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Estradiol alters the gut microbiota response to high-fat diet

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ABSTRACT

Post-menopausal women have an increased risk of developing metabolic disorders, such as obesity and type-two diabetes. Estradiol, a type of estrogen, which is primarily produced by ovaries and declines in postmenopausal women, protects against diet-induced obesity in women and female mice. However, the mechanisms by which estradiol prevents diet-induced obesity are not completely understood. Host diet has been shown to shift the compositional pattern of the gut microbiota, and both diet and the gut microbiome have been linked to weight gain and obesity. We hypothesized that one mechanism by which estradiol-treated mice resist diet-induced weight gain is through the gut microbiota. For my thesis, I investigated the effects of estradiol on the gut microbiota to a diet change and analyzed the fecal gut microbiota from fourteen adult C57BL6 mice that were ovariectomized and subcutaneously implanted with capsules containing either 17β-estradiol (E2) or oil (vehicle; control; Veh). All mice were fed a standard rodent diet for 10 days and then switched to a high-fat diet (HFD) for 25 days. To identify and compare microbial community composition of samples across treatments, we analyzed the longitudinal 16S rRNA gene data from fecal pellets. I observed that E2 treatment altered the gut microbiota response and structure following the diet change. Specifically, the gut microbiota in E2 mice response to the diet change was attenuated compared to the Veh mice. Moreover, HFD-fed mice were characterized by increased relative abundances of *Firmicutes* and *Proteobacteria*, but these HFD-induced shifts were conservative for the E2-treated group. With the diet change and the lack of E2 treatment, fecal endotoxin levels and taxa associated with inflammatory and metabolic diseases increased greatly, such as *Helicobacter* and *Peptostreptococcaceae*. Comparatively, the diet change had no effect on endotoxin levels and induced slight increases in abundance of these inflammatory and metabolic disease associated taxa in E2 mice. Microbes characterized as

metabolizers of plant-based diets, such as *Ruminococcaceae and S24*-7, were, however, associated with and in higher abundance in E2 mice than Veh mice during HFD. In summary, HFD disturbs the gut microbiota diversity and composition, but the HFD associated changes are lessened with E2 treatment. The reduced response and alterations in the gut microbiota to dietary change in E2 mice may be a critical component in understanding the complexities by which estradiol protects against obesity.

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INTRODUCTION

Estrogens, a class of sex hormones, regulate energy homeostasis and metabolism

Estrogens, a group of sex hormones, are involved in female reproduction. The circulating levels of estrogens increase at the onset of puberty, are at their highest during the reproductive years, fluctuating with ovulatory cycles, and decline after the menopause (Shapiro, 2001) (Figure 1). In addition to reproduction, estrogens have a profound role in regulating and maintaining energy homeostasis and mediating metabolism (as reviewed in Clegg, 2012; Lopez and Tena-Sempere, 2015; Mauvais-Jarvis et al., 2013). The decline of estrogens is associated with disruptions in energy homeostasis and metabolism that results in the increase of insulin resistance, fat accumulation, and circulatory pro-inflammatory markers (Lovejoy et al., 2008; Pfeilschifter et al., 2002; Sites et al., 2002). Post-menopausal women also have an increased pre-disposition for metabolic syndrome, type-2 diabetes, and obesity and an increased risk of cancer, and cardiovascular diseases (Al-Safi and Polotsky, 2015; Carr, 2003; Davis et al., 2012; Davis et al., 2015; Jouyandeh et al., 2013; Simpson and Brown, 2013). Estrogen replacement therapy has been shown to improve the symptoms of metabolic syndrome such as insulin sensitivity and reduce the likelihood of type-2 diabetes and obesity (Davis et al., 2012).

Ovariectomized (OVX) mice exhibit similar metabolic and inflammatory outcomes to menopausal women as a response to high-fat diet. OVX mice serve as a good models for studying natural and surgical menopause in women, since the surgical removal of ovaries reduces the circulating estrogen levels (Brinton, 2012). OVX mice feeding on a high-fat diet display an increase in body mass, are glucose intolerant (Riant et al., 2009) and have increased pro-inflammatory markers (Bhardwaj et al., 2015; Blasco-Baque et al., 2012; Pettersson et al., 2012). These factors remained significant even when food intake and high-fat diet were

considered as confounding factors (Rogers et al., 2009; Stubbins et al., 2012). Administration of estradiol ameliorates the obesity associated symptoms in OVX mice (Riant et al., 2009), similarly to estrogen replacement therapy in women. Current molecular mechanisms by which estradiol protects against symptoms of metabolic challenges and energy homeostasis disruptions are incomplete and not fully resolved (Bryzgalova et al., 2008; Camporez et al., 2013).

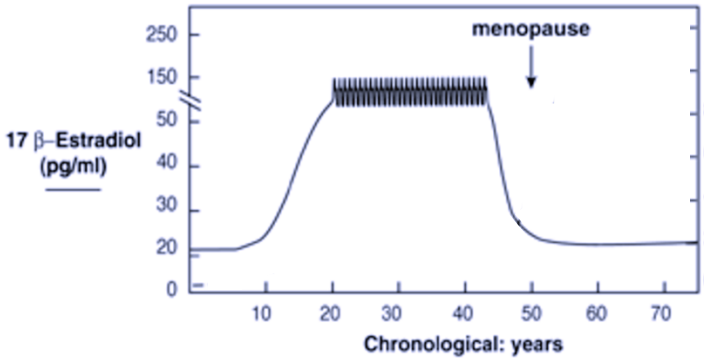


Figure 1**.** In pre-menopausal women, levels of 17 ß-estradiol (E2), the main circulating estrogen, **oscillate according to the menstrual cycles from the start of puberty until menopause when the levels decline. Figure adapted from (Shapiro, 2001).**

Gut microbiota,and inflammatory and metabolic diseases

The gut microbiota is a complex assemblage of microbes that inhabit the gut at densities of about 10^{11} to 10^{12} making the gut one of the most densely colonized surfaces of the human body. The gut microbiota contributes to a variety of inflammatory and metabolic diseases such as obesity, metabolic syndrome, liver disease, inflammatory bowel disease and colorectal cancer and is thus intricately involved in host health and disease (Marchesi et al., 2016).

The gut microbiota can modulate energy homeostasis and host metabolism, which are linked to inflammatory and metabolic diseases (Clarke et al., 2014; Rosenbaum et al., 2015). Germ-free mice have been shown to exhibit reduced adiposity and glucose intolerance compared to colonized (conventionally raised) mice, despite a greater energy consumption by germ-free mice (Backhed et al., 2004). The transfer of the gut microbiota of conventional mice (conventionalization) into germ-free mice induces an increase in body mass, insulin intolerance, and modified energy balance as compared to germ-free mice without transplantation (Backhed et al., 2004). Expression of genes associated with energy homeostasis and lipid metabolism differs between colonized mice and germ-free in that these genes are downregulated in colonized mice compared to germ-free mice (Larsson et al., 2012). Germ-free mice conventionalized with the microbiota of genetically obese donors have increased body fat and capacity to extract energy compared to mice conventionalized with the microbiota of lean donors (Turnbaugh et al., 2006). Together these studies illustrate the importance of gut microbiota in energy metabolism.

In addition to regulating the metabolic activities of the host, the gut microbiome plays a profound role in the etiology of obesity, a low-grade inflammatory disease (Ridaura et al., 2013). The conventionalization of germ-free mice with the microbiota of an obese human donor results in an obese phenotype. However, the transfer of the microbiota of a lean donor results in no

change in phenotype for the recipient mice (Ridaura et al., 2013), indicating that the donor phenotype is transferable via the gut microbiota. Conventionalized obese mice observe reduced adiposity and invasion of their gut microbiota by lean phenotype associated microbes when cohoused, which allows for the transfer of microbiota via coprophagy, with conventionalized lean mice (Ridaura et al., 2013). This observation occurs only when mice feed on a low-fat diet (Ridaura et al., 2013). Moreover, the switch in phenotype invasion is not evidenced in the conventionalized lean mice (Ridaura et al., 2013). This supports that there is a tight linkage between the invasion of the gut microbiota and the phenotype, which is highly dependent on diet.

Diet, a primary modulator of the gut microbiota

A diet high in fats, a hallmark of Western culture diet, is linked to increased fat accumulation and low-grade inflammation, which gives rise to diseases such as obesity, metabolic syndrome, and type 2 diabetes (Cani et al., 2008a; Fava et al., 2013; Lam et al., 2012). Interestingly, the lack of gut microorganisms, such as in germ-free mice, reduces these high-fat diet associated alterations on the host physiology compared to conventional mice (Backhed et al., 2004), indicating that the propensity of inflammatory and metabolic diseases is enabled through the dietary modulation of the gut microbiota.

A shift to high-fat diet (HFD) restructures the gut microbial diversity and composition and abundance of community members, and these changes in the gut microbiota are associated with increased weight gain (Daniel et al., 2014; Turnbaugh et al., 2008). Specifically, community diversity is similar amongst individuals consuming the same diet (Daniel et al., 2014; Turnbaugh et al., 2008). The HFD also induces phylum level taxonomic changes with high abundance of *Firmicutes* and low abundance of *Bacteroidetes* on the HFD (Daniel et al., 2014; Turnbaugh et al., 2008; Zhang et al., 2012). This taxonomic trend is also observed in obese mice (Ley et al.,

2005; Murphy et al., 2010b), and in mice that are genetically resistant to diet-induced obesity fed a HFD (Hildebrandt et al., 2009). While most studies have reported an increase ratio of *Firmicutes* to *Bacteroidetes* in obese individuals, several studies have contradicted these findings. For example, Duncan and co-workers (2008) did not find any difference in the ratio of *Firmicutes* to *Bacteroidetes* in the fecal microbiome of obese and lean individuals. HFD-induced effects on community diversity, interestingly, are maintained even with life-style changes such as reduced energy consumption or increased energy expenditure in HFD-fed mice (Liou et al., 2013; Zhang et al., 2013). Furthermore, the characteristic effect of HFD on the gut microbiota diversity are primarily dependent on HFD and not upon differences in individual genetics (Carmody et al., 2015). Diet is one of the primary environmental factors in shaping the gut microbial ecology.

Dietary changes can alter the microbial community dynamics. The gut microbiota of mice and humans is responsive to a diet change within 1 to 2 days (David et al., 2014; Turnbaugh et al., 2009; Zhang et al., 2012). Furthermore, despite differences in other environmental factors, such as host genetics, the response of the microbial community to a Western diet is consistent (Carmody et al., 2015). This dietary impact on the community diversity occurs before other host phenotypic changes become prevalent, such as weight gain (Carmody et al., 2015; Turnbaugh et al., 2009; Zhang et al., 2012). Essentially, the rapid microbial ecological adaptation to the change in nutrients is further correlational evidence of the gut microbiota's role in contributing to associated inflammatory and metabolic diseases and phenotypes. Interestingly, constant dietary shifts induce mirrored shifts in the gut microbial diversity in mice and humans (Carmody et al., 2015; David et al., 2014). This illustrates the

potential of dietary modulation of inflammatory and metabolic diseases through the gut microbiota.

The abundances of gut microbial community members are associated with host health and disease; low abundances of *Bacteroides* spp. is linked to a pro-inflammatory status (Boulange et al., 2016). High abundance of *Faecalibacterium prausnitzii* is found in healthy individuals and linked to anti-inflammatory status (Boulange et al., 2016; Miquel et al., 2013). Currently, this bacterium is proposed as a novel probiotic for inflammatory diseases (Miquel et al., 2013). Diet can alter taxonomic abundances of individual members over time. For example, members of the *Ruminococcaceae*, have a defined ecological niche as metabolizers of plant-based material (Flint et al., 2012; Ze et al., 2012). These members are tightly linked to diet high in resistant starch and changes in dietary nutrients results in swift shifts in the abundance of these members (Walker et al., 2011). Similarly, increases in the relative abundance of *Firmicutes* decreases and decreases in *Bacteroidetes* are characterized as HFD-induced (Carmody et al., 2015; Hildebrandt et al., 2009; Ley et al., 2005; Murphy et al., 2010b). Members of *Firmicutes* are the most diverse and highly abundant phylum in the gut (Rajilic-Stojanovic and de Vos, 2014). The *Bacteroidetes* phylum is characterized as the most stable phylum in the microbiome of healthy adults. An altered abundance of this phylum has been associated to obesity, irritable bowel disease and type 1 and 2 diabetes (Larsen et al., 2010; Ley, 2010; Rajilic-Stojanovic et al., 2011; Rajilic-Stojanovic et al., 2012). Species of this phylum have a diverse metabolic profile; for example, *Bacteroidetes* ferment complex plant polysaccharides, metabolize urea as nitrogen source, and deconjugate host metabolites such as bile acids (Rajilic-Stojanovic and de Vos, 2014).

