

OPEN

Estradiol and high fat diet associate with changes in gut microbiota in female *ob/ob* mice

Kalpana D. Acharya^{1*}, Xing Gao¹, Elizabeth P. Bless¹, Jun Chen² & Marc J. Tetel¹

Estrogens protect against diet-induced obesity in women and female rodents. For example, a lack of estrogens in postmenopausal women is associated with an increased risk of weight gain, cardiovascular diseases, low-grade inflammation, and cancer. Estrogens act with leptin to regulate energy homeostasis in females. Leptin-deficient mice (*ob/ob*) exhibit morbid obesity and insulin resistance. The gut microbiome is also critical in regulating metabolism. The present study investigates whether estrogens and leptin modulate gut microbiota in ovariectomized *ob/ob* (obese) or heterozygote (lean) mice fed high-fat diet (HFD) that received either 17 β -Estradiol (E2) or vehicle implants. E2 attenuated weight gain in both genotypes. Moreover, both obesity (*ob/ob* mice) and E2 were associated with reduced gut microbial diversity. *ob/ob* mice exhibited lower species richness than control mice, while E2-treated mice had reduced evenness compared with vehicle mice. Regarding taxa, E2 was associated with an increased abundance of the S24-7 family, while leptin was associated with increases in Coriobacteriaceae, *Clostridium* and *Lactobacillus*. Some taxa were affected by both E2 and leptin, suggesting these hormones alter gut microbiota of HFD-fed female mice. Understanding the role of E2 and leptin in regulating gut microbiota will provide important insights into hormone-dependent metabolic disorders in women.

Estrogens profoundly influence energy homeostasis^{1–3}, as well as reproductive physiology and behavior^{4–6}. Estrogens reduce food intake, attenuate body weight gain and adiposity, and increase physical activity in humans and rodents^{1,2}. Postmenopausal women have lower levels of circulating estrogens and an increased tendency to gain fat weight, which increases their risk for obesity, cardiovascular disease, stroke, and type 2 diabetes^{7–9}. Similarly, in mice on a high-fat diet (HFD), ovariectomy increases energy intake and obesity, while estradiol (E2) treatment prevents weight gain^{2,10–13}, indicating that estrogens protect against HFD-induced obesity.

Leptin is a peptide hormone secreted primarily by adipocytes, which acts primarily in the brain to stimulate metabolism, promote satiety, and regulate fat storage^{14–16}. A mutation in the *ob* gene that encodes leptin results in mice lacking the hormone (*ob/ob*)¹⁷. While phenotypically normal at birth, *ob/ob* mice quickly develop obesity and diabetes¹⁸. Additionally, *ob/ob* mice exhibit increased food intake and decreased physical activity, energy metabolism, and body temperature compared to lean controls, making *ob/ob* mice an excellent genetic model of obesity^{19–21}. Administering leptin to adult *ob/ob* mice reverses these effects by decreasing food intake, increasing energy output and decreasing circulating levels of glucose and insulin^{19,22}.

Leptin and estrogen signaling pathways interact to influence reproduction and energy metabolism. High levels of E2 are associated with increased leptin sensitivity in both male and female rodents²³. In contrast, decreased estrogens in ovariectomized mice and postmenopausal women increases leptin resistance, resulting in obesity^{24,25}. While female *ob/ob* mice do not have an estrous cycle, leptin administration restores fertility, including successful ovulation, pregnancy, and birth, indicating the profound effects of leptin on reproduction²⁶. While these findings indicate functional interactions between E2 and leptin, primarily through the convergence of their central pathways^{25,27}, these hormonal effects on peripheral regulators of energy metabolism are not well understood.

The gut microbiome, which is composed primarily of the bacteria in the intestinal tract and their metabolites, has profound effects on energy metabolism²⁸. The gut microbiome aids in digestion and absorption of macro- and micro-nutrients from food²⁹. Gut microbiota are also essential modulators of host immune homeostasis. Protective polysaccharides produced from the break-down of dietary fibers attenuate inflammation^{30,31}. Gut microbiota can also synthesize and metabolize neurotransmitters and hormones to alter host physiology^{32–34}.

¹Neuroscience Department, Wellesley College, Wellesley, MA, 02481, USA. ²Department of Health Sciences Research & Center for Individualized Medicine, Mayo Clinic, Rochester, MN, 55905, USA. *email: kacharya@wellesley.edu

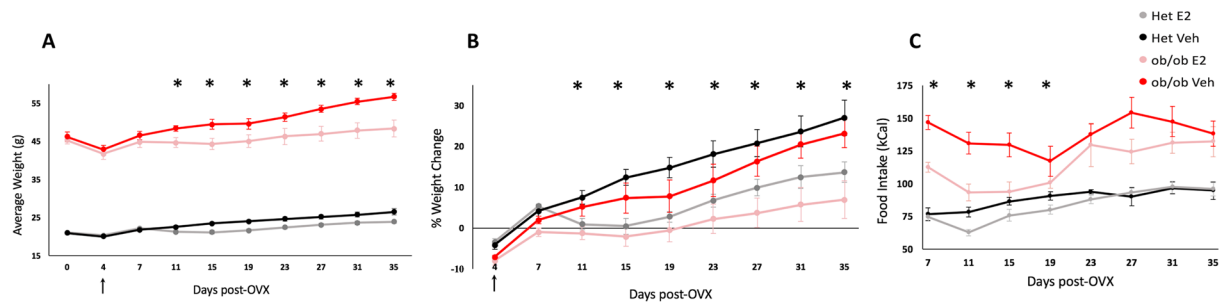


Figure 1. Estradiol and leptin alter weight gain and food intake in adult female *ob/ob* and lean control mice. **(A)** Average weight, **(B)** percent weight gain and **(C)** food intake (2 mice/cage) of heterozygote controls (Het) E2 ($n = 13$), Het Veh ($n = 12$), *ob/ob* E2 ($n = 8$) and *ob/ob* Veh ($n = 7$) with arrow indicating the start of high fat diet. The effects of genotype on average weight **(A)** and food intake **(C)** were present on all days. Days with effects of E2 are denoted by * ($p < 0.05$; ANOVA). Error bars indicate \pm SEM.

Furthermore, changes in body weight have been associated with changes in gut microbial diversity in humans and rodents³⁵. For example, the transfer of gut microbiota from obese human or mice donors results in an obese phenotype in recipient mice^{36–38}. Similarly, depletion of gut microbiota using antibiotics increases adiposity in mice³⁹.

A variety of factors, including host genetics, diet, stress, and gonadal hormones can alter the gut microbiome^{33,34,40–42}. Sex differences in gut microbiota have been reported in humans and rodents^{41,43}. In a European population, higher levels of Bacteroidetes and Prevotella were observed in men compared to women⁴⁴. Male mice exhibited higher abundances of Lachnospiraceae (phylum Firmicutes) and *Parabacteroides* spp. (phylum Bacteroidetes) and Proteobacteria than female mice^{33,45}. Additionally, testosterone administration to female neonatal rats decreased gut microbial diversity during adulthood, and increased the ratio of the two most abundant phyla, Firmicutes and Bacteroidetes⁴⁰. The hormone-dependent changes in gut microbiota were more robust compared to the diet-induced changes in these rats⁴⁰. Ovariectomy also alters gut microbial diversity in adult mice^{33,45,46}. While obesity and *ob/ob* genotype are associated with a reduction in Bacteroidetes/Firmicutes ratio in humans and male mice, respectively^{47–50}, the effects of leptin on gut microbiota have not been studied in females. Since estrogens and leptin interact to profoundly affect female metabolic homeostasis^{10,24}, it is important to understand the effects of leptin and its interaction with estrogens. Using a mouse model of obesity (*ob/ob*), the present study investigated if estradiol and leptin associate with longitudinal changes in gut microbiota and energy homeostasis in female mice on a HFD.

Results

Estradiol and *ob/ob* genotype altered weight gain and food intake in female mice on a HFD.

Body weights were collected every four days from the four experimental groups: control E2 ($n = 13$), control Veh ($n = 12$), *ob/ob* E2 ($n = 8$), and *ob/ob* Veh ($n = 8$). Longitudinal analysis of weight change showed that E2 decreased weight gain ($F = 15.67$, $p < 0.001$; ANOVA) in female mice fed a HFD. E2 treatment attenuated weight gain from day 11 through the end of the study (Fig. 1A,B). Within-genotype comparison showed that E2 attenuated weight gain in Het mice from days 11–35, compared to Veh mice. For *ob/ob* mice, E2 reduced weight gain on days 11, 15 and 27–35, and showed strong trends on days 19 ($p = 0.059$) and 23 ($p = 0.063$). Because the Het and *ob/ob* mice had different weights at the beginning of the study, the % weight gain was also analyzed to remove the bias due to existing differences in weights prior to hormone and diet manipulation. Similar to the effects on body weight, E2 treatment reduced % weight gain from days 11–35. In particular, E2-treated Het mice gained less % weight than Veh Het mice on days 11–35 (Fig. 1B). Within the *ob/ob* mice, E2 decreased % weight gain on days 7–15 and 27–35.

