**Nrf2 knockout and phenethyl isothiocyanate (PEITC) alters gut microbiome**

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**Abstract**

Many internal and external factors including host genotype and diets could impact the human microbiome. Recent studies have demonstrated that different genotypes and diets affect the gut microbiota profiles and influence the microenvironment of human gut. Such events could lead to either improve or damage the gastrointestinal tract, nutrient metabolism and immune responses. In our study, we explored the gut microbiome alterations induced by dietary intervention of phenethyl isothiocyanate (PEITC), a bioactive phytochemical found abundantly in cruciferous vegetables, in both wide-type (WT) and Nrf2 knockout (KO; a master regulator of oxidative stress and inflammation) C57BL/6 mice for 4 weeks. 16s ribosomal RNA sequencing was utilized to evaluate the microbiome alterations of mice fecal samples from 3 timepoints. Results showed that both, alpha and beta diversity differences were associated with genotype and time progression. Specifically, alpha diversity was higher in the Nrf2 KO mice compared to the WT. The average ratio of *Bacteroidetes* to *Firmicutes*, the two major bacterial phylum that combined represented between 65.7% and 96.0% of all bacterial operational taxonomic units (OUT) in the samples, varied between 1.43 and 2.15 in the Nrf2 KO samples across all 3 timepoints but was around 1 in the WT at weeks 1 and 4. Principal component analysis (PCA) indicated that Nrf2 KO group on average had higher relative abundance of *Actinobacteria* and *Proteobacteria* but lower abundance of *Verrucomicrobia* phylum. *Clostridia* and *Bacilli* were one of the most influential classes of *Firmicutes* (6.8% to 45.6% and 0.3% to 23.8% of the samples respectively) while the majority of phylum *Bacteroidete* was comprised of the class *Bacteroidia* (32.8% to 67.6% of the samples). Overall, our study shows gut microbiome altered dependently on both genotype and PEITC diet, and results could link to probiotic intervention on health improvement and therapy development.

**Keywords**

Gut microbiome; Nrf2 KO; PEITC diet; 16s ribosomal RNA sequencing

**1. Introduction**

The most typical example of symbiosis in nature is the mutual benefits between gut microbiota and host. A large quantity of bacteria are distributed in human gastrointestinal tract and affect the human health (Dethlefsen, McFall-Ngai, & Relman, 2007; Frank & Pace, 2008; Ley et al., 2008; Ley, Peterson, & Gordon, 2006). Gut microbiota have been appreciated by the potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system (Maslowski & Mackay, 2011; Ramakrishna, 2013; Rowland et al., 2018). For instance, *Butyricicoccus pullicaecorum* produces butyrate, an essential metabolite for human homeostasis and disease prevention{(Geirnaert et al., 2017); *Lactobacillus* is involved in essential vitamins metabolism (LeBlanc et al., 2013) and human sleep quality improvement (Aizawa et al., 2018); *Bifidobacterium* is able to influence human emotions like depression, reduce painful feeling, and alter brain activity during stress (Cryan & Dinan, 2012; Desbonnet et al., 2010; McKernan, Fitzgerald, Dinan, & Cryan, 2010; Schmidt, 2015; Tillisch et al., 2012). Previous studies have demonstrated the gut microbiome abundance and diversity are highly correlated with internal and external factors such as high fat diet, inflammatory bowel disease and health status (Butel, 2014; Cani et al., 2008; Daniel et al., 2014; Eom, Kim, Choi, Sadowsky, & Unno, 2018; K. A. Kim, Gu, Lee, Joh, & Kim, 2012; Sekirov, Russell, Antunes, & Finlay, 2010; Shim, 2013). However, the underlying molecular mechanism of how gut microbiome being regulated and respond to stimuli are poorly understood.