High-fat diet also selectively promotes the growth of microbes, specifically pro-inflammatory gram-negative microorganisms (Cani et al., 2008a). These microbes can alter immune homeostasis and have been associated with inflammation, characteristic of obesity and type 2 diabetes (Cani et al., 2008b; Devkota et al., 2012). Mechanistically, chronic, low-grade inflammation is triggered by lipopolysaccharides, a cell-wall component of gram-negative bacteria which interact with the immune system (Saad et al., 2016). Clearly, diet-induced dynamics of microbial taxa are extremely relevant in health and disease.

Gut microbiota and other environmental factors

Though the gut microbiota is highly influenced by diet change, other environmental factors can also impact the diversity and structure of gut microbiota. For example, while diet is a strong modulator of the gut microbiota, the response differs in mice with different genetic background (Carmody et al., 2015). This work and others suggests that the compositional abundance and dynamics are impacted by additional host factors to diet, such as genetics, host lifestyle, and the gut microbiota itself (Carmody et al., 2015; Liou et al., 2013; Ridaura et al., 2013). In total, diet and other secondary environmental factors are drivers of gut microbial ecology. The effect of these variables has major impacts on the gut microbiota, which are associated to a variety of inflammatory and metabolic diseases (Boulange et al., 2016).

While estrogens and gut microbiota play significant roles in inflammatory and metabolic diseases independent of each other, there is mounting evidence of crosstalk between microbiota and hormones. In pregnant women during hormone level fluctuations, the gut microbiome undergoes profound changes in microbial community composition and function (Koren et al., 2012). In female rats bred to have low and high running capacity, ovariectomy can alter the

composition of gut microbiota compared to sham controls (Cox-York et al., 2015). This work suggests that estrogens are an environmental factor that can modulate the gut microbiota. However, the microbial community and individual microorganisms can also impact the levels of hormones through metabolism (Adlercreutz et al., 1984; Clarke et al., 2014; Flores et al., 2012; Plottel and Blaser, 2011; Rose et al., 2014). Furthermore, in postmenopausal women, gut microbiota diversity is positively associated with the ratio of estrogen metabolites in urine, suggesting that these metabolites are derived by microbiota (Fuhrman et al., 2014; Goedert et al., 2015). Taken together, these studies provide strong evidence that the estrogens affect microorganisms (Beury-Cirou et al., 2013; Kornman and Loesche, 1982) and that microorganisms affect the concentration and levels of hormones (Adlercreutz et al., 1984; Clarke et al., 2014; Flores et al., 2012; Plottel and Blaser, 2011; Rose et al., 2014).

Several studies have shown that estradiol prevents HFD-induced obesity in female mice (Blasco-Baque et al., 2012; Bless et al., 2014; Riant et al., 2009). Although the changes of the gut microbiota associated with diet and hormones have been investigated in the past (Cox-York et al., 2015; Moreno-Indias et al., 2016; Org et al., 2016), less attention has been given to the dynamics of microbiota undergoing diet change under different hormone conditions. Characterizing the gut microbial community dynamics to diet has major implications on women's health in relation to inflammatory and metabolic diseases.

Study aims and hypothesis

For my thesis, I focused on understanding the ecological effects of estradiol on the gut microbiota dynamics. It has been shown that estradiol protects against high-fat diet induced weight gain and that the gut microbiota is involved in the modulation of the high-fat diet induced phenotype of weight gain and endotoxin production. We hypothesize that one mechanism

through which estradiol protects against diet-induced obesity is through the modulation of the gut microbiota. To test this hypothesis, we asked a series of questions: (1) does the treatment of estradiol influence the diet-driven changes in the gut microbiota diversity and composition, (2) are there specific taxa that are associated to the estradiol treatment, and (3) does the treatment of estradiol modulate gram-negative microbial dynamics and fecal endotoxin levels during HFD?

MATERIALS AND METHODS

Overview

We used culture-independent methods to profile the fecal microbial community of female mice. Fecal samples were collected from ovariectomized female mice estradiol-treated or vehicle-treated mice over a 35-day period study, with ten days of standard rodent-chow feeding and 25 days of high-fat diet feeding. Weight and food intake measurements were made about every four days through the study. Fecal samples collected were extracted for microbial genomic DNA and polymerase chain reaction (PCR) was used to amplify and sequence a portion of the 16S ribosomal RNA gene. The 16S rRNA gene sequences were processed to identify taxa for each sample and associated relative abundances per sample. These data were further analyzed using ecological metrics and statistical analyses and analysis to reveal longitudinal community diversity and compositional differences by treatment.

Animals and study design

All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fourteen eight-week old C57BL/6 female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained on a 12:12 light:dark cycle, co-housed and fed standard rodent chow for one week adjustment period to the new environment. Mice were bilaterally ovariectomized and implanted subcutaneously with a silastic capsule (Ingberg et al., 2012) containing either 50 µg 17ß-estradiol (E2, n=7) dissolved in 25 µl 5% ethanol/sesame oil (Kudwa et al., 2009; Rissman et al., 2002) or vehicle (5% ethanol/sesame oil, Veh, n=7). After surgery, mice were housed individually.

Prior to the surgery, mice were fed standard rodent chow (SD) (Purina, cat. #5001; Table 1). After the surgery, mice continued to receive SD for additional ten days. On the eleventh day post ovariectomy, mice were switched to a high fat diet (HFD) (containing 58% Kcal from fat in the form of lard) (Harlan Teklad, cat. #03584; Table 1) for 25 days. Food intake and animal weights were measured every four days during HFD; initial base-line weigh measurement was made on the day of surgery. Fecal samples were collected daily during the SD or every four days during HFD near the food source area and stored immediately at -80 ºC until downstream analyses. Mice were sacrificed at the end of the study, 25 days after the start of HFD. See Figure 2 for experimental timeline schematic.

Measurement of LPS levels in fecal samples

Fecal endotoxin levels were quantized for randomly selected fecal samples from SD and HFD for both groups. The levels were determined using the Pyrosate limulus amoebocyte lysate (LAL) assay (Associates of Cape Cod, Falmouth, MA) using ET-free glass, plasticware and reagents for all steps. LAL uses an enzyme derived from the horseshoe crab blood cells, which in the presence of endotoxin, catalyzes a reaction to produce a gel clot. Briefly, 250 mg of fecal sample was resuspended in 750 µl of LAL reagent water and centrifuged at 12,000 rpm for 12 min. Samples were then diluted in tubes containing LAL reagents, incubated at 37˚C and then tubes were removed and inverted once. When a gel was formed, the test was scored as positive.

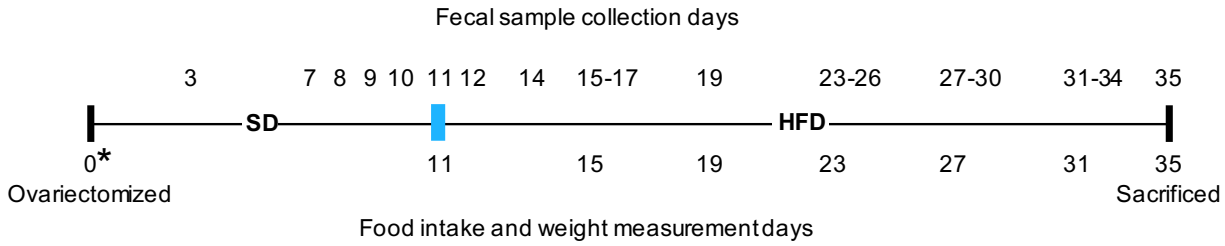


Figure 2. Timeline of experimental design.
Eight-week old female C57BL6 mice were ovariectomized and implanted with capsules containing either 50mg of 17ß-estradiol or vehicle on day 0. Animals were individually housed and given the SD (day 0 to day 10) and then HFD (day 11 to 35). Fecal samples were collected as the indicated time points above the timeline for microbial 16S rRNA analysis. Food intake and weight measurements were taken on days listed below the timeline. * indicates that no measurement for food intake was made for that day.

Table 1. Diet Composition.

% Kcal from	**SD**	**HFD**
Protein	29.8	15
Fat	13.4	26.6
Carbohydrate	56.7	58.4

DNA extraction and sequencing of fecal samples

DNA from each sample (2-3 fecal pellets, ~200 mg) was extracted using the PowerSoil® DNA Isolation Kit (MoBio, Valencia, CA) with minor adjustments to the manufacturer's protocol to increase DNA yield and concentration. The adjustments included a 5 min incubation time after adding 60 µl of C6 (elution buffer) to the spin column. DNA extracts were stored at -20 ˚C until further processing.

The 16S rRNA gene is of interest for determining gut microbial diversity and composition. This gene is universal in bacteria which gives a view on bacterial membership (Clarridge, 2004). Gene has conserved and variable regions which allows for the classification of bacterial sequences to a highly differential taxonomic level (such as the genus level) (Clarridge, 2004) where the highly variable regions provide greater phylogentic insight than conserved regions (Soergel et al., 2012). Hence, it is a viable marker gene because it can quantify the bacterial composition as relative abundances and the phylogentic diversity in a given environment (Pace, 1997; Ward et al., 1990). 16S rRNA gene has a ~1500 base pairs and the gene encodes for catalytic rRNA portion of the small ribosomal subunit. The length of the sequencing read has little effect on the accuracy of taxnomic classification and relative abundance measures (Huse et al., 2008), but the selection of primers for short sequence reads greatly limits the taxnomic classification of the reads at a highly diffrential taxonomic level (Huse et al., 2008; Soergel et al., 2012). Hence, the primers were carefully selected based on previous procedures that were complementary to highly conserved regions of the gene (Caporaso et al., 2011).

The V3-V4 region (Figure 3) of the 16S rRNA gene of the DNA extracts from each sample was amplified using the following universal 16S rRNA gene primers: 341F (5'-

CCTACGGGAGGCAGCAG-3') and reverse 806R (5'-GGACTACHVGGGTWTCTAAT-3') with sequence adapters on both primers and sample-specific Golay barcodes on the reverse primer following the procedure of Caporaso et al. (2011). Briefly, the PCR amplicons were quantified by PicoGreen (Invitrogen, Carlsbad, CA) using a plate reader. After quantification, amplicons were pooled in equal concentrations, cleaned up using UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA), and quantified using the Qubit (Invitrogen, Carlsbad, CA). This pool was then sequenced using paired-end v2 chemistry on an Illumina MiSeq (Illumina, San Diego, CA) at Forsyth Institute, Cambridge, MA.

The Illumina MiSeq platform is high-throughput method of sequencing 16S rRNA gene amplicons. An adapter sequence is added to each DNA fragment that is unique for each sample and as a results samples are pooled together. The DNA fragments are sequenced by *in situ* PCR using fluorescently labeled nucleotides and emission imaging. The adaptor sequence, sequenced with the 16S rRNA gene segment is used to distribute the amplicons according to their sample of origin. As a result, numerous samples can be sequenced together, with high number of amplicons in each sample.