Genotype also affected weight gain ($F = 1119.79$, $p < 0.001$; ANOVA) and % weight gain on longitudinal measures ($F = 4.23$, $p = 0.047$; ANOVA). *ob/ob* mice weighed more than control Het mice at all time points regardless of E2 treatment. At the start of the study, *ob/ob* mice weighed approximately twice that of the Het mice, which persisted throughout the study. Finally, there was an interaction of E2 and genotype on body weight ($F = 4.21$, $p = 0.048$; ANOVA), with E2-treated Het mice gaining more % weight than E2-treated *ob/ob* mice on days 4 and 7 (Fig. 1B). The Veh mice did not differ in % weight gain between the two genotypes.

E2 treatment reduced HFD consumption ($F = 11.04$, $p = 0.002$; ANOVA) from days 7 to 19 (Fig. 1C). Analysis within each genotype showed that E2 attenuated food intake in *ob/ob* mice, but not in Hets, on days 7, 11 and 15. Comparison between lean Hets and *ob/ob* mice showed that obesity profoundly increased food intake ($F = 110.5$, $p < 0.001$, ANOVA). Het mice consumed less calories than *ob/ob* mice throughout the study. Within the E2-treated groups, an increase in food intake in *ob/ob* groups compared to E2 Het mice was observed only at the beginning (days 7 and 11) and end (days 27, 31 and 35) of the study. There was an interaction between genotype and treatment on food intake ($F = 5.52$, $p = 0.029$; ANOVA). We also observed a decrease in food intake on day 19 in *ob/ob* Veh mice, but not other groups, following surgery on day 16 for removal of BrdU osmotic pumps.

Estradiol and *ob/ob* genotype alter gut microbial diversity during HFD. To assess the impact of E2 treatment and obesity on α -diversity of the gut microbiota, 16S rDNA from fecal samples from *ob/ob* and Het mice were analyzed. The data set contained 137 samples after removing samples with less than 2,000 reads. The 16S rDNA targeted sequencing yielded 25,399 reads/sample on average (range 11,154–82,733). Clustering of

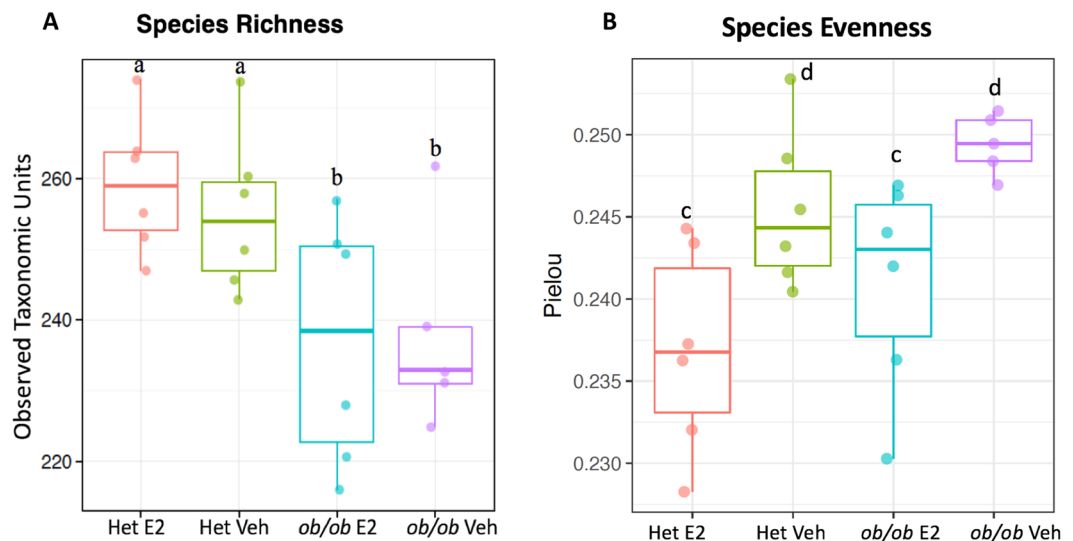


Figure 2. Estradiol and obesity reduce gut microbiota alpha diversity. **(A)** *ob/ob* genotype is associated with lower species richness as measured by Observed Taxonomic Units. **(B)** E2 treatment is associated with lower species evenness as measured by the Pielou's evenness index. "a" and "b" indicate groups with different species richness while "c" and "d" indicate groups with different species evenness ($p < 0.05$, linear regression). Het E2 ($n = 6$), Het Veh ($n = 6$), *ob/ob* E2 ($n = 6$) and *ob/ob* Veh ($n = 6$).

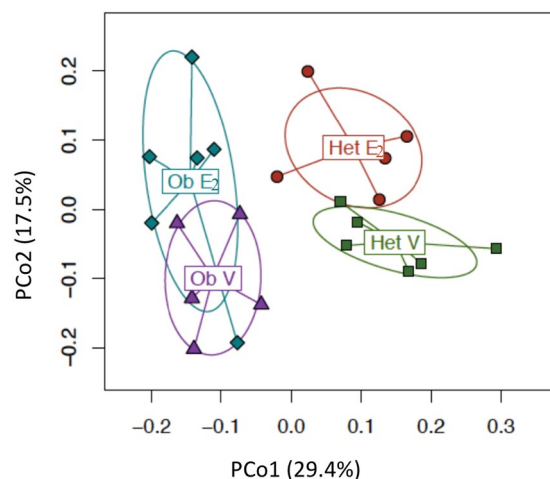


Figure 3. Gut microbial communities distinctly cluster as an effect of estradiol treatment and leptin. PCo1 and PCo2 clustering of each group. Bray-Curtis distance was used to calculate principal coordinates 1 and 2 and PERMANOVA, to calculate the association with leptin or E2 on aggregate data over all days. Ob E2 = E2-treated *ob/ob* ($n = 6$), Ob V = Vehicle *ob/ob* ($n = 6$), Het E2 = E2-treated Het ($n = 6$), and Het V = Vehicle Het mice ($n = 6$).

these 16S sequence tags produced 473 OTUs at 97% similarity level. The identified OTUs belonged to 10 phyla, 42 families and 62 genera based on the Greengenes database. During HFD, *ob/ob* mice had a lower number of total identified OTUs than Het mice, indicating that *ob/ob* mice had lower species richness than control mice (Fig. 2A; $p = 0.002$, linear regression). In addition, E2 treatment was associated with lower species evenness in both genotypes, suggesting that E2-treated mice have a more heterogeneous distribution of gut microbial communities than Veh mice (Fig. 2B; $p = 0.0008$).

The ordination plot based on Bray-Curtis distance, which measures differences due to relative abundances and composition, showed distinct clustering of microbial communities from the four experimental groups (Fig. 3; $p = 0.004$, PERMANOVA). The first and second principal coordinates (PCo1 and PCo2) explain 29.4% and 17.5% of the variation in the microbial communities, respectively. Genotype, most likely through obesity, accounted for most of the difference observed as reflected by PCo1 ($p = 4E-8$, t-test, Fig. 3). There was also a clear effect of E2 treatment, represented by PCo2 ($p = 0.001$, t-test, Fig. 3).

Day	Day 4	Day 7	Day 15	Day 23	Day 31	Day 35
Treatment	0.095	0.234	0.011*	0.044*	0.023*	0.022*
Genotype	0.081	0.004*	0.009*	0.001*	0.06	0.011*

Table 1. PERMANOVA P-values for individual days based on Bray-Curtis distance. *Indicate a p-value of <0.05.

Similar analyses were performed using Bray-Curtis distance measures on each day to characterize the temporal changes in gut microbiota. The effect of E2 on β -diversity was robust and continuously observed from the 2nd week of OVX (days 15–35). The effect of genotype was also present on days 7, 15, 23 and 35, and a trend was observed on the remaining two days (4 and 31) (Table 1).

Estradiol treatment alters relative abundances of intestinal microbiota. Gut microbial community composition was further analyzed on aggregate data from all days to identify the relative abundances at phylum, family and genus levels (Fig. 4). E2-mediated shifts in relative abundances was evident on many of these taxa levels. In all four experimental groups, the most prevalent phyla (>90%) were Firmicutes, Bacteroidetes, Actinobacteria and Tenericutes (Fig. 4A). The two most abundant families were S24-7 and Lachnospiraceae, within Bacteroidetes and Firmicutes, respectively (Fig. 4B). At the genus level, *Coprococcus* and *Ruminococcus* (both within phylum Firmicutes) were the most abundant (Fig. 4C).

To identify whether the differences in community structure were driven by differences in the relative abundances of particular microbial taxa, a differential abundance analysis based on overdispersed Poisson regression model was run on aggregate data to uncover the effects of treatment (Fig. 5A) and genotype (Fig. 5B). In the cladogram, the nodes in the first circle represent phyla, and the extending outer nodes in each level represent lower taxa within each phylum. A total of 26 taxa were differentially associated between E2 and Veh groups (Fig. 6A). Firmicutes were more associated with Veh treatment, while Bacteroidetes were associated with E2 treatment (Figs. 5A and 6A). Within Firmicutes, the class Erysipelotrichi, including *Allobaculum* and *Coprobacillus* spp., were more abundant in Veh than E2 mice. Similarly, the relative abundances of the class Bacilli, including its families Lactobacillaceae and Peptostreptococcaceae, were greater in Veh than E2 groups. Another family, Streptococcaceae, including *Lactococcus* spp., were also more associated with Veh mice. In contrast, the family Ruminococcaceae was more abundant in E2 mice. Within Bacteroidetes, the class Bacteroidia, and its lower taxa Bacteroidales and S24-7, were more abundant in E2 than Veh mice (Figs. 5A and 6A).

