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RESEARCH PAPER/REPORT

Effects of exposure to bisphenol A and ethinyl estradiol on the gut microbiota of parents and their offspring in a rodent model

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ABSTRACT

Gut dysbiosis may result in various diseases, such as metabolic and neurobehavioral disorders. Exposure to endocrine disrupting chemicals (EDCs), including bisphenol A (BPA) and ethinyl estradiol (EE), especially during development, may also increase the risk for such disorders. An unexplored possibility is that EDC-exposure might alter the gut microbial composition. Gut flora and their products may thus be mediating factors for the disease-causing effects of these chemicals. To examine the effects of EDCs on the gut microbiome, female and male monogamous and biparental California mice (*Peromyscus californicus*) were exposed to BPA (50 mg/kg feed weight) or EE (0.1 ppb) or control diet from periconception through weaning. 16s rRNA sequencing was performed on bacterial DNA isolated from fecal samples, and analyses performed for P_0 and F_1 males and females. Both BPA and EE induced generational and sex-dependent gut microbiome changes. Many of the bacteria, e.g. *Bacteroides*, Mollicutes, Prevotellaceae, Erysipelotrichaceae, *Akkermansia, Methanobrevibacter, Sutterella*, whose proportions increase with exposure to BPA or EE in the P_0 or F_1 generation are associated with different disorders, such as inflammatory bowel disease (IBD), metabolic disorders, and colorectal cancer. However, the proportion of the beneficial bacterium, *Bifidobacterium*, was also elevated in fecal samples of BPA- and EE-exposed F_1 females. Intestinal flora alterations were also linked to changes in various metabolic and other pathways. Thus, BPA and EE exposure may disrupt the normal gut flora, which may in turn result in systemic effects. Probiotic supplementation might be an effective means to mitigate disease-promoting effects of these chemicals.

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Introduction

Gut dysbiosis is increasingly being recognized to be a key initiator of many disease processes, ranging from metabolic, cardiovascular, reproductive, and neurobehavioral disorders.[1-3] Consequently, it has been proposed that various axes linked to the gut and microbiome exist, such as a gut-microbiome-brain axis.[1] Dysbiosis linked to changes in the intestinal flora could be due to direct effects of the bacteria, bacterial products, including metabolites and virulence factors (e.g., lipopolysaccharide- LPS), bacterial-induced changes in the host intestines, including increased gut permeability, or through production of hormone-like compounds. It is uncertain whether endocrine disruptors and other environmental chemicals may disrupt normal gut microbial populations.

To date, exposure to arsenic, nanoparticles, and most recently, lead (Pb) are the few environmental chemicals reported to alter the gut microbiome.[4-8] Perinatal exposure of mice to Pb alters the gut microbiota, which has been correlated with body weight changes in males but not females.[9] Estrogenic compounds can change the composition of the vaginal flora.[10,11] Depletion of estrogen due to ovariectomy leads to gut microbial shifts in low and high aerobic capacity rats.[12] Women with elevated hydroxylated

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estrogen metabolites possess a more diverse gut microbiome.[13] Thus, EDCs, including BPA and EE might affect the gut and other microbiomes.

BPA is in a wide assortment of household and everyday use items, including plastic storage containers, cardboard products, and credit card receipts. Current BPA production is estimated at ~15 billion pounds annually and is anticipated to rise dramatically in coming years.[14] Its inability to breakdown easily in the environment and prevalence[15] ensure long term exposure.[16] BPA is measurable in the urine of 93% of the US population,[17] and it has also been identified in fetal plasma, placental tissue,[18] and breast milk.[19] EE is the estrogenic chemical present in birth control pills. The un-metabolized form can be excreted in the urine, and thereby, may also accumulate in various environmental sources.[20] BPA and EE exposure, especially during the perinatal period, is linked to a variety of diseases, including cardiovascular, metabolic, reproductive, and neurobehavioral disorders.[21-23] To our knowledge, no prior studies have considered the impact of these chemicals on the gut microbiome, even though speculation exists that environmental chemical-induced gut microbiome shifts may serve as the underlying etiology for metabolic and possibly other diseases.[24]

To address this critical gap in our understanding, we exposed male and female California mice P_0 parents (*Peromyscus californicus*) to BPA or EE through the diet. This species was selected as their genetic outbred state and social organization, monogamous and biparental, might better reflect most human societies. Additionally, we have previously shown that developmental exposure to BPA and EE can lead to various sex-dependent behavioral deficits, including in exploration, territorial marking, parenting ability, and decreased voluntary physical activity.[25-27] Further, California mice may serve as a good animal model for human metabolic disorders, in particular T2M.[28] When F_1 pups were weaned at 30 d of age and prior to all pups being placed on the control diet, fecal samples were collected from P_0 parents and F_1 sons and daughters to determine whether generational differences in exposure affect the gut microbiota composition. Thus, the fecal samples were collected at the time when the pups were exposed to the varying respective diets (BPA, EE, or control). Figure 1 provides a model of the study design.