Sequence processing and analysis of microbial communities

A total of 1,610,282 sequences reads were processed, with 805,141 paired-end read pairs. Paired-end reads were joined using Flash software (Magoc and Salzberg, 2011). Only read pairs that had perfectly matching overlapping region were retained. Paired reads were then demultiplexed meaning the total sequences were distributed based on the reads adaptor sequence according to their sample origin. The sequences were then filtered based on the phred quality score. The phred quality score (q-score) is the probability of that a nucleotide base of the

sequence is incorrect. A high q-score is indicative of high-quality sequences. Reads that had a q-score <20 were removed from further downstream analysis as recommended by previous work and guidelines (Bokulich et al., 2013). Lastly, chimeric sequences were identified using UCHIME's usearch61 *de novo* based chimera detection algorithm (Edgar et al., 2011), and removed from the quality-filtered sequences. Chimeric sequences are artificial sequences produced during PCR. Demultiplexing and quality filtering were done using Quantitative Insights into Microbial Ecology (MacQIIME v1.9.0 (Caporaso et al., 2010b; Caporaso et al., 2012) and MacQIIME was used for additional processing of the data unless otherwise indicated.

Although the 16S rRNA gene is highly conserved, there are variable regions that allow for the distinction between different taxa (Figure 3). Universal primers, which are primers targeting conserved regions across as many different bacterial taxa, are used to amplify a variable region which is then used for taxon identification. We used V3-V4 region. Sequences are often clustered into operational taxonomic units (OTUs) based on similarity. For example, groups of sequences clustered based upon 97% sequence similarity threshold are taxonomically approximate to species (Konstantinidis and Tiedje, 2005). In general, taxonomic thresholds are rough estimates of taxonomic levels as they can differ for based on the hypervariable region of the 16S rRNA used and for different taxa (Konstantinidis and Tiedje, 2005; Yang et al., 2016). Chimera-free and filtered sequences were clustered at 97% sequence similarity level using open-reference OTU picking approach based on the most abundant sequence from each cluster was selected as a representative sequence. In this open-reference approach, sequences are aligned and clustered at 97% threshold based on sequences in a reference database. Sequences that have less than 97% sequence similarity are clustered *de novo* in reference to one another rather than a database.

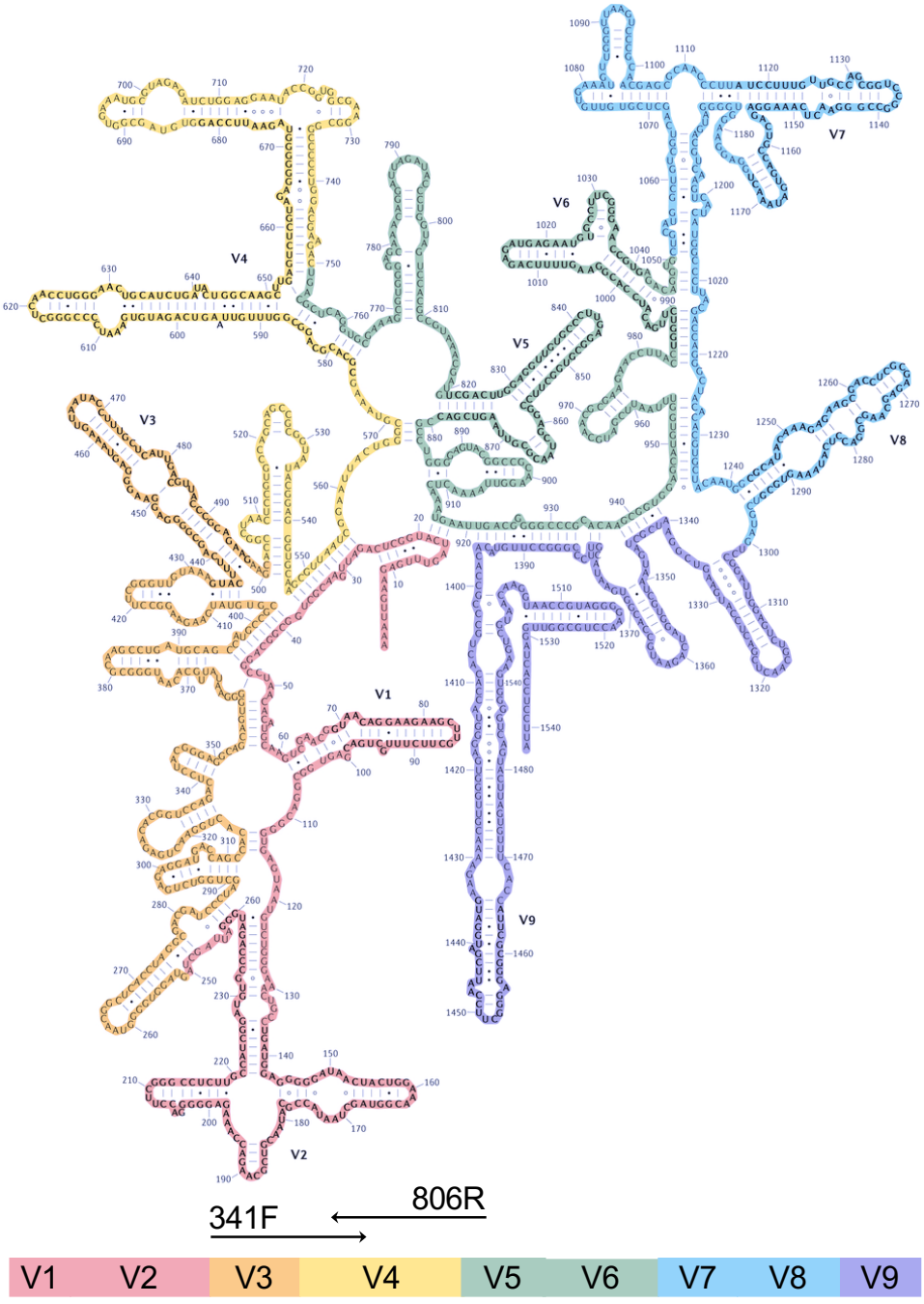


Figure 3. Hypervariable regions of the 16S rRNA gene and primer selected regions. Secondary structure and illustration of 16S rRNA of *Escherichia coli* and associated hypervariable regions. 341F and 806R primers used for our study are marked. Figure adapted from (Yarza et al., 2014).

All representative sequences were aligned using PyNAST (Caporaso et al., 2010a). The RDP classifier (Wang et al., 2007) was used to assign the Greengenes2013 database taxonomy to the representative sequences. Taxonomic classification of the representative sequence was inherited by the OTU cluster. A phylogenic tree for subsequent phylogenetic analyses was built using FastTree (Price et al., 2010). OTUs with ≤2 sequences, sequences belonging to chloroplasts and mitochondria were removed as they would not be relevant for downstream analyses.

An important distinction should be made between taxa and OTUs. Although the clustering of sequences is maintained at a 97% sequence threshold, it is important to note that classification of the OTUs may not be complete. In that OTUs may be taxonomically assigned up to class level and may lack taxonomic designation at family and genus level. There are two likely reasons for this cutoff in taxonomic classification, the first is that there is no phylogenetic information to that level within the database (Goodrich et al., 2014). The second reason is that there is that there is no consensuses as to which phylogenetic class the OTU should be assigned to for a given taxonomic level (Goodrich et al., 2014). Thus, a large proportion of OTUs will not be identified at the species taxonomic level. Taxa with a genus-level designation, for example, are thus a mix of OTUs that were not identified at the species level.

Often the number of sequences per sample varies and can lead to artificial estimates of microbiota diversity. To account for this variability, rarefaction approach is used. An equal number of sequence are randomly selected from each sample at a given sequencing depth. This random selection ensures that the population of sequences are represented in the sampling population of sequences after rarefaction. This is a conservation procedure, however, which emphasizes highly abundant sequences over rarer ones. Samples were rarefied to 9,000

sequences and samples with less than 9,000 sequences were excluded from analysis. In the end, a total of 166 samples was kept. After processing of the data, we ended up with sequences with 405 base pairs and an average of 9,472 (SD ±148) sequences in each sample.

Alpha and beta diversity

The microbiome diversity can be described using alpha and beta diversity. Alpha diversity can provide insight as whether HFD (for example) promotes diversity within a community equally with and without E2-treatment. Essentially, alpha diversity provides insight as to how the community as a whole is impacted by altered environmental factors. There are many different metrics for describing a community's alpha diversity, which can be qualitative or quantitative and species-based or divergence-based. The qualitative measure considers the presence and absence of taxa within samples, while the quantitative measure accounts for abundances of the taxa. Traditional species-based diversity measures from ecology have been adapted for surveying the taxa diversity in microbial ecology. Essentially, a species-based diversity measure treats all taxa equally and does not account for phylogenetic relationships between taxa, whereas a divergence-based measure takes into account the evolutionary relationships.

In this study, we determined the Chao1 and Shannon diversity indices (Table 2). The Chao1 index is a qualitiative, non-parametric, species-based measure of alpha diversity that provides the lower bound of species richness. Essentially, this metric defines the community richness and is reflected by the number of taxa present in a community. The Shannon index takes into account both richness and eveness (characterized by how representative a taxa is within a community though abundance values). This is quantitative, species based measure that accounts for the presence and abundance of the taxa. Low values of richness and/or evenness mean less

diversity. The Chao1 indices for each sample were generated using QIIME and Shannon indices were determined for each sample using the vegan (diversity) package in R. The Kruskal-Wallis test (kruskal.test) in R was used to determine differences in diversity by treatment and diet.

Beta diversity is defined as a measure of dissimilaity between communities. This measure of diversity is powerful in that we can assess how a microbial community changes over time and with different treatments. We used Unique Fraction metric (UniFrac) which relies on the premise that microbial communities that differ from one another have taxa with more unique evolutional lineages (Lozupone and Knight, 2005a). This metric incorporates phylogenetic information from the phylogenetic trees of the 16S rRNA gene of each sample in determining the similarity between communities (Lozupone and Knight, 2005a). The UniFrac metric provide qualitative (use presence absence information) and quantitative (presence/absence and abundance information) measures of beta diversity (Lozupone and Knight, 2005a; Lozupone et al., 2007). UniFrac values for both weighted and unweighted metric were calculated as a pair-wise comparison of similarity between samples. A distance matrix is produced with all the pairwise comparisons. We determined the UniFrac diversity values for each sample using QIIME. The weighted UniFrac values from the distance matrix were visualized as a boxplot to determine similarity between and within samples by treatment and diet and assessed for significance using Bonferroni-corrected Student's *t*-test in R and QIIME.

Table 2. Overview of different indices of community diversity.

Metric	Description	Reference
Chao1	Qualitative measure of alpha diversity; richness	(Chao, 1984)
Shannon	Quantitative measure of alpha diversity; richness and evenness	(Shannon and Weaver, 1949)
UniFrac, weighted	Incorporates the presence/absence and abundance data in addition to phylogenetic information; beta diversity metric	(Lozupone et al., 2007)

Visualizing the pairwise comparisons is challenging due to the high dimensionaly of the data. Hence, ordination methods, such as principal coordinates analysis that we implemented, reduce the the dimensionality so that the data can be visualized in a two dimensional space. In this two dimensional projection, distances between points, which correspond to each sample, represent how similar the points are to one another based on the weighted UniFrac diversity metric. Essentially, sample points closer together on the two dimensional plane are have similar community structures (i.e. similar composition in terms of presence/absence and abundance data) that sample points that are far apart. By applying this dimension reduction technique, we can visualize the high dimsional UniFrac diversity output for our dataset, while retaining the pairwise comparison information. We can also gain meaningful ecological interpretations about the samples by plotting the dimension that explains the greatest amount of variation between sample communities (principal component axis one) over time.

Dimensionality reduction of the UniFrac weighted distance matrix was done in QIIME and visualized using the ggplot2 package in R. Statistical significance for of beta diversity of samples by diet and treatment was determined using ANalysis Of SIMilaity (ANOSIM). This is an ANOVA-like hypothesis test used often in multivariate statistics to evaluate signficance of samples from a dissimiliaty matrix. The first principal component axis was plotted over time in R using ggplot2 package. Kruskal-Wallis test (kruskal.test) in R was used to determine significance of community diversity over time with the assumption that samples are independent from one another.

Comopsitional and temporal dynamics

Compositional temporal dynamics provide insight as to how environmental factors influence the community and its members over time. Longitudinal analyses allow for a nuanced understanding of how environmental factors drive microbial ecology.