For the taxa that differed across groups as detected from the aggregated data, the effect of E2 treatment on relative abundances was analyzed for each day. A comprehensive list of the taxa, adjusted p-values (q-values) and fold changes between E2 and Veh mice for each day are provided in Supplemental Table 1. E2-treated mice resisted a decrease in relative abundances of Bacteroidetes compared to Veh mice on days 23 (q = 0.007) and 31 (q = 0.09) (Fig. 7A). A main driver of the shifts in Bacteroidetes was the family S24-7. E2-treated mice resisted a decrease in S24-7 abundances compared to Veh mice on day 23 (q = 0.015) (Fig. 7B).

Compared to Veh mice, E2-treated animals resisted changes in relative abundances of the phylum Firmicutes, with lower relative abundances of Firmicutes on days 23 (q = 0.01), 31 (q = 0.06) and 35 (q = 0.04) compared to Veh mice (Fig. 8A). Within Firmicutes, *Lactobacillus* spp. showed a significant reduction on day 31 (q = 0.05) in E2-treated mice (Fig. 8B).

Estradiol also altered the relative abundance of the phylum Actinobacteria from days 15 to 35. On these days, E2-treated mice resisted an increase in the relative abundances of Actinobacteria compared to Veh mice (Fig. 9A). The E2-mediated effect on Actinobacteria was due mostly to the family Coriobacteriaceae which resisted this increase also on days 15 (q = 0.001), 23 (q < 0.001) and 35 (q = 0.03) (Fig. 9B).

Obesity is associated with changes in relative abundances of gut microbiota. As the gut microbial communities of the female mice clustered separately based on obesity (in *ob/ob* genotype) (Fig. 3), we further analyzed the effects of obesity on relative abundances at the taxa levels (Fig. 5B). A total of 26 identified taxa showed a differential association between *ob/ob* and Het mice (Fig. 6B). Comparison of the gut microbial communities between genotypes revealed that Firmicutes, including its families Lactobacillaceae, Turibacteraceae, Peptostreptococcaceae, Dehalobacteriaceae, and Ruminococcaceae were more abundant in Het mice than *ob/ob* mice. At the genus level, *Lactobacillus*, *Turibacter*, *Dehalobacterium*, *Dorea*, *Ruminococcus*, and *Oscillospira* were more abundant in Het controls than *ob/ob*. Similarly, the phylum Actinobacteria and its family Coriobacteriaceae showed a greater abundance in control Het mice. In contrast, *Enterococcus*, *Coprococcus*, *Lachnospira*, *Anaerotruncus* and *Coprobacillus* spp. of the phylum Firmicutes were more abundant in *ob/ob* mice compared to Het mice (Figs. 5B and 6B).

Differential abundance analysis across days showed that genotype affected the relative abundances of multiple taxa (Supplemental Table 2). Leptin was associated with increases in the relative abundances of Bacteroidetes and its family S24-7 in Het mice at the beginning (day 4; q = 0.04), but by day 23 (after 19 days on HFD; q = 0.03), these were lower than in *ob/ob* mice (Fig. 7). Within Firmicutes, *Clostridium*, *Lactobacillus* and *Lactococcus* spp. were altered by genotype (Fig. 8). *Lactobacillus* and *Clostridium* spp. were more abundant in Het mice. The relative abundance of *Lactobacillus* increased in Het mice compared to *ob/ob* mice on days 4 (q = 0.01), 7 (q < 0.001) and 23 (q = 0.001) (Fig. 8B). Similarly, *Clostridium* was higher in Het mice on days 4 (q = 0.04), 7 (q = 0.01) and 15 (q < 0.001) than *ob/ob* mice (Fig. 8C). In contrast, *Lactococcus* was more abundant in *ob/ob* mice on days 7 (q = 0.01), 15 (q = 0.002) and 35 (q = 0.007) (Fig. 8D).

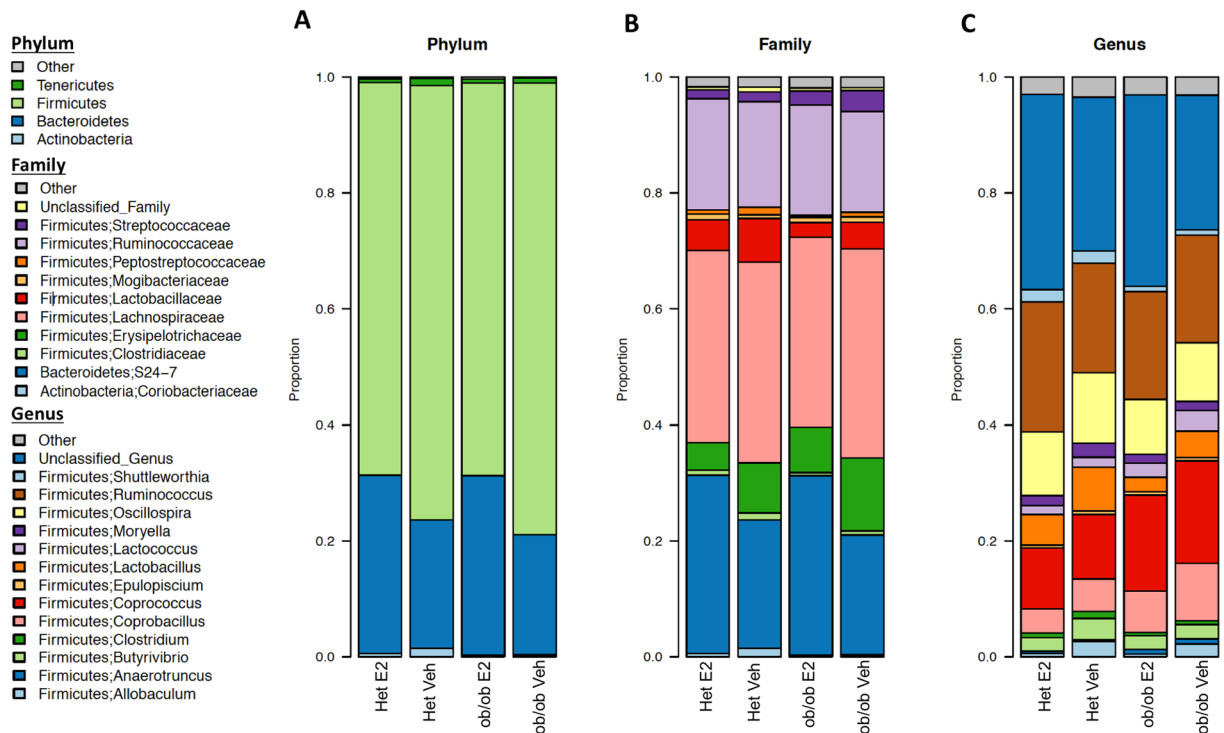


Figure 4. Gut microbiota associate with estradiol treatment and obesity at multiple taxa levels. Microbiota community structure at the (A) phylum, (B) family and (C) genus level, separated by estradiol treatment and *ob/ob* genotype. Data are shown as relative proportion of the taxa identified. Taxa with prevalence of >10% or with a maximum proportion of >0.2% were included. Het E2 (n = 6), Het Veh (n = 6), *ob/ob* E2 (n = 6), and *ob/ob* Veh mice (n = 6). An overdispersed Poisson regression was used to calculate associations of taxa with leptin and E2.

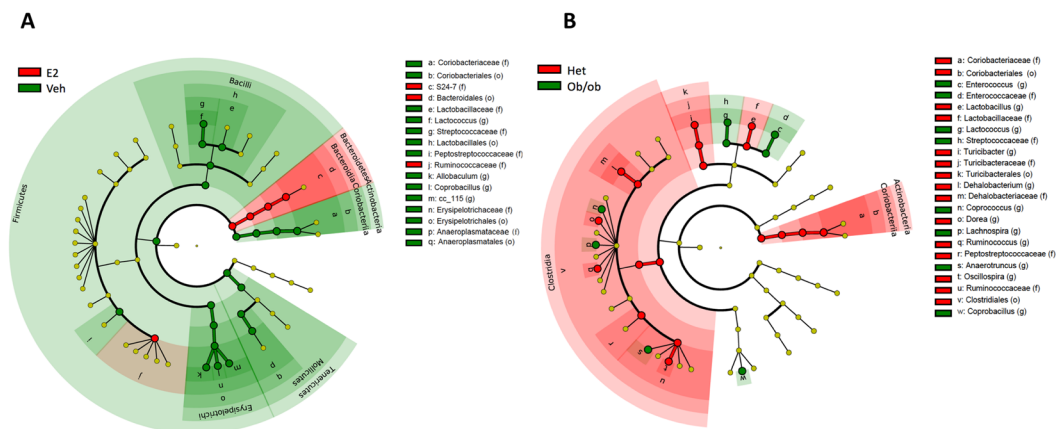


Figure 5. Estradiol and obesity associate with changes in the gut microbial composition. (A) Phyla and lower level taxa associated with estradiol (E2) or vehicle (Veh) treatment. (B) Phyla and lower level taxa associated with *ob/ob* or Het genotype. Het E2 (n = 6), Het Veh (n = 6), *ob/ob* E2 (n = 6) and *ob/ob* Veh (n = 6).