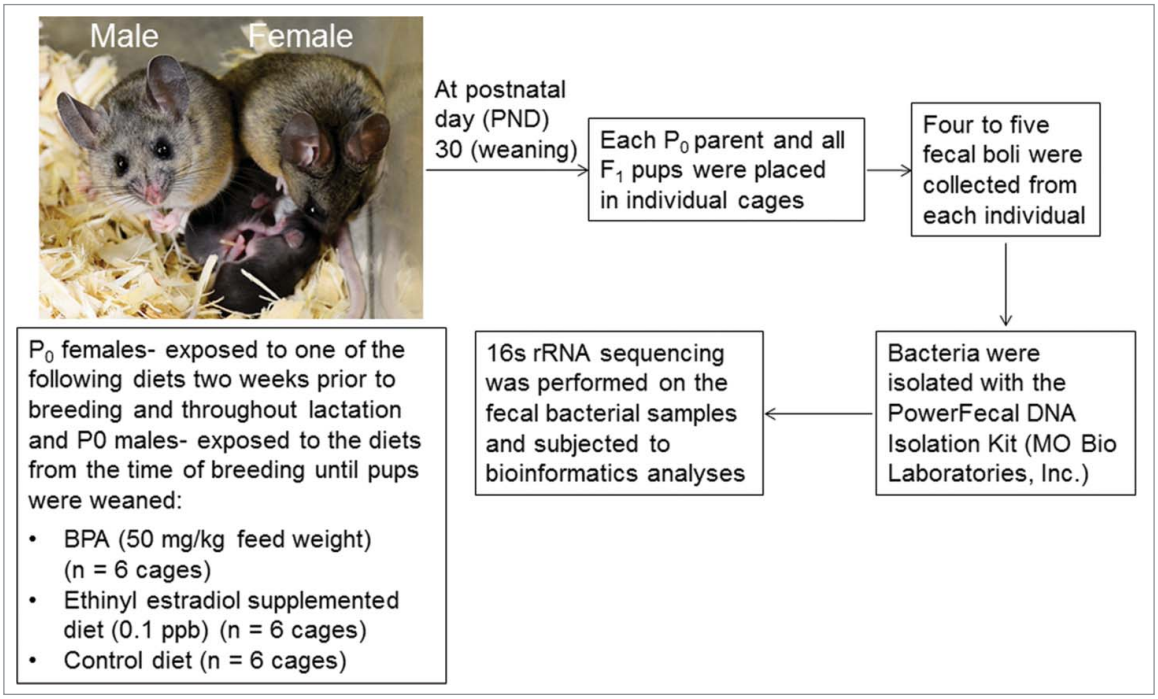

Figure 1. Experimental model design. P_0 California mice females were placed on the respective diets 2 weeks prior to breeding to to P_0 California mice males, and both parents were maintained on the respective diets throughout the perinatal period. At the time, pups were weaned, each animal was placed in a separate cage and fecal samples were collected from P_0 parents and one F_1 male and female pup in each litter. Thus, samples were collected prior to all F_1 pups being placed post-weaning on the control diet, and fecal samples were only collected at this one time point. Bacteria were then isolated from each sample, 16s rRNA sequencing done, and bioinformatics analyses performed.

Results

Absence of a cage or litter effect on the gut microbiota

To determine whether the P_0 and F_1 generational microbiota data should consider potential litter effects or whether it was sufficient to consider each individual as the statistical unit, we first determined whether there were any cage or litter effects. Using PERMANOVA of PCoA analyses, we failed to observe a significant effect ($p \leq 0.05$) of the cage upon the relationship between samples, either within treatment (Control: $p = 0.495$, BPA: $p = 0.279$, EE: $p = 0.296$) or when all treatments were analyzed together ($p = 0.108$). Thus, all of the remaining analyses detailed below use the individual animal as the statistical unit.

Influence of exposure to BPA and EE on the gut microbiota of P_0 females and males

When the 16S rRNA sequencing results were compared using Greengenes Version 13_8 (which is available through QIIME, http://qiime.org/home_static/dataFiles.html ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz), no clear distinctions were evident in the various bacterial classes based on treatment in either P_0 females or males (Fig. 2A and B). The PCoA analysis revealed no overt differences between the 3 groups in P_0 females or males (Fig. 2C and D, PERMANOVA for P_0 females = 0.3518 and PERMANOVA for P_0 males = 0.5917. Additionally, measures of α-diversity, including Chao1 and Shannon indices as well as rarefaction analysis, were similar among P_0 and F_1 (Fig. S1).

