64 | 0.573 | |
| Isoleucine | Amino Acids | HMDB0000172 | C00407 | 29859.25 (9379.66) | 27427.91 (6134.5) | 28011.08 [25206.815,35457.952] | 28049 [25026.994,30871.183] | 1 | 0.633 | |
| Citrulline | Amino Acids | HMDB0000904 | C00327 | 11482.89 (4008.55) | 10771.02 (3780.22) | 13312.67 [7862.718,14331.876] | 9644.25 [8503.452,12879.399] | 0.72 | 0.696 | |
| Creatinine | Amino Acids | HMDB0000562 | C00791 | 1042.7 (371.07) | 1043.09 (539.93) | 910.15 [812.907,1275.789] | 857.26 [619.733,1613.56] | 0.94 | 0.696 | |
| Glutamate | Amino Acids | HMDB0000148 | C00025 | 63265.08 (13838.32) | 64361.31 (13159.41) | 60406.55 [53211.758,71382.105] | 59828.46 [55216.557,73134.446] | 0.99 | 0.696 | |
| Proline | Amino Acids | HMDB0000162 | C00148 | 15497.69 (3321.26) | 15665.25 (2096.2) | 15401.69 [12886.993,16354.303] | 14761.56 [14043.299,17625.585] | 0.96 | 0.762 | |
| Phenylalanine | Amino Acids | HMDB0000159 | C00079 | 11743.28 (3412.88) | 12465.8 (1631.01) | 12463.19 [8793.642,14205.647] | 11825.69 [11295.593,13677.722] | 0.95 | 0.829 | |
| Urocanate | Azoles | HMDB0000301 | C00785 | 1219.58 (586.79) | 1136.2 (186.55) | 1144.76 [855.035,1679.083] | 1150.18 [1071.348,1220.884] | 1 | 1 | |
| Fucose | Carbohydrates | HMDB0012327 | C02781 | 985.08 (965) | 2035.9 (1307.22) | 1022.64 [5.732,1680.216] | 2001.5 [1148.011,3072.124] | 1.96 | 0.146 | |
| Xylose | Carbohydrates | HMDB0000098 | C00181 | 455.4 (678.86) | 1306.67 (1205.19) | 6.2 [5.093,865.914] | 1319.87 [276.344,1740.862] | 212.92 | 0.274 | |
| Ribose | Carbohydrates | HMDB0000283 | C00121 | 5.18 (0.91) | 3934.39 (4586.77) | 5.2 [4.436,5.902] | 1629.6 [4.198,7385.835] | 313.68 | 0.315 | |
| Galactose | Carbohydrates | HMDB0000143 | C00984 | 183.26 (503.2) | 1249.67 (1869.35) | 5.58 [4.954,6.106] | 4.27 [3.351,2431.327] | 0.76 | 0.515 | |
| Glucose | Carbohydrates | HMDB0000122 | C00031 | 997.29 (1901.09) | 3469.44 (5240.8) | 5.63 [4.954,769.257] | 750.93 [4.198,4721.212] | 133.42 | 0.633 | |
| Butyrate | Fatty Acids | HMDB0000039 | C00246 | 5316.03 (3043.7) | 7726.15 (5722.9) | 4943.33 [3178.675,6498.124] | 5930.76 [4381.518,9169.392] | 1.2 | 0.315 | |
| Propionate | Fatty Acids | HMDB0000237 | C00163 | 13224.69 (7047.59) | 16277.37 (4693.5) | 12063.15 [8313.261,18645.382] | 16693.09 [15998.882,18050.491] | 1.38 | 0.36 | |
| Isovalerate | Fatty Acids | HMDB0000718 | C08262 | 4193.37 (2359.34) | 3664.31 (1649.71) | 4134.17 [2648.348,5618.437] | 3241.47 [2471.145,4892.59] | 0.78 | 0.573 | |
| Valerate | Fatty Acids | HMDB0000892 | C00803 | 4427.75 (1347.95) | 3997.47 (1119.28) | 4342.96 [3399.86,5270.449] | 3960.92 [3438.842,4786.935] | 0.91 | 0.633 | |
| 2-methylbutyrate | Fatty Acids | HMDB0002176 | C18319 | 4144.31 (2178.44) | 3871.43 (1540.37) | 3683.22 [2769.844,5832.408] | 3706.59 [2683.016,4949.79] | 1.01 | 0.829 | |
| Isobutyrate | Fatty Acids | HMDB0001873 | C02632 | 2265.29 (796.38) | 2179.33 (608.86) | 2336.37 [1459.505,2814.65] | 2088.28 [1681.321,2625.118] | 0.89 | 0.965 | |

**Supplementary Table S6.** Cont.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Xanthine | Nucleotide | HMDB0000292 | C00385 | 5596.43 (1837.81) | 6676.28 (2127.25) | 5719.51 [5077.497,6496.786] | 6345.42 [5353.594,8747.441] | 1.11 | 0.36 |
| 2'-Deoxyguanosine | Nucleotide | HMDB0000085 | C00330 | 5.18 (0.91) | 150.92 (214.34) | 5.2 [4.436,5.902] | 4.27 [3.13,272.728] | 0.82 | 0.696 |
| 2'-Deoxyuridine | Nucleotide | HMDB0000012 | C00526 | 5.18 (0.91) | 177.52 (242.56) | 5.2 [4.436,5.902] | 4.27 [3.13,369.629] | 0.82 | 0.696 |
| 2'-Deoxyinosine | Nucleotide | HMDB0000071 | C05512 | 5.18 (0.91) | 254.95 (326.08) | 5.2 [4.436,5.902] | 42.52 [3.376,520.845] | 8.18 | 0.829 |
| Succinate | Organic Acids | HMDB0000254 | C00042 | 10030.08 (11563.14) | 4236.89 (1515.49) | 6138.96 [3960.743,8682.426] | 4315.57 [3320.048,4953.708] | 0.7 | 0.101 |
| Fumarate | Organic Acids | HMDB0000134 | C00122 | 133.42 (170.18) | 56.23 (65.33) | 93.17 [35.452,132.963] | 31.55 [4.057,86.807] | 0.34 | 0.203 |
| Malonate | Organic Acids | HMDB0000691 | C04025 | 1038.88 (539.23) | 1356.76 (465.6) | 901.26 [664.377,1502.826] | 1358.11 [954.523,1642.165] | 1.51 | 0.203 |
| Phenylacetate | Organic Acids | HMDB0000209 | C07086 | 953.16 (504.43) | 726.5 (229.04) | 915.54 [803.346,1046.528] | 788.14 [588.565,865.161] | 0.86 | 0.203 |
| 2-Hydroxyisovalerate | Organic Acids | HMDB0000407 | NA | 779.58 (663.48) | 808.95 (375.44) | 519.69 [382.833,802.401] | 720.81 [621.44,777.02] | 1.39 | 0.237 |
| Pyruvate | Organic Acids | HMDB0000243 | C00022 | 243.28 (216.13) | 369.57 (224) | 153.42 [101.417,412.677] | 367.8 [211.129,504.003] | 2.4 | 0.237 |
| 2-Oxoglutarate | Organic Acids | HMDB0000208 | C00026 | 761.87 (552.63) | 1370.72 (1603.95) | 583.62 [393.813,910.246] | 590.4 [470.111,1133.093] | 1.01 | 0.46 |
| Taurine | Organic Acids | HMDB0000251 | C00245 | 19565.55 (7107.57) | 15557.65 (5296.45) | 17566.39 [15906.771,19672.931] | 15786.9 [11102.664,19933.544] | 0.9 | 0.46 |
| Malate | Organic Acids | HMDB0000156 | C00149 | 254.13 (492.51) | 1036.78 (1492.16) | 5.92 [5.093,173.493] | 4.27 [3.13,1755.329] | 0.72 | 0.633 |
| 4-Hydroxyphenylacetate | Organic Acids | HMDB0000020 | C00642 | 1644.21 (826.34) | 1389.99 (509.47) | 1413.28 [1141.532,2142.113] | 1425.21 [1007.744,1727.096] | 1.01 | 0.696 |
| Citrate | Organic Acids | HMDB0000094 | C00158 | 684.56 (497.58) | 565.6 (354.72) | 486.79 [449.694,802.304] | 498.5 [341.853,719.753] | 1.02 | 0.696 |
| Lactate | Organic Acids | HMDB0000190 | C00256 | 50259.61 (46276.96) | 55886.66 (38143.53) | 36665.27 [17929.326,73008.364] | 50962.65 [19782.651,86376.547] | 1.39 | 0.696 |
| Formate | Others | HMDB0000142 | C00058 | 4405.52 (7063.45) | 2566.73 (1821.56) | 1134.43 [621.05,3770.088] | 2120.72 [1144.483,3107.663] | 1.87 | 0.315 |
| Methylamine | Others | HMDB0000164 | C00218 | 504.81 (233.79) | 398.57 (146.71) | 546.48 [314.492,714.968] | 387.96 [317.854,463.228] | 0.71 | 0.315 |
| Serine | Others | HMDB0062263 | C00716 | 11574.58 (7949.72) | 15471.5 (7951) | 9646.08 [6233.12,15278.115] | 13439.64 [9106.461,20899.144] | 1.39 | 0.315 |
| sn-Glycero-3-phosphocholine | Others | HMDB0000086 | C00670 | 145.27 (134.9) | 88.36 (44.27) | 116.11 [45.59,216.46] | 90.87 [68.951,94.671] | 0.78 | 0.408 |
| Ethanol | Others | HMDB0000108 | C00469 | 5353.08 (1716.07) | 8592.84 (7515.35) | 5101.52 [4550.712,6641.379] | 6842.94 [3748.678,8755.244] | 1.34 | 0.515 |
| Taurocholic acids | Others | HMDB0000036 | C05122 | 12532.04 (2989.6) | 12828.51 (4230.84) | 12853.71 [12320.71,13507.48] | 13291.17 [9661.131,15717.089] | 1.03 | 0.696 |
| Isopropanol | Others | HMDB0000863 | C01845 | 1790.57 (751.14) | 1536.7 (707.63) | 1585.93 [1228.24,1977.519] | 1702.94 [954.575,2074.905] | 1.07 | 0.762 |
| Choline | Others | HMDB0000097 | C00114 | 256.55 (191.99) | 304.11 (245.36) | 207.57 [152.804,284.821] | 197.41 [140.297,437.023] | 0.95 | 0.965 |
| Galacturonate | Others | HMDB0002545 | C08348 | 157.6 (431.08) | 646.02 (829.87) | 5.58 [4.436,6.106] | 145.91 [4.057,1117.11] | 26.13 | 0.965 |
| p-Cresol | Phenols | HMDB0001858 | C01468 | 451.15 (285.56) | 514.26 (220.41) | 396.83 [297.652,669.221] | 504.57 [317.652,691.996] | 1.27 | 0.762 |

**Supplementary Table S7.** Univariate analysis (Mann–Whitney U test) of the high fat and high fat containing EC 20mg/Kg metabolomic data

| Name | Class | HMDBID | KEGGID | HFE20.MeanSD | HF.MeanSD | HFE20.MedianIQR | HF.MedianIQR | FC | P | |
|---|---|---|---|---|---|---|---|---|---|---|
| Alanine | Amino Acids | HMDB0000161 | C00041 | 58246.93 (8483.17) | 66715.08 (10520.3) | 58270.5 [51700.869,63430.84] | 67157.37 [58032.744,72263.212] | 1.15 | 0.087 | up |
| Ribose | Carbohydrates | HMDB0000283 | C00121 | 6.12 (0.94) | 5.18 (0.91) | 5.76 [5.559,6.838] | 5.2 [4.436,5.902] | 0.9 | 0.049 | down |
| Valerate | Fatty Acids | HMDB0000892 | C00803 | 2913.73 (1693.61) | 4427.75 (1347.95) | 2472.97 [2209.19,4190.267] | 4342.96 [3399.86,5270.449] | 1.76 | 0.051 | |
| Isobutyrate | Fatty Acids | HMDB0001873 | C02632 | 3059.57 (1043.36) | 2265.29 (796.38) | 2863.21 [2350.69,3953.708] | 2336.37 [1459.505,2814.65] | 0.82 | 0.086 | |
| Cytosine | Nucleotide | HMDB0000630 | C00380 | 640.63 (438.87) | 1302.59 (415.23) | 619.47 [280.033,870.115] | 1318.93 [1138.844,1518.962] | 2.13 | 0.0049 | |
| 2'-Deoxyguanosine | Nucleotide | HMDB0000085 | C00330 | 6.12 (0.94) | 5.18 (0.91) | 5.76 [5.559,6.838] | 5.2 [4.436,5.902] | 0.9 | 0.049 | |
| 2'-Deoxyinosine | Nucleotide | HMDB0000071 | C05512 | 6.12 (0.94) | 5.18 (0.91) | 5.76 [5.559,6.838] | 5.2 [4.436,5.902] | 0.9 | 0.049 | |
| 2'-Deoxyuridine | Nucleotide | HMDB0000012 | C00526 | 6.12 (0.94) | 5.18 (0.91) | 5.76 [5.559,6.838] | 5.2 [4.436,5.902] | 0.9 | 0.049 | |
| Uridine | Nucleotide | HMDB0000296 | C00299 | 6.12 (0.94) | 5.18 (0.91) | 5.76 [5.559,6.838] | 5.2 [4.436,5.902] | 0.9 | 0.049 | |
| 3-(3-Hydroxyphenyl)propanoate | Organic Acids | HMDB0000375 | C11457 | 220.74 (141.54) | 5.18 (0.91) | 231.66 [159.539,295.512] | 5.2 [4.436,5.902] | 0.02 | 0.00095 | |
| Citrate | Organic Acids | HMDB0000094 | C00158 | 259.08 (146.58) | 684.56 (497.58) | 222.76 [124.259,390.953] | 486.79 [449.694,802.304] | 2.19 | 0.048 | |
| 4-Hydroxyphenylacetate | Organic Acids | HMDB0000020 | C00642 | 2364.85 (847.62) | 1644.21 (826.34) | 2149.58 [1881.051,2353.559] | 1413.28 [1141.532,2142.113] | 0.66 | 0.089 | |
| 2,3-Butanediol | Alcohols | HMDB0003156 | C00265 | 753.62 (996.81) | 4204.41 (7032.86) | 7.56 [5.743,1597.981] | 1419.77 [6.381,4565.103] | 187.82 | 0.21 | |
| Glycerol | Alcohols | HMDB0000131 | C00116 | 1934.32 (4086.29) | 934.84 (536.3) | 755.91 [413.737,897.57] | 933.55 [466.892,1285.725] | 1.23 | 0.463 | |
| Propylene glycol | Alcohols | HMDB0001881 | C00583 | 848.02 (549.1) | 746.2 (1038.36) | 770.62 [447.17,1373.463] | 349.43 [6.381,984.072] | 0.45 | 0.807 | |
| Leucine | Amino Acids | HMDB0000687 | C00123 | 37329.67 (7095.09) | 31058.9 (8594.68) | 34860.89 [31883.365,43639.95] | 31142.54 [25089.717,35511.207] | 0.89 | 0.12 | |
| Tyramine | Amino Acids | HMDB0000306 | C00483 | 5637.08 (1820.81) | 3522.67 (3306.38) | 6124.31 [4408.075,7142.034] | 2240.47 [1250.525,5791.971] | 0.37 | 0.135 | |
| Tryptophan | Amino Acids | HMDB0000929 | C00078 | 2719.42 (685.27) | 2293.93 (539.92) | 2615.66 [2228.04,3095.175] | 2471.53 [2073.401,2631.786] | 0.94 | 0.16 | |
| Ornithine | Amino Acids | HMDB0000214 | C00077 | 1438.62 (629.11) | 1958.47 (936.04) | 1655.88 [1338.747,1765.882] | 2340.49 [1884.123,2412.399] | 1.41 | 0.204 | |
| Aspartate | Amino Acids | HMDB0000191 | C00049 | 57686.68 (9769.31) | 47524.93 (19698.89) | 56440.4 [52461.788,63052.99] | 43454.61 [40132.916,55800.01] | 0.77 | 0.213 | |
| Glycine | Amino Acids | HMDB0000123 | C00037 | 35838.11 (6389.17) | 41451.22 (10705.98) | 36853.24 [32270.418,40136.557] | 40302.59 [38203.044,46353.356] | 1.09 | 0.218 | |
| Creatinine | Amino Acids | HMDB0000562 | C00791 | 855.94 (226.85) | 1042.7 (371.07) | 835.99 [674.546,880.66] | 910.15 [812.907,1275.789] | 1.09 | 0.237 | |
| Phenylalanine | Amino Acids | HMDB0000159 | C00079 | 13463.33 (2557.84) | 11743.28 (3412.88) | 13688.23 [11305.865,15576.726] | 12463.19 [8793.642,14205.647] | 0.91 | 0.258 | |
| Alpha-ketoisovalerate | Amino Acids | HMDB0000019 | C00141 | 108.14 (72.29) | 379.54 (641.57) | 148.5 [33.313,155.874] | 140.55 [87.488,290.373] | 0.95 | 0.272 | |
| Methionine | Amino Acids | HMDB0000696 | C00073 | 13801.33 (3473) | 11885.34 (3844.34) | 13684.53 [12164.065,15393.702] | 13122.2 [8561.484,14375.295] | 0.96 | 0.291 | |
| Valine | Amino Acids | HMDB0000883 | C00183 | 52578.2 (9757.18) | 47408.84 (11605.23) | 51218.66 [44743.921,59000.598] | 49507.01 [40847.387,53385.78] | 0.97 | 0.331 | |
| 2-Oxoisocaproate | Amino Acids | HMDB0000695 | C00233 | 6.12 (0.94) | 614.47 (1723.61) | 5.76 [5.559,6.838] | 5.2 [4.436,6.106] | 0.9 | 0.351 | |
| 4-Aminobutyrate | Amino Acids | HMDB0000112 | C00334 | 1063.31 (652.24) | 1269.08 (308.44) | 1155.84 [821.08,1420.723] | 1271.38 [1060.047,1473.4] | 1.1 | 0.393 | |
| Creatine | Amino Acids | HMDB0000064 | C00300 | 475.98 (234.98) | 582.19 (296.98) | 485.97 [339.881,533.627] | 657.52 [458.28,833.929] | 1.35 | 0.424 | |
| Threonine | Amino Acids | HMDB0000167 | C00188 | 6815.34 (4919.79) | 8037.66 (4426.64) | 5588.4 [4441.394,8413.132] | 7791.96 [5523.154,10163.833] | 1.39 | 0.587 | |
| Glutamine | Amino Acids | HMDB0000641 | C00064 | 3788.62 (1521.13) | 3440.03 (1856.99) | 3806.66 [2955.103,4282.84] | 2511.14 [2358.09,4162.815] | 0.66 | 0.675 | |
| Tyrosine | Amino Acids | HMDB0000158 | C00082 | 8513.31 (4673.38) | 9432.5 (5075.36) | 9040.83 [4097.738,12827.659] | 10431.93 [4861.76,12643.906] | 1.15 | 0.698 | |
| Glutamate | Amino Acids | HMDB0000148 | C00025 | 61133.72 (7142.12) | 63265.08 (13838.32) | 60750.81 [58748.87,64001.632] | 60406.55 [53211.758,71382.105] | 0.99 | 0.701 | |
| Asparagine | Amino Acids | HMDB0000168 | C00152 | 1606.45 (554.78) | 1673.33 (533.85) | 1658.42 [1286.542,2092.153] | 1666.13 [1262.001,2090.714] | 1 | 0.799 | |
| Isoleucine | Amino Acids | HMDB0000172 | C00407 | 30817.57 (5778.43) | 29859.25 (9379.66) | 29548.66 [26263.709,33707.097] | 28011.08 [25206.815,35457.952] | 0.95 | 0.805 | |
| 5-Aminopentanoate | Amino Acids | HMDB0003355 | C00431 | 3114.81 (997.54) | 3375.98 (2853.47) | 3022.13 [2554,3543.799] | 2281.54 [2045.001,3021.252] | 0.75 | 0.811 | |
| Citrulline | Amino Acids | HMDB0000904 | C00327 | 11127.57 (2692.03) | 11482.89 (4008.55) | 10368.17 [9193.728,12820.671] | 13312.67 [7862.718,14331.876] | 1.28 | 0.834 | |
| Lysine | Amino Acids | HMDB0000182 | C00047 | 23148.45 (7401.89) | 22643.96 (7257.37) | 22726.06 [21993.442,25956.229] | 22933.45 [19886.164,26761.348] | 1.01 | 0.886 | |
| Histidine | Amino Acids | HMDB0000177 | C00135 | 3615.19 (1047.27) | 3687.99 (1180.54) | 3348.91 [3251.915,4122.494] | 3833.03 [3062.453,4274.154] | 1.14 | 0.893 | |
| Proline | Amino Acids | HMDB0000162 | C00148 | 15657.22 (2418.59) | 15497.69 (3321.26) | 15372.32 [13968.148,16906.802] | 15401.69 [12886.993,16354.303] | 1 | 0.911 | |
| Urocanate | Azoles | HMDB0000301 | C00785 | 1086.25 (526.29) | 1219.58 (586.79) | 1076.7 [704.669,1360.225] | 1144.76 [855.035,1679.083] | 1.06 | 0.624 | |
| Xylose | Carbohydrates | HMDB0000098 | C00181 | 6.12 (0.94) | 455.4 (678.86) | 5.76 [5.559,6.838] | 6.2 [5.093,865.914] | 1.08 | 0.103 | |
| Fucose | Carbohydrates | HMDB0012327 | C02781 | 1849.9 (1432.77) | 985.08 (965) | 2450.67 [321.181,2709.706] | 1022.64 [5.732,1680.216] | 0.42 | 0.147 | |
| Glucose | Carbohydrates | HMDB0000122 | C00031 | 489.95 (1057.44) | 997.29 (1901.09) | 6.51 [5.743,7.623] | 5.63 [4.954,769.257] | 0.86 | 0.514 | |
| Galactose | Carbohydrates | HMDB0000143 | C00984 | 99.88 (296.3) | 183.26 (503.2) | 5.83 [5.623,7.364] | 5.58 [4.954,6.106] | 0.96 | 0.687 | |
| Butyrate | Fatty Acids | HMDB0000039 | C00246 | 3995.59 (1369.78) | 5316.03 (3043.7) | 4038.11 [3353.509,4746.475] | 4943.33 [3178.675,6498.124] | 1.22 | 0.284 | |
| Isovalerate | Fatty Acids | HMDB0000718 | C08262 | 5132.7 (2036.28) | 4193.37 (2359.34) | 4981.04 [4163.565,6384.153] | 4134.17 [2648.348,5618.437] | 0.83 | 0.388 | |
| 2-methylbutyrate | Fatty Acids | HMDB0002176 | C18319 | 5003.58 (2001.13) | 4144.31 (2178.44) | 4638.12 [3976.569,5625.311] | 3683.22 [2769.844,5832.408] | 0.79 | 0.403 | |
| Propionate | Fatty Acids | HMDB0000237 | C00163 | 11531.56 (2858.26) | 13224.69 (7047.59) | 12037.22 [9313.511,12908.767] | 12063.15 [8313.261,18645.382] | 1 | 0.539 | |
| Xanthine | Nucleotide | HMDB0000292 | C00385 | 4948.45 (2098.16) | 5596.43 (1837.81) | 4565.95 [3754.654,5477.718] | 5719.51 [5077.497,6496.786] | 1.25 | 0.495 | |
| Uracil | Nucleotide | HMDB0000300 | C00106 | 7648.02 (1398.92) | 7131.52 (2313.69) | 7366.92 [6960.249,8218.195] | 6806.46 [5842.153,8524.463] | 0.92 | 0.59 | |
| Hypoxanthine | Nucleotide | HMDB0000157 | C00262 | 5046.29 (1042.95) | 5336.82 (1441.03) | 5012.52 [4328.787,5410.439] | 5119.53 [4213.796,6062.723] | 1.02 | 0.64 | |
| nicotinate | Organic Acids | HMDB0000208 | C00026 | 434.42 (212.46) | 761.87 (552.63) | 377.96 [247.478,555.545] | 583.62 [393.813,910.246] | 1.54 | 0.149 | |
| Phenylacetate | Organic Acids | HMDB0000209 | C07086 | 725.94 (171.44) | 953.16 (504.43) | 722.81 [678.489,838.557] | 915.54 [803.346,1046.528] | 1.27 | 0.256 | |
| Fumarate | Organic Acids | HMDB0000134 | C00122 | 58.7 (53.51) | 133.42 (170.18) | 54.81 [6.082,102.439] | 93.17 [35.452,132.963] | 1.7 | 0.266 | |
| Taurine | Organic Acids | HMDB0000251 | C00245 | 24027.38 (10164.88) | 19565.55 (7107.57) | 22598.05 [18662.09,28287.953] | 17566.39 [15906.771,19672.931] | 0.78 | 0.291 | |