Obesity was also associated with altered relative abundance of the phylum Actinobacteria over time. Compared to *ob/ob* mice, Het mice had an increase in the relative abundance of Actinobacteria from days 7 to 35 (Fig. 9A). This leptin-dependent increase in Actinobacteria in Hets was due mostly to Coriobacteriaceae (family), also from days 7 to 35 (Fig. 9B), suggesting this microbial family is influenced by leptin or obesity status during HFD.

A subset of microbial taxa was affected by both E2 and leptin. In particular, Streptococcaceae, *Lactococcus* and *Coprobacillus* were decreased, whereas Ruminococcaceae were increased by E2 and leptin. In addition, these two hormones exerted opposing effects on other families, such that E2 decreased, and leptin (in Het controls) increased, abundances of Coriobacteriaceae, Lactobacillaceae and Peptostreptococcaceae.

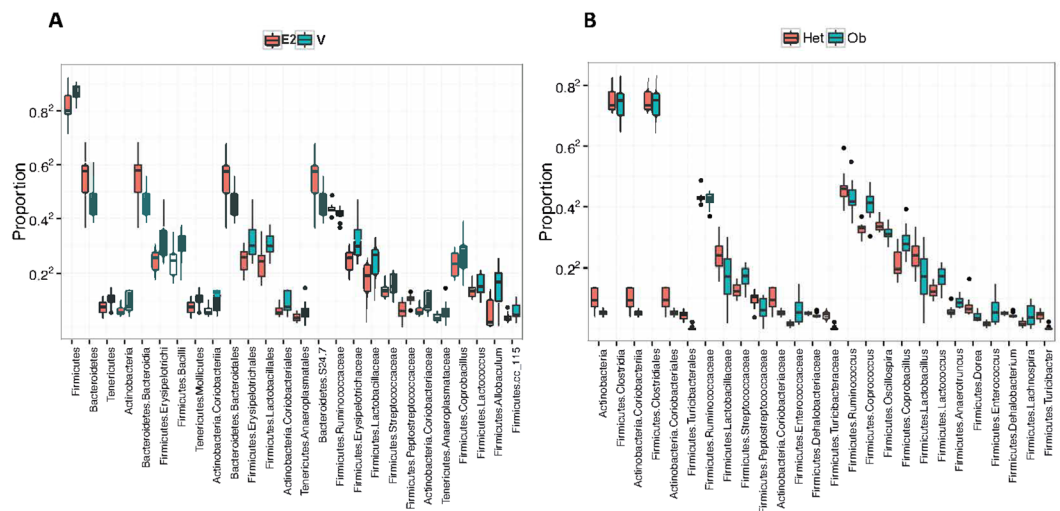


Figure 6. Gut microbiota associate with estradiol treatment and obesity at multiple taxa levels. Boxplots showing microbial taxa that are significantly altered by (A) Estradiol (E2) treatment and (B) genotype (overdispersed Poisson regression). Analysis was done on aggregate data from all 6 day points. Data are shown as relative proportion of the taxa identified. Taxa with prevalence of >10% or with a maximum proportion of >0.2% were included. E2-treated *ob/ob* (n = 6), E2-treated Het (n = 6), Veh *ob/ob* (n = 6), vehicle Het mice (n = 6).

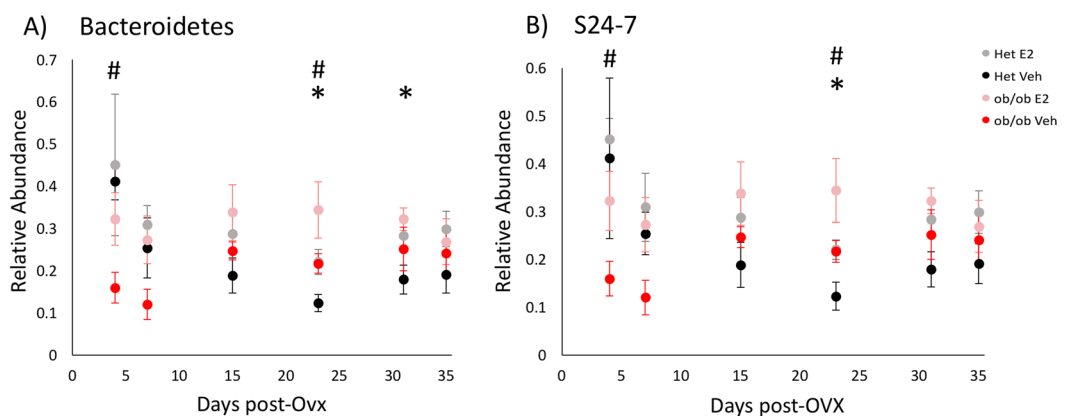


Figure 7. Estradiol and obesity associate with changes in the relative abundances of Bacteroidetes (phylum) and S24-7 (family) following the start of HFD. Relative abundances of the (A) the phylum Bacteroidetes and (B) its family S24-7 over time. *Indicates effects of E2 and # indicates effects of genotype ($q < 0.1$, overdispersed Poisson regression). Het E2 (n = 6), Het Veh (n = 6), *ob/ob* E2 (n = 6) and *ob/ob* Veh (n = 6). Error bars indicate ± SEM.

Discussion

Mounting evidence indicates profound effects of estrogens, leptin and gut microbiota on energy homeostasis^{10,25,51,52}. The present study investigated if estradiol and leptin-mediated energy regulation is associated with temporal changes in gut microbiota of females. Using leptin-deficient *ob/ob* female mice fed a HFD, we investigated the effects of estradiol and obesity (due to leptin deficiency) on body weight, energy intake, and gut microbiota in the present study. We found that E2 treatment decreases food intake and protects against HFD-induced weight gain in both *ob/ob* (obese) and Het (lean) females. *ob/ob* mice had increased HFD intake and greater body weight, compared to the Het controls. The differences in food intake and body weight between the two genotypes are reflected in their changes in gut microbiota. Both E2 treatment and genotype were associated with altered gut microbiota diversity. *ob/ob* female mice exhibited lower species richness, which supports previous studies that link obesity with a reduced gut microbiota diversity in humans and male mice^{53–55}. Interestingly, E2 treatment was associated with lower species evenness compared to Veh mice. At the phylum level, E2 treatment slowed down a HFD-induced decrease in Bacteroidetes and increases in Firmicutes and Actinobacteria compared to Veh controls. Leptin also was associated with changes in Bacteroidetes and Firmicutes and a profound increase in Actinobacteria. Many taxa were also associated with both leptin and E2. These findings suggest that E2 and leptin

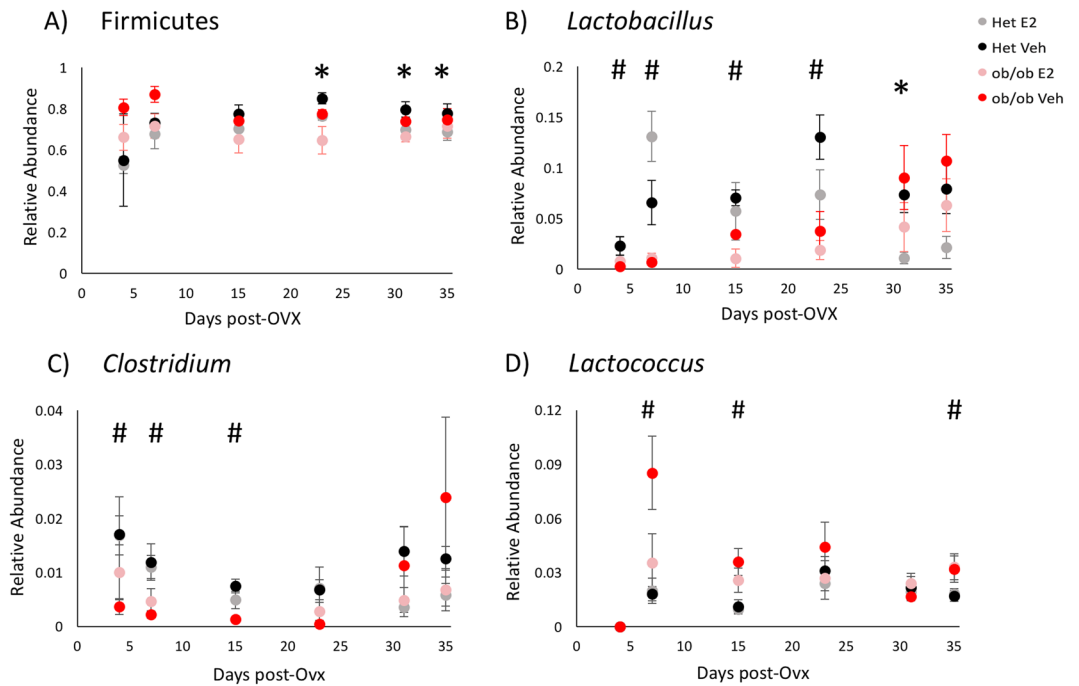


Figure 8. Estradiol and obesity associate with changes in the relative abundances of the phylum Firmicutes and its lower taxa. Relative abundances over time of the (A) phylum Firmicutes and genera (B) *Clostridium*, (C) *Lactobacillus* and (D) *Coprococcus*. *Indicates effects of E2 and # indicates effects of genotype ($q < 0.1$, overdispersed Poisson regression). Het E2 (n = 6), Het Veh (n = 6), ob/ob E2 (n = 6) and ob/ob Veh (n = 6). Error bars indicate \pm SEM.