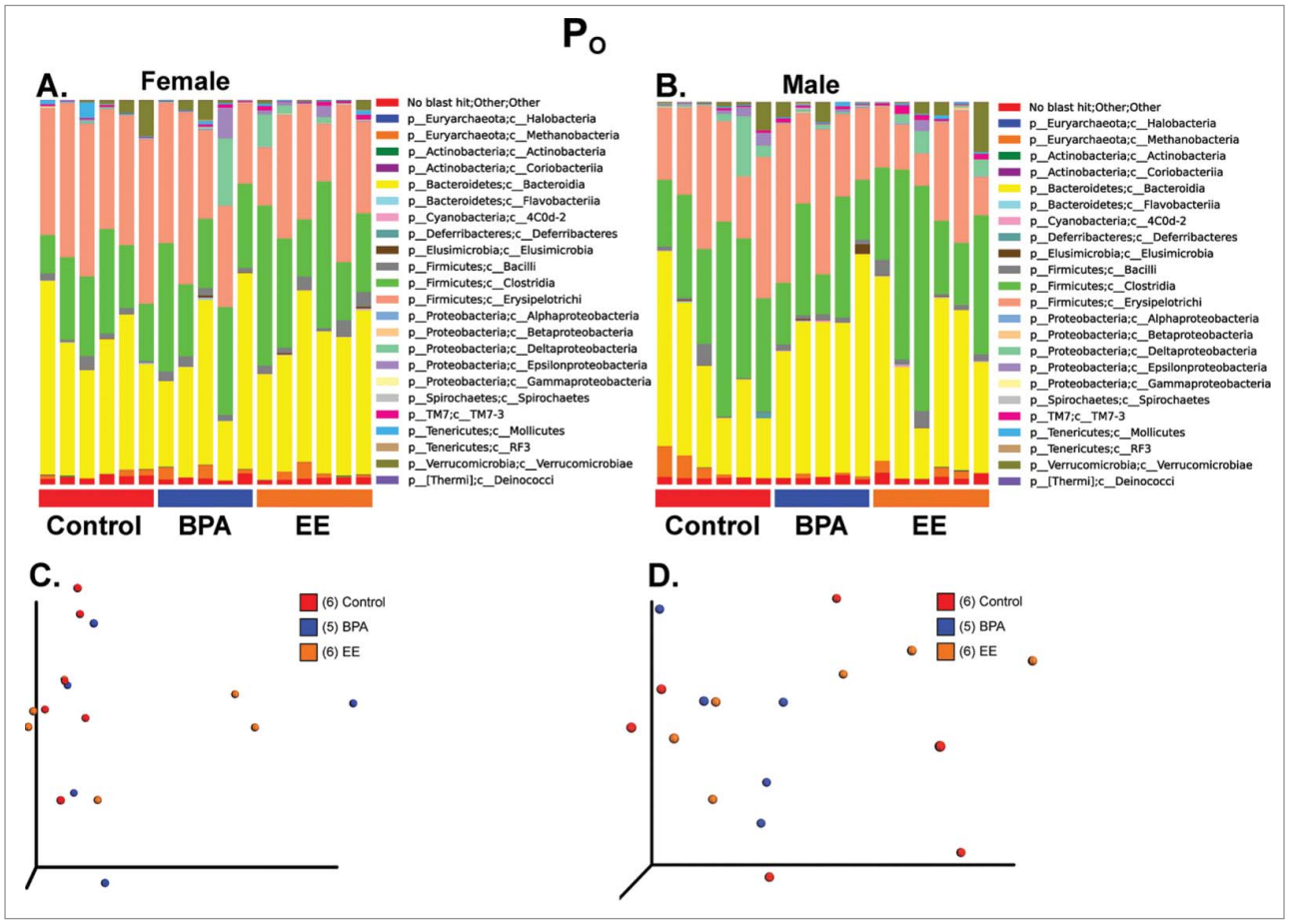

Figure 2. Bar plot and PCoA analysis of fecal microbiome data from P_0 females and males. A) Bar plot analysis of the most abundant bacterial classes in all 3 treatment groups for P_0 females. B) Bar plot analysis of the most abundant bacterial classes in all 3 treatment groups for P_0 males. C) PCoA analysis for P_0 females. PERMANOVA p value = 0.3518. D) PCoA analysis for P_0 males. PERMANOVA p value = 0.5917.

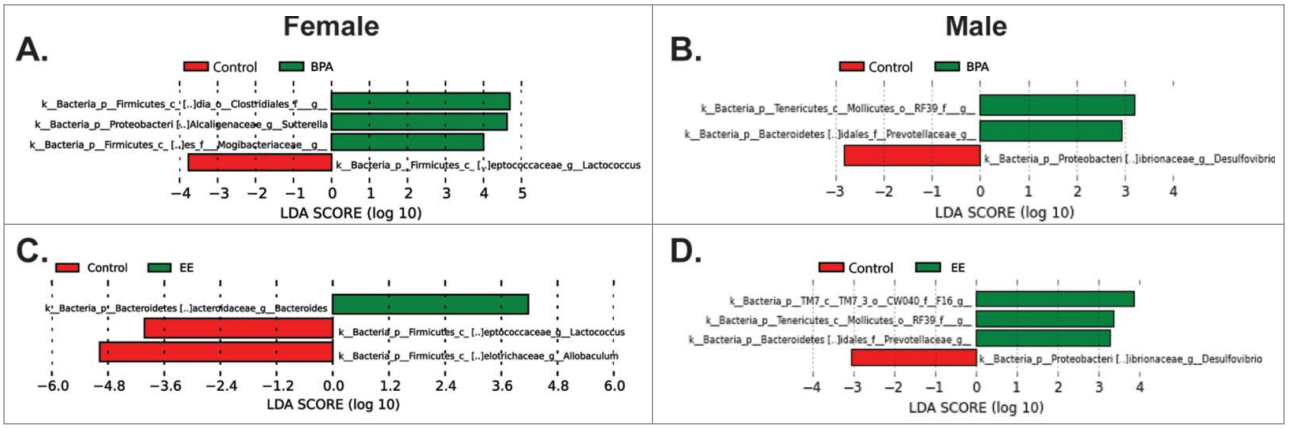

Figure 3. LEfSe analysis of fecal microbiome data from P_0 females and males. A) Comparison of BPA females to control females. The linear discriminant analysis (LDA) score of *Bacteroides* (green bar) was greater in BPA compared to control females. B) Comparison of BPA males and control males. LDA scores that are greater in BPA males are shown in green, whereas the one (*Delsufiovibrio*) LDA score elevated in control males is depicted in red. C) Comparison of EE females to control females. LDA scores that are greater in EE females are shown in green, whereas the LDA scores elevated in control females are in red. C) Comparison of EE males to control males. LDA scores that are greater in EE males are shown in green, whereas the LDA scores elevated in control males are in red. Only LDA scores ≥ 2 are listed.

To examine for subtle genera differences, P_0 data were analyzed by using linear discriminant analysis effect size (LEfSe) analysis.[29] When comparing control females to BPA females, *Lactococcus spp*. was increased in the control group; whereas, Mogibacteriaceae, *Sutterella spp*, and Clostridiales were increased in the BPA group (Fig. 3A). Comparison of control males to BPA males, revealed that Mollicutes and Prevotellaceae were characteristic of BPA males; whereas, *Desulfovibrio spp* was more representative of control males (Fig. 3B). When control females were compared to EE-exposed females, *Bacteroides spp* was increased in EE females. In contrast, *Lactococcus spp* and *Allobaculum spp* were distinctive of control females (Fig. 3C). Mollicutes and Prevotellaceae were representative of EE-exposed males compared to control males, but *Desulfovibrio spp* was characteristic of control males (Fig. 3D).

Based on the genera that differed between BPA/EE versus control P_0 females and males, correlation analyses were performed for various KEGG pathways. Comparison of control females to BPA females revealed that decreased abundance of *Sutterella spp*. and Clostridiales was inversely associated with histidine metabolism, tryptophan metabolism, lysine degradation, tropane-piperidine- pyridine alkaloid biosynthesis, stilbenoid-diarylheptanoid-gingerol biosynthesis, nitrogen metabolism, NOD-like receptor signaling pathway, antigen processing and presentation, linoleic acid metabolism, arachidonic acid metabolism, riboflavin metabolism, and polycyclic aromatic hydrocarbon degradation; whereas, these bacterial changes in controls were positively associated with glycolysis/gluconeogenesis, starch and sucrose metabolism, butanoate metabolism, pentose-glucoronate interconversions, carbohydrate digestion and absorption, transcription related proteins, and phosphanate and phosphanate metabolism to list a few examples (Fig. S2). While the bacterial differences between control and BPA females was associated with several trends for metabolic and other differences in the BPA group, none of them reached statistical significance (Fig. S2).

Assessments of P_0 control *vs*. BPA males revealed that a decrease in Prevotellaceae abundance in the former group was correlated with several up and down-regulated metabolic pathways; whereas this OTU was only associated with increased glycerophosholipid metabolism in the BPA group (Fig. S3). Alterations in *Desulfovibrio* and Mollicutes abundance in control males were also correlated with several pathway alterations. In BPA males, increased abundance in Mollicutes positively correlated with changes in secretion.