**Supplementary Table S7.** Cont.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Malate | Organic Acids | HMDB0000156 | C00149 | 678.93 (1133.48) | 254.13 (492.51) | 5.76 [5.559,1100.834] | 5.92 [5.093,173.493] | 1.03 | 0.306 |
| 3-Methyl-2-oxovalerate | Organic Acids | HMDB0000491 | C03465 | 50.65 (97.87) | 550.99 (1305.62) | 5.83 [5.559,7.623] | 6.24 [5.242,273.416] | 1.07 | 0.315 |
| Tartrate | Organic Acids | HMDB0000956 | C00898 | 3564.11 (3130.91) | 2417.4 (2316.75) | 2337.19 [1580.376,3782.88] | 1315.5 [1170.24,2690.917] | 0.56 | 0.385 |
| Lactate | Organic Acids | HMDB0000190 | C00256 | 33557.84 (34033.25) | 50259.61 (46276.96) | 20196.87 [7697.804,62860.247] | 36665.27 [17929.326,73008.364] | 1.82 | 0.41 |
| Succinate | Organic Acids | HMDB0000254 | C00042 | 6445.26 (1880.74) | 10030.08 (11563.14) | 6600.17 [5632.021,7062.892] | 6138.96 [3960.743,8682.426] | 0.93 | 0.413 |
| Nicotinate | Organic Acids | HMDB0001488 | C00253 | 888.95 (246.38) | 806.72 (245.11) | 911.75 [707.1,1032.812] | 838.73 [670.998,963.357] | 0.92 | 0.491 |
| 2-Hydroxyisovalerate | Organic Acids | HMDB0000407 | NA | 622.01 (462.61) | 779.58 (663.48) | 567.57 [257.431,955.621] | 519.69 [382.833,802.401] | 0.92 | 0.579 |
| 3-Phenylpropionate | Organic Acids | HMDB0000764 | C05629 | 522.28 (464.97) | 659.42 (613.1) | 455.88 [265.62,626.763] | 448.72 [238.39,1176.487] | 0.98 | 0.61 |
| Malonate | Organic Acids | HMDB0000691 | C04025 | 915.32 (620.3) | 1038.88 (539.23) | 1010.95 [448.66,1389.369] | 901.26 [664.377,1502.826] | 0.89 | 0.658 |
| 4-Hydroxyphenyllactate | Organic Acids | HMDB0000755 | C03672 | 223.94 (140.21) | 246.12 (84.77) | 191.03 [157.235,225.361] | 251.26 [186.333,316.082] | 1.32 | 0.684 |
| 4-Hydroxybenzoate | Organic Acids | HMDB0000500 | C00156 | 259.84 (124.75) | 263.55 (185.24) | 291.65 [176.925,345.164] | 240.29 [186.333,287.836] | 0.82 | 0.962 |
| Pyruvate | Organic Acids | HMDB0000243 | C00022 | 247.37 (130.54) | 243.28 (216.13) | 217.53 [158.82,333.644] | 153.42 [101.417,412.677] | 0.71 | 0.963 |
| Serine | Others | HMDB0062263 | C00716 | 6857.23 (2900.92) | 11574.58 (7949.72) | 6654.71 [4775.789,9381.286] | 9646.08 [6233.12,15278.115] | 1.45 | 0.147 |
| Isopropanol | Others | HMDB0000863 | C01845 | 2304.06 (697.18) | 1790.57 (751.14) | 2105.18 [1942.676,2549.307] | 1585.93 [1228.24,1977.519] | 0.75 | 0.158 |
| Taurocholic acids | Others | HMDB0000036 | C05122 | 15447.75 (5537.73) | 12532.04 (2989.6) | 13880.7 [11419.777,16837.958] | 12853.71 [12320.71,13507.48] | 0.93 | 0.175 |
| Lactadehyde | Others | HMDB0003052 | C00424 | 838.14 (338.57) | 1318.12 (951.39) | 729.36 [582.844,1110.703] | 973.07 [632.609,1592.833] | 1.33 | 0.209 |
| Dimethylamine | Others | HMDB0000087 | C00543 | 663.36 (114.8) | 569.17 (179.45) | 653.28 [570.488,713.903] | 554.1 [447.359,728.525] | 0.85 | 0.223 |
| Methanol | Others | HMDB0001875 | C00132 | 790.48 (113.43) | 684.25 (213.05) | 774.97 [727.249,872.017] | 657.52 [558.749,727.481] | 0.85 | 0.231 |
| Galacturonate | Others | HMDB0002545 | C08348 | 6.12 (0.94) | 157.6 (431.08) | 5.76 [5.559,6.838] | 5.58 [4.436,6.106] | 0.97 | 0.353 |
| Formate | Others | HMDB0000142 | C00058 | 7295.8 (7995.23) | 4405.52 (7063.45) | 4731.08 [1272.349,8468.892] | 1134.43 [621.05,3770.088] | 0.24 | 0.428 |
| sn-Glycero-3-phosphocholine | Others | HMDB0000086 | C00670 | 194.7 (143.16) | 145.27 (134.9) | 177.41 [122.92,286.521] | 116.11 [45.59,216.46] | 0.65 | 0.464 |
| Trimethylamine | Others | HMDB0000906 | C00565 | 941.66 (701.4) | 1139.16 (627.02) | 738.44 [614.918,905.212] | 906.2 [665.986,1789.229] | 1.23 | 0.538 |
| Ethanol | Others | HMDB0000108 | C00469 | 6147.97 (3845.26) | 5353.08 (1716.07) | 4730.1 [3424.584,9463.066] | 5101.52 [4550.712,6641.379] | 1.08 | 0.569 |
| Acetate | Others | HMDB0000042 | C00033 | 373711.36 (46230.92) | 363282.66 (54107.73) | 384521.76 [344837.636,396734.637] | 382046.7 [309920.974,402420.453] | 0.99 | 0.672 |
| Choline | Others | HMDB0000097 | C00114 | 291.74 (244.78) | 256.55 (191.99) | 202.35 [168.384,304.93] | 207.57 [152.804,284.821] | 1.03 | 0.737 |
| Methylamine | Others | HMDB0000164 | C00218 | 521.6 (186) | 504.81 (233.79) | 456.34 [393.594,679.001] | 546.48 [314.492,714.968] | 1.2 | 0.871 |
| p-Cresol | Phenols | HMDB0001858 | C01468 | 314.58 (95.9) | 451.15 (285.56) | 314.24 [239.029,369.818] | 396.83 [297.652,669.221] | 1.26 | 0.23 |

**Supplementary Table S8.** Spearman correlation analysis between the microbiome (Phylum) and metabolomics data for the high fat diet versus control (only Spearman R values >0.5 or <-0.5 are reported)

| Phylum | Metabolite | Spearman R | P value |
|---|---|---|---|
| Verrucomicrobia | 3-Phenylpropionate | -0.822 | 2.80E-05 |
| Verrucomicrobia | Cytosine | -0.626 | 5.40E-03 |
| Verrucomicrobia | Uracil | 0.684 | 1.70E-03 |
| Verrucomicrobia | Uridine | 0.754 | 3.00E-04 |
| Verrucomicrobia | Dimethylamine | -0.723 | 6.90E-04 |
| Verrucomicrobia | 3-(3-Hydroxyphenyl)propanoate | -0.705 | 1.10E-03 |
| Verrucomicrobia | Hypoxanthine | 0.635 | 4.70E-03 |
| Verrucomicrobia | 4-Hydroxybenzoate | -0.556 | 0.017 |
| Verrucomicrobia | Alanine | 0.697 | 1.30E-03 |
| Verrucomicrobia | Tryptophan | -0.598 | 8.80E-03 |
| Verrucomicrobia | Tartrate | -0.569 | 0.014 |
| Bacteroidetes | 3-Phenylpropionate | -0.55 | 0.018 |
| Bacteroidetes | Cytosine | -0.688 | 1.60E-03 |
| Bacteroidetes | Uracil | 0.643 | 4.00E-03 |
| Bacteroidetes | 4-Hydroxybenzoate | -0.515 | 0.029 |
| Bacteroidetes | 4-Hydroxyphenyllactate | -0.62 | 6.00E-03 |
| Tenericutes | Cytosine | -0.628 | 5.20E-03 |
| Tenericutes | Uracil | 0.736 | 5.00E-04 |
| Tenericutes | Hypoxanthine | 0.577 | 0.012 |
| Tenericutes | 4-Hydroxybenzoate | -0.513 | 0.03 |
| Tenericutes | 4-Hydroxyphenyllactate | -0.55 | 0.018 |
| Epsilonbacteraeota | Cytosine | -0.67 | 2.40E-03 |
| Epsilonbacteraeota | Uracil | 0.688 | 1.60E-03 |
| Epsilonbacteraeota | Hypoxanthine | 0.544 | 0.02 |
| Epsilonbacteraeota | 4-Hydroxybenzoate | -0.505 | 0.033 |
| Epsilonbacteraeota | 5-Aminopentanoate | -0.511 | 0.03 |
| Chlamydiae | Cytosine | -0.678 | 2.00E-03 |
| Chlamydiae | Uracil | 0.626 | 5.40E-03 |
| Deinococcus-Thermus | 3-Phenylpropionate | 0.536 | 0.022 |
| Deinococcus-Thermus | Uracil | -0.521 | 0.027 |
| Deinococcus-Thermus | Uridine | -0.637 | 4.50E-03 |
| Deinococcus-Thermus | Dimethylamine | 0.748 | 3.60E-04 |
| Deinococcus-Thermus | 3-(3-Hydroxyphenyl)propanoate | 0.552 | 0.018 |
| Deinococcus-Thermus | Hypoxanthine | -0.509 | 0.031 |
| Deinococcus-Thermus | 5-Aminopentanoate | 0.556 | 0.017 |
| Deinococcus-Thermus | Lactadehyde | -0.536 | 0.022 |
| Deinococcus-Thermus | 3-Methyl-2-oxovalerate | -0.595 | 9.10E-03 |
| Deinococcus-Thermus | 2-Oxoisocaproate | -0.662 | 2.80E-03 |
| Nanoarchaeaeota | Cytosine | -0.653 | 3.30E-03 |
| Nanoarchaeaeota | Uracil | 0.571 | 0.013 |
| Latescibacteria | Cytosine | -0.536 | 0.022 |
| Latescibacteria | Uracil | 0.536 | 0.022 |
| Hydrogenedentes | 4-Hydroxyphenyllactate | -0.554 | 0.017 |
| Nitrospirae | Cytosine | -0.54 | 0.021 |
| Nitrospirae | 4-Hydroxyphenyllactate | -0.637 | 4.50E-03 |
| Euryarchaeota | Cytosine | -0.585 | 0.011 |
| Euryarchaeota | Uracil | 0.511 | 0.03 |
| Euryarchaeota | 2-Oxoisocaproate | 0.503 | 0.034 |
| Fibrobacteres | Cytosine | -0.517 | 0.028 |
| Fibrobacteres | Uracil | 0.536 | 0.022 |

**Supplementary Table S9.** Spearman correlation analysis between the microbiome (Phylum) and metabolomics data for the high fat diet versus high fat + EC 20mg/Kg body weight (only Spearman R values >0.5 or <-0.5 are reported)

| Phylum | Metabolite | Spearman R | P value |
|---|---|---|---|
| Nitrospirae | 3-(3-Hydroxyphenyl)propanoate | 0.785 | 1.10E-04 |
| Nitrospirae | Cytosine | -0.695 | 1.40E-03 |
| Nitrospirae | 2'-Deoxyguanosine | 0.523 | 0.026 |
| Nitrospirae | 2'-Deoxyinosine | 0.523 | 0.026 |
| Nitrospirae | 2'-Deoxyuridine | 0.523 | 0.026 |
| Nitrospirae | Ribose | 0.523 | 0.026 |
| Nitrospirae | Uridine | 0.523 | 0.026 |
| Thaumarchaeota | 3-(3-Hydroxyphenyl)propanoate | 0.651 | 3.40E-03 |
| Thaumarchaeota | Cytosine | -0.513 | 0.03 |
| Thaumarchaeota | Citrate | -0.628 | 5.20E-03 |
| Acidobacteria | 3-(3-Hydroxyphenyl)propanoate | 0.686 | 1.70E-03 |
| Acidobacteria | Cytosine | -0.695 | 1.40E-03 |
| Acidobacteria | Citrate | -0.647 | 3.70E-03 |
| Acidobacteria | 2'-Deoxyguanosine | 0.515 | 0.029 |
| Acidobacteria | 2'-Deoxyinosine | 0.515 | 0.029 |
| Acidobacteria | 2'-Deoxyuridine | 0.515 | 0.029 |
| Acidobacteria | Ribose | 0.515 | 0.029 |
| Acidobacteria | Uridine | 0.515 | 0.029 |
| Entotheonellaeota | 3-(3-Hydroxyphenyl)propanoate | 0.802 | 8.30E-03 |
| Entotheonellaeota | Cytosine | -0.585 | 0.011 |
| Entotheonellaeota | Citrate | -0.631 | 5.00E-03 |
| Entotheonellaeota | 2'-Deoxyguanosine | 0.521 | 0.027 |
| Entotheonellaeota | 2'-Deoxyinosine | 0.521 | 0.027 |
| Entotheonellaeota | 2'-Deoxyuridine | 0.521 | 0.027 |
| Entotheonellaeota | Ribose | 0.521 | 0.027 |
| Entotheonellaeota | Uridine | 0.521 | 0.027 |
| Elusimicrobia | 3-(3-Hydroxyphenyl)propanoate | 0.721 | 7.30E-04 |
| Elusimicrobia | Cytosine | -0.742 | 4.20E-04 |
| Firmicutes | 3-(3-Hydroxyphenyl)propanoate | 0.864 | 3.90E-06 |
| Firmicutes | Cytosine | -0.542 | 0.02 |
| Firmicutes | 2'-Deoxyguanosine | 0.515 | 0.029 |
| Firmicutes | 2'-Deoxyinosine | 0.515 | 0.029 |
| Firmicutes | 2'-Deoxyuridine | 0.515 | 0.029 |
| Firmicutes | Ribose | 0.515 | 0.029 |
| Firmicutes | Uridine | 0.515 | 0.029 |
| GAL15 | 3-(3-Hydroxyphenyl)propanoate | 0.546 | 0.019 |
| GAL15 | Citrate | -0.581 | 0.011 |
| Gemmatimonadetes | 3-(3-Hydroxyphenyl)propanoate | 0.56 | 0.016 |
| Gemmatimonadetes | Cytosine | -0.672 | 2.30E-03 |
| Gemmatimonadetes | Citrate | -0.614 | 6.70E-03 |
| Actinobacteria | 3-(3-Hydroxyphenyl)propanoate | -0.88 | 1.40E-06 |
| Actinobacteria | Cytosine | 0.544 | 0.02 |
| Actinobacteria | 2'-Deoxyguanosine | -0.61 | 7.20E-03 |
| Actinobacteria | 2'-Deoxyinosine | -0.61 | 7.20E-03 |
| Actinobacteria | 2'-Deoxyuridine | -0.61 | 7.20E-03 |
| Actinobacteria | Ribose | -0.61 | 7.20E-03 |
| Actinobacteria | Uridine | -0.61 | 7.20E-03 |
| Deinococcus-Thermus | 3-(3-Hydroxyphenyl)propanoate | -0.542 | 0.02 |
| Deinococcus-Thermus | Cytosine | 0.538 | 0.021 |
| Euryarchaeota | 3-(3-Hydroxyphenyl)propanoate | 0.622 | 5.80E-03 |
| Euryarchaeota | Cytosine | -0.604 | 8.00E-03 |
| Euryarchaeota | 2'-Deoxyguanosine | 0.595 | 9.10E-03 |
| Euryarchaeota | 2'-Deoxyinosine | 0.595 | 9.10E-03 |
| Euryarchaeota | 2'-Deoxyuridine | 0.595 | 9.10E-03 |
| Euryarchaeota | Ribose | 0.595 | 9.10E-03 |
| Euryarchaeota | Uridine | 0.595 | 9.10E-03 |
| Rokubacteria | 3-(3-Hydroxyphenyl)propanoate | 0.608 | 7.50E-03 |
| Rokubacteria | Cytosine | -0.562 | 0.015 |
| Rokubacteria | Citrate | -0.531 | 0.023 |
| Bacteroidetes | Cytosine | -0.581 | 0.011 |
| Latescibacteria | 3-(3-Hydroxyphenyl)propanoate | 0.624 | 5.60E-03 |
| Latescibacteria | Cytosine | -0.577 | 0.012 |
| Latescibacteria | 2'-Deoxyguanosine | 0.583 | 0.011 |
| Latescibacteria | 2'-Deoxyinosine | 0.583 | 0.011 |
| Latescibacteria | 2'-Deoxyuridine | 0.583 | 0.011 |
| Latescibacteria | Ribose | 0.583 | 0.011 |
| Latescibacteria | Uridine | 0.583 | 0.011 |

# Chapter 4

## (-)-Epicatechin exerts positive effects on anxiety in high fat diet-induced obese mice through multi-genomic modifications in the hippocampus

**(-)-Epicatechin exerts positive effects on anxiety in high fat diet-induced obese mice through multi-genomic modifications in the hippocampus**

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**Abstract**

Obesity is associated with increased occurrence of cognitive and mood disorders. While consumption of high-fat diets (HFD) and associated obesity could have a detrimental impact on the brain, dietary bioactives may mitigate these harmful effects. We previously observed that (-)-epicatechin (EC) can mitigate HFD-induced anxiety-associated behaviors in mice. The aim of our study is to investigate the molecular mechanisms of EC actions in the hippocampus which underlies its anti-anxiety effects in HFD-fed mice using a multi-genomic approach. Healthy eight-weeks old male C57BL/6J mice were fed for 24 weeks either: A) a control diet containing 10% total calories from fat; B) a HFD containing 45% total calories from fat; or C) the HFD supplemented with 20 mg EC/kg body weight. Hippocampi were isolated for genomic analysis using Affymetrix arrays, followed by in-depth bioinformatic analyses. Genomic analysis demonstrated that EC induced significant changes in mouse hippocampal global gene expression. We observed changes in the expression of 1001 protein-coding genes, 241 miRNAs, and 167 long non-coding RNAs. Opposite gene expression profiles were observed when the gene expression profile obtained upon EC supplementation was compared to the profile obtained after consumption of the HFD. Functionality analysis revealed that the differentially expressed genes regulate processes involved in neurofunction, inflammation, endothelial function, cell-cell adhesion, and cell signaling. In summary, the capacity of EC to mitigate anxiety-related behaviors in HFD-induced obese mice can be in part explained by its capacity to exert complex genomic modifications in the hippocampus, counteracting changes driven by consumption of the HFD and/or associated obesity.

## 1. Introduction

More than 2.1 billion adults are estimated to be overweight or obese worldwide, of which 640 million are obese [1]. It is predicted that in the US nearly 1 in 2 adults will be obese and 1 in 4 adults will be severely obese by 2030 [2]. Obesity is a serious public health concern given that it raises risks for several diseases including type 2 diabetes, cardiovascular diseases, nonalcoholic fatty liver disease, and certain types of cancer. Obesity is also a risk factor for the development of metabolic and vascular disorders, which have emerged as risk factors to mood and cognitive disorders [3-5]. HFD and/or associated obesity induce chronic low-grade inflammation and the circulating pro-inflammatory molecules can cross the blood-brain-barrier (BBB), eliciting neuroinflammation [3, 6]. Systemic inflammation can also disrupt the BBB, leading to infiltration of inflammatory molecules into the central nervous system and subsequently neuroinflammation, which can contribute to alterations in cognition and mood [5, 7].

While HFD and obesity can have detrimental effects on the brain, consumption of select flavonoids have neuroprotective effects [4, 8, 9]. (-)-Epicatechin (EC) is one of the most widely consumed flavan-3-ols, being abundantly found in cocoa, berries, apples, and tea [10]. A considerable body of evidence supports the beneficial effects of dietary EC at the nervous system, which includes its capacity to improve cognition and mood [9, 11-14]. For example, in healthy adults (50-75 years), EC improves hippocampal-dependent learning suggesting that EC consumption may be associated with increased memory function in age-related cognitive decline [15].

We recently observed that HFD-fed obese mice show significantly increased anxiety-related behaviors which were mitigated by EC consumption (under revision). The capacity of EC to improve anxiety was in part explained by its capacity to modulate BDNF- and glucocorticoids (GC)-regulated signaling and to mitigate HFD-induced dysbiosis. However, a full understanding of EC actions at the hippocampus is missing. Previous evidence showed that EC protects brain vascular endothelial cell

integrity and reduces the risk of neurodegenerative conditions by exerting multi-genomic actions, modulating the expression of protein-coding genes as well as non-coding genes, particularly microRNA (miRNA) and long non-coding RNA (lncRNA) [16, 17].

The use of cutting edge untargeted genomic methodologies represents a significant breakthrough in nutrigenomics, as these methods enable detailed insights into the involved molecular mechanisms. Moreover, the implementation of multi-omics approaches allows integration of different levels of regulation of cellular functions and to obtain a comprehensive understanding of the molecular mechanisms of action of polyphenols. miRNAs are small noncoding RNA molecules, in average 22 nucleotides long, that act as post-transcriptional regulators of gene expression, in most cases by degradation or inhibition of translation of their target mRNAs. miRNAs regulate a wide spectrum of biological processes and are therefore involved in the physiopathology of diseases, including age-related and neurodegenerative diseases [18]. LncRNAs are single strand RNAs with over 200 nucleotides in length. Although they do not directly encode proteins, LncRNAs are involved in the regulation of cellular functions through various mechanisms, such as regulation of gene expression by sequestering miRNAs and subsequently reducing the number of miRNAs available for their target mRNA [19]. LncRNAs play an important role in the pathophysiology of diseases, such as cardiovascular [20], cardiometabolic [21], and neurodegenerative diseases [22]. The expressions of lncRNAs can be affected by diet. For example, in mice fed a HFD, changes in the expression of 52 lncRNAs are proposed to play a significant role in diabetes mellitus [23]. Recent studies also showed that polyphenols can affect the expressions of non-coding genes [16, 17, 24]. Taken together, integrated multi-genomic analysis coupled with bioinformatics represent an insightful approach to obtain detailed information about underlying molecular mechanisms of action of dietary components.