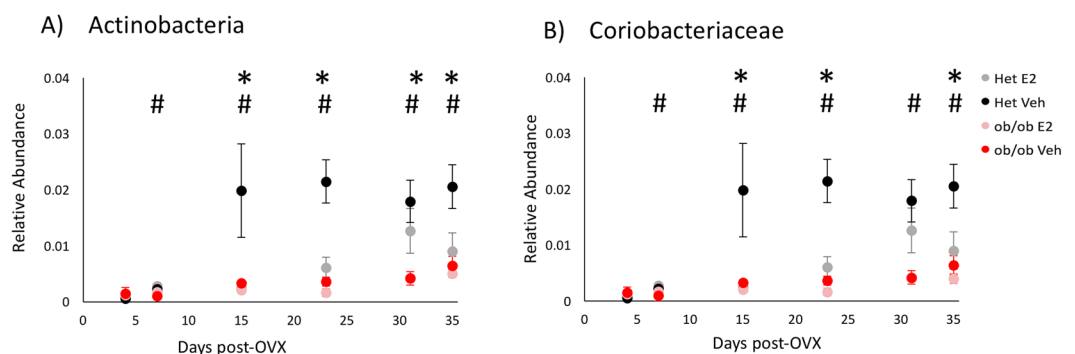


Figure 9. Estradiol and obesity resist increases in the relative abundances of the phylum Actinobacteria and its lower taxa. Relative abundances of the (A) phylum Actinobacteria and (B) family Coriobacteriaceae over time. *Indicates effects of E2 and # indicates effects of genotype ($q < 0.1$ overdispersed Poisson regression). Het E2 (n = 6), Het Veh (n = 6), ob/ob E2 (n = 6) and ob/ob Veh (n = 6). Error bars indicate \pm SEM.

can act independently, or interact together, to modulate gut microbiota to mediate energy regulation during HFD intake in females.

We and others have shown that E2 protects female mice from HFD-induced obesity^{10,11,13,56–59}. Estrogens exert protective effects by acting directly on brain^{10–12,60}, pancreas⁶¹, liver^{56,57,59,62}, adipose tissue⁵⁹, and muscle^{63,64} to regulate energy production and utilization. Moreover, long-term E2 treatment improves glucose tolerance and insulin sensitivity and attenuates lipid synthesis in liver in ob/ob female mice⁶⁵. The present findings suggest that the modulation of gut microbiota is another mechanism by which E2 mediates energy homeostasis. While E2 was associated with a decrease in the gut microbial evenness in the current study, an increase in microbial diversity has been reported in cycling rats and E2-treated female mice^{40,66}. These differences across studies could be due to fluctuating estrogens and other ovarian hormones in the cycling rats and/or species differences⁴⁰, and a much higher dose of estradiol (2.5 mg/day, consistent with levels at pregnancy) used in the mice⁶⁶. Alternatively, while a lower microbial diversity is usually associated with obesity and metabolic disorders^{67,68}, a change in relative abundance without any changes in microbial richness or evenness can also alter microbial homeostasis⁴⁷.

E2 attenuated a longitudinal shift in the two major phyla Bacteroidetes and Firmicutes, compared to the Veh controls. In particular, the order Bacteroidales, and its families S24-7 and Ruminococcaceae were positively associated with E2 treatment, whereas *Allobaculum* spp. were negatively associated. Many microbes that belong to S24-7 produce short chain fatty acids (SCFA; fermented products of dietary fibers) that protect against inflammation^{69,70}. In particular, SCFA, including butyrate, maintain a low pH in the gastrointestinal tract and aid in nutrient absorption and pathogen inactivation⁷¹. Interestingly, S24-7 is downregulated in inflammatory conditions, including Crohn's disease, colitis and type I diabetes⁷²⁻⁷⁴. While the current findings suggest that E2 provides protection against diet-induced metabolic disorders in females by maintaining healthy levels of microbes belonging to S24-7, it will be important in future studies to selectively manipulate these microbes to test their functions in energy regulation.

In the present study, E2 was associated with changes in Firmicutes, which is generally associated with obesity in rodents and humans⁷⁵. Studies in males have shown that a high caloric diet profoundly affects Firmicutes^{36,76,77}. Consistent with these findings in males, in the present study, Veh female mice on a HFD gained weight and had an increase in relative abundances of Firmicutes. However, E2-treated mice resisted this HFD-induced increase, suggesting that modulation of these microbes contributes to the regulation of energy homeostasis by estrogens. Firmicutes contain more OTUs that are efficient energy producers than Bacteroidetes, leading to increased calorie absorption and weight gain⁵². Additionally, some members of Firmicutes promote lipid droplet formation increasing fatty acid absorption and weight gain⁷⁸. In the current study, E2 was associated with changes in relative abundances of Firmicutes in both Het and *ob/ob* mice, suggesting that E2 affects this phylum independent of body weight and leptin levels.

The obese leptin-deficient (*ob/ob*) mice had less diverse microbiota as observed by species richness, suggesting that leptin is required for an enriched microbial ecosystem. In support, obesity is associated with a decreased microbial diversity in humans⁵³. Furthermore, a Danish study on men and women found that higher levels of leptin in obese populations were associated with a lower richness of the gut microbial communities, suggesting that optimum levels of leptin are associated with metabolic health and a more diverse gut microbiota⁵⁴. Taken together, the present results suggest that changes in leptin levels are associated with disruptions in metabolic and microbial homeostasis.

While *ob/ob* mice weighed almost twice that of Het mice in the beginning, the percent weight gain was greater in Veh Hets compared to Veh *ob/ob* mice. Interestingly, the relative abundance of the phylum Actinobacteria was greater in Hets compared to *ob/ob* mice, primarily due to an increase in its family Coriobacteriaceae. Actinobacteria is increased in obese humans and is associated with ulcerative colitis^{53,68}. More than two-thirds of the obesity-related human gut microbes belong to Actinobacteria^{53,79}. In particular, Coriobacteriaceae have been positively associated with increased cholesterol absorption in hamsters and obese humans^{80,81}. A profound increase in the relative abundance of Coriobacteriaceae in Veh Het mice, the group that showed the highest percent weight gain, but not in E2 Hets, suggests this OTU is associated with HFD-induced weight gain.

Estrogens elicit many effects on physiology and behavior by binding to their intracellular and membrane receptors⁸²⁻⁸⁵. While estrogen receptor- α (ER α) and ER β have been implicated in the effects of estrogens on metabolism⁸⁶⁻⁸⁸, ER α appears to be the primary contributor to energy balance^{86,89-91}. ER α knock-out mice exhibit increased visceral adiposity, impaired glucose tolerance and elevated insulin levels⁹¹. Furthermore, systemic activation of ER α , but not ER β , decreases food intake and body weight in female rats⁹⁰. While the effects of ER α on gut microbiota have not been investigated directly, a study using ER β knock-out female mice suggests that ER β influences gut microbiota in a diet-specific manner⁹². In support of this finding, ER β is expressed in human and mouse colon epithelium^{93,94}. Alternatively, given that estrogens reduce food intake¹⁰, it is also possible that E2 influences gut microbiota by altering nutrition availability.

Estrogens may also act as direct substrates for gut microbiota. For example, microbes with β -glucuronidase and β -glucosidase enzymes, including *Lactobacillus*, *Bifidobacterium*, and *Clostridium* spp., convert inactive estrogens into their active forms through deconjugation⁹⁵⁻⁹⁷. In addition, estrogens may alter metabolism and immune responses through direct actions on gut microbial metabolites. For example, E2 treatment protects against HFD-induced metabolic disorders by blocking the activation of lipopolysaccharides (LPS), the endotoxins produced by gram-negative microbes⁹⁸⁻¹⁰⁰. Additionally, E2 upregulates intestinal alkaline phosphatase, a protective enzyme in the gut epithelium that functions by attenuating pro-inflammatory signals⁴⁵. These studies, taken together with the present findings, suggest that estrogens can alter gut microbiota through multiple direct and indirect mechanisms to protect from metabolic disorders.