Comparison of P_0 control to EE females revealed that decreased abundance of Bacteroides in the former was positively correlated with changes in amino acid metabolism, citrate cycle (TCA cycle), and lipid biosynthesis proteins; whereas, amino sugar and nucleotide sugar metabolism, peptidases were negatively correlated with reductions in this bacterium

(Fig. S4). *Allobaculum* abundance was increased in control relative to EE females, and this change was negatively associated with flavone and flavonol biosynthesis, and starch and sucrose metabolism. Increased abundance of *Bacteroides spp* in P_0 EE females was positively associated with arginine and proline metabolism, glycine, serine, and threonine metabolism, butirosin and neomycin biosynthesis, citrate cycle (TCA cycle), metabolism of cofactors and vitamins, lipoic acid metabolism, and limonene and pinene degradation to list a few examples (Fig. S4). In contrast, amino sugar and nucleotide sugar metabolism, peptidases, phosphotransferase system (PTS), and nucleotide metabolism were inversely associated with an increase in this bacterium for EE females. Decreased abundance of Allobaculum also led to several pathway associations.

When comparing control to EE males, changes in the abundance of Prevotellaceae, Delsulfovibrio, an uncharacterized bacterium, and Mollicutes in the former were positively and negatively associated with several pathways (Fig. S5). Significant pathway changes, however, were only correlated with an uncharacterized bacterium in EE-exposed males.

Influence of exposure to BPA and EE on the gut microbiota of F_1 females and males

No overt divisions were evident in the various bacterial classes based on treatment in either F_1 females or males (Fig. 4A and B). The PCoA analysis revealed no extreme differences between the 3 groups in F_1 females or males (Fig. 4C and D, PERMANOVA for F_1 females = 0.5492 and PERMANOVA for F_1 males = 0.3936). To examine for subtle differences, F_1 results were then further analyzed with LEfSe.[29] Abundance of *Oxalobacter spp* was increased in F_1 control females relative to F_1 BPA females, who showed

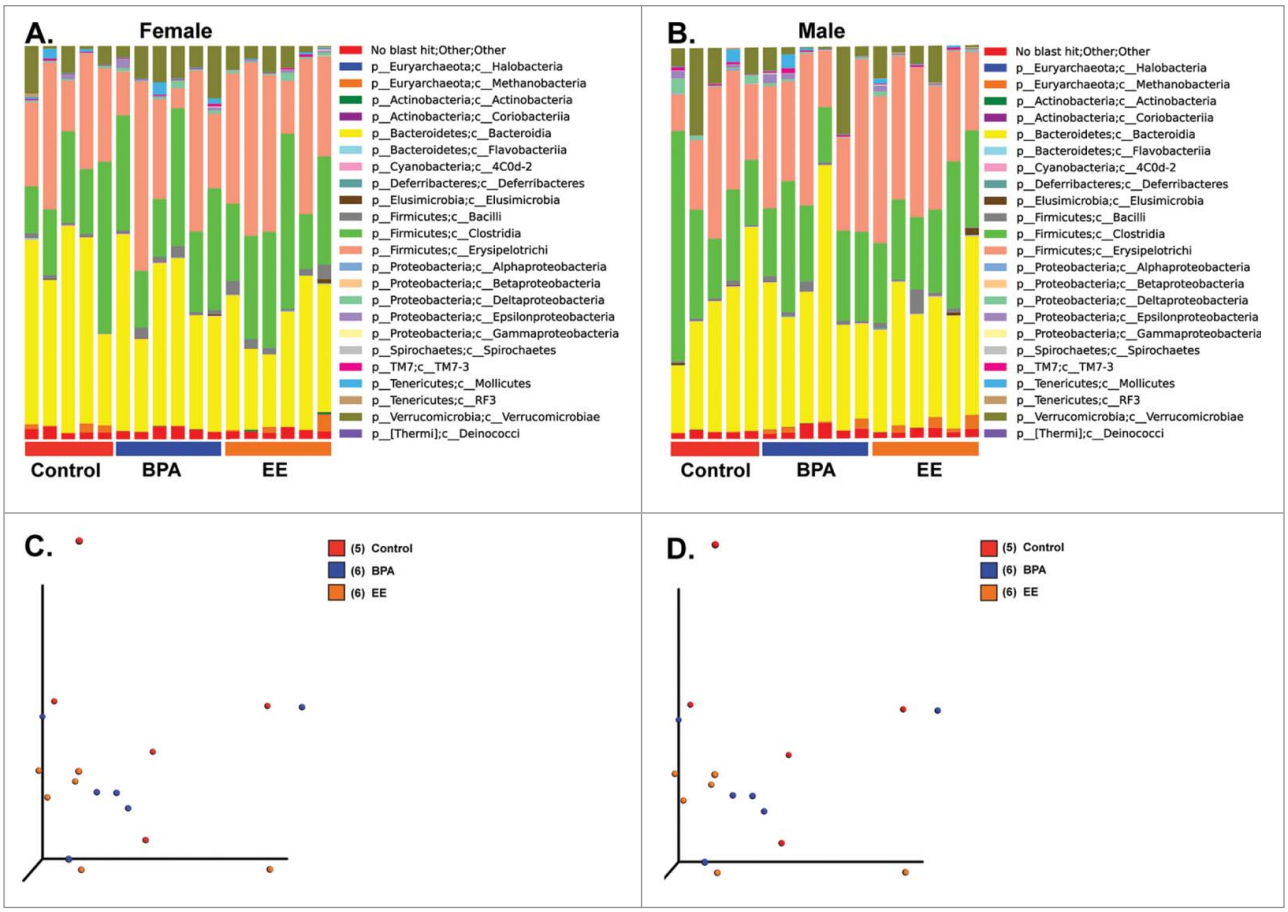

Figure 4. Bar plot and PCoA analysis of fecal microbiome data from F_1 females and males. A) Bar plot analysis of the most abundant bacterial classes in all 3 treatment groups for F_1 females. B) Bar plot analysis of the most abundant bacterial classes in all 3 treatment groups for F_1 males. C) PCoA analysis for F_1 females. PERMANOVA p value = 0.5492. D) PCoA analysis for F_1 males. PERMANOVA p value = 0.3936.