In the present work, we investigated the molecular mechanisms underlying the neuroprotective effects of EC against HFD- and obesity-induced anxiety behaviors in mice. For this purpose, we evaluated

EC-mediated multi-genomic modifications, including changes in the expression of protein-coding and non-coding genes in the hippocampus. We characterized affected molecular pathways and key regulators to better understand the neuroprotective actions of EC.

## 2. Material 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) were fed for 24 weeks either: a diet containing 10% total calories from fat (C group) (TD.06416, Envigo, Indianapolis, IN), a diet containing 45% total calories from lard fat (HF group) (TD.06415, Envigo, Indianapolis, IN), and the HFD supplemented with 20 mg EC/kg body weight (HFE group). 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. 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. Hippocampi were dissected and flash frozen in liquid nitrogen and then stored at −80 °C for further analysis.

### 2.2. RNA extraction from the hippocampus

Total RNA was extracted from the right hippocampus from three animals per experimental group (C, HF, HFE) using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), and small residual amounts of DNA

were removed using an on-column RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted RNA was assessed by NanoDrop One (Thermo Scientific, Waltham, MA), and integrity by 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quality and integrity assessments showed that A260/A280 ratios were 2 or higher and the RNA integrity number (RIN) was 8 or higher for all samples.

**2.3. Microarray hybridization and transcriptome analysis**

Clariom D mouse assays (Affymetrix, Santa Clara, CA) were used for transcriptomics analysis. Total RNA (200 ng per sample) for 9 RNA samples (3 samples per group) was used to prepare cRNA (15 ug) and then sscDNA (5.5 ug) using GeneChip WT PLUS Reagent Kit (Thermo Scientific, Waltham, MA). Purified sscDNA (5.5 ug) was fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and labeled by terminal deoxynucleotidyl transferase (TdT) using the DNA Labeling Reagent that is covalently linked to biotin. Fragmented and labeled sscDNA samples were submitted to the UC Davis Comprehensive Cancer Center's Genomics Shared Resource (GSR) core for hybridization, washing, staining, and scanning using the GeneChip Hybridization, Wash, and Stain Kit (Thermo Scientific, Waltham, MA) following the manufacturer's instruction. Hybridization of fragmented and labeled sscDNA samples was done using GeneChip Hybridization oven, and the arrays were washed then stained using GeneChip Fluidics Station. The arrays were scanned using GeneChipTM Scanner 3000 7G (Thermo Fisher Scientific, Santa Clara, CA). Quality control of the microarrays was performed using the Affymetrix Transcriptome Analysis Console (TAC) 4.0.2. (Thermo Fisher Scientific, Santa Clara, CA).

**2.4. Bioinformatic analysis**

*2.4.1. Comparisons of gene expression profiles*

The TAC software was used to determine differential gene expression of HF vs. C and HFE vs. HF groups using the SST-RMA normalization method and to create volcano plots. All genes from microarray with $p < 0.05$ and ±1.1-fold change was considered as differentially expressed. Partial Least-Squares Discriminant Analysis (PLS-DA) plot was built using MetaboAnalyst (https://www.metaboanalyst.ca; [25]), and heatmap using Hmisc package in R (https://www.r-project.org/). Gene types of the differentially expressed genes (mRNA, miRNA, and lncRNA) were identified using ShinyGO v0.66 (http://bioinformatics.sdstate.edu/go; [26]). Steps of bioinformatic analyses are presented in **Figure 1**.

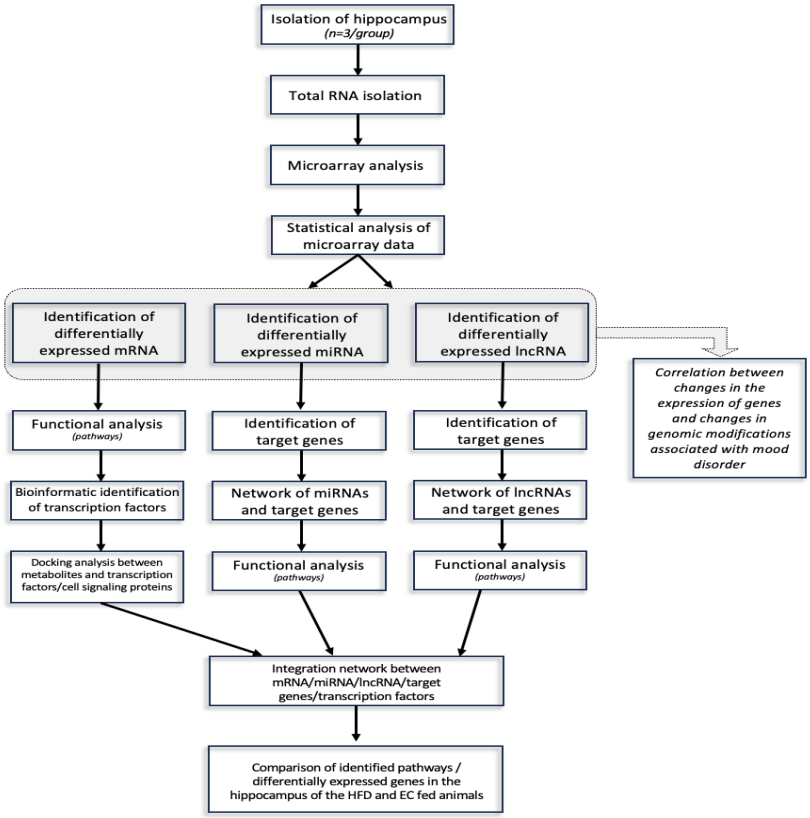



**Figure 1. A flow-chart describing the steps implemented in the genomic and bioinformatic analysis.** The hippocampus (n = 3/group) was isolated from control, HFD, and HFE groups for microarray analysis. For the differentially expressed mRNAs, functional pathways analysis was conducted followed by identification of transcription factors and docking analysis. Next, target genes of the differentially expressed miRNAs and lncRNAs were identified. Subsequently, network and functional analysis were

performed with the identified targets. Integrating them together, network analysis of mRNA, miRNA, lncRNA, target genes, and transcription factors was conducted. Differentially expressed genes and pathways in the hippocampus of the HFD-fed and EC supplemented animals were also compared. Lastly, the identified genomic profile upon EC supplementation was compared with genomic signatures identified in patients with generalized anxiety disorder.

*2.4.2. Database-predicted miRNA and lncRNA targets*

Validated target genes of the identified miRNAs were searched with miRWalk (http://mirwalk.umm.uni-heidelberg.de/; [27]). Network-based visualization of miRNA-gene target enrichment was performed with MIENTURNET (http://userver.bio.uniroma1.it/apps/mienturnet/; [28]). Target genes of the lncRNAs were identified using LncRRIsearch that enables retrieval of lncRNA-gene target interactions (http://rtools.cbrc.jp/LncRRIsearch/; [29]).

*2.4.3.* Pathway enrichment analysis

The pathways involving differentially expressed genes and targets of significantly regulated miRNAs and lncRNAs from HF vs. C and HFE vs. HF comparisons were obtained using Genetrail2. Over-representation analysis was used to obtain the pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and WikiPathways databases. Additionally, top 30 pathways identified by KEGG database search were added to the list of pathway enrichment analysis. Histograms were generated using Microsoft Excel and Venn diagrams by Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/). Interaction networks were constructed with Cytoscape software, version 3.7.2. (https://cytoscape.org; [30]).

*2.4.4. Transcription factor analysis*

Potential transcription factors which activity could be modulated by polyphenols were identified with bioinformatic tool Enrichr (https://amp.pharm.mssm.edu/Enrichr/) [31, 32]. TRRUST [33] and TRANSFAC [34] databases were used to search for the potential transcription factors.

*2.4.5. Docking analysis*

Potential binding interactions between identified transcription factors, their regulatory cell signaling proteins, and major circulatory EC metabolites were examined by molecular docking using the SwissDock docking analysis tool (http://www.swissdock.ch/docking). Protein 3D structures were obtained from UniProt Data Bank (https://www.uniprot.org) and chemical structures of metabolites from PubChem database (https://pubchem.ncbi.nlm.nih.gov).

*2.4.6. Associated and correlated diseases*

The association of identified differentially expressed genes with human diseases was analyzed using the Comparative Toxicogenomics Database (https:// ctdbase.org/) [35]. To explore correlations between the identified genes in the study and genes associated with mood disorder, we first searched the GEO (https://www. ncbi.nlm.nih.gov/gds) for suitable dataset of gene expression profiles in human with mood disorder. The available raw datasets were further analyzed with GEO2R. Pearson's correlation coefficients between gene expression profiles in patients with anxiety disorder and genes which expression had been identified as modulated by EC was calculated using R (https://www.r-project.org/).

## 3. Results

### 3.1. HFD modulates the expression of protein-coding and non-coding genes in the hippocampus.

To assess the molecular mechanisms involved in HFD-induced behavioral changes (under revision), the effects of the HFD on the global expression of genes in the hippocampus was evaluated

comparing data from the HF and the C groups. Gene expression analysis showed that the HFD caused significant changes in global hippocampal gene expression. There were 10,778 probes identified as differentially expressed. Volcano plot of the significantly up- or down-regulated genes in the hippocampus of HFD-fed mice showed the upregulation of 3295 probes and downregulation of 7483 probes compared to C mice **(Supplemental Figure 1A)**. Among them, 6510 genes were identified and classified by ShinyGO v0.66, 47% corresponded to protein-coding genes (3030 mRNAs), 10% to miRNAs (629 miRNAs), and 5% to lncRNAs (353 lncRNAs) **(Supplemental Figure 1B)**.

Gene expression analysis demonstrated that consumption of the HFD induced significant changes in the expression of 3030 hippocampal protein-coding genes. The fold-change values of protein-coding genes varied from -3.26 to 1.97 with an average fold-change of -1.29 for the downregulated genes and 1.24 for the upregulated genes. To understand the biological functions of identified genes, the differentially expressed mRNAs were used to perform enrichment analysis and obtain pathways related to the protein-coding genes. The analysis showed that they are involved in different biological processes such as neurofunction-related pathways, which include pathways involved in Alzheimer's disease, dopaminergic synapse, GABAergic synapse, glutamatergic synapse, and neurodegeneration. Genes identified in these pathways include *Atf4, Calm4, Calm5, Drd1, Gnb2, Gng7, Nos1*, and *Th*. The bioinformatic analysis also revealed that the differentially expressed protein-coding genes can impact inflammation-related pathways (chemokine signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, NF-κB signaling pathway, TNFα signaling pathway, Toll-like receptor signaling pathway), cell-cell adhesion (adherens junction, focal adhesion, gap junction, PI3K-Akt signaling pathway, tight junction), cell signaling pathways (insulin signaling pathway, MAPK signaling pathway, mTOR signaling pathway, PPAR signaling pathway), metabolic pathways (N-glycan biosynthesis, nuclear receptors involved in lipid metabolism and toxicity, TCA cycle), and other cellular processes (apoptosis,

endocytosis, oxidative phosphorylation, regulation of autophagy, ubiquitin mediated proteolysis) **(Supplemental Figure 1C)**.

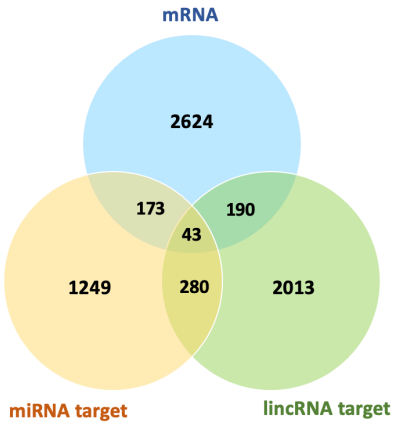
The expression of 629 miRNAs was also modulated upon consumption of the HFD. The fold-change values of miRNA non-coding genes varied from -2.27 to 2.61 with an average fold-change of -1.38 for the downregulated genes and 1.32 for the upregulated genes. The next step of our analysis was to identify target genes of observed differentially expressed miRNAs. MIENTURNET and miRWalk identified 1745 target genes of 68 differentially expressed miRNAs. Networks between differentially expressed miRNAs and their target genes shows a complex interconnectivity **(Supplemental Figure 1D).** Next, we performed enrichment analysis and obtained pathways in which identified targets of differentially expressed miRNAs are involved in. Pathway enrichment analysis showed that several functional pathways are modulated by the miRNAs target genes, being the most over-represented pathways those involved in axon guidance, neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, cytokine-cytokine receptor interaction, focal adhesion, PI3K-Akt signaling, apelin signaling, foxO signaling, mitogen-activated protein kinases (MAPK) signaling, apoptosis, cellular senescence, endocytosis, and non-odorant GPCRs **(Supplemental Figure 1E)**. Genes identified to be involved in these pathways include *Akt1, blc2, Ccr9, Cx3cr1, Gabrb2, Gsk-3β, Il1r1, Il18r1, L1cam, Mapk11, Nr3c1, Plcβ1, Sod2*, and *Stat1.*

In addition, consumption of the HFD induced changes in expression of 353 lncRNAs. The fold-change values of lncRNA non-coding genes varied from -1.74 to 1.63 with an average fold-change of -1.26 for the downregulated genes and 1.25 for the upregulated genes. Subsequently, using the differentially expressed lncRNAs identified in the microarray study, we retrieved 2588 target genes of the lncRNAs from LncRRIsearch database. Pathway enrichment analysis of the target genes showed that they can also regulate pathways involved in various biological processes. The most over-represented pathways include neuroactive ligand-receptor interaction, neurodegeneration, cytokine-cytokine receptor interaction, calcium signaling, focal adhesion, PI3K-Akt signaling, regulation of actin cytoskeleton, cAMP signaling,

MAPK signaling, endocytosis, and non-odorant GPCRs **(Supplemental Figure 1F)**. Key genes identified in these pathways include *C5ar1, Calm1, Camk2α, Ccr5, ccr9, Cxcr11, Egfr, Gabra2, Gsk-3β, Igfr1, Mapk10, Mc1r, Nos1*, and *Xcr1*.

There were 43 common genes among the differentially expressed mRNAs, target genes of the differentially expressed miRNAs, and target genes of differentially expressed lncRNAs **(Figure 2A)**. The common genes identified include *Stat1, Crp, Tyw3, Gmeb1, Hist4h4, Ceacam20, Ceacam1, Nav2, Ntrk3, Ncan, Ccr9, Neurod2, Cd300a, Atf7, Rab11b, Sema6a,* and *Tcf7l2*. Comparison of pathways identified for differentially expressed mRNAs, miRNAs target genes, and lncRNA target genes identified 54 common pathways. These pathways are related to neurofunction (Alzheimer's disease, axon guidance, cholinergic synapse, dopaminergic synapse, glutamatergic synapse, pathways of neurodegeneration), inflammation (chemokine signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, TNFα signaling pathway, Toll-like signaling pathway), cell-cell adhesion (adherens junction, focal adhesion, PI3K-Akt signaling pathway, Rap1 signaling pathway, Ras signaling pathway, regulation of actin cytoskeleton), cell signaling (insulin signaling pathway, MAPK signaling pathway, mTOR signaling pathway, Wnt signaling pathway), metabolism (purine metabolism, sphingolipid metabolism), and other cellular processes (apoptosis, endocytosis, non-odorant GPCRs, spliceosome, type II diabetes mellitus) **(Figure 2B).**

**A**



**B**

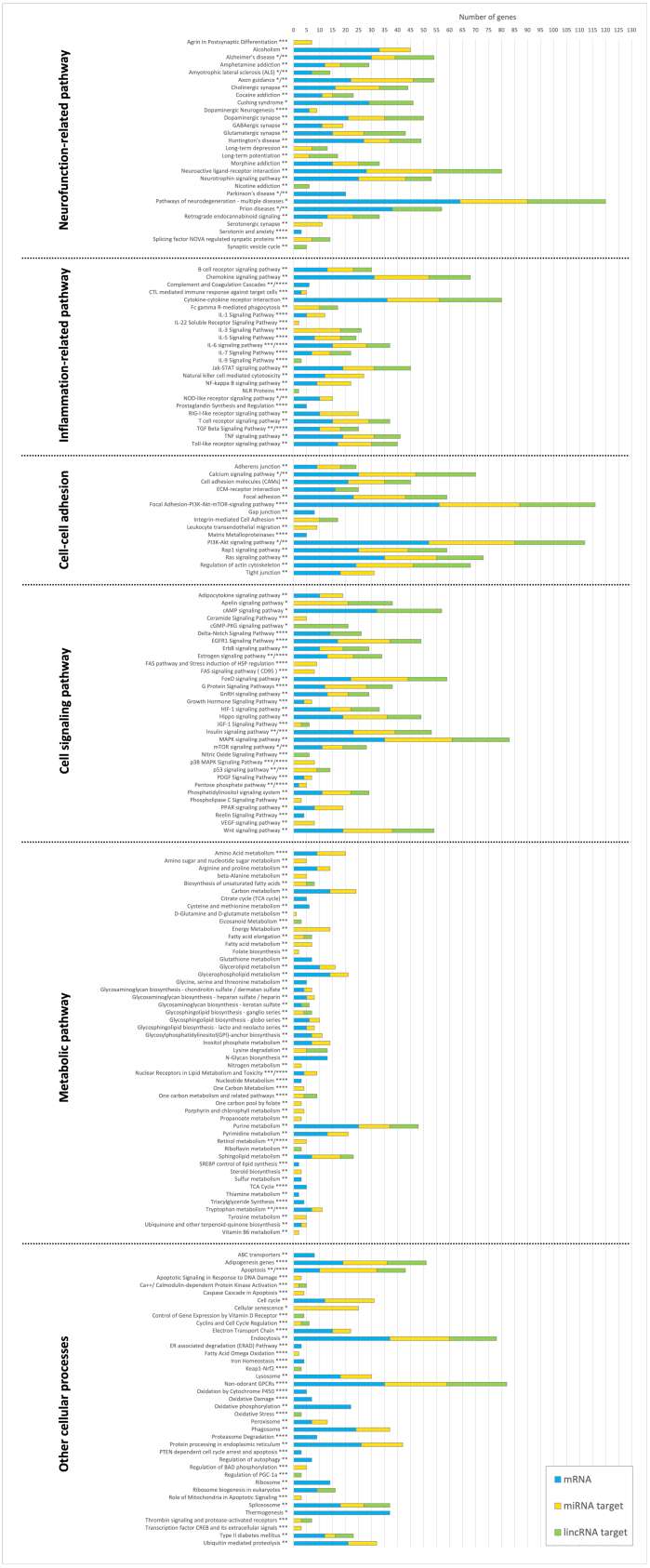

Number of genes
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130
Neurofunction-related pathway
Agrin in Postsynaptic Differentiation ***
Alcoholism **
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */***
Axon guidance */**
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic Neurogenesis ****
Dopaminergic synapse **
GABAergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Nicotine addiction **
Parkinson's disease */**
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Serotonergic synapse **
Serotonin and anxiety ****
Splicing factor NOVA regulated synaptic proteins ***
Synaptic vesicle cycle **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Complement and Coagulation Cascades **/****
CTL mediated immune response against target cells ***
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-1 Signaling Pathway ****
IL-22 Soluble Receptor Signaling Pathway ***
IL-3 Signaling Pathway ****
IL-5 Signaling Pathway ****
IL-6 signaling pathway ***/****
IL-7 Signaling Pathway ****
IL-9 Signaling Pathway ****
Jak-STAT signaling pathway **
Natural killer cell mediated cytotoxicity **
NF-kappa B signaling pathway **
NLR Proteins ****
NOD-like receptor signaling pathway */**
Prostaglandin Synthesis and Regulation ****
RIG-I-like receptor signaling pathway **
T cell receptor signaling pathway **
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Toll-like receptor signaling pathway **
Cell-cell adhesion
Adherens junction **
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Gap junction **
Integrin-mediated Cell Adhesion ****
Leukocyte transendothelial migration **
Matrix Metalloproteinases ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Adipocytokine signaling pathway **
Apelin signaling pathway *
cAMP signaling pathway *
Ceramide Signaling Pathway ***
cGMP-PKG signaling pathway *
Delta-Notch Signaling Pathway ****
EGFR1 Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **/****
FAS pathway and Stress induction of HSP regulation ****
FAS signaling pathway ( CD95 ) ***
FoxO signaling pathway **
G Protein Signaling Pathways ****
GnRH signaling pathway **
Growth Hormone Signaling Pathway ***
HIF-1 signaling pathway **
Hippo signaling pathway **
IGF-1 Signaling Pathway ***
Insulin signaling pathway **/****
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
p38 MAPK Signaling Pathway **/****
p53 signaling pathway **/***
PDGF Signaling Pathway ***
Pentose phosphate pathway **/****
Phosphatidylinositol signaling system **
Phospholipase C Signaling Pathway ***
PPAR signaling pathway **
Reelin Signaling Pathway ***
VEGF signaling pathway **
Wnt signaling pathway **
Metabolic pathway
Amino Acid metabolism ****
Amino sugar and nucleotide sugar metabolism **
Arginine and proline metabolism **
beta-Alanine metabolism **
Biosynthesis of unsaturated fatty acids **
Carbon metabolism **
Citrate cycle (TCA cycle) **
Cysteine and methionine metabolism **
D-Glutamine and D-glutamate metabolism **
Eicosanoid Metabolism ***
Energy Metabolism **
Fatty acid elongation **
Fatty acid metabolism **
Folate biosynthesis **
Glutathione metabolism **
Glycerolipid metabolism **
Glycerophospholipid metabolism **
Glycine, serine and threonine metabolism **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Glycosphingolipid biosynthesis - globo series **
Glycosphingolipid biosynthesis - lacto and neolacto series **
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis **
Inositol phosphate metabolism **
Lysine degradation **
N-Glycan biosynthesis **
Nitrogen metabolism **
Nuclear Receptors in Lipid Metabolism and Toxicity **/****
Nucleotide Metabolism ****
One Carbon Metabolism ****
One carbon metabolism and related pathways ****
One carbon pool by folate **
Porphyrin and chlorophyll metabolism **
Propanoate metabolism **
Purine metabolism **
Pyrimidine metabolism **
Retinol metabolism **/****
Riboflavin metabolism **
Sphingolipid metabolism **
SREBP control of lipid synthesis ***
Sterold biosynthesis **
Sulfur metabolism **
TCA Cycle ****
Thiamine metabolism **
Triacylglyceride Synthesis ***
Tryptophan metabolism **/****
Tyrosine metabolism **
Ubiquinone and other terpenoid-quinone biosynthesis **
Vitamin B6 metabolism **
Other cellular processes
ABC transporters **
Adipogenesis genes ****
Apoptosis **/****
Apoptotic Signaling in Response to DNA Damage ***
Ca++/ Calmodulin-dependent Protein Kinase Activation ***
Caspase Cascade in Apoptosis ***
Cell cycle **
Cellular senescence *
Control of Gene Expression by Vitamin D Receptor ***
Cyclins and Cell Cycle Regulation ***
Electron Transport Chain ****
Endocytosis **
ER associated degradation (ERAD) Pathway ***
Fatty Acid Omega Oxidation ****
Iron Homeostasis ****
Keap1-Nrf2 ****
Lysosome **
Non-odorant GPCRs ***
Oxidation by Cytochrome P450 ****
Oxidative Damage ****
Oxidative phosphorylation **
Oxidative Stress ****
Peroxisome **
Phagosome **
Proteasome Degradation ****
Protein processing in endoplasmic reticulum **
PTEN dependent cell cycle arrest and apoptosis ***
Regulation of autophagy **
Regulation of BAD phosphorylation ***
Regulation of PGC-1a ***
Ribosome **
Ribosome biogenesis in eukaryotes **
Role of Mitochondria in Apoptotic Signaling ***
Spliceosome **
Thermogenesis *
Thrombin signaling and protease-activated receptors ***
Transcription factor CREB and its extracellular signals ***
Type II diabetes mellitus **
Ubiquitin mediated proteolysis **
mRNA
miRNA target
lincRNA target

**Figure 2. Functional pathway analysis of differentially expressed genes in the hippocampus of HFD-fed mice compared to control diet-fed mice.** A) Venn diagram showing 43 common genes among the differentially expressed mRNAs, target genes of the differentially expressed miRNAs, and target genes of differentially expressed lncRNAs identified from comparing HFD-fed mice to control diet-fed mice. B) Comparison of pathways obtained from differentially expressed mRNAs (blue), miRNAs target genes (yellow), and lncRNA target genes (green) identified 54 common pathways. These pathways indicate that the identified genes are involved in various processes related to neurofunction, inflammation, cell-cell adhesion, cell signaling, metabolism, and other cellular processes. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

### 3.2. EC modulates the expression of protein-coding and non-coding genes in the hippocampus of HFD-fed mice.

A comparison of gene expression profiles in the hippocampus of mice fed the control diet, HFD, and HFD supplemented with 20 mg EC/kg body weight, showed a distinct separation among the three groups, as evaluated by PLS-DA **(Figure 3A)**. Gene expression analysis also showed that EC supplementation caused significant changes in hippocampal gene expression in mice fed the HFD (HFE vs. HF groups). There were 5085 probes identified as differentially expressed, and of these, 3927 probes were identified as upregulated and 1158 as downregulated **(Figure 3B)**. Among them, 2635 genes were identified and classified by ShinyGO v0.66, 38% corresponded to protein-coding genes (1001 mRNAs), 9% to miRNAs (241 miRNAs), and 6% to lncRNAs (167 lncRNAs) **(Figure 3C)**.