The mechanisms by which leptin affects gut microbiota are not well understood. Intestinal epithelium expresses leptin receptors (LepR), but ablation of these receptors does not alter gut microbiota or body weight in male mice¹⁰¹. In females, leptin may act in concert with ER β in the gut epithelium^{93,94}. While genotype effects may be primarily dependent on leptin, it is important to note that *ob/ob* mice are obese and Het mice are lean. Obesity is an independent modulator of gut microbiota. Obese and lean twins exhibit differences in gut microbial diversity⁵³, and the obese phenotype can be transferred to lean recipients through the transplant of gut microbiota from obese donors^{36,37}. Alternately, HFD and obesity directly alter the host transcriptome and epigenome by differentially activating enhancers and promoters in the intestinal epithelium¹⁰². In addition, female *ob/ob* mice have reduced estrogen levels and are sterile, and thus have altered hormone-dependent development^{26,103}. These findings suggest that leptin, acting directly, or indirectly via alteration of obesity status, affects host physiology, including gut microbial homeostasis. In future studies, it will be important to test the effects of leptin administration on the gut microbiota in HFD-fed ovariectomized *ob/ob* mice.

This study provides further insights into the potential roles of E2 and leptin in HFD-induced obesity and gut microbiota. While the identification of taxa that differ between treatments is the first step, their potential actions on energy metabolism will need to be explored in functional studies that manipulate the microbiota using fecal microbiota transplant, selective administration, or depletion of the target microbes. Given that many

metabolic diseases are characterized by disturbances in the gut microbiome, investigating how estradiol and leptin-dependent energy regulation associates with disruption in microbial homeostasis provides a foundation for future functional studies on the role of these hormones and the gut microbiome in metabolic disorders in women.

Materials and Methods

Animals. Seven week-old lean heterozygote (Het) and obese *ob/ob* (leptin-deficient) mice (24 mice/genotype) were purchased from Jackson Laboratory (Bar Harbor, Maine), kept on a 12:12 light:dark cycle and allowed to acclimate at the Wellesley College animal facility for a week. Het mice, which have a metabolic phenotype similar to wildtype mice as evidenced by normal serum glucose and insulin, body temperature, and energy expenditure¹⁹, were used as controls²¹ to account for genetic background. While phenotypically very similar, Het mice have lower leptin levels than wildtype mice¹⁰⁴. All mice were ovariectomized (OVX) and subcutaneously implanted with a silastic capsule containing 17 β -Estradiol (E2, #E8875, Sigma; 50 μ g in 25 μ l of 5% ethanol/sesame oil) or Veh (5% ethanol/sesame oil) resulting in the following four experimental groups: control E2, 2) control Veh, 3) *ob/ob* E2, and 4) *ob/ob* Veh. Following surgery, mice were pair-housed with a mouse of the same genotype and treatment.

As part of another study to identify newborn cells, on day 7 mice underwent intracranial surgery for the implantation of an intraventricular cannula attached to a subcutaneous osmotic pump filled with bromodeoxyuridine. The osmotic pumps were removed after 10 days. On day 35, animals received an intraperitoneal injection of leptin (5 mg/kg) 45 minutes prior to being euthanized for assessment of acute leptin response in the brain, unrelated to the present study. While possible, it is unlikely that leptin administration 45 minutes prior to sacrifice would have effects on gut microbiota. All procedures were approved by the Institutional Animal Care and Use Committee of Wellesley College and performed in accordance with National Institutes of Health Animal Care and Use Guidelines.

Food intake and body weight measurements and fecal sample collection. Following OVX (day 0), all mice were maintained on a standard diet for three days (13.5% calories from fat, Purina, #5001) before switching to a HFD (58.4% calories from fat, Teklad, #03584) on day 4 and maintained on a HFD for the remainder of the study. Throughout the experiment, food intake and body weights were recorded every four days from heterozygote control (Het) E2 (n = 13), Het Veh (n = 12), *ob/ob* E2 (n = 8) and *ob/ob* Veh (n = 7) mice. Food intake was calculated per cage, from a total of Het E2 (n = 6), Het Veh (n = 6), *ob/ob* E2 (n = 6) and *ob/ob* Veh (n = 6) cages, with each cage containing two mice of the same treatment. For cages in which one mouse died, the amount of food eaten by the remaining mouse was doubled to match with cages containing two mice. Similarly, for microbiota analysis, fecal samples from each cage was counted as n = 1, resulting in Het E2 (n = 6), Het Veh (n = 6), *ob/ob* E2 (n = 6) and *ob/ob* Veh (n = 6). Fecal samples were collected on days 4, 7, 15, 23, 31, and 35 and immediately stored at -80°C .

DNA extraction from fecal samples, 16S rDNA sequencing and bioinformatics processing.

DNA was extracted from fecal samples using a MO BIO PowerSoil DNA Isolation Kit (Valencia, CA) with minor adjustments to the manufacturer's protocol. A 5-minute incubation with the elution buffer before centrifugation was added to increase the DNA yield. The quality and quantity of the DNA samples were measured using Nanodrop (Thermo Scientific, Waltham, MA). The samples were stored at -20°C until sequencing.

The V3-V4 region of the 16S rDNA was amplified using the following universal 16S rDNA primers: forward 341F (5'-CCTACGGGAGGCAGCAG-3') and reverse 806R (5'-GGACTACHVGGGTWTCTAAT-3') with sequence adapters on both primers and sample-specific Golay barcodes on the reverse primer¹⁰⁵. The PCR products 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 (MO BIO, Carlsbad, CA), and again quantified using the Qubit (Invitrogen, Carlsbad, CA). The pooled samples were then sequenced using paired-end v2 chemistry using Illumina Miseq sequencing technology (Illumina, San Diego, CA) at the Microbiome Core, Mayo Clinic (Rochester, Minnesota).

Paired R1 and R2 sequence reads were then processed via the *hybrid-denovo* bioinformatics pipeline, which clustered a mixture of good-quality paired-end and single-end reads into operational taxonomic units (OTUs) at 97% similarity level¹⁰⁶. OTUs were assigned taxonomy using the RDP classifier trained on the Greengenes database (v13.5)¹⁰⁷. A phylogenetic tree based on FastTree algorithm was constructed based on the OTU representative sequences¹⁰⁸. Singleton OTUs as well as samples with less than 2,000 reads were removed from downstream analysis.

Statistical analysis. *Food intake and body weight data analysis.* Repeated measures ANOVA (SPSS, v.24) was performed to examine the effects of treatment and genotype on food intake and body weight over time. After a main effect was confirmed, ANOVA without corrections was performed on measures from each day to identify the days with effects of one or both variables. One-way ANOVAs were then conducted to identify differences between specific groups when effects of treatment or genotype were present. Differences were considered statistically significant at $p < 0.05$.

16S rDNA sequence analysis. Analyses were first performed on the aggregated data, in which sequence reads from each mouse across all days were aggregated. To study specific longitudinal trends, stratified analyses on individual days were also performed if needed.

Diversity analysis. Both α -diversity and β -diversity were analyzed on the rarefied OTU data. α -diversity (within-sample diversity) reflects species richness and evenness within the microbial populations. Two representative α -diversity measures were investigated: the observed number of OTUs, an index of the species richness, and the Pielou's evenness index¹⁰⁹. A multiple linear regression model ("lm" function in R) was used to test the association between α -diversity (outcome) and treatment/genotype (covariates, both included in the model).

β -diversity (between-sample diversity) reflects the shared diversity between bacterial populations in terms of ecological distances; pair-wise distance measure allows quantification of the overall compositional difference between samples. Different β -diversity measures provide distinctive views of the community structure. The β -diversity measures were calculated using Bray-Curtis dissimilarity, which measures differences in bacterial composition based on taxa abundances ("vegdist" function in the R "vegan" package, v2.4-3). To test the association between β -diversity measures and treatment or genotype, we used PERMANOVA (999 permutations, "adonis" function in the R "vegan" package, v2.4-3) when adjusting the effect of the other covariate. Ordination plots were generated using principal coordinate analysis (PCoA) on the distance matrix ("cmdscale" function in R)¹¹⁰.

Taxa analysis. Differential abundance analyses were performed at the phylum, class, order, family and genus levels. Taxa with prevalence less than 10% or with a maximum proportion less than 0.2% were excluded from analysis to reduce the number of tests. An overdispersed Poisson model was fitted to the taxa counts with treatment and genotype as covariates ("glm" function in R)¹¹¹. Wald test was used to assess significance. To account for variable sequencing depths, the GMPR size factor was estimated and used as an offset (log scale) in the regression model¹¹². False discovery rate (FDR) control (B-H procedure, 'p.adjust' in R) was used to correct for multiple testing at each taxonomical level, and FDR-adjusted p-values (q-values) < 0.1 were considered significant¹¹³. The differential taxa were visualized on a cladogram using GraPhlAn¹¹⁴. All statistical analyses were performed in R (v. 3.3.2, R Development Core Team).