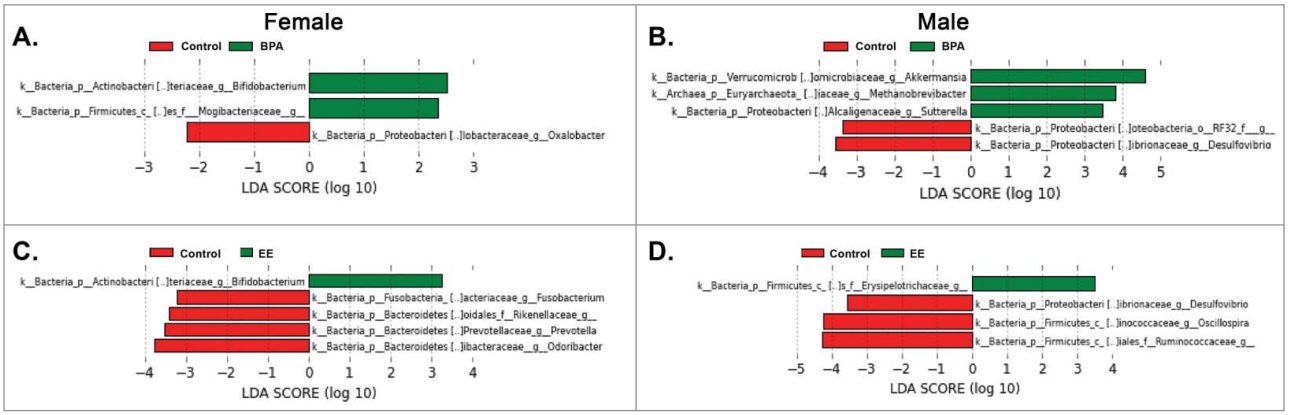

Figure 5. LEfSe analysis of fecal microbiome data from F_1 females and males. A) Comparison of BPA females to control females. LDA scores that are greater in BPA females are shown in green; whereas, the one (*Oxalobacter*) elevated LDA score in control females is depicted in red B) Comparison of BPA males and control males. LDA scores that are greater in BPA males are shown in green, whereas LDA scores increased in control males are depicted in red. C) Comparison of EE females to control females. The one LDA score (*Bifidobacterium*) that is greater in EE females is shown in green, whereas those LDA scores elevated in control females are in red. C) Comparison of EE males to control males. The one LDA score (Erysipelotrichaceae) higher in EE males is shown in green; whereas, those LDA scores elevated in control males are in red. Only LDA scores $\geq$ 2 are listed.

increased proportion of *Bifidobacterium spp* and Mogibacteriaceae (Fig. 5A). Comparison of the bacterial genera identified in F_1 BPA males and F_1 control males revealed several differences. *Akkermansia spp, Methanobrevibacter spp, Sutterella spp* abundance increased in the former. However, the abundance of a Proteobacteria and *Desulfovibrio spp* was greater in the latter (Fig. 5B).

Bifidobacterium spp abundance was increased in F_1 EE females compared to control females, who in contrast had increased abundance of *Fusobacterium spp*, Rikenellaceae, *Prevotella spp, Odoribacter spp* (Fig. 5C). Erysiopelotrichaceae abundance was greater in F_1 EE males compared to F_1 control males, where *Delsulfovibrio spp, Oscillospira spp*, and Ruminococcaceae levels were elevated (Fig. 5D).

As with the P_0 data, correlation analyses were performed for various KEGG pathways based on the microbiota that differed between controls and BPA/EE females and males. Comparison of F_1 control to BPA-exposed females revealed that an increased abundance of *Oxalobacter* in the former was positively linked with galactose metabolism, RIG-I-like receptor signaling pathway, and ether lipid metabolism; whereas, tropane, piperidine, and pyridine alkaloid biosynthesis, other ion-coupled transporters, and nitrogen metabolism were negatively associated with in the abundance of this bacterium (Fig. S6). Increased abundance of *Bifidobacterium* and Mogibacteriaceae in BPA-exposed females resulted in both positive and negative correlations for various pathways. For instance, fatty acid and arachidonic acid metabolism were positively associated with an elevation in *Bifidobacterium* abundance in this group.

Comparison of F_1 control to BPA-exposed males demonstrated changes in Methanobrevibacter and *Desulfiovibrio* abundances correlated with several up and downregulated metabolic and other pathways in both groups (Fig. S7). In F_1 BPA-exposed males, an increase in *Akkermansia* abundance associated with several pathways, such as caffeine, insulin signaling pathway, sulfur metabolism, and steroid hormone biosynthesis positively correlated with this bacterium's abundance.

Comparison of F_1 EE to control females revealed that an increase in *Bifidobacterium* abundance in the former was positively associated with renin-angiotensin system but negatively correlated with secretion system (Fig. S8). Increased abundance of *Fusobacterium spp*, Rikenellaceae, *Prevotella spp, Odoribacter spp* in control females led to both positive and negative associations. For instance, an increase in *Odoribacter spp* abundance was negatively correlated with caffeine metabolism, steroid biosynthesis, and fatty acid elongation in mitochondria.

When F_1 control male results were compared to EE-exposed males, an increase in *Desulfiovibrio* abundance in the former was linked to several both positive and negative pathway associations (Fig. S9). In

contrast, increased Erysipelotrichaceae abundance in F_1 EE-exposed males associated with several negative pathway changes, including penicillin and cephalosporin biosynthesis, caffeine metabolism, ubiquitin system, steroid biosynthesis, and fatty acid elongation in mitochondria.

Comparison of microbiota in BPA and EE exposed groups for P_0 and F_1 generations

To determine whether there were any microbiota differences between BPA and EE-exposed animals, these 2 groups were directly compared for the P_0 and F_1 generations. There were no microbiota differences between P_0 BPA and P_0 EE females. In P_0 males, the only difference was that *Ruminococcus spp*, were greater in the EE compared to BPA group (Fig. S10A). In F_1 females, *Lachnobacterium spp*. and *Prevotella spp* were increased in the BPA compared to EE group (Fig. S10B). In F_1 males, *Parabacteroides spp*. and *Sutterella spp*, were more abundant in the BPA compared to EE group (Fig. S10C).