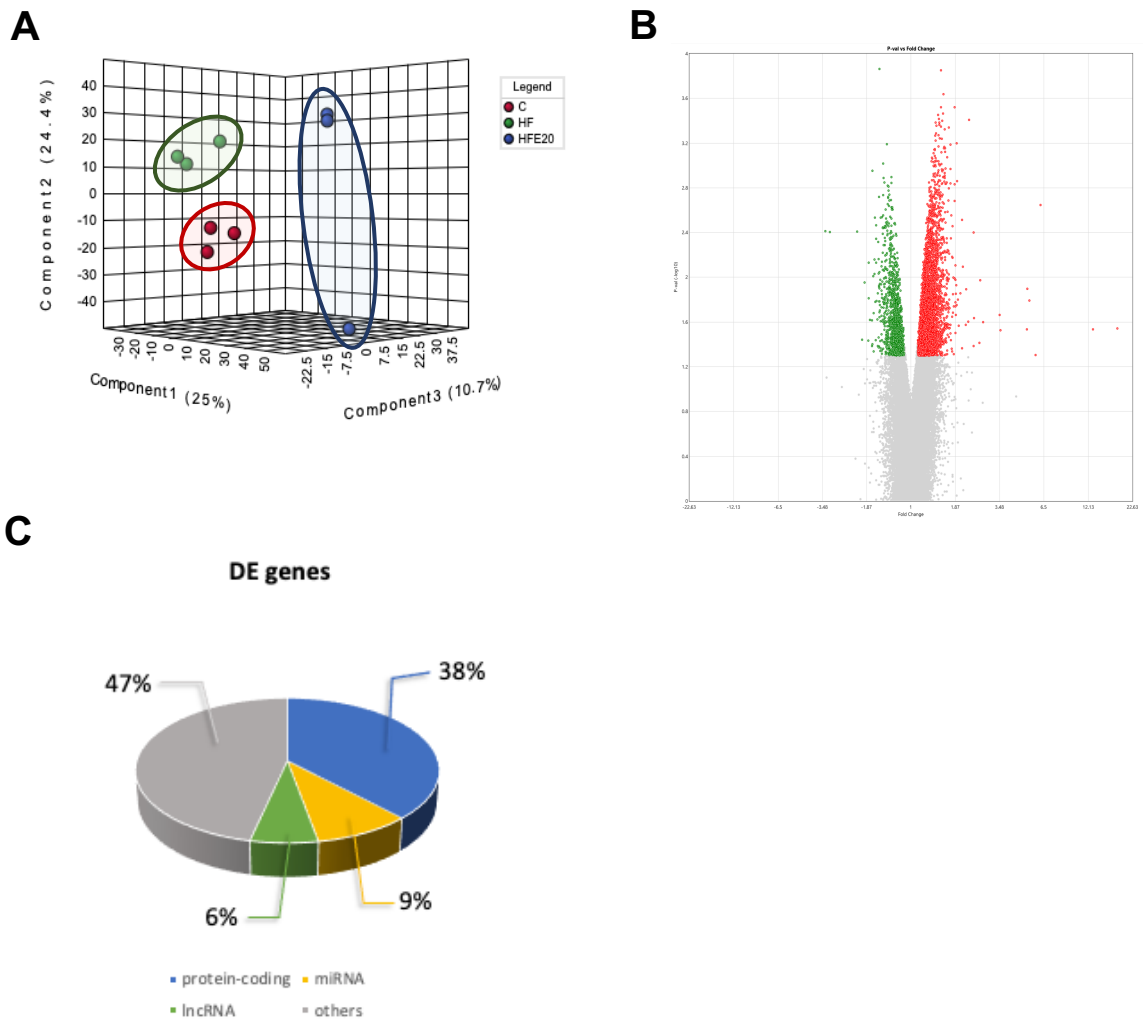



**Figure 3. Global genomic modifications induced by EC supplementation in the hippocampus of HFD-fed mice.** A) 3D Partial least squares discriminant analysis (PLS-DA) plot of the genomic profiles obtained in samples from 3 experimental groups, control, HFD, and HFD supplemented with (20 mg EC/kg body weight), showing a distinct group separation. B) Volcano plot representing the distribution of differentially expressed genes, mapping the up- (red) and down-regulated (green) genes in the hippocampus of EC supplemented HFD-fed mice when compared to the genes obtained from the hippocampus of HFD-fed mice. C) Pie chart showing the distribution of different types of RNAs that are identified as differentially expressed in EC supplemented mice compared to the non-supplemented mice.

*3.2.1 EC modulates the expression of protein-coding genes in the hippocampus of HFD-fed mice.*

Gene expression analysis showed that EC supplementation caused significant changes in expressions of 1001 hippocampal protein-coding genes. The fold-change values of protein-coding genes varied from -1.57 to 18.27 with an average fold-change of -1.23 for the downregulated genes and 1.33 for the upregulated genes. Functionality analysis revealed that the differentially expressed mRNAs mainly regulate processes involved in neurofunction (alcoholism, Alzheimer's disease, amyotrophic lateral sclerosis, neuroactive ligand-receptor interaction, neurodegeneration), inflammation (chemokine signaling pathway, complement and coagulation cascade, cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway), cell-cell adhesion (focal adhesion-PI3K-Akt-mTOR signaling pathway), cell signaling (foxO signaling pathway), and other cellular processes (non-odorant GPCRs, phagosome) **(Figure 4)**. Genes involved in the pathways include *Calm5, Ccl2, Ccr4, Drd1, Gabrr1, Gngt2, Irs1, Kras, Slc2a4, Stat1,* and *Psma2.*

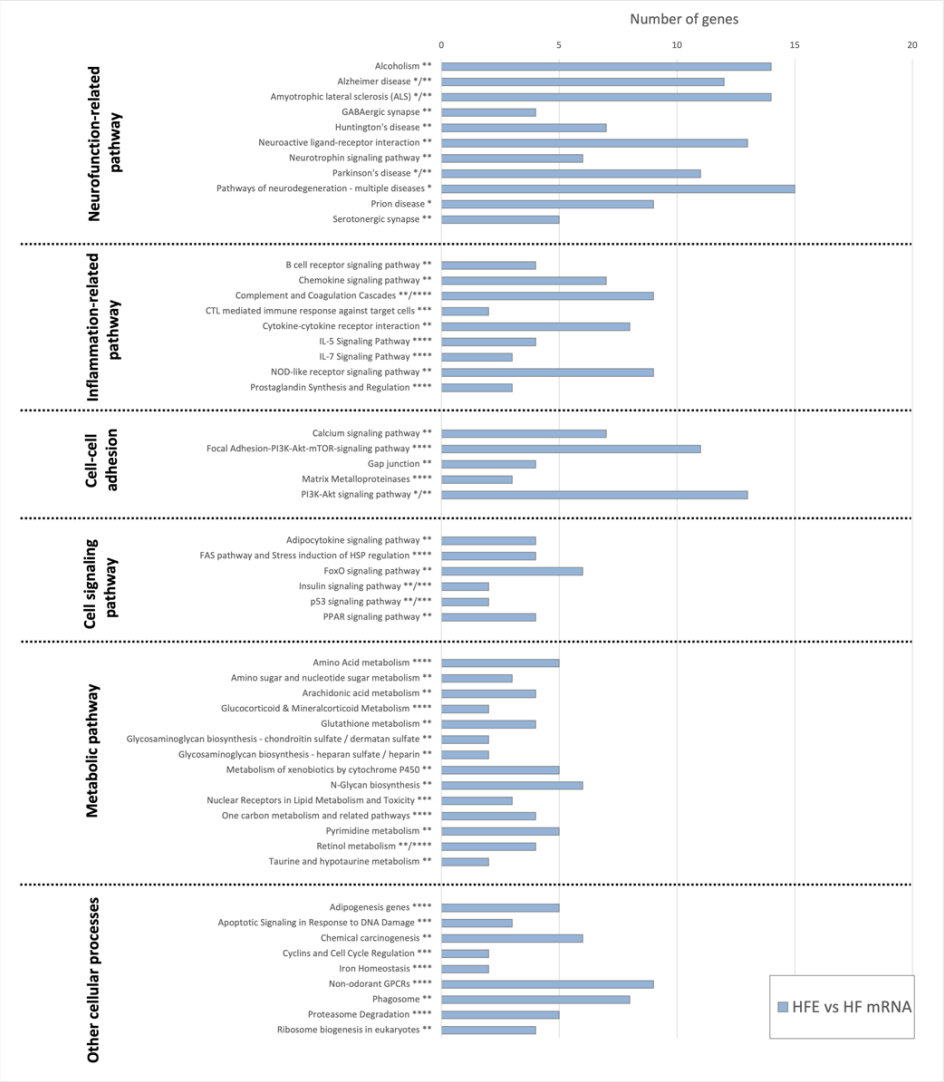
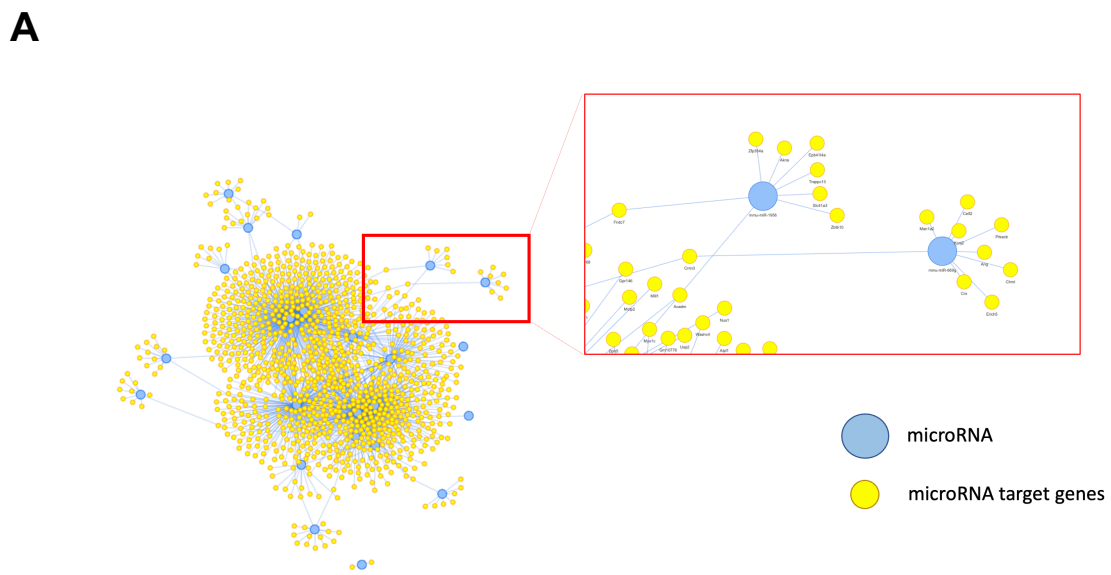


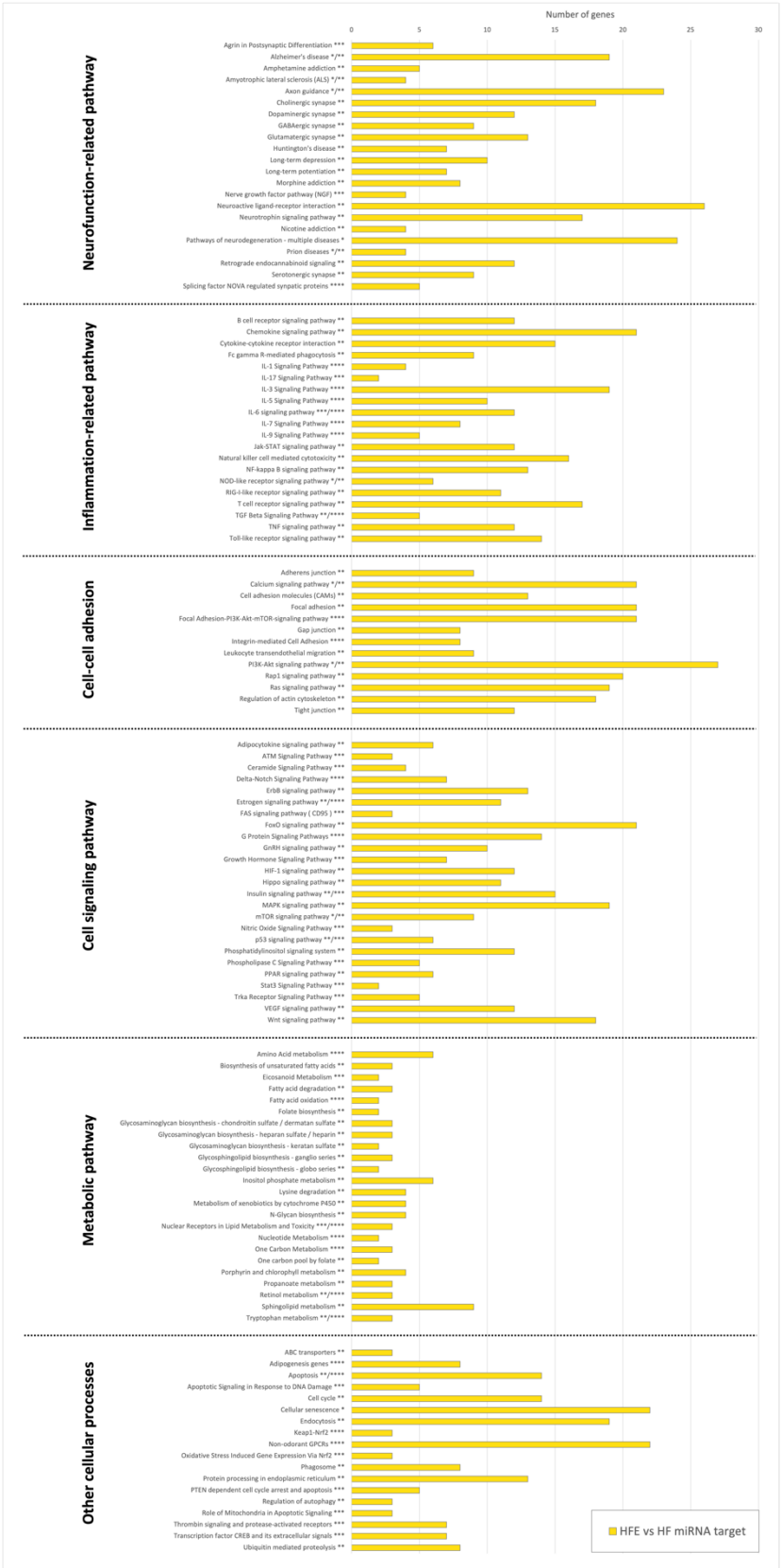

**Figure 4. Histogram of pathway subsets identified from differentially expressed protein-coding genes in the hippocampus of HFD-fed mice supplemented with EC.** Pathways were identified using differentially expressed genes in the hippocampus from the EC supplemented mice compared to the hippocampus from HFD-fed mice. Functional pathway analysis revealed that the differentially expressed mRNAs mainly regulate processes involved in neurofunction, inflammation, cell-cell adhesion, cell signaling, and other cellular processes. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

*3.2.2 EC modulates the expression of miRNAs in the hippocampus of HFD-fed mice.*

Modulation of hippocampal miRNA expression was also identified upon EC supplementation. A total of 241 miRNA were differentially expressed with a fold-change range of -1.49 to 2.41 and an average fold-change of -1.25 for the downregulated genes and 1.38 for the upregulated genes. Next, MIENTURNET and miRWalk database analysis identified a total of 1177 target genes of differentially expressed 30 miRNAs. Networks between differentially expressed miRNAs and their target genes forms a complex interconnectivity **(Figure 5A)**. Pathway enrichment analysis of the identified target genes of differentially expressed miRNAs shows that the target genes are involved in the regulation of various processes including Alzheimer's disease, axon guidance, neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, interleukin-3 signaling, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, calcium signaling, focal adhesion, PI3K-Akt signaling, Rap1 signaling, Ras signaling, foxO signaling, MAPK signaling, Wnt signaling, cellular senescence, endocytosis, and non-odorant GPCRs **(Figure 5B)**. Modulated target genes include *Blc2, Ccr9, Cd4, Cnr1,Cx3cr1, Gabra2, Gsk-3ϐ, Il3, Insr, L1cam, Mapk1, Mapk11, Mylk, Nr3c1, Plcϐ1, Rac1, Stat1,* and *Tacr1.*



**B**


Number of genes
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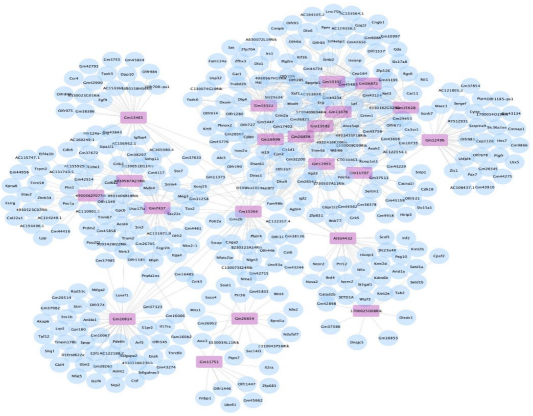
5
10
15
20
25
30
Neurofunction-related pathway
Agrin in Postsynaptic Differentiation ***
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Axon guidance */**
Cholinergic synapse **
Dopaminergic synapse **
GABAergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Nerve growth factor pathway (NGF) ***
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Nicotine addiction **
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Serotonergic synapse **
Splicing factor NOVA regulated synpatic proteins ****
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-1 Signaling Pathway ***
IL-17 Signaling Pathway ***
IL-3 Signaling Pathway ****
IL-5 Signaling Pathway ****
IL-6 signaling pathway ***/****
IL-7 Signaling Pathway ****
IL-9 Signaling Pathway ****
Jak-STAT signaling pathway **
Natural killer cell mediated cytotoxicity **
NF-kappa B signaling pathway **
NOD-like receptor signaling pathway */**
RIG-I-like receptor signaling pathway **
T cell receptor signaling pathway **
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Toll-like receptor signaling pathway **
Cell-cell adhesion
Adherens junction **
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Gap junction **
Integrin-mediated Cell Adhesion ****
Leukocyte transendothelial migration **
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Adipocytokine signaling pathway **
ATM Signaling Pathway ***
Ceramide Signaling Pathway ***
Delta-Notch Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **/****
FAS signaling pathway ( CD95 ) ***
FoxO signaling pathway **
G Protein Signaling Pathways ****
GnRH signaling pathway **
Growth Hormone Signaling Pathway ***
HIF-1 signaling pathway **
Hippo signaling pathway **
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
p53 signaling pathway **/***
Phosphatidylinositol signaling system **
Phospholipase C Signaling Pathway ***
PPAR signaling pathway **
Stat3 Signaling Pathway ***
Trka Receptor Signaling Pathway ***
VEGF signaling pathway **
Wnt signaling pathway **
Metabolic pathway
Amino Acid metabolism ****
Biosynthesis of unsaturated fatty acids **
Eicosanoid Metabolism ***
Fatty acid degradation **
Fatty acid oxidation ****
Folate biosynthesis **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Glycosphingolipid biosynthesis - globo series **
Inositol phosphate metabolism **
Lysine degradation **
Metabolism of xenobiotics by cytochrome P450 **
N-Glycan biosynthesis **
Nuclear Receptors in Lipid Metabolism and Toxicity ***/****
Nucleotide Metabolism ****
One Carbon Metabolism ****
One carbon pool by folate **
Porphyrin and chlorophyll metabolism **
Propanoate metabolism **
Retinol metabolism **/****
Sphingolipid metabolism **
Tryptophan metabolism **/****
Other cellular processes
ABC transporters **
Adipogenesis genes ****
Apoptosis **/****
Apoptotic Signaling in Response to DNA Damage ***
Cell cycle **
Cellular senescence *
Endocytosis **
Keap1-Nrf2 ****
Non-odorant GPCRs ****
Oxidative Stress Induced Gene Expression Via Nrf2 ***
Phagosome **
Protein processing in endoplasmic reticulum **
PTEN dependent cell cycle arrest and apoptosis ***
Regulation of autophagy **
Role of Mitochondria in Apoptotic Signaling ***
Thrombin signaling and protease-activated receptors ***
Transcription factor CREB and its extracellular signals ***
Ubiquitin mediated proteolysis **
HFE vs HF miRNA target

**Figure 5. Modulation of hippocampal miRNA expression in the hippocampus of HFD-fed mice upon EC supplementation.** A) Network presentation of differentially expressed miRNAs (blue circles) and their potential target genes (yellow circles). B) Histogram of pathway subsets identified with miRNA target genes. The target genes are involved in the regulation of various processes such as Alzheimer's disease, axon guidance, neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, interleukin-3 signaling, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, calcium signaling, focal adhesion, PI3K-Akt signaling, Rap1 signaling, Ras signaling, foxO signaling, MAPK signaling, Wnt signaling, cellular senescence, endocytosis, and non-odorant GPCRs. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

*3.2.3 EC modulates expression of long non-coding RNAs in mouse hippocampus*

In addition, EC supplementation of HFD-fed mice caused changes in expression of 167 lncRNAs. The fold-change values of lncRNA non-coding genes varied from -1.60 to 2.06 with an average fold-change of -1.27 for the downregulated genes and 1.26 for the upregulated genes. LncRRIsearch database analysis retrieved 1481 lncRNA target genes from the identified differentially expressed lncRNAs. The differentially expressed lncRNAs and their target genes together forms a complex network **(Figure 6A)**. Pathway enrichment analysis of the target genes reveals that the most over-represented pathways include neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, cytokine-cytokine receptor interaction, calcium signaling, focal adhesion, PI3K-Akt signaling, regulation of actin cytoskeleton, cAMP signaling, MAPK signaling, and non-odorant GPCRs **(Figure 6B)**. The target genes identified for these pathways include Atp2b2, Bace1, Cacna1c, *Ccr4, Ccr5,* Creb5, *Cx3cr1, Grin2a Gsk-3β, Lpl, Igfr1, Mapk10, Mylk4, Nos1*, Ryr3, *Tacr1, Taok2, Taok3,* and *Tnr.*