We plotted the relative abundance of all taxa at the phylum taxanomic level and the genus taxanomic level from the abundance tables generated after sequence procressing in QIIME over time as area graphs. The abudances were averaged by treatment for each timepoint. Kruskal-Wallis test (kruskal.test) in R was used to determine significance of abundance over time with the assumption that samples are independent from one another for each taxon.

In the net comparison analysis, we sought to determine the magnitude of difference in abundance for each taxon between E2 and Veh mice for each time point. Essentially this analysis defines if the bacterial taxa is more abundant under the E2- or Veh-treatment and by what percent the taxa are higher or lower in abundance. The net difference in abundances for each taxon were calculated using the following formula:

$$Net\ = avg(\mathrm{E2}) - \ avg(\mathrm{Veh})$$

The abundances for each taxon were averaged by day for E2 and Veh mice. The difference in the averages was determined for each day as the net difference. Similarily, the net difference in abundances was determined for SD and HFD period. Based on this analysis, positive values indicate enrichment of the taxa in E2 treatment over Veh treatment. Negative values indicate that taxa abundance is higher in Veh mice than E2 mice. We determined significance of the net differences values between SD and HFD using the Wilcoxon Rank Sum test.

We used the linear discriminant analysis of effect size (LEFSe) pipeline on the galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/) to identify bacterial taxa that uniquely

associated to E2- and Veh-treatment over time and for SD and HFD time periods (Segata et al., 2011). Both the net comparison analysis and LEFSe analysis provide an estimate of the effect size between the two treatment groups over time and by diet, however the LEFSe analysis provides greater biological interpretation by characterizing taxa as biomarkers that can discriminate between treatments (Segata et al., 2011). There are two analysis in the pipeline, the Kruskal-Wallis test and Linear Discriminant Analysis (LDA). The significance of the associations between taxa abundance and treatment are first determined by Kruskal-Wallis test. Then a ranking of the taxa is generated using LDA. LDA is similar to the principal component analysis in that it is reduces the dimensionality of the abundance data to a linear axis. This axis for LDA, unlike the principal coordinate analysis, is orientated to maximize the difference between groups of interest (i.e. E2 and Veh treatments). In total, taxa with a high LDA value for a taxon will indicates that the taxon best discriminates between the groups. The sign of the LDA score for comparing two groups is indicative of the group.

The methods described thus far for analyzing temporal and compositional dynamics do not do a good job of describing the longitudinal changes in less abundant taxa. Characterizing the temporal dynamics of rare taxa is highly important as many rare taxa contribute to the temporal variability in microbial diversity disproportionately to abundant taxa (Shade et al., 2014). Horizon plots visualize the changes in relative abundance of taxa over a time course relative to the its median abundance for all days. Hence these plots allow for the comparative visualization of the temporal dynamics of rare and abundant taxa. The data used to generate the plots were abundance values for each taxon averaged by day for E2 and Veh treatments times series. Graphs were generated by first centering each OTU (with the relative abundances >1%) time series around its median, followed by dividing the curve into colored bands whose width is the median

absolute deviation. Then, the colored bands were overlaid with negative values mirroring upwards. Regions with warmer colors and cooler colors indicate a range where an OTU exceeds or falls below its median abundance, respectively.

Not all temporal dynamics are ecologically driven changes due to environmental factors. Abundance data, due to the technical aspects of sequencing rare taxa, for example, contains random noise. In other words, changes in abundance for some taxa may be transient and not due to the environmental factors. Hence, we used Augmented Dickey-Fuller (ADF) test to characterize dynamics of individual OTUs (Said and Dickey, 1984), to identify if individual taxa are at equilibrium or fluctuating non-randomly. The test was run by using the tseries (adf.test) package in R. The ADF test was set with a null hypothesis that the time series for a given taxon follows a random trend. Rejection of the null hypothesis means that the taxon is not at equilibrium.

RESULTS

Estradiol prevents high-fat diet induced weight gain in female mice

Eight-week-old C57BL/6J female mice were ovariectomized to remove their endogenous source of estrogens and treated with either E2 (estradiol) or Veh (control). E2-treated and Veh-treated females adapted to a defined standard diet (SD) were switched to a high-fat diet (HFD) on day 11 after ovariectomy (Table 1). During the HFD feeding period, Veh mice significantly gained body weight (Figure 1; $p<0.05$; ANOVA) and after 24 days had gained 35% more weight than on the first day of HFD. In contrast, E2 mice, did not show significant body weight gain throughout the experiment and maintained their lean phenotype until the end of the experiment on day 35 (Figure 1). As observed in previous studies (Blaustein et al., 1976; Blaustein and Wade, 1976; Eckel, 2011), food intake was higher in Veh-treated mice than E2-treated mice, and in our study, food intake was 22% higher in Veh-treated mice (Supplementary Figure 1). This increase of HFD-intake alone cannot explain the extent of the observed weight gain in Veh-treated mice.

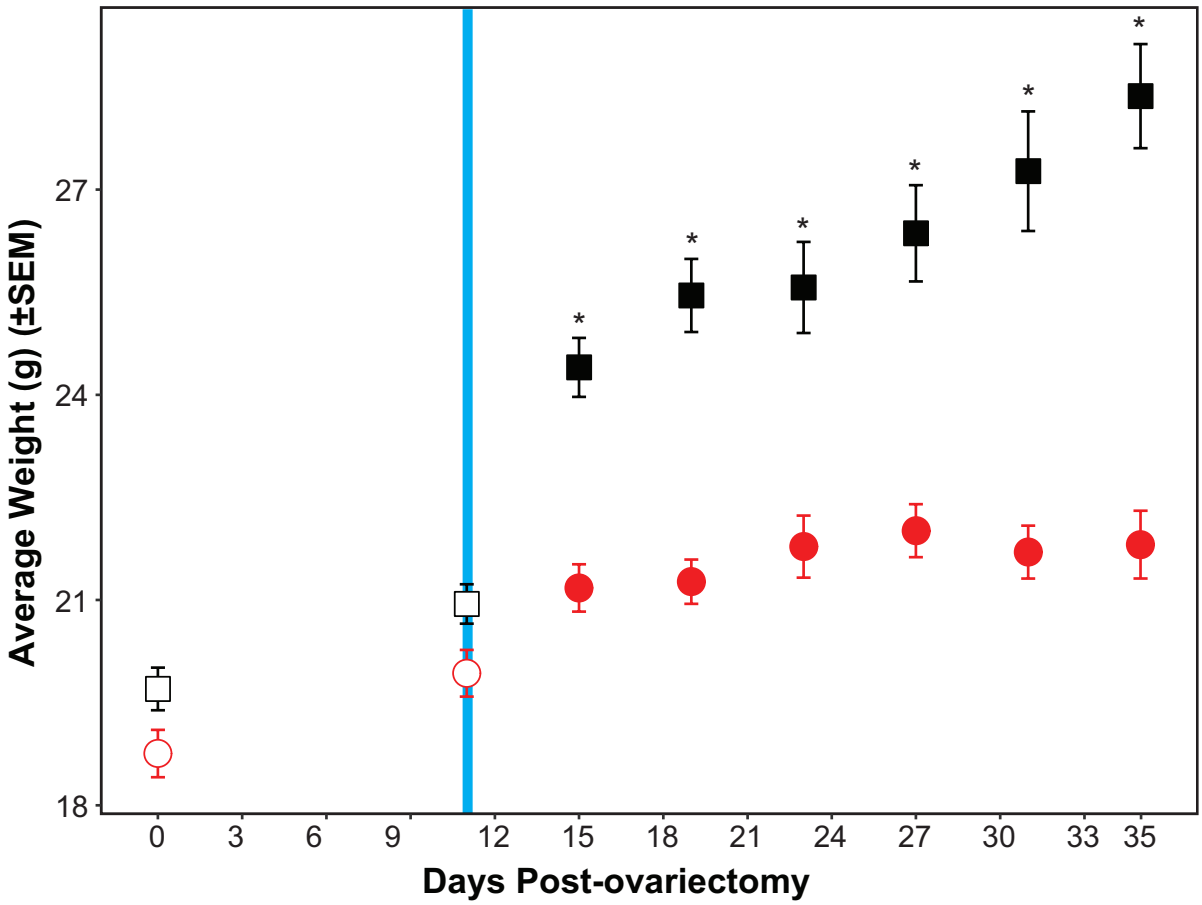


Figure 4. E2-treated mice gain less weight than Veh-treated mice. Mice (n=7 per treatment group) were measured for weight and sampled for fecal samples during SD (days 0-10 post-ovariectomy) and during HFD (days 11-35). Solid blue line indicates start of HFD. Red and black symbols indicate E_2 and Veh samples, respectively. Significant difference in weight between Veh- and E2-treated mice as measured by ANOVA ($p<0.05$) is indicated by *.

Microbial community in E2 and Veh mice respond differently to HFD

To assess the impact of E2 and diet on the female mouse fecal microbiota, we sequenced the V3-V4 amplicons of 16S rRNA gene from E2- and Veh-treated mice. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using Greengenes database.

The richness (the number of different taxa defined by OTUs in a sample) and evenness (the relative abundances of taxa in a sample) are often calculated to compare and characterize the sampled community to other communities. Based on Kruskal-Wallis test ($p<0.05$), fecal microbiota from HFD-fed mice had significantly different Chao1 and Shannon index values from SD-fed mice. For the HFD-fed mice, E2 treatment reduced both richness and evenness of the microbial community as evidenced from lower Chao1 index and Shannon diversity index values compared to Veh treatment (Table 3).

Table 3. Average Chao1 and Shannon index values by diet and treatment group.

	Diet	Treatment	N	Average	Standard Error of Mean
Chao1 index	HFD	E2	45	1946	77
		Veh	38	2099	86
	SD	E2	34	2231	79
		Veh	35	2249	62
Shannon index	HFD	E2	38	2	0
		Veh	32	2	0
	SD	E2	34	1	0
		Veh	35	1	0

Beta-diversity analysis based on weighted Unifrac distance metric was used to compare bacterial communities (Lozupone and Knight, 2005b; Lozupone et al., 2011). Weighted Unifrac metric is obtained by incorporating information on relative abundances and phylogenetic diversity observed in samples. To identify major sources of variation in our dataset, we used a correspondence analysis, a technique that allows for the identification of potential relationships between variables when there are no *a priori* expectations of the nature of the relationships. The weighted-Unifrac principal coordinate analysis (PCoA) revealed that most fecal microbiota from both Veh and E2 groups clustered together during SD (open symbols in Figure 5A). However, the switch to HFD altered the composition of the fecal microbiota in both groups, as shown by the clear separation of groups from the SD cluster (closed symbols in Figures 5A, $p<0.001$, ANOSIM). Pair-wise comparison of bacterial community composition within and between diet and treatment categories revealed that variation in microbial diversity was the smallest for Veh and E2 groups fed a SD, and the largest between Veh mice fed SD and HFD ($p<0.05$; Bonferroni-corrected Student's *t*-test; Figure 5B). To characterize temporal patterns of the bacterial community in the Veh and E2 mice, we plotted the first principal coordinate 1 (PC1) weighted-Unifrac values against time (Figure 5C). The PC1 captured 42% of variation in the community. Within three days of switching to the HFD, the fecal microbiota of both Veh and E2 mice differed from their respective SD microbiota and remained different for all subsequent days ($p<0.05$; Kruskal-Wallis test; Figure 5C). The greatest difference in weighted Unifrac PC-1 values between bacterial communities from E2 and Veh mice was observed on day 15 (Figure 5C), and then decreased between treatment groups after day 15 (Figure 5C). Importantly, the bacterial community response to the new diet was smaller and slower in E2 mice, implying that estradiol attenuates the HFD-induced shift in the gut community structure.

To determine if there is any relationship between the community structure and body weight, we plotted the PC1 values against mouse weight (Figure 5D). Weight-gain was linked to HFD and bacterial community composition (Figure 5D). The slope determined by PC1 and weight gain was different for Veh and E2 groups, indicating a differential correlation between weight gain and microbial communities in the two treatment groups ($R^2 = 0.7$ for E2 group, $R^2 = 0.8$ for Veh group; $p<0.01$; Figure 5D).