Comparison of generational differences to the different diets

To determine whether the different diets induced generation-dependent differences that varied according to sex, gut microbiota populations were compared between P_0 vs. F_1 males and females exposed to the same diet. Surprisingly, even the control diet led to generation-dependent genera differences for both females and males. For females in this group, Actinobacteria, *Bifidobacterium spp*., *Streptococcus spp*., and *Lactococcus spp*., were more abundant in the P_0 generation; whereas, Fusobacteriaceae, *Prevotella spp*., and *Anaeroplasma spp*., were more plentiful in the F_1 generation (Fig. S11A). In control males, Coriobacteriaceae and Christensenellaceae were greater in the P_0 generation (Fig. S11B). In contrast, *Clostridium spp*., Prevotellaceae, and Rikenellaceae were more abundant in F_1 control males.

When comparing across generations, there were no differences in P_0 *vs.* F_1 females in the BPA-supplemented group. However, several genera were different in P_0 vs. F_1 males in the BPA group (Fig. S11C). For instance, Alphaproteobacteria, Mollicutes, and Cyanobacteria were enriched in P_0 BPA males; whereas Fusobacteriales, *Sutterella spp*., and *Akkermansia spp*. were greater in F_1 BPA males. Comparison of P_0 to F_1 females exposed to EE revealed that only genera in the P_0 group were elevated, and these included Mollicutes and Cyanobacteria (Fig. S11D). In EE-exposed males, Alphaproteobacteria, *Oxalobacter spp*, and Christensenellaceae were more abundant in the P_0 generation; whereas Erysipelotrichaceae was elevated in the F_1 generation (Fig. S11E).

Comparison of sex differences in the P_0 and F_1 generations in response to the different diets

To determine whether there were sex-dependent differences in both generations to the different diets, the gut microbiota of males and females within the same generation and exposed to the same diet were compared with LEfSe analysis.[29] In the P_0 generation, the control diet resulted in males showing more abundance of Desulfovibrionaceae, Christensenellaceae, and *Ruminococcus spp*. (Fig. S12A). In contrast, P_0 control females possessed greater amounts of Rickenellaceae, Prevotellaceae, and *Allobaculum spp*. No differences were detected between P_0 males and females exposed to BPA. In P_0 EE-exposed individuals, *Oxalobacter spp*. and another Proteobacteria were more abundant in males, but no bacteria were identified as being greater in females compared to males in this group Fig. S12B).

In the F_1 generation control group, a Proteobacteria, Ruminococcaceae, and *Oscillospira spp* were more abundant in males, but no genera were greater in females relative to males in this group (Fig. S12C). For F_1 BPA-exposed individuals, *Methanobrevibacter spp*. was more abundant in males, whereas, a Cyanobacterium was more plentiful in females (Fig. S12D). No differences were detected between F_1 males and females exposed to EE.

Discussion

The main goal of the current study was to examine how generational exposure to BPA and EE affect the gut microbiota at respective doses that have already been shown to lead to later behavioral and metabolic disruptions in F_1 offspring.[25-27] The 2 attendant goals were to determine 1) whether similar microbiome changes occur in the P_0 parents and F_1 offspring and 2) whether these chemicals would induce sex-dependent differences in both generations.

In regards to the primary goal, gut microbiota differences were observed in BPA- and EE-exposed P_0 and

F_1 males and females compared to non-exposed control counterparts. To our knowledge, these are the first set of studies to show that parental exposure to environmentally relevant concentrations of BPA and EE causes changes in microbial composition in unexposed offspring. Other environmental chemicals, including lead, arsenic and nanoparticles appear to alter the gut microbiome in rodent models.[4-8] Estrogenic compounds and metabolites affect the composition and diversity of the gut and vaginal microbiota.[10-13] EDC-induced gut dysbiosis may trigger metabolic and other diseases, as postulated previously.[24] By altering the gut microbiome, EDCs may increase the permeability of the intestinal barrier. This pathological change may increase the likelihood that bacterial pathogens, their virulence factors and metabolites will penetrate and enter the systemic circulation, whereupon other target organs, including the brain, may be affected.[1,30,31] Perinatal exposure of rats to BPA affects the intestinal barrier function and gut nocioception.[32] Gut microbes might also be transmitted to the brain via the enteric nervous system and vagal nerve (Reviewed in[33,34]). Bacterial-derived metabolites, such as spermidine, urea, short-chained fatty acids (SCFA), and 4-ethylphenylsulfate (4-EPS), might negatively impact various systems, including the central nervous system (Reviewed in[1]). Other mechanisms by which gut microbes might influence host function are through production of neuroendocrine factors, neurotransmitters, and modulation of the host epigenome (Reviewed in[1]).

In P_0 females, Mogibacteriaceae, *Sutterella spp*, and Clostridiales were increased in the BPA-exposed individuals relative controls; whereas, *Bacteroides* levels were elevated in EE-exposed females relative to controls. Abundance of *Bacteroides* species is negatively correlated with the ratio of hydroxylated estrogen metabolites to parental estrogen compounds (estrone and estradiol) in postmenopausal women.[13] In P_0 males, both BPA and EE increased the abundance of Mollicutes and Prevotellaceae compared to controls. Mollicutes and Erysipelotrichaceae (abundances increased in EE-exposed F_1 males) are more abundant in the gut microbiome of male mice exposed to valproic acid, which is a considered a murine model for autism spectrum disorders.[35]

F1 females exposed to BPA or EE had increased amounts of *Bifidobacterium*. This genus is considered a beneficial inhabitant within the gut flora, and based on this premise is in many probiotic formulations, including those administered to pre-term and neonatal infants.[36-38] The abundance of *Mogibacteriaceae* was increased in the gut microbiome of BPA-exposed F_1 females. Scant information is available on the potential health implications of the presence of this bacterial family in the gut flora. *Mogibacteriaceae* tend to cluster with other microorganisms associated with lower body mass index (BMI) in mice and humans[39] and non-obese diabetic (NOD) mice fed a cellulose, pectin, and xylan-rich diet.[40] For F_1 males, BPA- and EE-exposure led to unique changes in the bacterial intestinal flora. Levels of *Akkermansia* were elevated in the gut microbiome of BPA-exposed males. *Akkermansia* levels are up-regulated in humans and mouse models stricken with colon cancer,[41-43] and this bacterium has been proposed as a target for probiotic treatment.[44] *Methanobrevibacter* levels were also elevated in the gut of F_1 BPA-exposed males. These Archaea, such as *M. smithii*, possess heightened ability to metabolize dietary substrate with resulting increased host energy intake and weight gain.[45] *Erysipelotrichaceae* was the only OTU elevated in the fecal samples of F_1 EE-exposed males compared to AIN males. This bacterial family is linked with various diseases and appears to be highly responsive to dietary shifts (Reviewed in[46]). Comparison of microbiota that were differentially expressed between BPA *vs*. EE exposed males and females in both generations revealed no differences in P_0 females and only isolated bacterial differences in P_0 males and F_1 females and males. Notwithstanding, the findings may suggest that BPA and EE can induce differential effects on the gut microbiota, even though BPA is considered a weak estrogen.[47]