Received: 9 August 2019; Accepted: 12 December 2019;

Published online: 27 December 2019

References

- Asarian, L. & Geary, N. Modulation of appetite by gonadal steroid hormones. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **361**(1471), 1251–1263 (2006).
- Wade, G. N. & Gray, J. M. Gonadal effects on food intake and adiposity: a metabolic hypothesis. *Physiol. Behav.* **22**(3), 583–593 (1979).
- Clegg, D. J. Minireview: the year in review of estrogen regulation of metabolism. *Mol. Endocrinol.* **26**(12), 1957–1960 (2012).
- McCarthy, M. M. Estradiol and the developing brain. *Physiol. Rev.* **88**(1), 91–124 (2008).
- Pfaff, D., Waters, E., Khan, Q., Zhang, X. & Numan, M. Minireview: estrogen receptor-initiated mechanisms causal to mammalian reproductive behaviors. *Endocrinology* **152**(4), 1209–1217 (2011).
- Blaustein, J. D. Neuroendocrine regulation of feminine sexual behavior: lessons from rodent models and thoughts about humans. *Annu. Rev. Psychol.* **59**, 93–118 (2008).
- Roquer, J., Campello, A. R. & Gomis, M. Sex differences in first-ever acute stroke. *Stroke* **34**(7), 1581–1585 (2003).
- Carr, M. C. The emergence of the metabolic syndrome with menopause. *J. Clin. Endocrinol. Metab.* **88**(6), 2404–2411 (2003).
- Guo, S. S., Zeller, C., Chumlea, W. C. & Siervogel, R. M. Aging, body composition, and lifestyle: the Fels Longitudinal Study. *Am. J. Clin. Nutr.* **70**(3), 405–411 (1999).
- Bless, E. P., Reddy, T., Acharya, K. D., Beltz, B. S. & Tetel, M. J. Oestradiol and diet modulate energy homeostasis and hypothalamic neurogenesis in the adult female mouse. *J. Neuroendocrinol.* **26**(11), 805–816 (2014).
- Bless, E. P. *et al.* Adult neurogenesis in the female mouse hypothalamus: Estradiol and high-fat diet alter the generation of newborn neurons expressing estrogen receptor alpha. *eNeuro.* **3**(4), 1–11 (2016).
- Brown, L. M. & Clegg, D. J. Central effects of estradiol in the regulation of food intake, body weight, and adiposity. *J. Steroid. Biochem. Mol. Biol.* **122**(1–3), 65–73 (2010).
- Xu, Y. *et al.* Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell. Metab.* **14**(4), 453–465 (2011).
- Erickson, J. C., Hollopeter, G. & Palmiter, R. D. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science* **274**(5293), 1704–1707 (1996).
- Vaisse, C. *et al.* Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat. Genet.* **14**(1), 95–97 (1996).
- Sahu, A. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Front. neuroendocrinology* **24**(4), 225–253 (2003).
- Green, E. D. *et al.* The human obese (OB) gene: RNA expression pattern and mapping on the physical, cytogenetic, and genetic maps of chromosome 7. *Genome Res.* **5**(1), 5–12 (1995).
- Coleman, D. L. Diabetes-Obesity Syndromes in Mice. *Diabetes* **31**, 1–6 (1982).
- Pelleymounter, M. A. *et al.* Effects of the obese Gene Product on Body Weight Regulation in ob/ob Mice. *Science* **269**, 540–543 (1995).
- Mistry, A. M., Andrew, G., Swick, A. G. & Romsos, D. R. Leptin Rapidly Lowers Food Intake and Elevates Metabolic Rates in Lean and ob/ob Mice. *J. Nutr.* **127**(10), 2065–2072 (1997).
- Shimomura, K. *et al.* Is leptin a key factor which develops obesity by ovariectomy? *Endocr. J.* **49**(4), 417–423 (2002).
- Steppan, C. M., Crawford, D. T., Chidsey-Frink, K. L., Ke, H. & Swick, A. G. Leptin is a potent stimulator of bone growth in ob/ob mice. *Regul. Pept.* **92**(1–3), 73–78 (2000).
- Clegg, D. J., Riedy, C. A., Smith, K. A., Benoit, S. C. & Woods, S. C. Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* **52**(3), 682–687 (2003).
- Clegg, D. J., Brown, L. M., Woods, S. C. & Benoit, S. C. Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes* **55**(4), 978–987 (2006).
- Gao, Q. & Horvath, T. L. Cross-talk between estrogen and leptin signaling in the hypothalamus. *Am. J. Physiol. Endocrinol. Metab.* **294**(5), E817–826 (2008).
- Chehab, F. F., Lim, M. E. & Lu, R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat. Genet.* **12**(3), 318–320 (1996).

27. Ainslie, D. A. *et al.* Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. *Int. J. Obes. Relat. Metab. Disord.* **25**(11), 1680–1688 (2001).
28. Soto, M. *et al.* Gut microbiota modulate neurobehavior through changes in brain insulin sensitivity and metabolism. *Mol. Psychiatry* (2018).
29. Ley, R. E. *et al.* Evolution of mammals and their gut microbes. *Science* **320**(5883), 1647–1651 (2008).
30. Round, J. L. & Mazmanian, S. K. The gut microbiome shapes intestinal immune responses during health and disease. *Nat. Rev. Immunology* **9**(5), 313–323 (2009).
31. Kang, C. *et al.* Gut Microbiota Mediates the Protective Effects of Dietary Capsaicin against Chronic Low-Grade Inflammation and Associated Obesity Induced by High-Fat Diet. *mBio.* **8**(3) (2017).
32. Clarke, G. *et al.* Minireview: Gut microbiota: the neglected endocrine organ. *Mol. Endocrinol.* **28**(8), 1221–1238 (2014).
33. Markle, J. G. *et al.* Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* **339**(6123), 1084–1088 (2013).
34. Tetel, M. J., de Vries, G. J., Melcangi, R. C., Panzica, G. & O'Mahony, S. M. Steroids, Stress, and the Gut Microbiome-Brain Axis. *J. Neuroendocrinol.* (2017).
35. Cani, P. D. Human gut microbiome: hopes, threats and promises. *Gut* **67**(9), 1716–1725 (2018).
36. Turnbaugh, P. J. *et al.* The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* **1**, 6ra14 (2009).
37. Ridaura, V. K. *et al.* Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **341**(6150), 1241214 (2013).
38. Ellekilde, M. *et al.* Transfer of gut microbiota from lean and obese mice to antibiotic-treated mice. *Sci. Rep.* **4**, 5922 (2014).
39. Cho, I. *et al.* Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* **488**(7413), 621–626 (2012).
40. Moreno-Indias, I. *et al.* Neonatal Androgen Exposure Causes Persistent Gut Microbiota Dysbiosis Related to Metabolic Disease in Adult Female Rats. *Endocrinology* **157**(12), 4888–4898 (2016).
41. Org, E. *et al.* Sex differences and hormonal effects on gut microbiota composition in mice. *Gut. microbes* **0** (2016).
42. Foster, J. A. & McVey Neufeld, K. A. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci.* **36**(5), 305–312 (2013).
43. Jasarevic, E., Morrison, K. E. & Bale, T. L. Sex differences in the gut microbiome-brain axis across the lifespan. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **371**(1688), 20150122 (2016).
44. Mueller, S. *et al.* Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl. Env. Microbiol.* **72**(2), 1027–1033 (2006).
45. Kaliannan, K. *et al.* Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. *Microbiome* **6**(1), 205 (2018).
46. Yurkovetskiy, L. *et al.* Gender bias in autoimmunity is influenced by microbiota. *Immunity* **39**(2), 400–412 (2013).
47. Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proc. Natl Acad. Sci. USA* **102**(31), 11070–11075 (2005).
48. Murphy, E. F. *et al.* Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut* **59**(12), 1635–1642 (2010).
49. Ley, R. E., Turnbaugh, P. J., Klein, S. & Gordon, J. I. Microbial ecology: human gut microbes associated with obesity. *Nature* **444**(7122), 1022–1023 (2006).
50. Yang, M. *et al.* Gut Microbiota Composition and Structure of the Ob/Ob and Db/Db Mice. *Int. J. Endocrinol.* **2019**, 1394097 (2019).
51. Blaustein, J. D. & Wade, G. N. Ovarian influences on the meal patterns of female rats. *Physiol. Behav.* **17**(2), 201–208 (1976).
52. Turnbaugh, P. J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**(7122), 1027–1031 (2006).
53. Turnbaugh, P. J. *et al.* A core gut microbiome in obese and lean twins. *Nature* **457**(7228), 480–484 (2009).
54. Le Chatelier, E. *et al.* Richness of human gut microbiome correlates with metabolic markers. *Nature* **500**(7464), 541–546 (2013).
55. Ravussin, Y. *et al.* Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obes. (Silver Spring, Md.)* **20**(4), 738–747 (2012).
56. Camporez, J. P. *et al.* Cellular mechanism by which estradiol protects female ovariectomized mice from high-fat diet-induced hepatic and muscle insulin resistance. *Endocrinology* **154**(3), 1021–1028 (2013).
57. Riant, E. *et al.* Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology* **150**(5), 2109–2117 (2009).
58. Mamounis, K. J., Hernandez, M. R., Margolies, N., Yasrebi, A. & Roepke, T. A. Interaction of 17beta-estradiol and dietary fatty acids on energy and glucose homeostasis in female mice. *Nutr. Neurosci.* **1–14** (2017).
59. Bryzgalova, G. *et al.* Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am. J. Physiol. Endocrinol. Metab.* **295**(4), E904–912 (2008).
60. Gao, Q. & Horvath, T. L. Neurobiology of feeding and energy expenditure. *Annu. Rev. Neurosci.* **30**, 367–398 (2007).
61. Tian, J. P. & Mauvais-Jarvis, F. Molecular mechanisms of estrogen receptors' suppression of lipogenesis in pancreatic beta-cells. *Endocrinology* **153**(7), 2997–3005 (2012).
62. Yasrebi, A., Rivera, J. A., Krumm, E. A., Yang, J. A. & Roepke, T. A. Activation of Estrogen Response Element-Independent ERalpha Signaling Protects Female Mice From Diet-Induced Obesity. *Endocrinology* **158**(2), 319–334 (2017).
63. Ribas, V. *et al.* Skeletal muscle action of estrogen receptor α is critical for the maintenance of mitochondrial function and metabolic homeostasis in females. *Sci. Transl. Med.* (2016).
64. Campbell, S. E., Mehan, K. A., Tunstall, R. J., Febbraio, M. A. & Cameron-Smith, D. 17beta-estradiol upregulates the expression of peroxisome proliferator-activated receptor and lipid oxidative genes in skeletal muscle. *J. Mol. Endocrinol.* (2003).
65. Gao, H. *et al.* Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol. Endocrinol.* **20**(6), 1287–1299 (2006).
66. Benedek, G. *et al.* Estrogen protection against EAE modulates the microbiota and mucosal-associated regulatory cells. *J. Neuroimmunol.* **310**, 51–59 (2017).
67. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. Diversity, stability and resilience of the human gut microbiota. *Nature* **489**(7415), 220–230 (2012).
68. Peters, B. A. *et al.* A taxonomic signature of obesity in a large study of American adults. *Sci. Rep.* **8**(1), 9749 (2018).
69. Ormerod, K. L. *et al.* Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. *Microbiome* **4**(1), 36 (2016).
70. Rios-Covián, D. *et al.* Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Front Microbiol.* **7** (2016).
71. Macfarlane, G. T. & Macfarlane, S. Bacteria, colonic fermentation, and gastrointestinal health. *J. AOAC Int.* **95**(1), 50–60 (2012).
72. Krych, L., Nielsen, D. S., Hansen, A. K. & Hansen, C. H. Gut microbial markers are associated with diabetes onset, regulatory imbalance, and IFN-gamma level in NOD mice. *Gut microbes* **6**(2), 101–109 (2015).
73. Rooks, M. G. *et al.* Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J.* **8**(7), 1403–1417 (2014).
74. Morgan, X. C. *et al.* Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* **13**(9), R79 (2012).