**A**



**B**

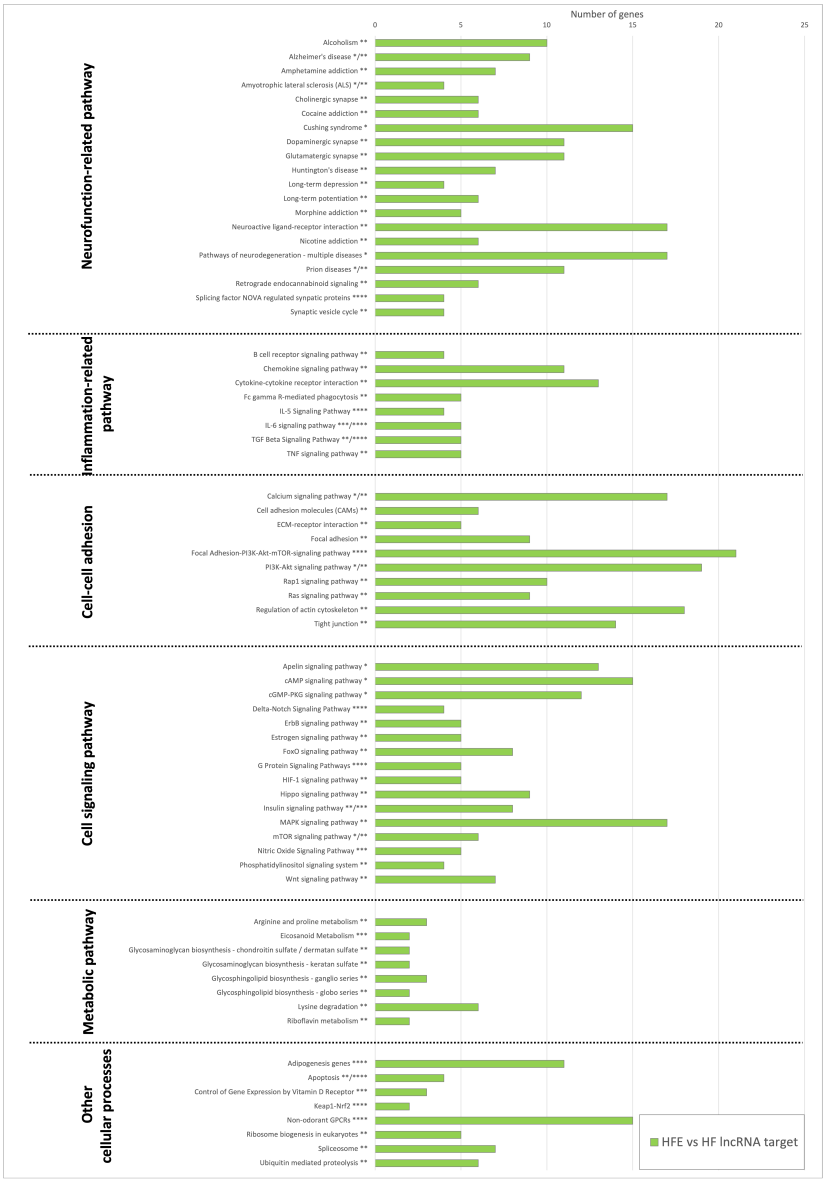

Number of genes
0
5
10
15
20
25
Neurofunction-related pathway
Alcoholism **
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Neuroactive ligand-receptor interaction **
Nicotine addiction **
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Splicing factor NOVA regulated synaptic proteins ****
Synaptic vesicle cycle **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-5 Signaling Pathway ****
IL-6 signaling pathway ***/****
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Cell-cell adhesion
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Apelin signaling pathway *
cAMP signaling pathway *
cGMP-PKG signaling pathway *
Delta-Notch Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **
FoxO signaling pathway **
G Protein Signaling Pathways ****
HIF-1 signaling pathway **
Hippo signaling pathway **
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
Phosphatidylinositol signaling system **
Wnt signaling pathway **
Metabolic pathway
Arginine and proline metabolism **
Eicosanoid Metabolism ***
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Glycosphingolipid biosynthesis - globo series **
Lysine degradation **
Riboflavin metabolism **
Other cellular processes
Adipogenesis genes ****
Apoptosis **/****
Control of Gene Expression by Vitamin D Receptor ***
Keap1-Nrf2 ****
Non-odorant GPCRs ****
Ribosome biogenesis in eukaryotes **
Spliceosome **
Ubiquitin mediated proteolysis **
HFE vs HF lncRNA target

**Figure 6. Modulation of hippocampal lncRNA expression in the hippocampus of HFD-fed mice upon EC supplementation.** A) Network presentation of differentially expressed lncRNA (purple rectangles) and their potential target genes (blue circles). B) Histogram of pathway subsets identified with lncRNA target genes. The target genes are involved in processes including neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, cytokine-cytokine receptor interaction, calcium signaling, focal adhesion, PI3K-Akt signaling, regulation of actin cytoskeleton, cAMP signaling, MAPK signaling, and non-odorant GPCRs. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

*3.2.4 Transcription factors affected by EC and their interactions with EC metabolites*

Following identification of differentially expressed protein-coding genes, we used bioinformatic tools to identify potential transcription factors, which could be involved in the regulation of our identified genes and therefore could be affected by EC **(Figure 7A)**. Seven significant transcription factors were identified, NR5A2, RBPJ, GATA4, RARα, FLI1, HNF4α and SREBF1. Our next step was to assess if major circulating EC metabolites could interact with these transcription factors and modify their activities, by performing in-silico 3d docking analysis between the proteins and the metabolites. Using such approach, we observed that (−)-epicatechin-7-*O*-β-D-glucuronide (E7G) presents a high potential binding capacity to RARα transcription factor (-10.1 kcal/mol) **(Figure 7B)**. We also observed that (−)-epicatechin-3'-sulfate (E3'S) also presents binding capacity to RARα (-8.8 kcal/mol). Moreover, the docking analyses revealed that E3'S and (−)-epicatechin-5-*O*-β-D-glucuronide (E5G) could bind to HNF4α transcription factor, with binding capacity of -7.9 kcal/mol and -9.3 kcal/mol, respectively **(Figure 7B)**. Taken together, these bioinformatic analyses allowed identification of potential transcription factors involved in the genomic modifications induced by EC in the hippocampus by interacting with the major EC metabolites found in mice.

**A**

| Transcription factor | Name |
|---|---|
| NR5A2 | Nuclear receptor subfamily 5, group A, member 2 |
| RBPJ | Recombination signal binding protein for immunoglobulin kappa J region |
| GATA4 | GATA binding protein 4 |
| RARα | Retinoic acid receptor, alpha |
| FLI1 | Friend leukemia integration 1 |
| HNF4α | Hepatic nuclear factor 4, alpha |
| SREBF1 | Sterol regulatory element binding transcription factor 1 |
| NKX2-5 | NK2 Homeobox 5 |
| SRF | Serum Response Factor |
| CBEPA | CCAAT Enhancer Binding Protein Alpha |
| TBP | TATA-binding *protein* |
| NFYB | Nuclear Transcription Factor Y Subunit Beta |
| CPEB1 | Cytoplasmic Polyadenylation Element Binding *Protein* 1 |
| SOX2 | SRY-Box Transcription Factor 2 |
| SP1 | specificity *protein* 1 |
| NFKB1 | Nuclear Factor Kappa B |
| RELA | Nuclear Factor NF-Kappa-B P65 Subunit |
| TRP53 | Tumor Protein P53 |

**B**

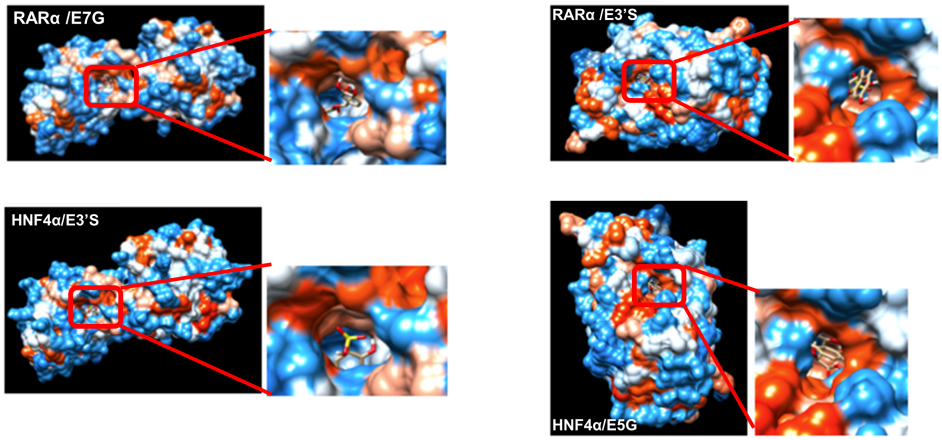



**Figure 7. Transcription factors involved in the observed genomic modifications and their potential interactions with EC metabolites.** A) List of identified potential transcription factors, which could be involved in the regulation of the identified differentially expressed protein-coding genes and therefore could be affected upon EC supplementation. B) In-silico docking analysis of interactions between EC metabolites and transcription factors showing that major circulating EC metabolites found in mice could interact with select identified transcription factors. RARα demonstrated energetically favorable binding to (-)-epicatechin-7-O-glucuronide (E7G) and (-)-epicatechin-3'-sulfate (E3'S), and HNF4α to E3'S and (-)-epicatechin-5-O-β -D-glucuronide (E5G).

*3.2.5 Integration of multi-genomic modifications by EC in the hippocampus*

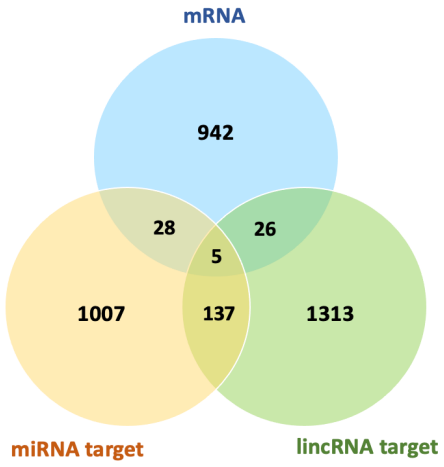
The next step of our analysis was to integrate data obtained from different genomic analysis. Comparison between differentially expressed protein-coding genes, targets of differentially expressed miRNAs and of differentially expressed lncRNAs identified 5 genes in common **(Figure 8A)**. Also, 28 of differentially expressed protein-coding genes were in common with targets of differentially expressed miRNAs and 26 were identified in common with targets of differentially expressed lncRNAs. These observations suggest that expression levels of the mRNA of these genes can be affected by the capacity of EC metabolites to regulate the expression of protein non-coding genes. Because EC could affect the activities of transcription factors which will result in changes in expression of genes and non-coding RNAs that can interact with mRNAs, we aimed to build a network of interactions between identified differentially expressed genes, gene targets, and potential transcription factors **(Figure 8B)**. This analysis allowed us to observe global interactivities between the studied types of RNAs and potential transcription factors, showing complex, multi-omic mode of action of EC in mice hippocampus *in vivo*.

Together with the comparison of identified differentially expressed genes and target genes of non-coding RNAs, we also compared the pathways identified as enriched in the 3 omic analyses. We observed that 3 pathways were in common between protein-coding genes and targets of lncRNAs and 40 pathways in common between targets of miRNAs and of lncRNAs **(Figure 8C)**. The identified pathways were involved in cellular functions including cell signaling pathways, neuronal cell function, and cellular metabolism. Moreover, 18 pathways were identified to be regulated by all three types of RNA which included pathways related to neurodegenerative diseases, like Alzheimer's disease, pathways regulating endothelial cell functions like focal adhesion, or pathways related to inflammation and cell signaling transduction. This suggests that these common pathways can be affected simultaneously by mRNA and targets of miRNAs and lncRNAs. An example presented is a pathway related to neurodegeneration where

we identified 15 differentially expressed genes, 24 targets of miRNAs, and 17 as targets of lncRNAs **(Figure 8D)**.

To further investigate the observed interactions between different omic effects, we also combined pathways identified for each omic analysis and grouped them into functional groups **(Figure 8E)**. We observed that these pathways are involved in the regulation of neurological functions and diseases, inflammation, cell-cell adhesion, cell signaling, and metabolic pathways. This suggests that, by regulating the expression of different types of RNAs, EC can affect pathways in the hippocampus regulating these cellular functions. For each functional pathway group, we identified differentially expressed genes and target genes involved in each pathway with the aim to construct a network. Example networks of neurofunction-related and cell-cell adhesion functional groups show that pathways and involved genes within each functional group are interconnected **(Figure 8E)**. This demonstrates that regulation of different types of RNAs can exert multitudinous effects on cellular functions.

**A**



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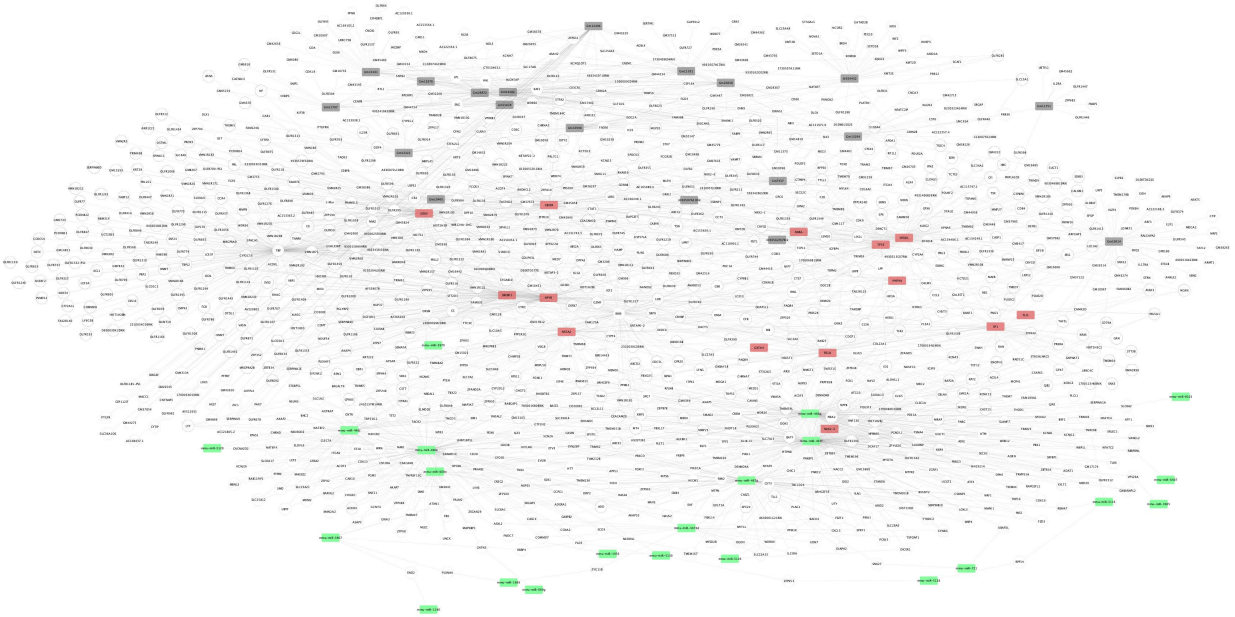

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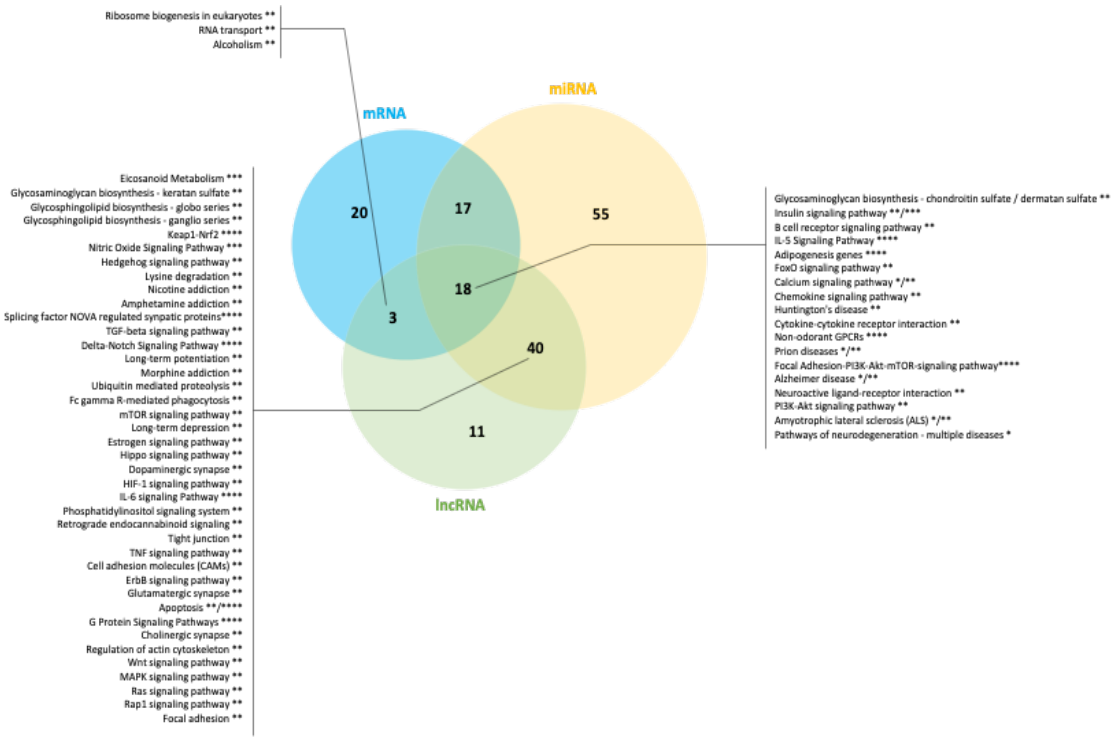
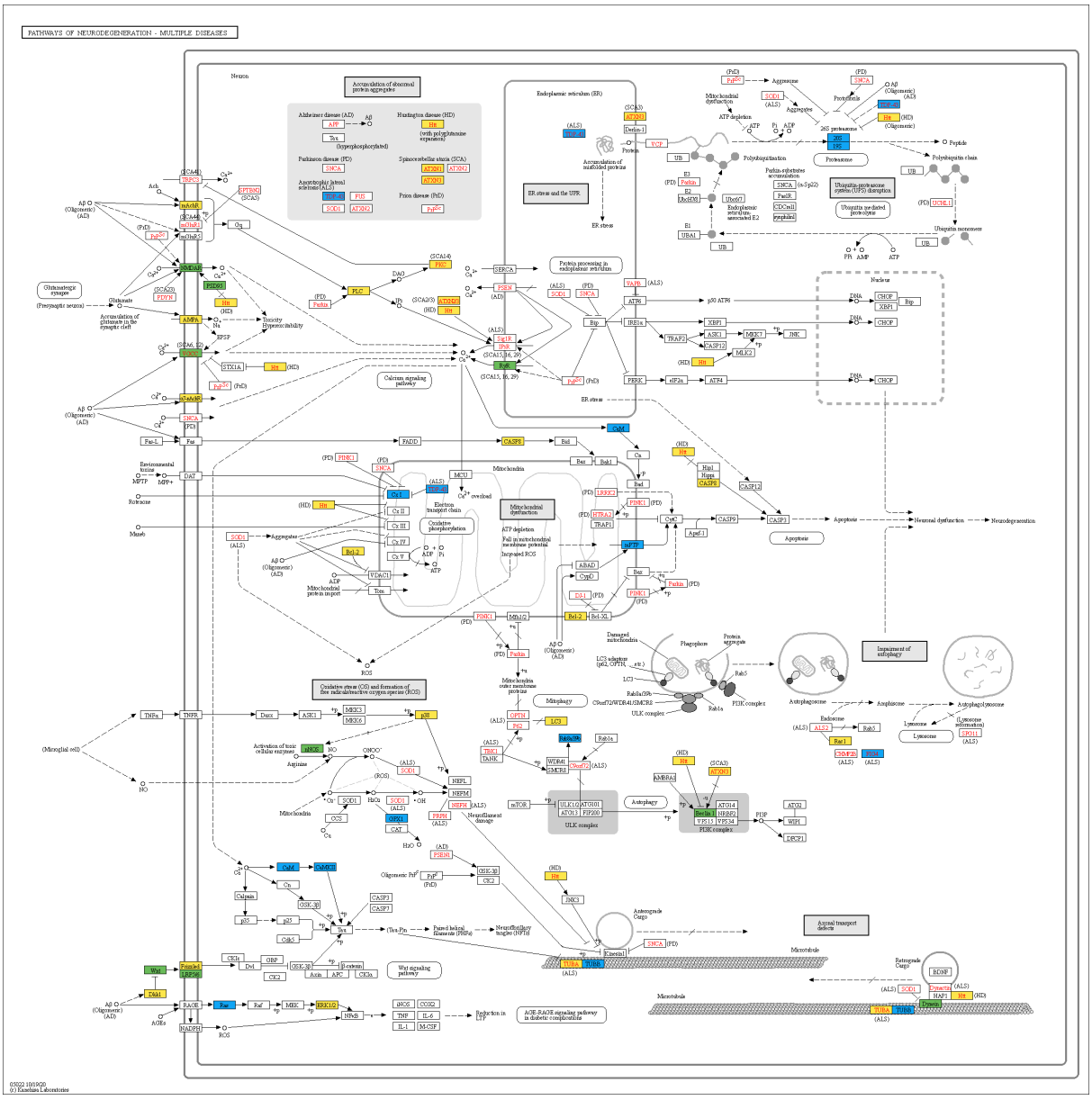
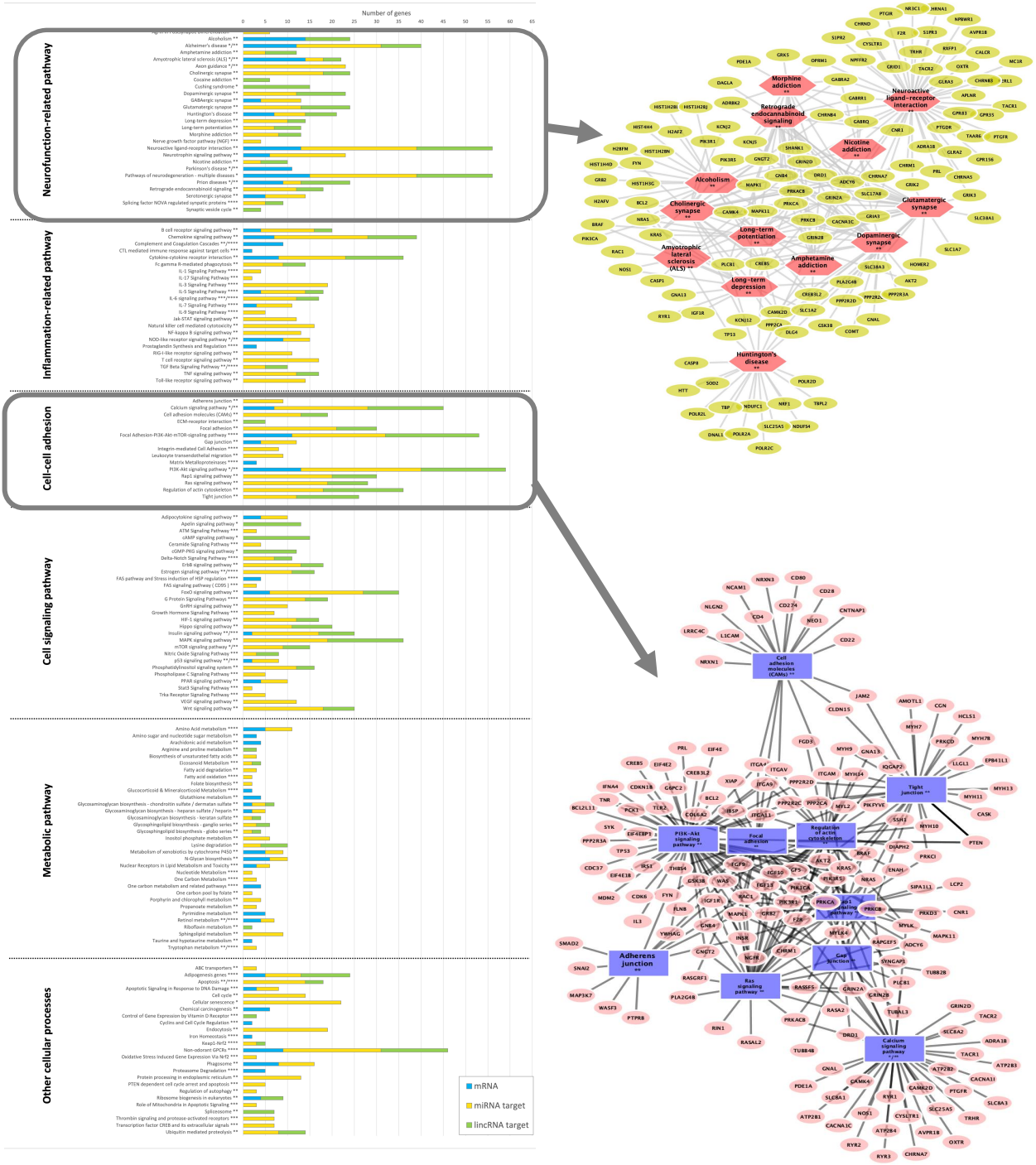