In P_0 and F_1 males and females, various genera are associated with metabolic and other pathway changes that were group-dependent (AIN, BPA, or EE). The bioinformatic analyses only predict which pathways might be affected, but such predictions can be tested by assaying for specific metabolite changes. Alterations in bacterial metabolite and other pathway changes may be another mechanism by which these EDCs can lead to various diseases.

In relation to attendant goal 1, bacterial gut colonization occurs at the time of birth. When neonates pass through the birth canal, they are innoculated with a complex mixture of maternal vaginal microorganisms.[48-50] In human infants, the intestinal microbiome resembles that of their mother up until about one year of age,

whereupon a distinct microbiome profile develops.[51,52] Thus, our prediction was that the gut microbiome changes in BPA or EE-exposed F_1 offspring would mirror that of their P_0 mothers. However, bacterial changes in the F_1 EDC-exposed generation varied from that of their P_0 mothers and fathers. The findings support the notion that generational exposure to BPA or EE differentially impacts the gut microbiome relative to adults. The etiology of these differences is unclear. It is interesting to note that even within the control diet, there were genera differences in males and females in the P_0 relative to the F_1 generation. Divergences in the F_1 progeny from their P_0 parents and across treatments could be attributed to the *in utero* environment or occur postnatally due to altered milk composition. In human infants, breastfeeding increases fecal *Bifidobacterium* compared to supplementation with standard formulas.[53] As detailed above, *Bifidobacterium* was more abundant in BPA- and EE-exposed F_1 females. Other studies provide additional evidence that the gut microbiome is vulnerable to infant dietary changes in humans and animal models.[54,55]

For attendant goal 2, clear sex differences were evident in both generations after exposure to BPA or EE. Even for the control diet, different genera varied between males and females in the 2 generations, suggesting that even a so-called control diet can lead to sex-dependent drift across generations in gut microbial populations. Neonatal NOD mice also show sex differences in the gut microbiome, which governs sex hormone levels and autoimmunity progression.[56] In males, resident microbiota increase serum testosterone concentrations and confer protection against type I diabetes. These phenotypes might be transmitted to juvenile females receiving fecal transplantation from adult males.

In summary, the current findings indicate EDC exposure results in gut microbiome changes and potentially accompanying changes in metabolic and other pathways. The genera altered by these treatments are dependent upon generation and sex. While some of the intestinal flora changes were similar in BPA- and EE-exposed females and males in both generations, others differed. The results suggest that even though BPA is a weak estrogen, it can induce effects outside of binding and engaging estrogen receptors. Overall, many of the genera whose abundances were elevated by EDC-exposure are associated with various diseases, including IBD, metabolic disorders, and colorectal cancer. The notable exception was the surge of *Bifidobacterium* in the fecal samples of BPA- and EE-exposed F_1 females. The escalation of this bacterium in the gut flora of these groups might be due to direct effects of generational exposure to one of the EDCs or mediated via changes in the *in utero* environment/milk composition in the P_0 exposed mothers. The F_1 males and females used in this study were also generated to examine for gene expression differences at adolescence, which is currently ongoing. Thus, one limitation of the current study is that we cannot link the gut microbiome changes in the P_0 and F_1 generations to phenotypic or molecular alterations. However, future studies will aim to do such correlation analyses and also test several doses of BPA and EE. Even so, the current study demonstrates that BPA and EE at doses that have previously been shown to result in behavioral and metabolic disturbances in F_1 California mice offspring[25-27] also leads to generational and sex-dependent changes in the gut microbiome that could initiate subsequent adverse effects to the host.

Materials and methods

Animal husbandry

Founder captive adult (60-90 d of age) California mouse females and males, free of common rodent pathogens, were purchased from the *Peromyscus* Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC). When they were shipped to the University of Missouri, they were placed in quarantine, along with sentinel mice, at the MU Lab Animal Center (LAC) for a minimum of 8 weeks to ensure that they did not carry any transmittable and zoonotic diseases. No diseases have been identified in any sentinel animals or colony animals. Once the animals were deemed pathogen-free, they were transported from the LAC to the Animal Sciences Research Center (ASRC). At this facility, we have established our own breeding colony established. Additional animals are purchased, though as needed, from the PGSC to maintain the outbred status of the line and similar procedures as previously mentioned are followed. All experiments were approved by University of Missouri Animal Care and Use Committee (Protocol #7753) and performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. To reduce any background BPA exposure, animals were housed in polypropylene cages (Allentown, NJ), provided glass water bottles, and BPA-free water.

Two weeks prior to breeding, virgin females, 8 to 12 wks of age were randomly assigned to receive one of 3 diets: 1) a low phytoestrogen AIN 93G diet supplemented with 7% by weight corn oil to minimize potential phytoestrogenic contamination (control), 2) the same diet supplemented with 50 mg BPA/kg feed weight, which we have reported results in internal serum concentrations approximating those measured in pregnant women unknowingly exposed to this chemical,[57,58] and 3) AIN93G diet supplemented with 0.1 parts per billion of EE, as the US Food and Drug Administration (FDA) required estrogen positive control for BPA studies.[59] The FDA has requested EE be included in BPA studies that may guide policy decisions based on the notion that BPA acts primarily as a weak estrogen.[47] Each generation (P_0 and F_1) included control females and males that were used as the as the base comparison within generation to their counterpart BPA-exposed and EE-exposed females and males, respectively. Treatments were initiated 2 weeks prior to breeding to span the peri-conceptional period. P_0 females were maintained on these diets throughout gestation and lactation, as described previously.[25-27,57,60] Exposure to these doses has previously been shown to induce behavioral and metabolic alterations in F_1 male and female California mice offspring.[25-27] Further, the BPA dose falls below the diet-administered maximum nontoxic dose for rodents (200mg/kg of body weight per day), which is within the presumptive NOAEL[61-65] and yields serum concentrations comparable to those identified in human populations.[58,66-70] P_0 males were exposed to the diets at the time from breeding up until weaning the F_1 pups. The F_1 generation sons and daughters were weaned at 30 d of age and fecal samples collected at this time. They were then placed on the control diet. The study design model is detailed in Figure 1.