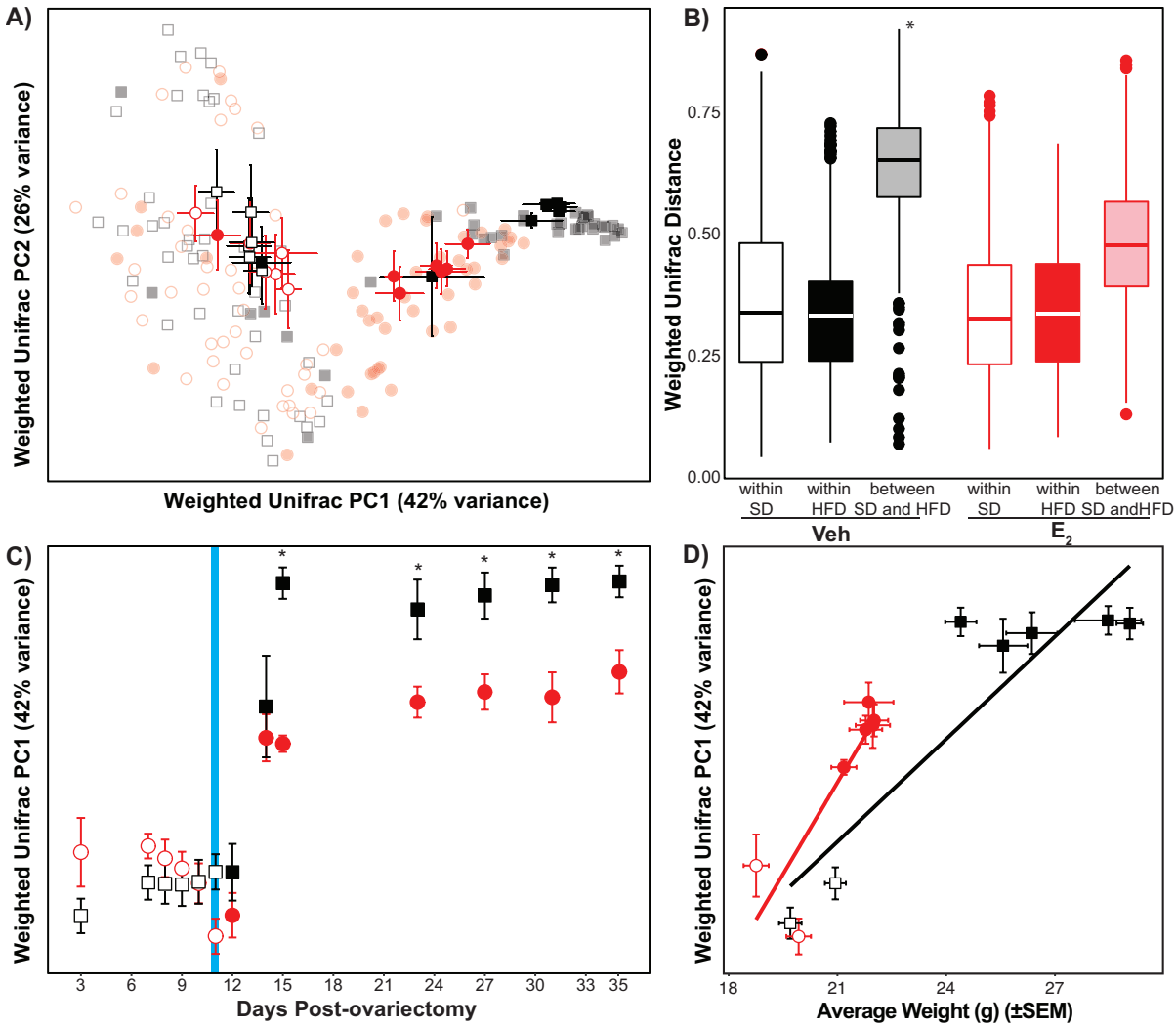


Figure 5. Responsiveness of Veh treated microbial community is robust to the diet change. **(A)** Weighted Unifrac-based PCoA of the fecal microbiota of Veh- (black) and E2- (red) treated mice during SD (open) and HFD (closed). **(B)** Comparison of weighted Unifrac distances of bacterial communities of Veh and E2 mice within and between diet groups. **(C)** Comparison of communities over time based on their position along PC1 of the weighted Unifrac-based PCoA. Solid blue line indicates start of HFD. Significant difference in microbial communities of Veh- and E2-treated mice as measured by Kruskal Wallis ($p<0.05$) is indicated by * **(D)** Comparison of communities over time based on their position along PC1 of the weighted Unifrac-based PCoA and weight in E_2 and Veh mice. Error bars represent standard error of the mean.

Identification of key taxa responsible for differences between Veh and E2 groups

Analysis of major taxonomic groups supported our finding that the switch to HFD differentially impacted the fecal microbiota of Veh and E2 groups. From 166 samples collected from 14 mice, we identified 136 bacterial taxa belonging to 12 phyla. The three most prevalent phyla in both treatment groups were *Bacteroidetes, Firmicutes,* and *Proteobacteria* (Figure 6A). Mean relative abundance level of *Bacteroidetes* was higher during SD (58.6% and 59.4% for Veh and E2 groups, respectively) than HFD (7.5% and 29.4% for Veh and E2 groups, respectively, $p<0.05$; Kruskal Wallis test, Figure 6A). In contrast, the relative abundance of *Firmicutes* during SD was lower (38.6% for Veh and 37.9% for E2 groups), when compared to HFD (86.7% for Veh and 65.8% for E2 groups, $p<0.05$; Kruskal Wallis test; Figure 6A). The mean relative abundance for the phylum *Proteobacteria* during SD was also lower (0.06% for Veh and 0.08% for E2) and increased under the HFD conditions to 1.01 % for Veh and 0.56% for E2 groups. The change in the relative abundances due to the shift in diet was the highest for *Proteobacteria* for both treatment groups and increased by 18 times for Veh mice and by 7 times for E2 mice on the HFD (Figure 6A).

Across all communities sampled, 27 taxa were found at greater than 1% relative abundances in at least one sample (Figure 6B and Supplementary Table 1). *S24-7* family belonging to the *Bacteroidales* was the most abundant in the SD-fed Veh (58.4%) and E2 (59.4%) mice (Figure 6B). The switch from SD to HFD, resulted in the decrease in the relative abundance of the OTU in the *S24-7* family in both treatment groups (Figure 6B). The mean relative abundance of *S24-7* across all HFD samples was 7.4% and 29.4% in Veh and E2 mice, respectively (Figure 6B). The only other OTU that decreased due to the diet switch was the one belonging to the *Clostridiales* family, *Lachnospiraceae*, but its decrease was the same across

both groups (Figure 6B). A total of 20 bacterial genera that significantly increased after the HFD switch belonged to the *Firmicutes* phylum, 9 of which had greater than 5% sequences in at least one sample (Figure 6B, Supplementary Table 1). These included some of the most abundant members of the order *Erysipelotrichales* such as *Allobaculum* spp.; order *Clostridiales* such as *Oscillospira, Dorea* spp. and unassigned members of *Lachnospiraceae* and *Ruminococcaceae* families*;* and order *Lactobacillales* such as *Lactobacillus* and *Streptococcus* spp. (Figure 6B; Supplementary Table 1).

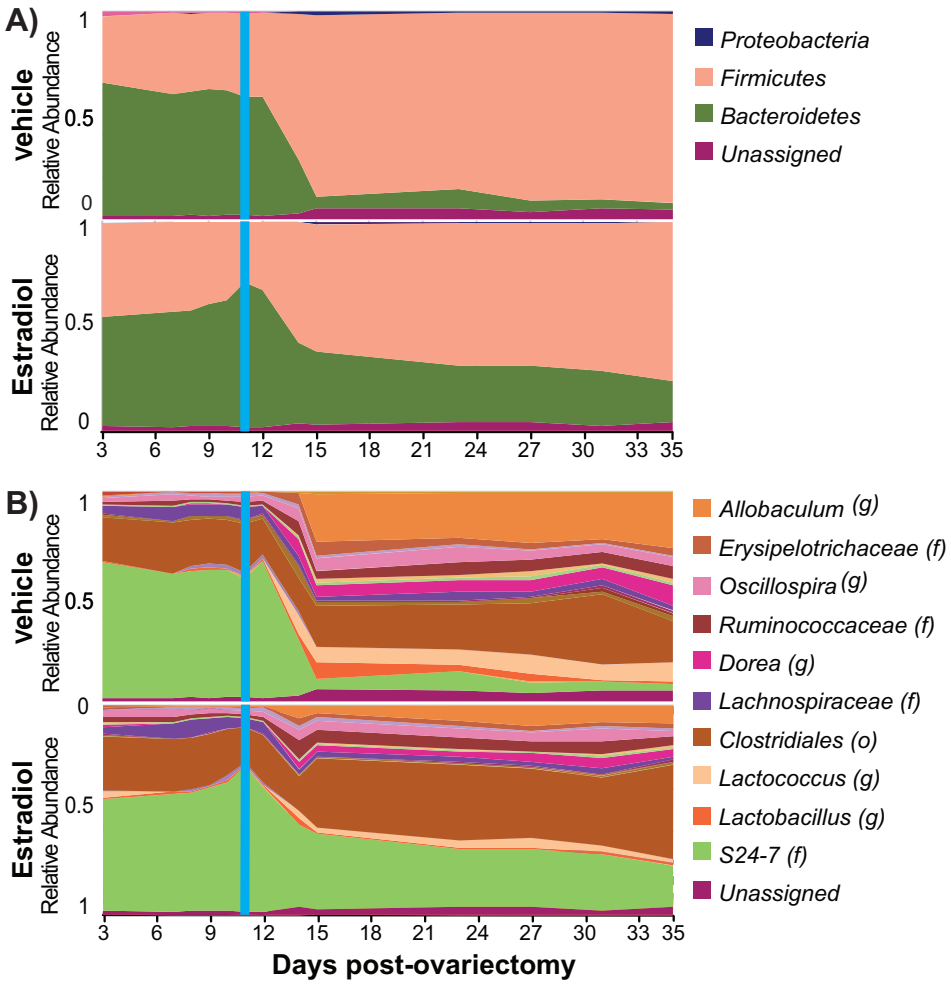


Figure 6. Diet-induced changes in the fecal microbiota composition are gradual in E2 treated mice relative to Veh treated mice. (A) Mean relative abundances of major phyla (>1% in at least one sample) in E_2 and Veh treatment groups. **(B)** Mean relative abundances of taxa (>1% in at least one sample) reported at the lowest identifiable level (o = order, f = family, g= genus) with legend showing taxa with relative abundance >5%. Solid blue line indicates start of HFD.

The observed differences in community structure between treatment groups during both SD and HFD (Figures 5 and 6), were driven by differences in the relative abundance of multiple bacterial taxa (Figure 4A). We identified these bacterial taxa by calculating the difference in the mean relative abundance for the taxa between E2- and Veh-treatment for each time point. Relative abundances of taxa in E2 and Veh groups during SD were similar and as a result we did not observe differences more than 5% between the two groups during SD (Figure 7A). The only taxa that significantly differed between the two groups during SD were *Peptostreptococcaceae*, two *Clostridiaceae* from the order *Clostridiales* and genus *Lactobacillus* (Benjamini-Hochberg corrected p-value < 0.05; Wilcoxon Rank Sum test). The relative abundances of these taxa were higher in Veh mice (Figure 7A). In contrast, 18 out of 27 taxa were significantly enriched in either E2 or Veh mice fed the HFD (Benjamini-Hochberg corrected p-value < 0.05; Wilcoxon Rank Sum test; Figure 7A). OTUs belonging to *S24*-7, one group of *Clostridiales*, and *RF39* were more abundant in the E2 versus Veh mice after the diet switch (Figure 7A). OTUs belonging to the phylum *Firmicutes* such as *Allobaculum*, *Lactococcus*, *Roseburia, Lactobacillus, Coprococcus*, *Peptostreptococcus*, *Erysipelotrichaceae,* and *Dorea* were more prevalent in Veh mice fed HFD (Figure 7A). Also, more prevalent in Veh mice were *Helicobacteraceae* from the phylum *Proteobacteria* and *Prevotella* spp. from the phylum *Bacteroidetes* (Figure 7A). The same four taxa that were significantly higher in Veh than E2 mice during SD, were also higher during HFD (Figure 7A). This suggests that E2, rather than the diet, influences the dynamics of these taxa in the gut.