Ribosome biogenesis in eukaryotes **
RNA transport **
Alcoholism **
mRNA
miRNA
lncRNA
20
17
55
3
18
40
11
Eicosanoid Metabolism ***
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - globo series **
Glycosphingolipid biosynthesis - ganglio series **
Keap1-Nrf2 ****
Nitric Oxide Signaling Pathway ***
Hedgehog signaling pathway **
Lysine degradation **
Nicotine addiction **
Amphetamine addiction **
Splicing factor NOVA regulated synpatic proteins****
TGF-beta signaling pathway **
Delta-Notch Signaling Pathway ****
Long-term potentiation **
Morphine addiction **
Ubiquitin mediated proteolysis **
Fc gamma R-mediated phagocytosis **
mTOR signaling pathway **
Long-term depression **
Estrogen signaling pathway **
Hippo signaling pathway **
Dopaminergic synapse **
HIF-1 signaling pathway **
IL-6 signaling Pathway ****
Phosphatidylinositol signaling system **
Retrograde endocannabinoid signaling **
Tight junction **
TNF signaling pathway **
Cell adhesion molecules (CAMs) **
ErbB signaling pathway **
Glutamatergic synapse **
Apoptosis **/****
G Protein Signaling Pathways ****
Cholinergic synapse **
Regulation of actin cytoskeleton **
Wnt signaling pathway **
MAPK signaling pathway **
Ras signaling pathway **
Rap1 signaling pathway **
Focal adhesion **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Insulin signaling pathway **/***
B cell receptor signaling pathway **
IL-5 Signaling Pathway ****
Adipogenesis genes ****
FoxO signaling pathway **
Calcium signaling pathway */**
Chemokine signaling pathway **
Huntington's disease **
Cytokine-cytokine receptor interaction **
Non-odorant GPCRs ****
Prion diseases */**
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway****
Alzheimer disease */**
Neuroactive ligand-receptor interaction **
PI3K-Akt signaling pathway **
Amyotrophic lateral sclerosis (ALS) */**
Pathways of neurodegeneration - multiple diseases *

**D**


PATHWAYS OF NEURODEGENERATION - MULTIPLE DISEASES
Neuron
Endoplasmic reticulum (ER)
Mitochondria
Nucleus
Apoptosis
Autophagy
Lysosome
Microtubule
Calcium signaling pathway
Wnt signaling pathway
ER stress
Oxidative stress (OS) and formation of free radicals/reactive oxygen species (ROS)
Neuronal dysfunction
Neurodegeneration

**E**


Number of genes
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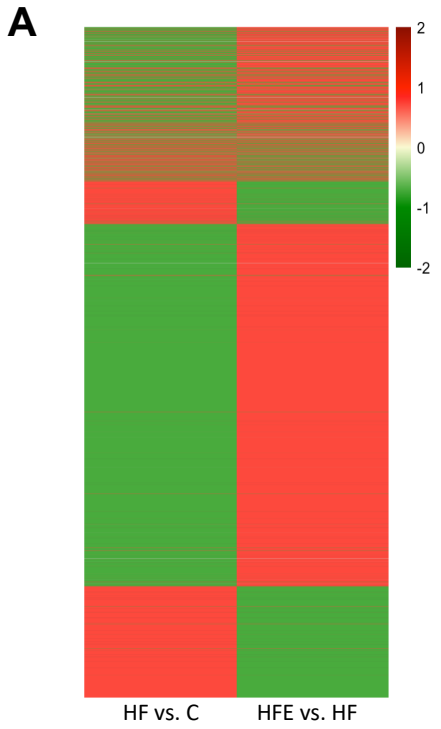
Neurofunction-related pathway
Alcoholism **
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Axon guidance */**
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic synapse **
GABAergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Nerve growth factor pathway (NGF) ***
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Nicotine addiction **
Parkinson's disease */**
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Serotonergic synapse **
Splicing factor NOVA regulated synaptic proteins ****
Synaptic vesicle cycle **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Complement and Coagulation Cascades **/****
CTL-mediated immune response against target cells ***
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-1 Signaling Pathway ****
IL-17 Signaling Pathway ***
IL-3 Signaling Pathway ****
IL-5 Signaling Pathway ****
IL-6 signaling pathway ***/****
IL-7 Signaling Pathway ****
IL-9 Signaling Pathway ****
Jak-STAT signaling pathway **
Natural killer cell mediated cytotoxicity **
NF-kappa B signaling pathway **
NOD-like receptor signaling pathway */**
Prostaglandin Synthesis and Regulation ****
RIG-I-like receptor signaling pathway **
T cell receptor signaling pathway **
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Toll-like receptor signaling pathway **
Cell-cell adhesion
Adherens Junction **
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Gap junction **
Integrin-mediated Cell Adhesion ****
Leukocyte transendothelial migration **
Matrix Metalloproteinases ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Adipocytokine signaling pathway **
Apelin signaling pathway *
ATM Signaling Pathway ***
cAMP signaling pathway *
Ceramide Signaling Pathway ***
cGMP-PKG signaling pathway *
Delta-Notch Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **/****
FAS pathway and Stress induction of HSP regulation ****
FAS signaling pathway ( CD95 ) ***
FoxO signaling pathway **
G Protein Signaling Pathways ****
GnRH signaling pathway **
Growth Hormone Signaling Pathway ***
HIF-1 signaling pathway **
Hippo signaling pathway **
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
p53 signaling pathway **/***
Phosphatidylinositol signaling system **
Phospholipase C Signaling Pathway ***
PPAR signaling pathway **
Stat3 Signaling Pathway ***
Trka Receptor Signaling Pathway ***
VEGF signaling pathway **
Wnt signaling pathway **
Metabolic pathway
Amino Acid metabolism ****
Amino sugar and nucleotide sugar metabolism **
Arachidonic acid metabolism **
Arginine and proline metabolism **
Biosynthesis of unsaturated fatty acids **
Eicosanoid Metabolism ***
Fatty acid degradation **
Fatty acid oxidation ****
Folate biosynthesis **
Glucocorticoid & Mineralcorticoid Metabolism ****
Glutathione metabolism **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Glycosphingolipid biosynthesis - globo series **
Inositol phosphate metabolism **
Lysine degradation **
Metabolism of xenobiotics by cytochrome P450 **
N-Glycan biosynthesis **
Nuclear Receptors in Lipid Metabolism and Toxicity ***
Nucleotide Metabolism ****
One Carbon Metabolism ****
One carbon metabolism and related pathways ****
One carbon pool by folate **
Porphyrin and chlorophyll metabolism **
Propanoate metabolism **
Pyrimidine metabolism **
Retinol metabolism **/****
Riboflavin metabolism **
Sphingolipid metabolism **
Taurine and hypotaurine metabolism **
Tryptophan metabolism **/****
Other cellular processes
ABC transporters **
Adipogenesis genes ****
Apoptosis **/****
Apoptotic Signaling in Response to DNA Damage ***
Cell cycle *
Cellular senescence *
Chemical carcinogenesis **
Control of Gene Expression by Vitamin D Receptor ***
Cyclins and Cell Cycle Regulation **
Endocytosis **
Iron Homeostasis ****
Keap1-Nrf2 ****
Non-odorant GPCRs ****
Oxidative Stress Induced Gene Expression Via Nrf2 **
Phagosome **
Proteasome Degradation ****
Protein processing in endoplasmic reticulum **
PTEN dependent cell cycle arrest and apoptosis ***
Regulation of autophagy **
Ribosome biogenesis in eukaryotes **
Role of Mitochondria in Apoptotic Signaling ***
Spliceosome *
Thrombin signaling and protease-activated receptors ***
Transcription factor CREB and its extracellular signals ***
Ubiquitin mediated proteolysis **
mRNA
miRNA target
lincRNA target
Morphine addiction **
Neuroactive ligand-receptor interaction **
Retrograde endocannabinoid signaling **
Nicotine addiction **
Alcoholism **
Cholinergic synapse **
Glutamatergic synapse **
Long-term potentiation **
Dopaminergic synapse **
Amyotrophic lateral sclerosis (ALS) **
Amphetamine addiction **
Long-term depression **
Huntington's disease **
Cell adhesion molecules (CAMs) **
Tight junction **
PI3K-Akt signaling pathway **
Focal adhesion **
Regulation of actin cytoskeleton **
Adherens junction **
Ras signaling pathway **
Gap junction **
Calcium signaling pathway **

**Figure 8. Integrative analyses of multi-genomic data and networks of gene obtained from the hippocampus of EC supplemented HFD-fed mice.** A) Venn diagram comparison of the differentially expressed mRNA, target genes of miRNA, and target genes of lncRNA identified from comparing EC supplemented HFD-fed mice to the HFD-fed mice. The comparison shows 5 genes were commonly modulated by all three types of RNAs upon EC supplementation. B) Integrative network showing interactions of differentially expressed protein-coding genes, transcription factors, miRNAs and lncRNAs. The network reveals global interactivities between the studied types of RNAs and potential transcription factors. Gray rectangles = lncRNAs; Red rectangles = transcription factors; Green rectangles = miRNAs; White circles = differentially expressed mRNAs, and miRNA and lncRNA targets. C) Venn diagram comparison of common pathways obtained from differentially expressed protein-coding genes, target genes of differentially expressed miRNAs and target genes of differentially expressed lncRNAs. This suggests that these common pathways can be affected simultaneously by mRNA and targets of miRNAs and lncRNAs. D) A representative integrated analysis of differentially expressed genes and target genes of differentially expressed miRNAs and lncRNA indicated in a pathway related to neurodegeneration. Blue = differentially expressed genes; Yellow = target genes of differentially expressed miRNAs, Green = target genes of differentially expressed lncRNAs. E) Integrated histogram of pathway subsets obtained from differentially expressed protein-coding genes, targets of differentially expressed miRNAs and lncRNAs. These pathways are involved in the regulation of neurological functions and diseases, inflammation, cell-cell adhesion, cell signaling, and metabolic pathways. Pathway and gene interaction networks implicated in neurofunction-related and cell-cell adhesion functional group show that pathways and involved genes within each functional group are interconnected. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

### 3.3 Comparison of the effect of HFD (HF vs. C) and EC (HFE vs. HF)

The expression profile of genes identified in the EC supplemented group was compared to the expression profile identified as differentially expressed by the HFD group. The heatmap analysis showed that the expressions of genes obtained following the EC supplementation have the opposite expression profile when compared to the gene expression profile obtained after consumption of the HFD **(Figure 9A)**. This implies that most of the genes identified as upregulated by the HFD were identified as downregulated by the EC consumption and vice versa. This finding suggests that EC consumption can reverse HFD-induced changes of protein-coding and non-coding gene expression profiles in the hippocampus. There was a total of 270 protein-coding genes in common that are differentially expressed by consumption of the HFD and EC **(Figure 9B)**. Expression profiles of the common protein-coding genes also demonstrate that EC consumption can reverse the effect of the HFD-induced changes in the protein-coding gene expressions in the hippocampus. In agreement with the finding, the fold changes of the differentially expressed genes induced by the EC consumption show strong inverse correlation with the ones differentially expressed by consumption of the HFD **(Figure 9C).**



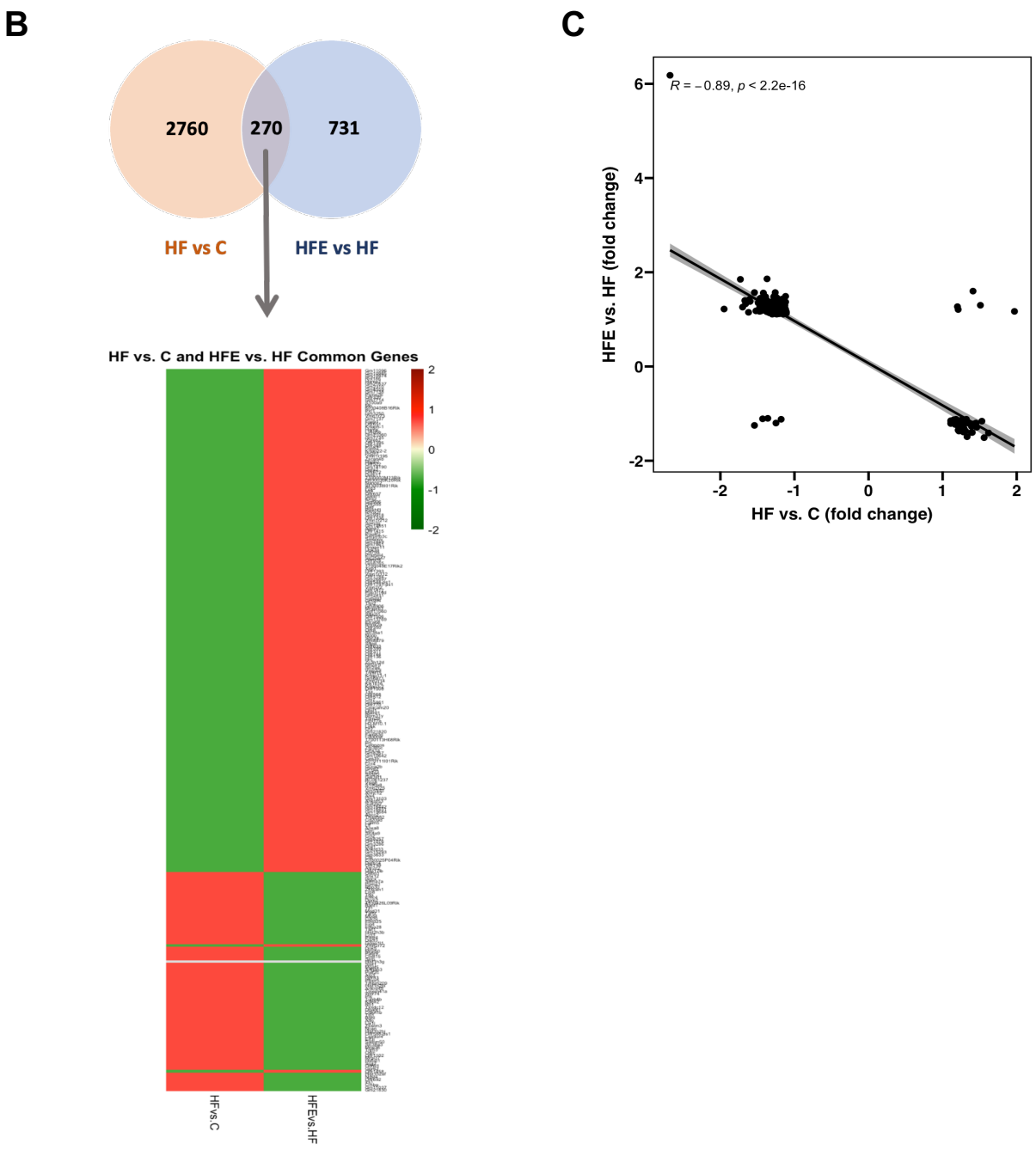
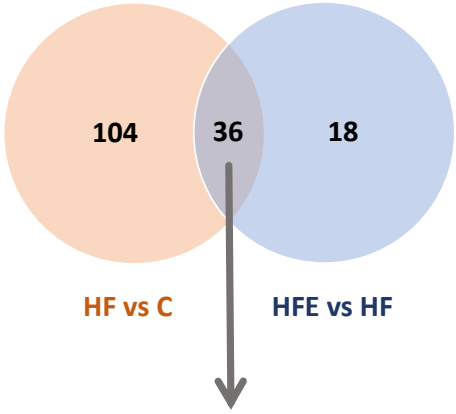



**Figure 9. Comparison of global genomic modifications induced by the HFD and by EC supplementation in the hippocampus.** The expression profile of genes identified in the EC supplemented group was compared to the expression profile identified as differentially expressed by the HFD group. A) Heatmap analysis shows that the expressions of genes obtained following the EC supplementation (HFE vs. HF) have the opposite expression profile when compared to the gene expression profile obtained after

consumption of the HFD (HF vs. C), suggesting EC consumption can reverse HFD-induced changes of protein-coding and non-coding gene expression profiles in the hippocampus. B) Venn diagram shows a total of 270 protein-coding genes in common that are differentially expressed by consumption of the HFD and EC. Expression profiles of the common protein-coding genes demonstrate that EC consumption can reverse the effect of the HFD-induced changes in the protein-coding gene expressions in the hippocampus. C) Correlation plot of differentially expressed protein-coding genes identified from HF vs. C and HFE vs. HF comparisons confirming the significant inverse relationship ($r = -0.89$, $p < 2.2x10^{-16}$).

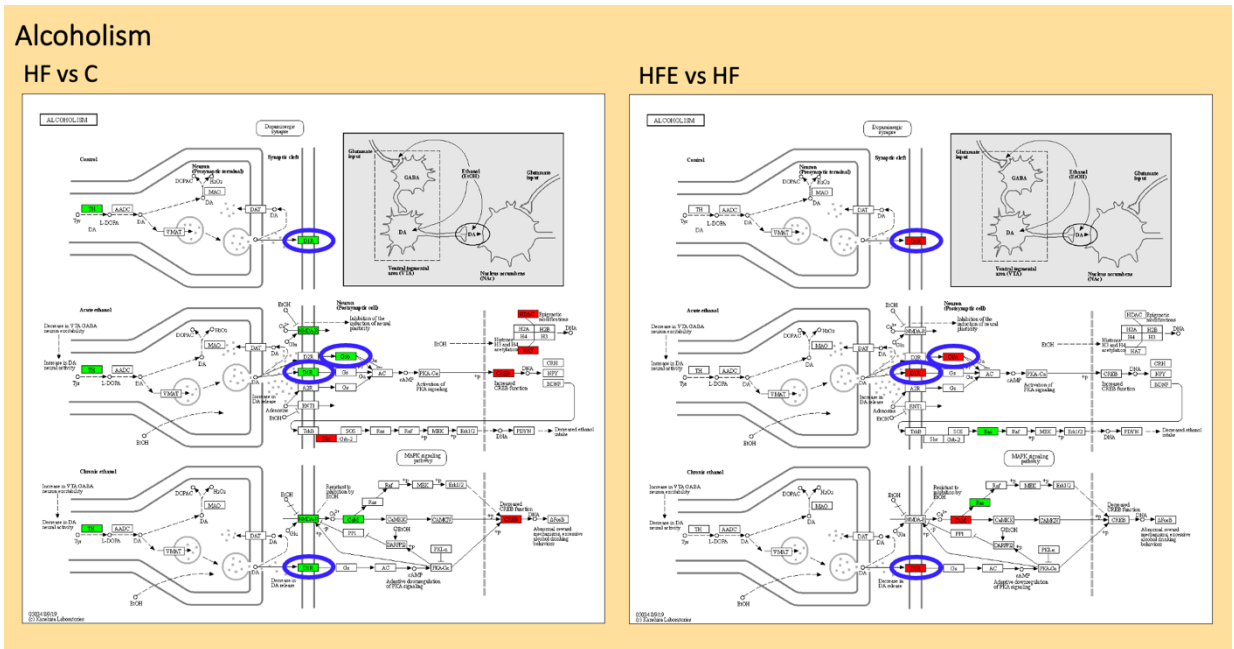
The pathway enrichment analysis indicated that 36 pathways were identified to be commonly modulated by both EC and HFD consumption **(Figure 10A)**. Consistent with the findings observed in the heatmap **(Figure 9A and B)**, expressions of select genes obtained upon EC supplementation show the opposite expression profile when compared to the ones obtained following consumption of the HFD. For example, *Drd1* and *Gngt2* genes implicated in the pathway related to neuronal consequences of alcoholism were downregulated in the hippocampus of mice fed the HFD while their levels were restored by EC supplementation **(Figure 10B)**. Similarly, a couple of genes implicated in the Alzheimer's disease pathway showed opposite expression profiles. *Psme2b* (26S proteasome), *Irs1, and Tubb4b* were upregulated and *Calm5* downregulated in the HFD group while they all show opposite expression profiles upon EC supplementation **(Figure 10C)**. In the insulin signaling pathway, the HFD downregulated the expression of *Slc2a4* and upregulated the expression of *Irs1* in the hippocampus while EC reversed these effects **(Figure 10D)**.