Collection of fecal samples and isolation of fecal microbial DNA

Prior to fecal collection at weaning, each animal was placed one per cage without any bedding. Four to 5 fecal boli were collected from each animal and placed in a 7ml polypropylene vial (Fisher Scientific, St. Louis, MO). The fecal microbial DNA was isolated using the PowerFecal DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) and in accordance with the manufacture's protocol. The quantity of DNA isolated was measured using Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY). Fecal samples were only collected once for the P_0 and F_1 generations. The number of replicates included in the final analysis for the P_0 and F_1 generations is listed in Supplemental Table 1. The number of replicates tested is comparable to those used in other maternal diet and offspring gut microbiome studies that showed such sample sizes can result in statistical differences between offspring groups.[71,72] While we tested both males and females in the P_0 pairings, one control male and one BPA female sample did not properly sequence, and thus, these were not included in the final analyses.

16s rRNA sequencing

The University of Missouri DNA Core Facility prepared bacterial 16S rDNA amplicons from extracted fecal DNA by amplification of the V4 hypervariable region of the 16s rDNA with universal primers (U515F/806R) flanked by Illumina standard adapter sequences.[73,74] Universal primer sequences are available at proBase (http://www.microbial-ecology.net/probebase/).[75] A forward primer and reverse primer with a unique 12-base index were used in each PCR reaction. PCR reactions (50μl) contained 100ng of genomic DNA, forward and reverse primers (0.2μM each), dNTPs (200μM each), and Phusion High-Fidelity DNA Polymerase (1U). PCR amplification was performed as follows: 98°C(3:00) + [98°C(0:15) + 50°C(0:30) + 72°C(0:30)] × 25 cycles + 72°C (7:00). Maximum sample volume was added to each PCR reaction for seminal fluid samples which contained <100 ng of input DNA. Amplified product (5μl) from each PCR reaction was combined and thoroughly mixed to prepare a single pool. Pooled amplicons were then purified by addition of Axygen AxyPrep MagPCR Clean-up beads (50μl) to an equal volume of 50μl of the amplicon library pool and incubated at room temperature for 15 minutes. Products were placed on a magnetic stand for 5 minutes and supernatant (95 μl) was removed and discarded. Each well was washed by addition of 200μl of freshly prepared 80% EtOH, incubation at room temperature for 30 seconds, and removal of supernatant. Wash steps were repeated once and plate was allowed to dry on magnetic stand for 15 minutes. The dried pellet was resuspended in Qiagen EB Buffer (32.5 μl), incubated at room temperature for 2 minutes, and then placed on the magnetic stand for 5 minutes. Supernatant (30

μl) was transferred to low binding microcentrifuge tube for storage. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified with the Qubit flourometer using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard protocol for sequencing on the MiSeq.

Bioinformatics and amplicon analyses

Paired-end Illumina MiSeq DNA reads were joined using FLASH.[76] Usearch7[77] was used to clean contigs and remove those with E > 0.5, as explained here: http://drive5.com/usearch/manual/exp_errs.html. Contigs were clustered to 97% identity against DNA sequences in the Greengenes database,[78] version 13_5, using the QIIME,[79] version 1.8, script pick_closed_reference_otus.py, which obviates chimera and PCR error detection. For α-diversity in P_0 and F_1 fecal samples, Chao1 (species richness) and Shannon (species diversity) values were calculated and plotted using the phlyoSeq R package.[80] Rarefaction metrics were calculated using the α_rarefaction.py script in the Qiime package[79] and plotted using Microsoft Excel (Fig. S1).

For the follow-up analyses, we first sought to determine whether the P_0 and F_1 generational microbiota data should consider potential litter effects or if it was instead appropriate to consider each individual as the statistical unit. The PERMNAVOVA method was employed by using the QIIME script compare_categories.py to measure the significance of differences among cages (litters) used across all treatments or within individual treatments. Based on these analyses, it was determined that no litter effects were evident, and thus, the individual animal was considered the statistical unit for the remaining analyses.

Measurements of β-diversity were facilitated by the QIIME script jackknifed_β_diversity, as implemented in QIIME.[79] LEfSe[29] was used to identify genera most characteristic of different sample types. LEfSe results were visualized using taxonomy bar-chart and cladogram plots, as implemented on the LEfSe website, http://huttenhower.sph.harvard.edu/galaxy/. Bacterial metabolic characterization of sample types was facilitated with PICRUSt,[81] version 1.0.0. To correlate the genera changes with metabolic characteristics of sample types, we used a custom R script provided as a gift from Dr. Jun Ma and Kjersti Aagaard-Tillery, Baylor College of Medicine, Houston, TX. In these figures, the correlation of the abundance of genera (from the OTU table) with their predicted metabolic function (from KEGG pathways as determined by PICRUSt), was calculated with the R stats function cor.test (https://cran.r-project.org/), using the Kendall method, a rank-based measure of association. The cor.test function outputs a matrix of correlation coefficients and a matrix of results of tests of their significance. The matrix of correlation values was visualized using an adaptation of the R package corrplot made by Ma et al.[71] The area and intensity change together so that larger, darker, circles represent correlation coefficients that are larger in magnitude. The scale to the right of each figure relates those shades of color to the value of the correlation coefficient. Those values whose correlation coefficients were found to be significant at the 0.05 level have a red square around them.

Abbreviations

BMI	Body mass index
BPA	Bisphenol A
EDCs	Endocrine disrupting chemicals
EE	Ethinyl estradiol
FDA	Food and drug administration
GF	Germ-free
IBD	Inflammatory bowel disease
LAC	Lab Animal Center
LPS	Lipopolysaccharide
NOD	Non-obese diabetic
OTU	Operational taxonomic unit
PGSC	Peromyscus genetic stock center
TCA	Citric acid cycle

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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