To identify bacterial taxa that were distinctly associated in E2 or Veh mice, we determined the associations between diet/treatment and bacterial taxa abundance by Linear Discriminant Analysis (LDA) effect size (LEfSe) algorithm (Segata et al., 2011; Figure 7B). The

same differentially abundant taxa detected by the Wilcoxon Rank Sum analysis (Figure 7A) were also detected with LEfSe (Figure 7B).

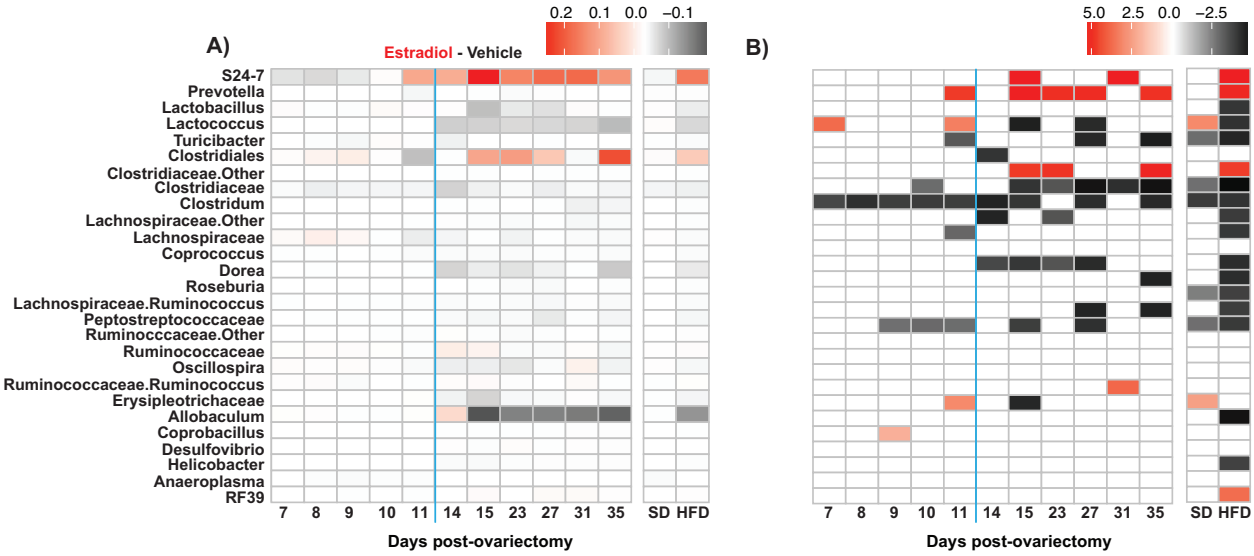


Figure 7. Microbial compositional changes in response to diet differ in Veh and E2 mice. **(A)** Difference between E2 and Veh mean relative abundance of taxa >1% grouped by time point, SD (days 7-11), and HFD (days 14-25). The graded color scale indicates the relative percent difference between E2 and Veh taxa highlighting taxa more abundant in E2 mice (red tiles) versus in Veh mice (black tiles). **(B)** Differentially associated taxa over time, SD (days 7-11), and HFD (days 14-25) as determined using the LEfSe analysis (alpha value for Kruskal-Wallis (KW) test <0.05, and Linear Discriminant Analysis (LDA) significant threshold >2). The graded color scale indicates LDA score (log 10). Comparison highlights taxa enriched in Veh mice (black tiles) and taxa enriched in E2 mice (red tiles). Solid blue line in both A) and B) indicates start of HFD.

To further characterize the temporal dynamics of the major microbial taxa for Veh and E2 treatment groups, we used horizon graphs. Horizon plots longitudinally visualize the deviations from the median relative abundance of each individual taxa within Veh and E2 groups. In both mice groups, taxa were differentially responsive to the diet change where some taxa increased and others decreased, as observed by the cool and warm shades, respectively (Figure 5B). However, taxa in Veh mice compared to E2 mice deviated from their respective median relative abundance more extremely as observed by the darker colors during HFD (Figure 5B). This suggests that the E2 treatment lessens the responsiveness of the taxa to the HFD. We used an Augmented Dickey-Fuller (ADF) test to characterize the dynamics of individual taxa and determine whether deviations from the median were random fluctuations or due to a diet change. Changes in relative abundance of 22 taxa during HFD in Veh and E2 mice were transient ($p<0.05$; Augmented Dickey-Fuller test; Figure 8). Twenty two out of 27 taxa continuously deviated from median relative abundance in Veh mice during HFD ($p<0.05$; Augmented Dickey-Fuller test; Figure 8B). Comparatively, only nine out of 27 taxa continuously shifted in relative abundance in E2 mice during HFD ($p<0.05$; Augmented Dickey-Fuller test; Figure 8B), suggesting that the fecal microbial community from E2 mice had not stabilized and continued to respond to the diet change throughout the HFD time course.

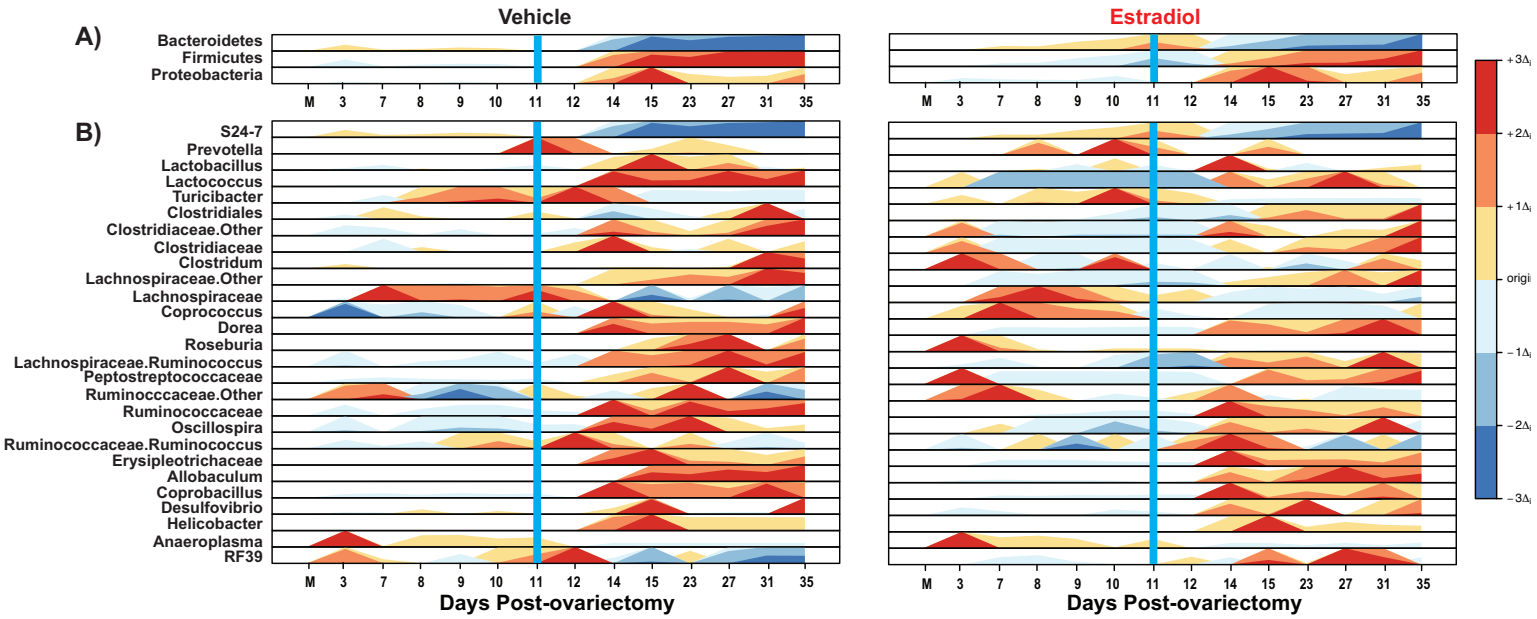


Figure 8 **Dynamics of fecal bacteria in Veh and E2 mice during SD and HFD. Horizon graphs of the most common OTU abundances (>1% relative abundance) over time. Origin of each taxon, indicated by M on the x-axis, in the horizon plot is the median relative abundance of the given taxon. Darkening of the colors indicate the median absolute deviation, where cool, blue, colors indicate ranges where a taxon falls below its median abundance. Warm, red, colors indicate ranges where taxon's median abundance is exceeded (the graded color scale indicates standard deviations from the median (the chart height of each taxon is cut in half and overlaid). Horizon plots at (A) the phylum level and (B) the lowest identifiable taxon level.**

Change in Bacteroidetes and Firmicutes abundance corresponds to weight gain

We characterized the abundance trends of highly abundant taxa and found that after the introduction to HFD, the relative abundance of predominant taxon in the *Bacteroidetes* phylum, *S24-7* family, decreased by 51% in Veh-mice and 33% in E2-mice (Figure 9A). The relative abundance of the *Allobaculum* genus, a dominant taxon in the *Firmicutes* phylum, increased by 25% in Veh-mice and 7.6% in E2-mice during HFD (Figure 9A). To explore the relationship of weight and relative abundances of *Allobaculum* and *S24-7* OTUs, we plotted relative abundances against weight (Figure 9B). Increase in relative abundance of *Allobaculum* spp. correlated with weight gain (R^2 was 0.9; $p<0.001$; Figure 9B) and it was the same for both E2 and Veh groups. In contrast, the decrease in relative abundance of *Bacteroidales S24-7* correlated with weight gain (R^2 was 0.7; $p<0.001$; Figure 9B), but the change in the relative abundances with the weight gain was different in E2 and Veh groups.