**A**




| | |
|---|---|
| **Alcoholism **** | Adipocytokine signaling pathway ** |
| **Alzheimer's disease */**** | FAS pathway and Stress induction of HSP regulation **** |
| GABAergic synapse ** | FoxO signaling pathway ** |
| Huntington's disease ** | **Insulin signaling pathway **/***** |
| Neuroactive ligand-receptor interaction ** | p53 signaling pathway **/*** |
| Neurotrophin signaling pathway ** | PPAR signaling pathway ** |
| Pathways of neurodegeneration - multiple diseases * | Amino Acid metabolism **** |
| Serotonergic synapse ** | Amino sugar and nucleotide sugar metabolism ** |
| B cell receptor signaling pathway ** | Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate ** |
| Chemokine signaling pathway ** | Glycosaminoglycan biosynthesis - heparan sulfate / heparin ** |
| CTL mediated immune response against target cells *** | One carbon metabolism and related pathways **** |
| Cytokine-cytokine receptor interaction ** | Pyrimidine metabolism ** |
| IL-5 Signaling Pathway **** | Retinol metabolism **/**** |
| IL-7 Signaling Pathway **** | Adipogenesis genes **** |
| NOD-like receptor signaling pathway */** | Apoptotic Signaling in Response to DNA Damage *** |
| Calcium signaling pathway */** | Cyclins and Cell Cycle Regulation *** |
| Focal Adhesion-PI3K-Akt-mTOR-signaling pathway **** | Non-odorant GPCRs **** |
| PI3K-Akt signaling pathway */** | Phagosome ** |

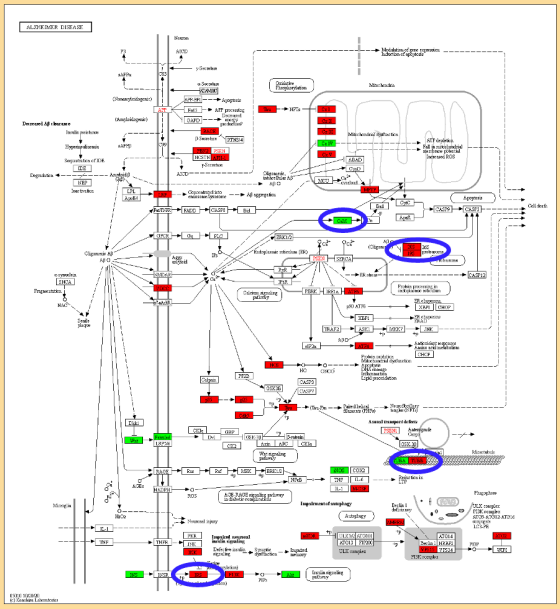
**B**



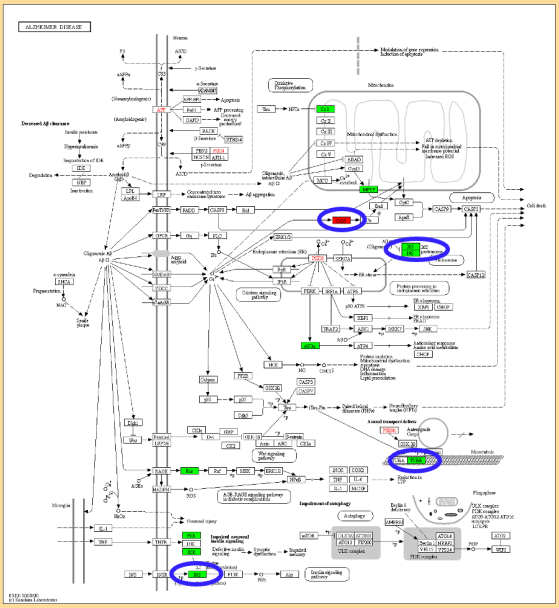
**C**

## Alzheimer Disease

HF vs C



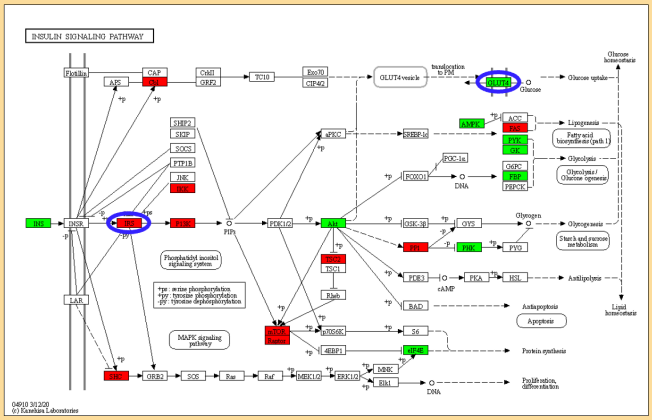
HFE vs HF



**D**

## Insulin Signaling Pathway

HF vs C



HFE vs HF

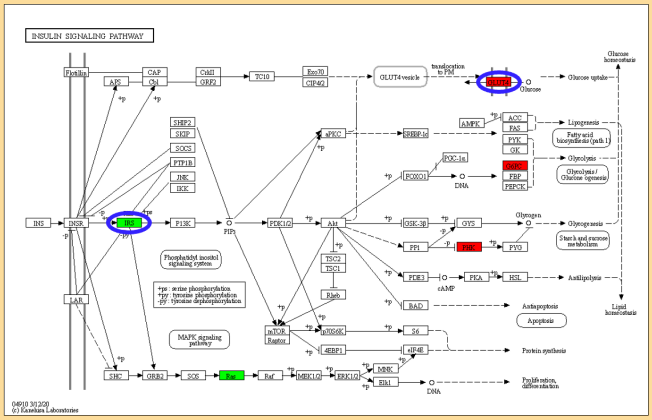


**Figure 10. Comparison of enriched pathways and protein-coding genes modulated by the HFD and by EC supplementation in the hippocampus.** A) Venn diagram and lists of the common enriched pathways identified in HF vs. C and HFE vs. HF comparison. The pathway enrichment analysis indicated that 36 pathways were identified to be commonly modulated by both EC and HFD consumption. *Drd1* and *Gngt2* genes implicated in the pathway related to neuronal consequences of B) alcoholism were downregulated (green) in the hippocampus of HFD-fed mice. In the C) Alzheimer's disease pathway, *Psme2b* (26S proteasome), *Irs1, and Tubb4b* were upregulated (red) and *Calm5* downregulated in the HFD group. In the D) insulin signaling pathway, the HFD downregulated the expression of *Slc2a4* and upregulated the expression of *Irs1.* Their levels were all reversed by EC supplementation.

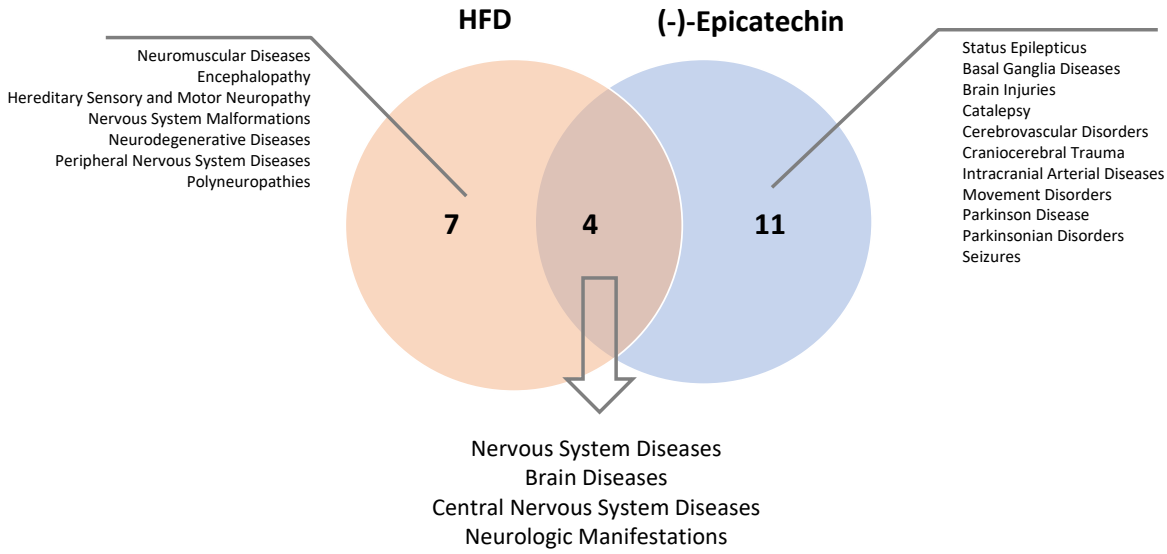
### 3.4. Association of the genomic data with human diseases

Next, we aimed to identify associations of the identified genes in the present study with known human diseases. The Comparative Toxicogenomics Database, a database that interrelates differentially expressed genes with diseases, was used to understand the potential roles of the identified genes in prevention or development of human diseases. Using this approach, we found that genes identified as differentially expressed by the HFD, compared to control diet, are shown to be involved in neurological diseases, such as nervous system malformations, neurodegenerative diseases, and peripheral nervous system diseases. Genes that were identified as modulated by EC on the HFD were also associated with diseases related to neurodegeneration, such as brain injuries, cerebrovascular disorders, intracranial arterial diseases, movement disorders or Parkinson's disease. Comparison of the diseases identified in the 2 groups revealed 4 neurological conditions in common which include nervous system diseases, brain diseases, central nervous system diseases, and neurologic manifestations **(Figure 11A)**.

As cognitive analyses performed on these mice identified anxiolytic effect of EC (under revision), we next aimed to assess how the global gene expression profile in the hippocampus induced by EC is

correlated with anxiety in humans. Correlation analysis of gene expression changes obtained in the hippocampus following EC supplementation and genomic signatures identified in patients with anxiety disorder indicated a significant inverse relationship (r =-0.15; p < 0.05) **(Figure 11B)**. This suggests that EC-induced changes in hippocampal gene expressions may explain its neuroprotective actions against anxiety-related behavior, an observation that is in accordance with our previous findings for the same animal set (under revision).

**A**




**B**

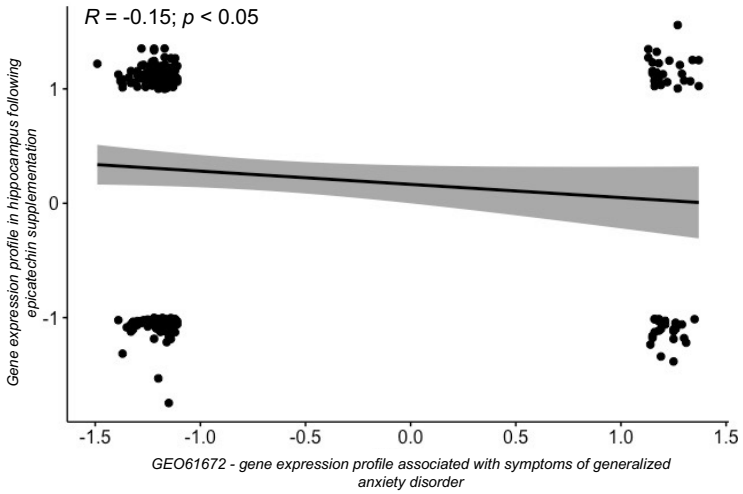


**Figure 11. Association of identified differentially expressed genes with human neurological disorders.** A) Comparison of neurological disorders in humans associated with differentially expressed genes following HFD and following EC diet. Comparison of the diseases identified in the diet groups revealed 4 neurological conditions in common which include nervous system diseases, brain diseases, central nervous system diseases, and neurologic manifestations. B) Correlation analysis between gene expression changes obtained in the hippocampus following EC supplementation and genomic signatures identified in patients with anxiety disorder. The correlation plot indicates a significant inverse relationship ($r = -0.15$; $p < 0.05$), suggesting that EC-induced changes in hippocampal gene expressions may in part explain its neuroprotective actions against anxiety-related behavior.

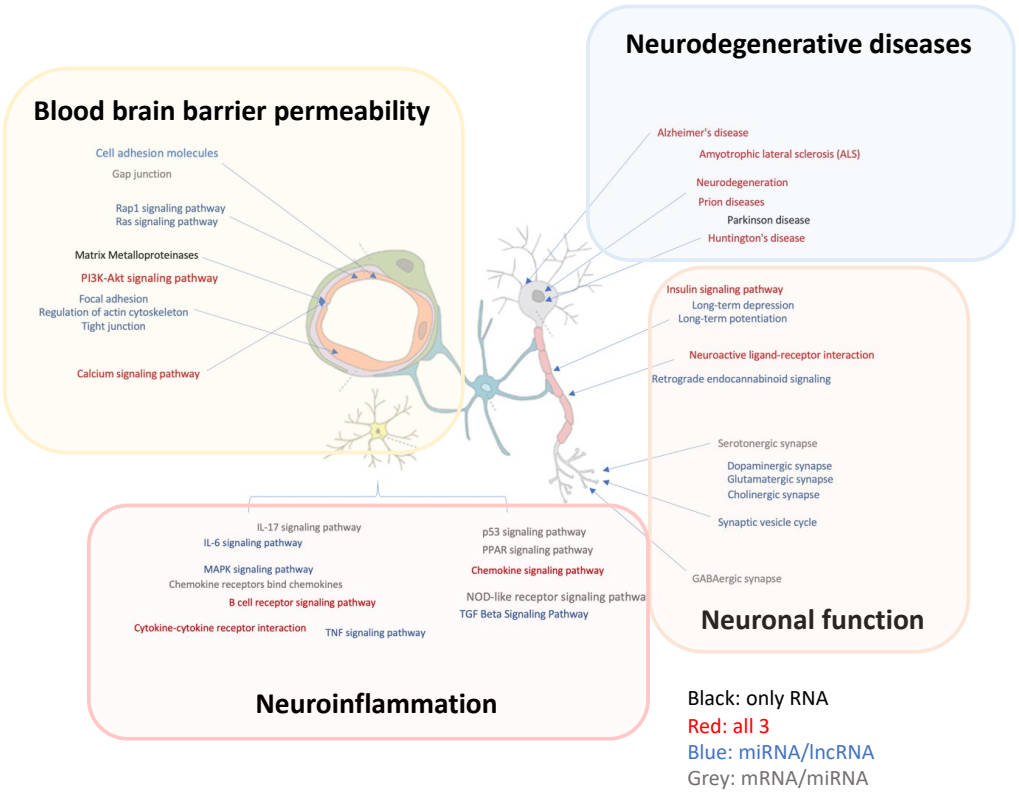



**Figure 12. Summary of functional analysis of multi-genomic data.** EC can modulate the expression of protein-coding and non-coding genes that are involved in various processes such as pathways regulating BBB permeability, neuroinflammation, neurodegeneration, and neuronal function which may in part explain the neuroprotective capacity of EC.

## 4. Discussion

HFD and associated obesity can contribute to cognitive and mood dysfunction [36]. We previously reported that chronic consumption of a HFD induced anxiety-related behavior in mice which was mitigated by EC supplementation (under revision). To better understand the underlying the anti-anxiety mechanisms of EC, the present study conducted multi-genomic analysis that included analyses of mRNAs, miRNA, and lncRNA in the hippocampus of HFD-fed obese mice. Both HFD consumption and EC supplementation induced significant changes in the expression of protein-coding and non-coding genes. For the first time, the capacity of EC to regulate the expression of hippocampal protein-coding and non-coding transcripts in a preclinical model of obesity was investigated. We observed a significant inverse relationship between hippocampal gene expression profiles of HFD-fed and EC-supplemented mice. This suggests that EC can counteract changes driven by consumption of the HFD and/or associated obesity in the hippocampus.

Several studies reported that EC can exert beneficial effects by triggering complex multi-omic modifications. Consumption of a cocoa flavanols drink (containing 64 mg EC) for one month improved vascular function in healthy middle-aged volunteers compared to control subjects [37]. This improvement was accompanied by significant changes in whole blood cell expression profiles of genes involved in the regulation of inflammation, cell adhesion, and chemotaxis of immune cells [38]. Similarly, in a 4-week randomized, double-blind, placebo-controlled crossover trial, EC supplementation (100 mg/day) downregulated groups of genes involved in inflammation, PPAR signaling, and adipogenesis in PBMCs from prehypertensive subjects aged between 30 and 80 [39]. This change in gene expression profiles was accompanied by EC-mediated decrease in plasma insulin and improvement in insulin resistance.

Our pathway enrichment analysis revealed that differentially expressed mRNAs and targets of differently expressed miRNAs and lncRNAs upon EC supplementation are involved in different biological processes regulating neurofunction, inflammation, and cell-cell adhesion, metabolism, and cell signaling

pathways. Interestingly, these transcripts were particularly enriched in the pathways regulating BBB permeability, neurofunction, inflammation, and neurodegeneration in the hippocampus. Recently, it has been shown that a western-type diet given to mice induces not only increased BBB permeability but also decreased cognitive function [40], phenotypic changes found to be associated with significant changes in the expression of protein-coding as well as non-coding RNAs in hippocampal microvasculature of male [41] and female mice [42]. On the other hand, studies have suggested the capacity of EC to mitigate BBB dysfunction and protect from neurodegeneration via multi-omic regulation. For instance, gut microbiome-derived EC metabolites, 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-O-glucuronide, exerted neuroprotective action in TNF-α-stimulated human brain microvascular endothelial cell by modulating the expressions of mRNAs, miRNAs, lncRNAs, and proteins involved in the regulation of cell adhesion, cytoskeleton organization, focal adhesion, and interaction with immune cells [17]. Similarly, in brain microvascular endothelial cells stimulated by lipid stress, a model of BBB dysfunction, structurally related EC metabolites (SREM) and gut microbiome-derived EC metabolites simultaneously modulated the expression of mRNA, miRNA, and lncRNAs involved in VEGF signaling, cell adhesion, and permeability [16]. An inverse correlation was identified between gene expression profiles of the lipid-stressed cells and cells exposed to EC metabolites mixtures, suggesting the capacity of EC to counteract the adverse effects of lipotoxic stress on gene expressions [16].

We also identified 36 common enriched pathways comparing HF vs. C and HFE vs. HF, and several genes involved in pathways modulated by HFD consumption that were inversely modulated by EC supplementation. This supports the capacity of EC supplementation to reverse the effect of HFD on gene expressions. In the pathway related to neuronal consequences of alcoholism, *Drd1* and *Gngt2* are downregulated following the consumption of the HFD while both were upregulated by EC supplementation. *Drd1* encodes the D1 subtype of the dopamine receptor. Interestingly, studies report robust antidepressant-like effects upon D1R activation [43]. For instance, levo-stepholidine (l-SPD), an

antipsychotic medication, has antidepressant effects on rats with depressive- and anxiety-like behaviors. The antidepressant effect was attributed to activation of D1R that leads to activation of downstream PKA/mTOR signaling pathway, which in turn increases synaptic proteins and enhances synaptic plasticity. Similarly, EC-induced restoration of *Drd1* may in part explain the observed anxiolytic effects of EC in HFD-fed mice. Although a direct relevance of *Gngt2*, a member of Gi/o family, in anxiety has not been established, it has been reported that Gi/o levels decrease with aging, particularly in age-vulnerable brain regions like the hippocampus [44]. Similarly, several genes implicated in the Alzheimer's disease pathway showed opposite expression profiles. *Psme2b* (26S proteasome), *Irs1, and Tubb4b* were upregulated and *Calm5* downregulated in the hippocampus of HFD group while they all showed opposite expression profiles upon EC supplementation. It has been shown that the proteosome [45] and IRS1 [46] play important role in the development of Alzheimer's disease. In the insulin signaling pathway, consumption of the HFD downregulated hippocampal gene expression of *Slc2a4,* which encodes GLUT4, an insulin-regulated glucose transporter that plays an important role in glucose uptake and utilization in the brain [47]. Reduced hippocampal GLUT4 expression has been proposed to be responsible for inducing anxiety-like behavior in diabetic rats [48]. The capacity of EC to reverse HFD-induced downregulation of *Slc2a4* also may in part explain its capacity to protect HFD-fed mice from anxiety. Interestingly, the HFD upregulated the expression of *Irs1* in the hippocampus while EC downregulated its expression. It is possible that the observed downregulation is due to the mitigation of HFD-induced increase in plasma insulin levels upon EC supplementation observed in this set of animals (under revision). Consistent with our result, EC-mediated improvements of plasma insulin were observed in HFD-fed mice [49, 50]. Similarly, downregulation of select genes involved in the insulin signaling cascade was found in immune cells of prehypertensive adults upon EC supplementation [39].

In addition to the modulation of protein-coding genes, we observed that EC modulated the expression of non-coding RNAs, including miRNAs and lncRNAs. Studies have shown that several miRNA

are modulated in patients with neurodegenerative diseases suggesting that non-coding RNA also play a role in the pathogenesis of neurodegeneration [18]. Indeed, miRNAs such as miR-15b, miR-127-3p, miR-let-7f-5p, miR-124, miR-219, miR-342-3p, and miR-455-3p are involved in the development of Alzheimer's diseases [18]. Similarly, several miRNAs, including miR-34a, miR-16, miR-34c, miR-182, and miR-124, are associated with the pathogenesis as well as treatment of depression and anxiety [51-55]. For instance, targeted deletion of the miR-34-family resulted in increased resilience to stress-induced anxiety in mice [52]. Similarly, chronic corticosterone administration induced depressive behavior and increased miR-34a levels in the hippocampus while miR-34a downregulation exerted antidepressant-like effects [53]. Among the miRNAs identified as differentially expressed by EC, miR-467 has been reported to promote inflammation and insulin resistance in mice [56]. EC supplementation downregulated miR-467 expression in the hippocampus of HFD-fed mice which may partly explain the beneficial effect of EC. Moreover, miR-669, which plays a protective role in ischemic stroke in mice [57], was increased by EC. EC also modulated lncRNAs expressions. Although the role of many lncRNAs in regulating biological functions are unknown, studies have suggested that lncRNAs are involved in the pathogenesis of Parkinson's disease [58], Alzheimer's [59], and depression [60]. Interestingly, a study reported that expression of the lncRNA Gm15628, which we identified as modulated by EC, was altered in the brain of mice following a stroke [61]. Taken together, our findings suggest that mRNAs, miRNAs and for the first time lncRNAs, may contribute to the neuroprotective properties of EC in the hippocampus.

In-silico docking analysis suggests that certain EC metabolites could bind to some of the identified transcription factors, potentially affecting their activity and the expressions of related genes. Among the identified transcription factors, RARα demonstrated energetically favorable binding to (-)-epicatechin-7-O-glucuronide (E7G) and (-)-epicatechin-3'-sulfate (E3'S), and HNF4α to E3'S and (-)-epicatechin-5-O-β-D-glucuronide (E5G). RARα modulates several pathways crucial for synaptic plasticity [62], which when disrupted increase susceptibility to depression [63]. It has been proposed that RARα has anti-

inflammatory effects and can promote clearance of amyloid beta (Aβ) [64]. HNF4α also plays a critical role in emotional behaviors. A TNFα inhibitor, infliximab, was effective in ameliorating depressive symptoms in patients, especially in those with a high inflammatory condition [65]. Genomic studies proposed that HNF4α anti-depressive effects are linked to the regulation of gluconeogenesis, lipid homeostasis, and serotonin metabolism [66, 67].

In conclusion, our multi-genomic data including transcriptomic, microRNomic, and lncRNomic of the hippocampus of EC supplemented HFD-fed mice revealed that EC can exert neuroprotective effects by regulating pathways that modulate BBB permeability, neurofunction, inflammation, and neurodegeneration in the hippocampus **(Figure 12)**. HFD and associated obesity can be detrimental to the brain, eliciting BBB dysfunction and neuroinflammation, and consequently cognitive impairment and mood disorders. Our results indicate that EC can counteract HFD-induced alterations of hippocampal gene expression profiles. Additionally, we observed that gene expression profiles upon EC supplementation are negatively correlated with gene expression profiles associated with symptoms of anxiety disorder in humans. This suggests that EC could elicit neuroprotective action and reduce the risk of HFD-induced or obesity-associated mood disorders. The present study does not establish a direct causal link between the observed changes in gene expression levels of protein-coding and non-coding genes and alteration in cognitions and mood. However, the bioinformatic analyses provide valuable insights and biological networks possibly affected by consumption of EC. Although the causal relationship was not investigated, protein-coding genes, miRNA, lncRNA, and transcription factors that have been described in neurofunction were identified. Further studies elucidating the direct role of the identified transcripts and proteins in mood and cognition upon EC consumption are warranted.

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**Conflict of Interest**

Authors have no conflict of interest.

**The authors' roles**

J.K. and D.M. performed bioinformatic analyses and wrote the manuscript. J.K., P.O. and D.M. designed the research; had primary responsibility for final content; participated in the interpretation of the data and editing of the manuscript. All authors read and approved the final manuscript.