75. Bäckhed, F. *et al.* The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl Acad. Sci. USA* **101**(44), 15718–15723 (2004).
76. Hildebrandt, M. A. *et al.* High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* **137**(5), 1716–1724 e1711-1712 (2009).
77. Zhang, C. *et al.* Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. *ISME J.* **6**(10), 1848–1857 (2012).
78. Semova, I. *et al.* Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host Microbe* **12**(3), 277–288 (2012).
79. Lepage, P. *et al.* Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* **141**(1), 227–236 (2011).
80. Martínez, I. *et al.* Diet-Induced Alterations of Host Cholesterol Metabolism Are Likely To Affect the Gut Microbiota Composition in Hamsters. *Appl. Environ. Microbiol.* (2013).
81. Zhang, H. *et al.* Human gut microbiota in obesity and after gastric bypass. *Proc. Natl Acad. Sci. USA* **106**, 2365–2370 (2009).
82. Jensen, E. V. *et al.* A two-step mechanism for the interaction of estradiol with rat uterus. *Proc. Natl Acad. Sci. USA* **59**(2), 632–638 (1968).
83. Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S. & Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl Acad. Sci. USA* **93**(12), 5925–5930 (1996).
84. Tetel, M. J. & Pfaff, D. W. Contributions of estrogen receptor- α and estrogen receptor- β to the regulation of behavior. *Biochim. Biophys. Acta* **1800**(10), 1084–1089 (2010).
85. Mani, S. K. & Oyola, M. G. Progesterone signaling mechanisms in brain and behavior. *Front. Endocrinol. (Lausanne)* **3**, 7 (2012).
86. Frank, A., Brown, L. M. & Clegg, D. J. The role of hypothalamic estrogen receptors in metabolic regulation. *Front. Neuroendocrinol.* **35**(4), 550–557 (2014).
87. Qiu, J. *et al.* A G-protein-coupled estrogen receptor is involved in hypothalamic control of energy homeostasis. *J. Neurosci.* **26**(21), 5649–5655 (2006).
88. Park, C. J. *et al.* Genetic rescue of nonclassical ER α signaling normalizes energy balance in obese ER α -null mutant mice. *J. Clin. Invest.* **121**(2), 604–612 (2011).
89. Santollo, J., Katzenellenbogen, B. S., Katzenellenbogen, J. A. & Eckel, L. A. Activation of ER α is necessary for estradiol's anorexigenic effect in female rats. *Horm. Behav.* **58**(5), 872–877 (2010).
90. Santollo, J., Wiley, M. D. & Eckel, L. A. Acute activation of ER α decreases food intake, meal size, and body weight in ovariectomized rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**(6), R2194–R2201 (2007).
91. Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor- α knockout mice. *Proc. Natl Acad. Sci. USA* **97**(23), 12729–12734 (2000).
92. Menon, R. *et al.* Diet complexity and estrogen receptor beta status affect the composition of the murine intestinal microbiota. *Appl. Environ. Microbiol.* **79**(18), 5763–5773 (2013).
93. Enmark, E. *et al.* Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J. Clin. Endocrinol. Metab.* **82**(12), 4258–4265 (1997).
94. Campbell-Thompson, M., Lynch, I. J., Bhardwaj, B. Expression of estrogen receptor (ER) subtypes and ER β isoforms in colon cancer. *Cancer Res* (2011).
95. McIntosh, F. M. *et al.* Phylogenetic distribution of genes encoding beta-glucuronidase activity in human colonic bacteria and the impact of diet on faecal glycosidase activities. *Environ. Microbiol.* **14**(8), 1876–1887 (2012).
96. Kwa, M., Plottel, C. S., Blaser, M. J. & Adams, S. The Intestinal Microbiome and Estrogen Receptor-Positive Female Breast Cancer. *J. Natl. Cancer Inst.* **108**(8) (2016).
97. Flores, R. *et al.* Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. *J. Transl. Med.* **10**, 253 (2012).
98. Cani, P. D. *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57**(6), 1470–1481 (2008).
99. Cani, P. D. *et al.* Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**(7), 1761–1772 (2007).
100. Blasco-Baque, V. *et al.* High-fat diet induces periodontitis in mice through lipopolysaccharides (LPS) receptor signaling: protective action of estrogens. *PLoS One* **7**(11), e48220 (2012).
101. Rajala, M. W. *et al.* Leptin acts independently of food intake to modulate gut microbial composition in male mice. *Endocrinology* **155**(3), 748–757 (2014).
102. Qin, Y. *et al.* An obesity-associated gut microbiome reprograms the intestinal epigenome and leads to altered colonic gene expression. *Genome Biol.* **19**(1), 7 (2018).
103. Ng, K. Y., Yong, J. & Chakraborty, T. R. Estrous cycle in ob/ob and ovariectomized female mice and its relation with estrogen and leptin. *Physiol. Behav.* **99**(1), 125–130 (2010).
104. Chung, W. K. *et al.* Heterozygosity for Lepob or Leprdb affects body composition and leptin homeostasis in adult mice. *Am. J. Physiol.* (1998).
105. Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA* **108**(Suppl 1), 4516–4522 (2011).
106. Chen, X. *et al.* Hybrid-denovo: a de novo OTU-picking pipeline integrating single-end and paired-end 16S sequence tags. *GigaScience* **7**(3), 1–7 (2018).
107. Cole, J. R. *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**(Database issue), D633–642 (2014).
108. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* **26**(7), 1641–1650 (2009).
109. Pielou, E. C. The measurement of diversity in different types of biological collections. *J. Theor. Biol.* **13**, 131–144 (1966).
110. McArdle, B. H. & Anderson, M. J. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**(1), 290–297 (2001).
111. Chen, J. *et al.* Altered gut microbiota in female mice with persistent low body weights following removal of post-weaning chronic dietary restriction. *Genome Med.* **8**(1), 103 (2016).
112. Chen, L. *et al.* GMPR: A robust normalization method for zero-inflated count data with application to microbiome sequencing data. *PeerJ* **6**, e4600 (2018).
113. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. B (Methodol.)* **57**, 289–300 (1995).
114. Asnicar, F., Weingart, G., Tickle, T. L., Huttenhower, C. & Segata, N. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. *PeerJ* **3**, e1029 (2015).

Acknowledgements

This work was funded by NIH R01 DK61935 (M.J.T.) and a Re-Entry award (E.P.B.) from the National Institute of Diabetes and Digestive and Kidney Diseases. The authors thank Dr. Cassandra Pattanayak, Director of the Quantitative Analysis Institute at Wellesley College, for input on statistical analysis.

Author contributions

K.D.A. conducted the study, analyzed data, wrote the manuscript. X.G. conducted the study, analyzed data, wrote the manuscript. E.P.B. designed and conducted the study and analyzed data. J.C. analyzed data and wrote the manuscript. M.J.T. designed the study, analyzed data, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-019-56723-1>.

Correspondence and requests for materials should be addressed to K.D.A.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019