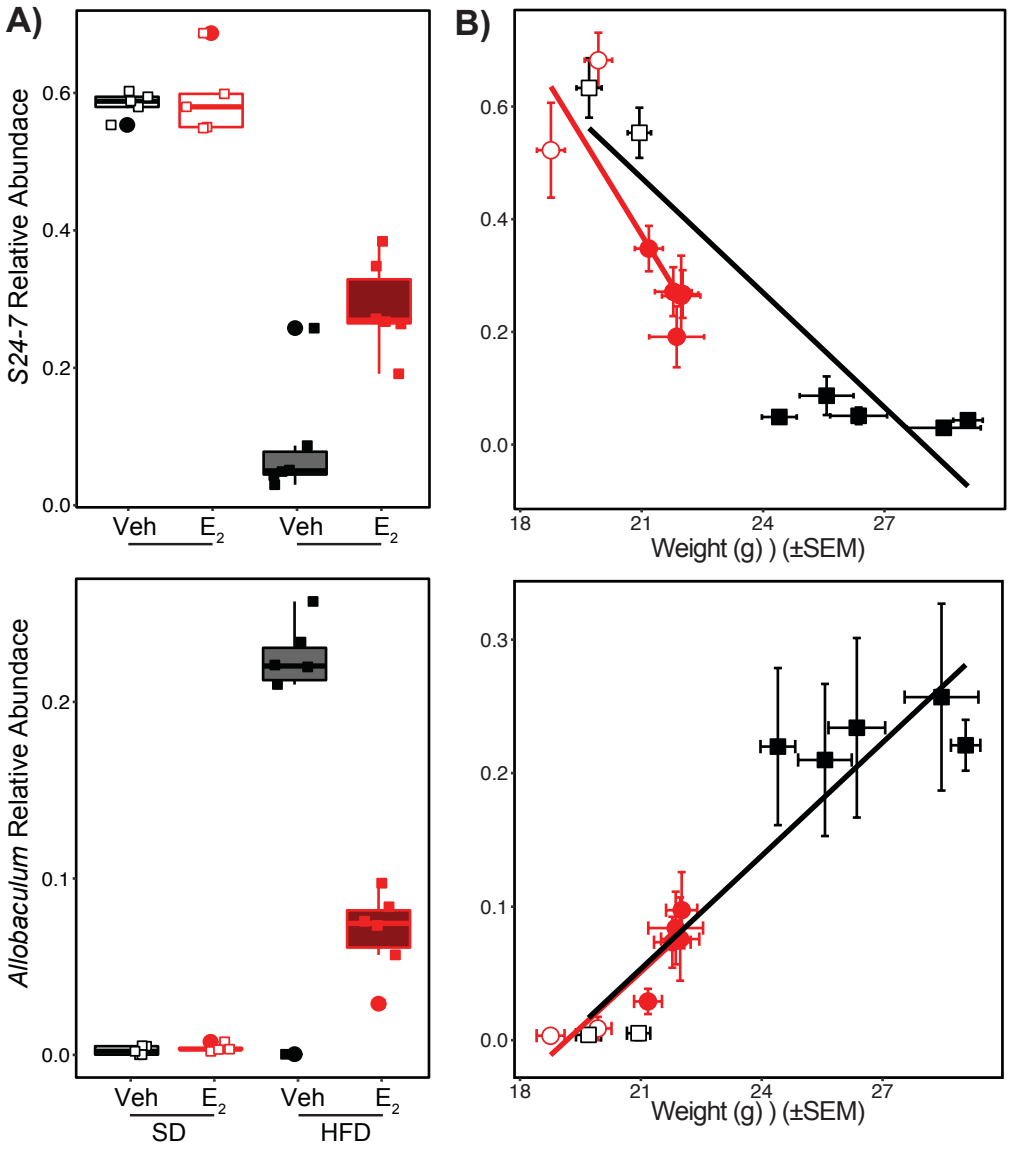


Figure 9. E2 treatment differentially alters the response of predominant taxa to the diet change. (A) Boxplots of relative abundance of two abundant taxa. **(B)** Correlation of relative abundance of the predominant taxa to weight. Error bars indicate standard error of the mean.

Estradiol protects against high-fat diet-induced endotoxins

Gram-negative bacteria contain lipopolysaccharide (LPS) as the major constituent in the outer leaflet of the outer cell membrane. These bacteria have variable regions of polysaccharide and oligosaccharide and a conserved lipid region. The lipid is the endotoxic and biologically active part of the LPS molecule responsible for septic shock. To determine if estradiol protected against endotoxins during HFD, we quantified the fecal lipopolysaccharide (LPS) levels for E2 and Veh mice during SD and HFD using the limulus amebocyte lysate (LAL) gel clot assay. We observed no differences in endotoxin levels between E2 and Veh mice fed SD (Figure 10). In HFD-fed mice, we found that fecal endotoxin concentrations were 10 times higher in Veh compared to E2 mice ($p<0.05$; Kruskal Wallis test; Figure 10). This HFD-induced increase in fecal endotoxin levels is consistent with an increase in gram-negative bacteria belonging to the phylum *Proteobacteria* in Veh-treated mice (Figure 6 and 10). Moreover, the reduced levels of LPS are also consistent with the reduced abundance of gram-negative bacteria in HFD-fed E2-treated mice (Figure 6 and 10).

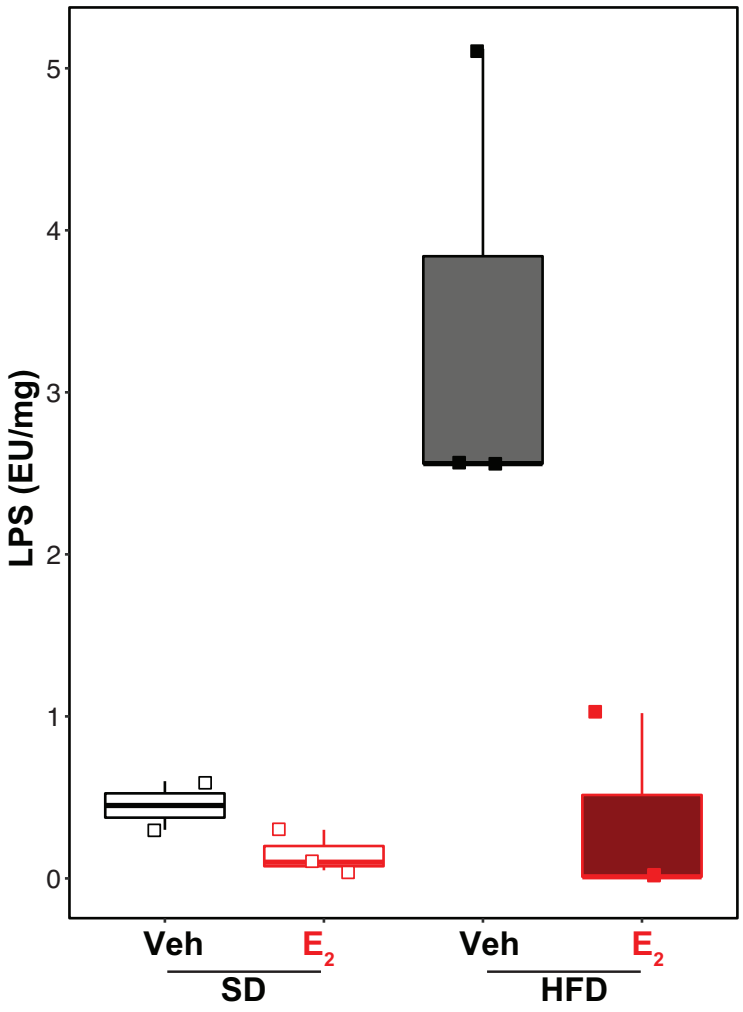


Figure 10 E2 treatment protects against HFD driven increases in fecal LPS levels. LAL assay was used to measure the fecal endotoxin concentration (EU/mg) for randomly selected fecal pellets (n=3) for E2- and Veh-treated mice during SD and HFD.

DISCUSSION

In this study, we tested the hypothesis that estradiol affects the composition of the gut microbiota of female mice and that estradiol affects the dynamics of the gut microbiota to changes in diet. We observed that a change in diet from the standard rodent chow to high-fat diet resulted in changes in the abundance and composition of the gut microbiota in ovariectomized female mice. This diet change also induced a differential community response between Veh- and E2-treated mice. Additionally, HFD-fed mice were characterized by increased relative abundances of *Firmicutes* and *Proteobacteria*, but these HFD-induced shifts were conservative for the E2-treated group. Members of these phyla include opportunistic pathogens and gram-negative bacteria which were higher in Veh mice than E2 mice during HFD. In contrast, microbes associated to plant-based diets were higher in HFD-fed E2-treated mice (Flint et al., 2012; Ze et al., 2012). The lack of E2-treatment significantly increased the fecal LPS levels for HFD-fed mice. In general, HFD intake disrupts the gut microbiota structure and function, but this alteration can be impeded by administration of E2 treatment. This suggests that E2 is a protective factor to high-fat diet in shaping the gut microbial ecology.

Environmental factors that modulate the gut microbiota

Diet is a primary factor in modulating the gut microbiota (Carmody et al., 2015; Hildebrandt et al., 2009). We observed that with an introduction to HFD Veh-treated mice, which exhibited an obese phenotype, had different gut microbiota structure than E2-treated mice, which had a lean phenotype (Figure 5C). High-caloric diets, such as HFD, have been shown to induce shifts in the gut bacterial diversity and composition and increase body fat (Cani et al., 2008a; Turnbaugh et al., 2008; Zhang et al., 2012). Furthermore, although obesity can also

influence the gut microbiota, HFD can alter the gut microbiota composition of obese individuals independent of the obesity and host genetics in general (Carmody et al., 2015; Hildebrandt et al., 2009; Ley et al., 2005; Murphy et al., 2010b). Specifically, *Bacteroidetes* decreases and *Firmicutes* increases were determined as HFD-induced shifts and independent of obesity phenotype and host genetics (Carmody et al., 2015; Hildebrandt et al., 2009; Ley et al., 2005; Murphy et al., 2010b). We observe these same shifts with diet change in Veh and E2 mice, however the shifts are attenuated in E2 mice (Figure 6A). In total this suggests that, despite a difference in phenotype between Veh- and E2-treated mice the changes observed with the introduction of HFD are primarily due to the HFD.

Food intake can also have an effect on the murine gut microbiota, specifically low-fat diet into a unique community state after 21 weeks of calorie restriction compared to *ad libitum* controls (Zhang et al., 2013). However, subtler differences were observed for shorter periods of calorie restriction in the gut microbiota between calorie-restricted and *ad libitum* controls (Mai et al., 2007). If E2-treatment induced an appreciable difference in reducing food intake during the 10 days of SD, then we would have observed an effect of food intake on the gut microbiota of E2-treated and Veh-treated mice during SD based on (Zhang et al., 2013). Though food intake was higher in Veh-treated than E2-treated mice by 22% during HFD, similar to other studies (Blaustein et al., 1976; Blaustein and Wade, 1976; Eckel, 2011), this difference would not be impactful on the gut microbiota of either treatment during HFD based on (Zhang et al., 2013). In general, the gut microbial structure and response is not affected by reduced energy consumption (Liou et al., 2013). In sum, the attenuated HFD-induced changes in the gut microbiota for E2-treated mice cannot be explained by a decrease in food intake, but instead due to the E2-treatment.

We observed that the change in gut microbiota community composition in E2 mice attenuates the influence of HFD as compared to Veh-treated mice (Figure 5C). This affects the gut microbial diversity in that the gut microbiota of E2 mice separates from Veh mice during HFD (Figure 5C). Menon and colleagues (Menon et al., 2013) also examined the longitudinal changes in beta diversity and observed a difference in microbial communities with respect to estrogen receptor beta status. By the end of the study this difference was no longer significant (Menon et al., 2013). Although the host-mediated effects of estrogens were taken into account (i.e. estrogen receptor beta status), the effects of circulating estradiol on the gut microbiota were not considered nor controlled (Menon et al., 2013). In another study, the gut microbiota diversity has been shown to differ for OVX and sham operated female mice on a high-fat high-sugar diet, in that the gut microbiota of sham mice cluster separately and uniquely from OVX mice (Org et al., 2016). However, this effect is only observed in two of three strains of mice used in the study, including in C57BL6J mice that we used in our study (Org et al., 2016). This study reinforces our finding that a E2-treatment HFD-fed mice have a different gut microbial community than Veh HFD-fed mice. Our findings indicate that the microbial community adaption to diet change and HFD over time are relevant for understanding the effect of estradiol on the gut microbiota.

Estradiol modulation of taxonomic composition

The switch from SD to a HFD diet resulted in an increase in *Firmicutes* and decrease in *Bacteroidetes* abundances (Figure 6B), similarly as reported in other studies that investigated dietary effects on the murine gut microbiota (Murphy et al., 2010b; Turnbaugh et al., 2008; Zhang et al., 2012). With the diet change, *Proteobacteria* increased by 18 times in Veh mice and by 7 times in E2 mice (Figure 6B). This phylum, associated with inflammation, is prevalent in

HFD-fed mice and European children consuming a Western diet (De Filippo et al., 2010; Everard et al., 2014; Shin et al., 2015; Wang et al., 2015; Zhang et al., 2012).The increase in inflammation-inducing, gram-negative *Proteobacteria* has been defined as an indicator of instability in the microbial community (dysbiosis) induced by environmental factors such as diet change (Shin et al., 2015). With the diet change, *Proteobacteria* was 158% more abundant in Veh mice than E2 mice (Figure 6B). This suggests that the gut microbial community of Veh mice is more unstable than the community of E2 mice due to the change in diet.