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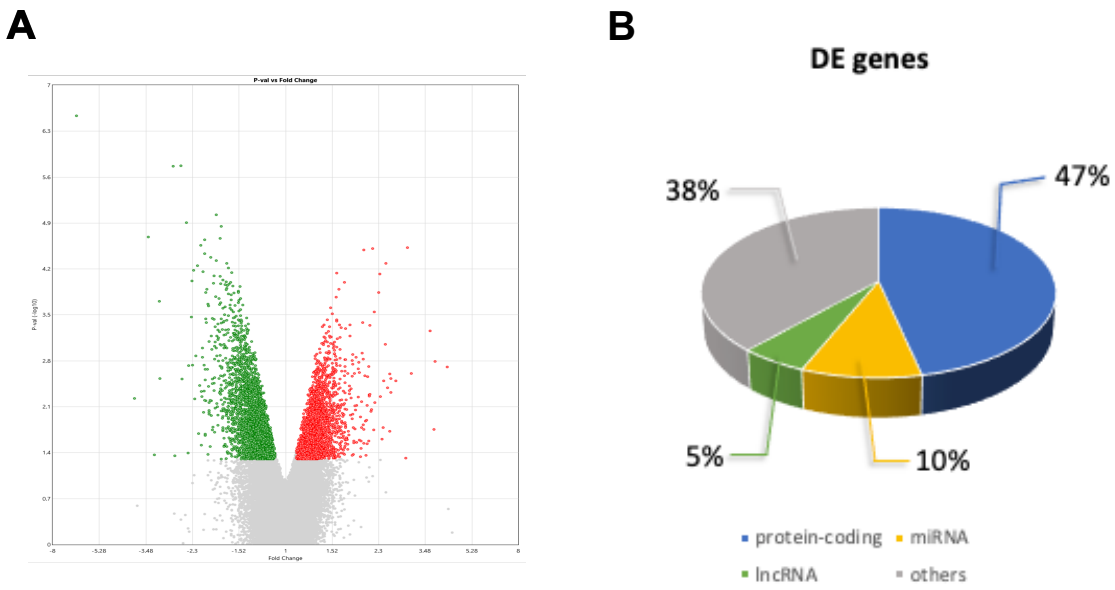
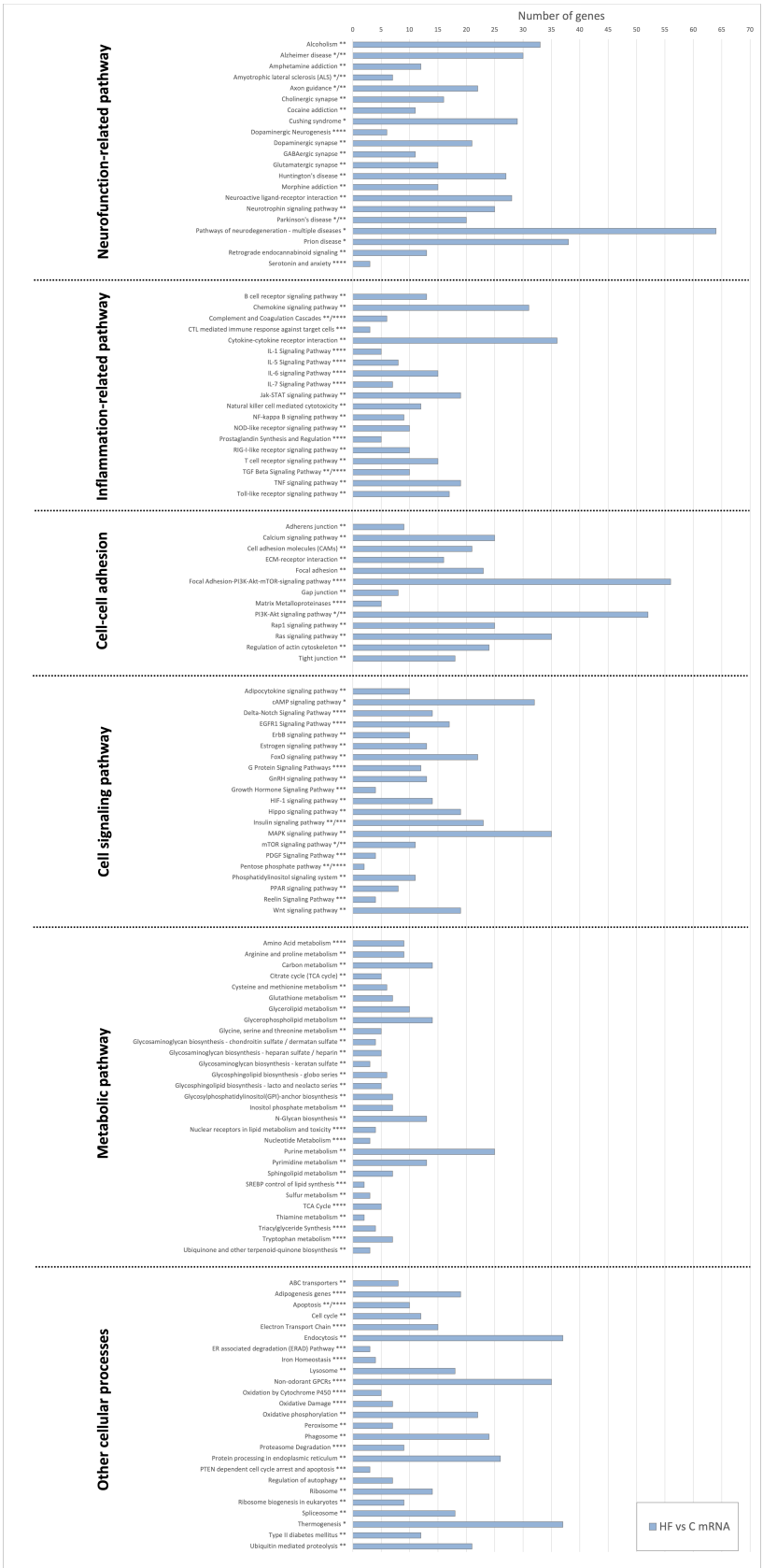
**Supplementary materials**

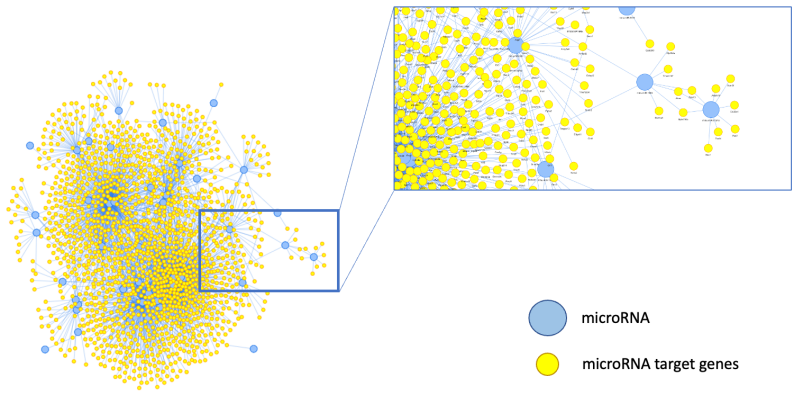
**Supplementary Figure S1. Genomic modifications induced by consumption of the HFD in the hippocampus.** (A) Volcano plot representing the distribution of differentially expressed genes, mapping the up- (red) and down- (green) regulated genes in the hippocampus of HFD-fed mice when compared to those obtained from the hippocampus of control diet-fed mice. (B) Pie chart showing the distribution of different types of RNAs that are identified as differentially expressed. (C) Histogram of a subset of pathways obtained from the differentially expressed protein-coding genes in the hippocampus of HFD-fed mice compared to the control mice. (D) Network of differentially expressed miRNAs (blue circles) and their target genes (yellow circles). Histogram of subset of significant gene pathways of (E) miRNA target genes and (F) lncRNA target genes identified in the hippocampus of HFD-fed mice compared to the ones identified in the control diet-fed mice. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.



**C**


Number of genes
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Neurofunction-related pathway
Alcoholism **
Alzheimer disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Axon guidance */**
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic Neurogenesis ****
Dopaminergic synapse **
GABAergic synapse **
Glutamatergic synapse **
Huntington's disease **
Morphine addiction **
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Parkinson's disease */**
Pathways of neurodegeneration - multiple diseases *
Prion disease *
Retrograde endocannabinoid signaling **
Serotonin and anxiety ****
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Complement and Coagulation Cascades **/****
CTL mediated immune response against target cells ***
Cytokine-cytokine receptor interaction **
IL-1 Signaling Pathway ****
IL-5 Signaling Pathway ****
IL-6 signaling Pathway ****
IL-7 Signaling Pathway ****
Jak-STAT signaling pathway **
Natural killer cell mediated cytotoxicity **
NF-kappa B signaling pathway **
NOD-like receptor signaling pathway **
Prostaglandin Synthesis and Regulation ****
RIG-I-like receptor signaling pathway **
T cell receptor signaling pathway **
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Toll-like receptor signaling pathway **
Cell-cell adhesion
Adherens junction **
Calcium signaling pathway **
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Gap junction **
Matrix Metalloproteinases ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Adipocytokine signaling pathway **
cAMP signaling pathway *
Delta-Notch Signaling Pathway ****
EGFR1 Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **
FoxO signaling pathway **
G Protein Signaling Pathways ****
GnRH signaling pathway **
Growth Hormone Signaling Pathway ***
HIF-1 signaling pathway **
Hippo signaling pathway **
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
PDGF Signaling Pathway ***
Pentose phosphate pathway **/****
Phosphatidylinositol signaling system **
PPAR signaling pathway **
Reelin Signaling Pathway ***
Wnt signaling pathway **
Metabolic pathway
Amino Acid metabolism ****
Arginine and proline metabolism **
Carbon metabolism **
Citrate cycle (TCA cycle) **
Cysteine and methionine metabolism **
Glutathione metabolism **
Glycerolipid metabolism **
Glycerophospholipid metabolism **
Glycine, serine and threonine metabolism **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - globo series **
Glycosphingolipid biosynthesis - lacto and neolacto series **
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis **
Inositol phosphate metabolism **
N-Glycan biosynthesis **
Nuclear receptors in lipid metabolism and toxicity ***
Nucleotide Metabolism ****
Purine metabolism **
Pyrimidine metabolism **
Sphingolipid metabolism **
SREBP control of lipid synthesis ***
Sulfur metabolism **
TCA Cycle ****
Thiamine metabolism **
Triacylglyceride Synthesis ****
Tryptophan metabolism ****
Ubiquinone and other terpenoid-quinone biosynthesis **
Other cellular processes
ABC transporters **
Adipogenesis genes ****
Apoptosis **/****
Cell cycle **
Electron Transport Chain ***
Endocytosis **
ER associated degradation (ERAD) Pathway ***
Iron Homeostasis ****
Lysosome **
Non-odorant GPCRs ****
Oxidation by Cytochrome P450 ***
Oxidative Damage ****
Oxidative phosphorylation **
Peroxisome **
Phagosome **
Proteasome Degradation ****
Protein processing in endoplasmic reticulum **
PTEN dependent cell cycle arrest and apoptosis ***
Regulation of autophagy **
Ribosome **
Ribosome biogenesis in eukaryotes **
Spliceosome **
Thermogenesis *
Type II diabetes mellitus **
Ubiquitin mediated proteolysis **
HF vs C mRNA

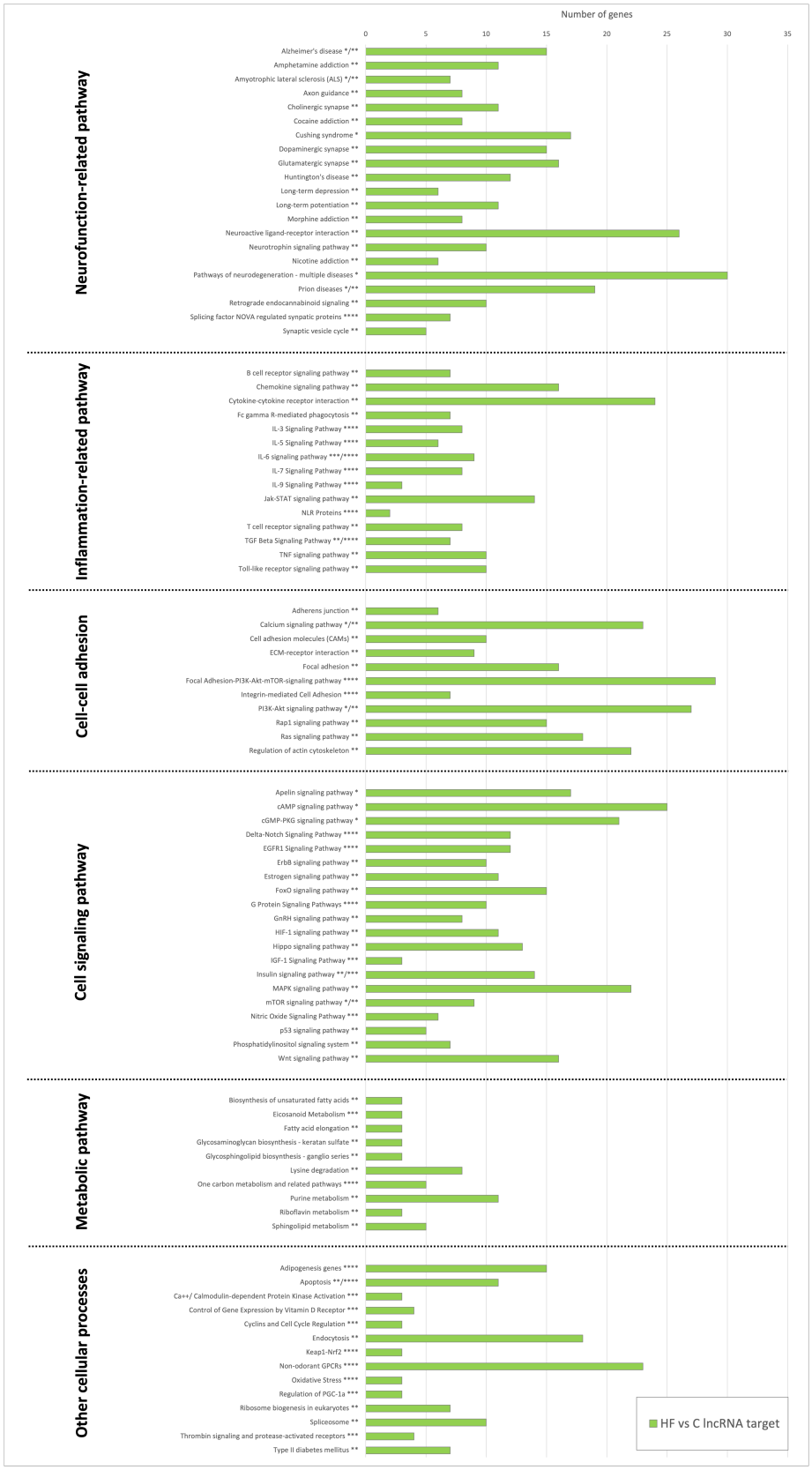
**D**


microRNA
microRNA target genes

**E**


Number of genes
Neurofunction-related pathway
Inflammation-related pathway
Cell-cell adhesion
Cell signaling pathway
Metabolic pathway
Other cellular processes
HF vs C miRNA target

**F**


Number of genes
0
5
10
15
20
25
30
35
Neurofunction-related pathway
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Axon guidance **
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Nicotine addiction **
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Splicing factor NOVA regulated synpatic proteins ****
Synaptic vesicle cycle **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-3 Signaling Pathway ****
IL-5 Signaling Pathway ****
IL-6 signaling pathway ***/****
IL-7 Signaling Pathway ****
IL-9 Signaling Pathway ****
Jak-STAT signaling pathway **
NLR Proteins ****
T cell receptor signaling pathway **
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Toll-like receptor signaling pathway **
Cell-cell adhesion
Adherens junction **
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Integrin-mediated Cell Adhesion ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Cell signaling pathway
Apelin signaling pathway *
cAMP signaling pathway *
cGMP-PKG signaling pathway *
Delta-Notch Signaling Pathway ****
EGFR1 Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **
FoxO signaling pathway **
G Protein Signaling Pathways ****
GnRH signaling pathway **
HIF-1 signaling pathway **
Hippo signaling pathway **
IGF-1 Signaling Pathway ***
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
p53 signaling pathway **
Phosphatidylinositol signaling system **
Wnt signaling pathway **
Metabolic pathway
Biosynthesis of unsaturated fatty acids **
Eicosanoid Metabolism ***
Fatty acid elongation **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Lysine degradation **
One carbon metabolism and related pathways ****
Purine metabolism **
Riboflavin metabolism **
Sphingolipid metabolism **
Other cellular processes
Adipogenesis genes ****
Apoptosis **/****
Ca++/ Calmodulin-dependent Protein Kinase Activation ***
Control of Gene Expression by Vitamin D Receptor ***
Cyclins and Cell Cycle Regulation ***
Endocytosis **
Keap1-Nrf2 ****
Non-odorant GPCRs ****
Oxidative Stress ****
Regulation of PGC-1a ***
Ribosome biogenesis in eukaryotes **
Spliceosome **
Thrombin signaling and protease-activated receptors ***
Type II diabetes mellitus **
HF vs C lncRNA target

# Chapter 5

# Discussion

This dissertation project investigated the neuroprotective potential of EC, particularly its capacity to mitigate HFD-/obesity-associated alterations in cognition and mood using preclinical models of obesity. The specific objectives of this thesis were to 1-assess the effects of EC on memory and learning and its capacity to mitigate neuroinflammation in the hippocampus of HFD-fed obese mice, 2- assess long-term effects (24 weeks) of EC on HFD-induced alterations in memory and mood and gut-microbiota in obese mice, and 3- investigate the underlying mechanisms of EC in the hippocampus using a multi-genomic and bioinformatic approach.

The flavan-3-ol EC was previously shown to have anti-inflammatory actions in various tissues/organs [1-5]. As obesity is characterized by a condition of low-grade chronic inflammation that can potentially lead to neuroinflammation and alterations in cognition [6-8], we first investigated the capacity of dietary EC (20 mg/kg body weight) to mitigate hippocampal inflammation and impaired learning and memory in HFD-fed (60% kcal from fat) mice. After 13 weeks on the dietary intervention, HFD-fed mice developed obesity, which was not affected by EC supplementation. On the other hand, EC supplementation significantly improved short-term recognition memory in HFD-fed mice while neither HFD consumption nor EC supplementation affected mouse spatial memory and learning. The HFD consumption caused metabolic endotoxemia and increases in parameters of inflammation in the hippocampus, which were all mitigated by EC supplementation. There was a strong positive correlation between plasma endotoxin concentration and hippocampal TLR4 mRNA levels, suggesting that the hippocampal inflammation induced by the HFD consumption can be, in part, explained by increased activation of LPS-mediated TLR4 signaling pathway while this was attenuated by EC supplementation. Moreover, BDNF mRNA levels were significantly increased in the EC supplemented groups compared to the groups without the supplementation, suggesting the capacity of EC to exert neuroprotective actions in part by upregulating this neurotrophic factor. This finding is in agreement with other previous report on EC regulating BDNF [9].

Although HFD consumption for 13 weeks impaired short-term object recognition memory, the HFD-fed mice did not show other major behavioral changes. To further understand the potential capacity of EC to mitigate obesity-induced changes in cognition, we conducted a longer dietary intervention study (24 weeks) with a HFD (45% kcal from fat) that is more comparable to the amount of fat consumed by humans. EC was supplemented at two levels, one that can be extrapolated to average human dietary consumption (2 mg/kg body weight) [10], and a higher amount (20 mg/kg body weight) that could be reached in humans by supplementation [11]. Consistent with the first study, the HFD consumption induced obesity while EC had no influence upon body weight and body composition. The HFD had adverse effects on metabolic parameters, and both doses of EC improved glucose homeostasis. Surprisingly, supplementation with EC had no beneficial impact upon learning and memory and did not mitigate HFD-induced spatial memory impairment. In fact, control mice supplemented with the higher amount of EC displayed even poorer object recognition memory compared to the non-supplemented controls suggesting that higher doses of EC might not always be better for brain function. Interestingly, this decline was absent in HFD animals indicating 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 restoring open field center exploration time back to the levels observed in control diet-fed mice. This implies that the mechanisms leading to learning and memory impairment are uncoupled from those associated with anxiety.

The neuroprotective effect of EC decreasing HFD-induced anxiety-related behavior may be, in part, explained by its capacity to mitigate alterations in glucocorticoid signaling in the hippocampus. In fact, the HFD consumption significantly decreased mRNA levels of both GR and MR, while EC mitigated these alterations. Consistent with our findings, high hippocampal MR expressions have been linked to a low-anxiety phenotype [12] while inhibition of the MR was linked to anxiety-like behavior as well as decreased adult hippocampal cell proliferation [13]. Although current evidence on the role of the

hippocampal GR on anxiety-related behavior is conflicting, upregulation of GR expression was correlated with decreased anxiety-related behavior [14]. EC also mitigated increased hippocampal mRNA level of 11β-HSD1. As inhibition of 11β-HSD1 has been proposed to provide neuroprotective effects in humans [15], its role in mood regulation in obesity would be an interesting area to further investigate. Moreover, hippocampal BNDF mRNA levels were positively correlated with center exploration time in the open field, consistent with the previous findings that the neuroprotective capacity of EC is partly mediated via BDNF upregulation [9, 16]. Additionally, we investigated whether the anxiolytic effects of EC could be explained by shifts in the microbiota. Similar to a previous 15-week dietary EC and HFD study [3], EC (20 mg/kg body weight) did not significantly affect the overall microbial community in HFD-fed mice. However, the long-term EC supplementation modulated select microbial species that have putative roles in mood regulation (i.e., *Enterobacter* and *Lactobacillus*) altered by the HFD consumption. This may be in part involved in EC-mediated improvement of the anxiety-related behavior.

The mechanisms affecting the CNS are mostly likely to be multifactorial and a full understanding of EC actions in the hippocampus remains to be elucidated. Thus, we conducted an untargeted genomic study to gain more detailed insights into the molecular mechanisms underlying the neuroprotective effects of EC against HFD- and obesity-induced anxiety in mice. Hippocampal gene expression following EC supplementation (20 mg/kg body weight) showed the opposite profile when compared to the gene expression profile obtained after consumption of the HFD. This suggests that EC could counteract HFD-induced alterations of hippocampal gene expression profiles. Additionally, we observed that gene expression profiles upon EC supplementation are negatively correlated with gene expression profiles associated with symptoms of anxiety disorder in humans. The multi-genomic data, including transcriptomic, microRNomic, and lncRNomic of the hippocampus of EC supplemented HFD-fed mice, revealed that EC could exert neuroprotective effects by regulating pathways that modulate BBB permeability, neurofunction, inflammation, and neurodegeneration.

Overall, this thesis contributes to the explanation of mechanisms related to the neuroprotective potential of EC in the context of HFD and associated obesity. Our results suggest that EC could mitigate HFD-induced alterations in memory and anxiety, in part, by ameliorating neuroinflammation, modulating BDNF- and glucocorticoids-regulated signaling, mitigating dysbiosis, and counteracting the effects of HFD on the hippocampus at a multi-genomic level. It is important to consider that the reported findings are entirely based on gene expression analyses and do not establish a direct causal link between the observed changes and alteration in cognition and mood. Therefore, further studies analyzing protein levels of the identified genes and functional validation of the proteins are warranted to support the underlying mechanisms of EC in the brain. Moreover, the fact that the composition of circulating EC metabolites found in humans differs from the composition found in mice [17] should be considered when interpretating the results obtained from the animal models. In addition, in humans, obesity results from complex interaction of individual factors (i.e., genetic background, medications, infection, gut-brain-hormone axis, sleep) and the environment (i.e., culture, social media, policies, food marketing, economic systems) [18]. Therefore, clinical studies will be essential to support the concept that consumption of EC could contribute to improvement in cognition and mood in obesity. Further research to understand safe EC doses is also warranted as the safety of long-term supplementation with high EC doses and its effects on the brain is not clear.

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