HFD increases an abundance of bacteria associated with adverse health outcomes such as opportunistic pathogens (Zhang et al., 2012). We, too, have observed the increase of these opportunistic pathogens when mice were switched to the HFD, but this increase was more prevalent in mice that had no estradiol. The lack of estrogen resulted in greater abundances in *Lachnospiraceae* family, specifically taxa belonging to the genera *Coprococcus* and *Dorea* (Figure 6B), similar to the study by Org and colleagues (Org et al., 2016). High abundances of *Coprococcus* and *Dorea* have been linked to *Campylobacter* infections (Kampmann et al., 2016). We also observed that the abundances of *Lactococcus* and *Helicobacter* were higher in Veh-treated mice than in E2-treated mice during HFD (Figure 6B). These OTUs have been associated with inflammatory diseases such as taxa belonging to *Lactococcus spp.* (Akhaddar et al., 2002; Versalovic, 2011), *Helicobacter spp.* (Versalovic, 2011), and *Dorea* spp. (Rajilic-Stojanovic et al., 2011; Saulnier et al., 2011).

Our analysis of fecal lipopolysaccharide (LPS), a pathogen associated molecular marker, levels showed an HFD-induced increase in fecal endotoxin load in Veh-treated mice (Figure 10). This observation has been shown in other studies for plasma and fecal endotoxin levels (Kim et al., 2012). The gram-negative microorganisms capable of producing this endotoxin include

members of the proteobacterial *Helicobacter* genus (Eidhin and Mouton, 1993; Kim, 2010; Whittle et al., 2001), and were higher in abundance for Veh-treatment than E2-treatment in HFD-fed mice (Figure 6B). The introduction of HFD has been shown to increase the proportion of gram-negative bacteria and plasma LPS load in male mice (Cani et al., 2007) of which LPS has been linked to the mechanism of HFD-induced obesity pathogenesis (Cani et al., 2008a). In general, the increase of LPS levels in HFD-fed mice have been attributed to increases in gram-negative microbes (Cani et al., 2008a; Kim et al., 2012; Serino et al., 2012).

However, it is difficult to ascertain whether estradiol directly modulates gram-negative microbes and thereby attenuates LPS levels or indirectly interacts with the host to modulate the microbiota. The robustness of E2-treatment associated microbes to HFD-induced changes suggests a close interaction between the bacteria and the female sex hormone. Estradiol has been shown to reduce the *in vitro* growth of *Helicobacter* strains by inhibiting coccoid cell formation in a dose-dependent manner (Fteita et al., 2014; Hosoda et al., 2011) indicative of the potential of estradiol to directly modifying gram-negative bacterial growth and proliferation. However, further *in vivo* studies are needed to understand the molecular link between the E2 modulation of the gut bacteria and reduced endotoxins levels. Estradiol has also been shown to attenuate the LPS-activated TLR4 pro-inflammatory pathway (Murphy et al., 2010a). A disruption of the TLR4 pathway as in TLR4-deficient and HFD-fed mice reduces plasma and fecal endotoxin levels (Kim et al., 2012). Further work is needed, however, to understand the role of estradiol's downregulation of the inflammatory response to HFD in modulating of gut microbes.

Estrogen replacement therapy has been shown to reduce symptoms of inflammatory bowel disease in post-menopausal women (Kane and Reddy, 2008). In rodents, estradiol treatment can also improve the symptoms of inflammatory bowel disease (Harnish et al., 2004).

Females have a lower incidence of infections than males specifically due to the immunomodulatory effects of estrogens (Ruggieri et al., 2016). We hypothesize that estradiol's protective role against inflammatory and infectious diseases involves the modulation of the gut microbes. The abundance of *Peptostreptococcaceae* was higher in mice with no estradiol, a finding also observed by Org et al. (2016). Members of the *Peptostreptococcaceae* spp. are associated with compromised health in that high abundance is associated to patients with ulcerative colitis and colorectal cancer (Chen et al., 2012; Rajilic-Stojanovic et al., 2013; Wang et al., 2012). *Clostridium difficile*, a member of the *Peptostreptococcaceae* family, is the prime example of the family's association to bad health outcomes in that at high abundance this pathogen has deleterious effects (Ozaki et al., 2004; Rajilic-Stojanovic and de Vos, 2014). When examining the interaction between sex and age factors in a population analysis of *C. difficile* patients, older women had a higher risk for *C. difficile* infections than younger women (Esteban-Vasallo et al., 2016). One molecular explanation for this lower risk in younger women may be due in part to circulating estrogens modulating the microbiota through the host and/or directly, however further work is needed to elucidate the molecular mechanism.

In our work, we show that abundances of *Clostridiales* class was linked to estradiol independent of the diet (Figure 6B). In post-menopausal women, non-ovarian, urinary estrogen metabolite levels were directly correlated to *Clostridiales* abundance (Flores et al., 2012; Fuhrman et al., 2014). At the order level, *Ruminococcaceae* in the *Clostridiales* class was abundant to in HFD-fed mice administered E2 treatment (Figure 6B) and directly associated with urinary estrogens (Fuhrman et al., 2014). This is surprising in that though *Ruminococcaceae* order had high abundances in E2-treated mice during HFD, the members this order metabolize plant-based material such as complex carbohydrates (Flint et al., 2012; Ze et al., 2012).

Similarly, the *S24-7* family, highly abundant during SD in both E2 and Veh mic, remained abundant in E2 mice during HFD (Figure 6B). This family has been characterized with the potential to degrade oxalate, which is primarily found in plant-based diets (Ormerod et al., 2016). Although some microbes are flexible to variable dietary nutrients, which can explain the resistance of the community to change, the fact that *Ruminococcaceae* and *S24-7* were responsive to the diet change in Veh mice suggests that these taxa are not tolerant of change in nutrients.

One possible explanation as to how estradiol induces microbial tolerance to diet change may be through quorum sensing. Quorum sensing is a cell-cell communication mediated by several signaling molecules such as acyl-homoserine lactones (AHLs) that allows cells to sense cell density and orchestrate gene expression. These molecules are used in *Proteobacteria* (Schuster et al., 2013). Estrogens inhibit quorum sensing regulated gene expression and thereby reducing AHLs accumulation (Beury-Cirou et al., 2013). We speculate that estradiol affects quorum sensing in the gut microbes attenuating the microbial response to an environmental change such as diet. Further work is need to verify and elucidate the role of estradiol on the gut microbiota's communication network.

Potential biases and future directions

In the present study, we used culture-independent 16S rRNA gene sequencing technique to characterize the role of estradiol on the gut microbiota of female mice. All aspects of our study were controlled and were consistently applied across all samples which allows for comparisons across treatments and diet. The equal treatment of samples also allows for viable temporal analysis. However, it is important to note the potential biases associated with DNA extraction and PCR amplification. Specifically, DNA extraction and PCR amplification protocols increase

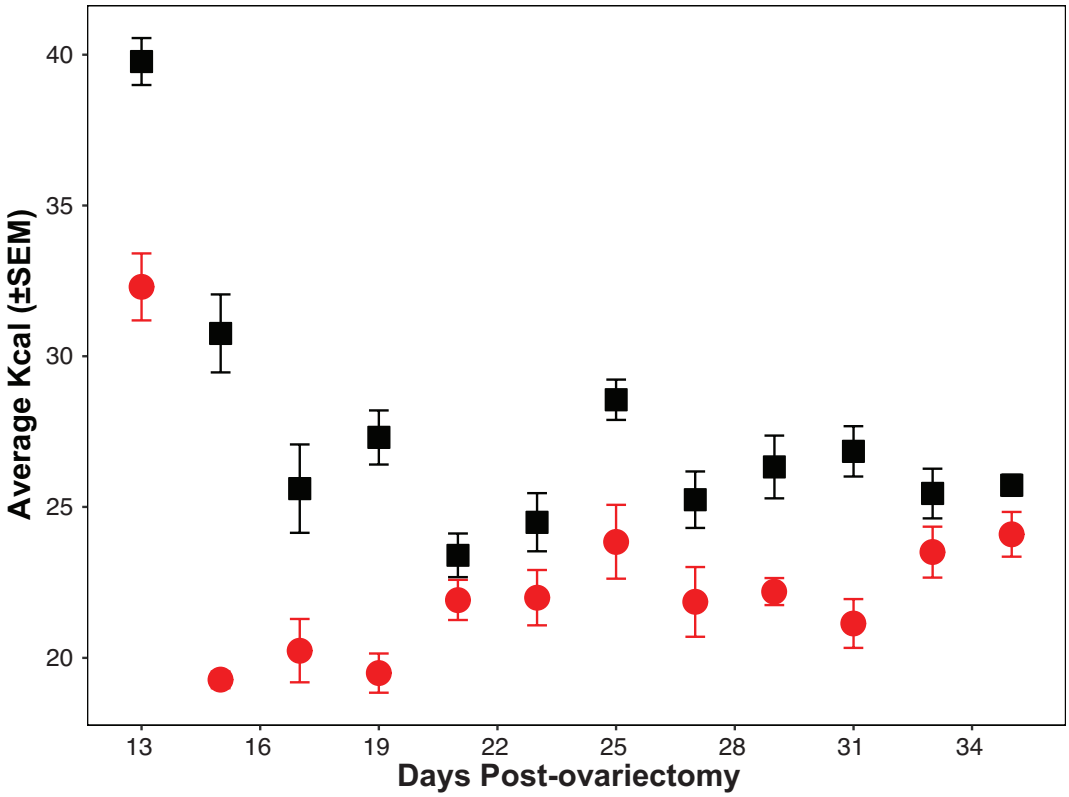
the likelihood of spurious or missed true correlations and in turn potential biases of the results (Brooks et al., 2015). The primers used for 16S rRNA gene amplification can over represent microbial taxa with multiple copies of 16S rRNA genes (Pei et al., 2010) or can preferentially anneal to 16S rRNA genes of some taxa and not others such as *Bifidobacterium,* an important gut microbiota member (Hayashi et al., 2004; Kuczynski et al., 2012). However, due to the studies already conducted that use similar study variables, our findings are validated despite some bias introduced by the 16S rRNA gene profiling.

Thus far we have purported, several host-dependent and host-independent mechanisms though which estradiol may be modulating the gut microbial diversity, response and composition to a diet change. For future studies, defining the role of the immune system in our mechanistic understanding of hormone, gut microbiota, and diet-induced obesity interplay would give us insight to the low incidence of inflammatory diseases in pre-menopause women. In addition, by focusing on estradiol's direct effect on the gut microbiota, it can increase our understanding of estradiol's role in shaping the microbial ecology independent of diet. Specifically, by focusing on quorum sensing, we will gain insight to the potential mechanism of how the gut microbial community and members resist the change in diet with E2 treatment. The focus on quorum sensing will also provide insight to anti effects on opportunistic pathogens such as *C. difficile* abundance. In total, E2-treatment likely reduces the ecological niche of certain phylogenetic groups such as opportunistic pathogens and endotoxin producing bacteria, while conserving the niche of microbes associated with a plant-based diet.

Conclusions

The results presented in this work characterize the role of estradiol on the gut microbiota structure and temporal dynamics during a diet change. We found that estradiol treatment limits

the HFD diet-induced effect on the gut microbiota structure. In addition, estradiol reduces the sensitivity of the gut microbial community response to a diet change. The gut microbes associated with bad health outcomes such as opportunistic pathogens and gram-negative bacteria were less prevalent with E2 treatment. Microorganisms, such as *Ruminococcaceae* and *S24-7*, associated with plant-based diet were resistant to HFD associated changes with E2 treatment. Lastly, we found E2-treatment protected against HFD-induced endotoxin increase. Our work, in total, describes estradiol as an additional factor that should be considered as a modulator of the gut microbiota to diet-induced changes. In addition, this work suggests to estradiol control of microbial ecology niches through speculative host-dependent and/or host-independent mechanisms. Understanding the mechanisms by which estradiol modulates the microbiota are relevant for understanding women's health before and after the onset of menopause. The future steps should focus on disentangling host-dependent from host-independent effects of estradiol on the gut microbiota using enrichment cultures of gut microbiota amended with different concentrations of estradiol.



Supplementary Figure 1. Mean Kcal intake of E2 (red) and Veh (black) treatment group during HFD (days 11 to 25). Mice were measured for intake every 2 days during HFD (days 11-35).

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