Studies on the gut microbiome alterations show that diet and host genotype play important roles in the host-microbiome interactions. For instance, gut microbiome is responding rapidly and consistently to low fat/high-plant-polysaccharide, and high fat/sugar diet on gene deficient mice (Carmody et al., 2015). The relative abundance of *Firmicutes* (*clostridiales, lactobacillales, turicibacterales*) and *Verrucomicrobia verrucomicrobiales* are observed to increases significantly. In contrast, *Bacterioidetes* *bacteroidales* significantly decrease in high fat/sugar diet group. Additionally, *F. Clostridiales* and *B. Bacterioidales* are significantly altered during the dietary shift between low fat/high-plant-polysaccharide diet and high fat/sugar diet. Low fat/high-plant-polysaccharide diet decrease the relative abundance of *Firmicutes erysipelotrichi*, *Firmicutes bacilli*, and increase the relative abundance of *B. Bacteroidetes* compared with high fat/sugar Western diet on gnotobiotic mouse with healthy human fecal transplantation. 28 healthy subjects are given 60 g of whole grain barley, brown rice or equal mixture of two ingredients every day for 4 weeks (Martinez et al., 2013). All three whole grain diets significantly increase the gut bacterial diversity (Shannon’s index and Simpson’s index) and phylum *Firmicutes*, while decrease the proportion of phylum *Bacterioidetes*. In addition, genus *Bacerioides* is significantly decreased by whole barley and brown rice mix diet but not affected by either of the single ingredient diet. In addition, genus *Roseburia*, *Bifidobacterium*, *Dialister* and *Odoribacter* are significantly altered by whole grain barley diet alone, and genus *Blautia* is altered by both, mix diet and whole grain barley diet.

Host genotype influences the human gut microbiota with some controversial opinions regarding its contribution due to the potential confounding factors such as the diet (Carmody et al., 2015; Ussar et al., 2015). For instance, gut microbiota alterations are genotype dependent when mouse were housed at the same facility and given the same diet, using automated ribosomal intergenic spacer analysis and length heterogeneity polymerase chain reaction (Kovacs et al., 2011). Similar results are reported on genetic defect of toll-like receptor 2 (TLR2)-deficient mouse study (Albert, Sommerfeld, Gophna, Marshall, & Gophna, 2009). The genus *Helicobacter* is significantly elevated in TLR2 knockout (KO) mice when compared to the wide-type (WT). In addition, gut microbiome changes are also correlated with gene deficiency related diseases. Mutation of NOD2 are highly susceptible to inflammatory bowel disease(Cuthbert et al., 2002). Studies have shown decreasing bacterial diversity, richness and phylum *Proteobacteria*, and increasing phylum *Bacteroidetes* on NOD2-/- mice, when compared with WT (Jess et al., 2011; Mondot et al., 2012). Gene ATG16L1 polymorphism, normally found increasing the risk of inflammatory bowel disease, alters the relative abundance of phylum *Bacteroidetes* (Lavoie et al., 2019). Phytochemicals are bioactive ingredients in our daily diet, which have been determined to improve our health status by interacting with our gut microbiome (Carrera-Quintanar et al., 2018; D. H. Kim, 2018; Yang et al., 2019; Yin, Li, Tian, Xi, & Liu, 2018; C. Zhang et al., 2016). In our study, we selected phenethyl isothiocyanate (PEITC), a strong anti-inflammatory phytochemical previously reported by us and others (Boyanapalli et al., 2016; Dey et al., 2010; C. Zhang et al., 2016), and tested its gut microbiome interaction on Nrf2 (a master regulatory gene in oxidative stress and inflammation) KO mouse. 16s ribosomal RNA (rRNA) sequencing technology was used to examine the alterations of gut microbiota between dietary treatment and genotype.

**2. Materials and Methods**

**2.1 Experimental design, animal and diet**

C57BL/6J WT mice were purchased from Jackson Laboratory (Bar Harbor, ME), and C57BL/6J KO mice were maintained in our laboratory (Lin et al., 2008; Shen et al., 2006). All mice were maintained at a controlled temperature (20-22°C) and humidity (45–55%) environment under 12-hour light and dark cycles at the Rutgers Animal Facility. Food and water were provided *ad libitum*. Mice were fed with AIN93M diet (Research Diets, Inc. NJ) for a 2-week period to “equalize” the gut microbiome, then assigned semi-randomly by genotype alone: WT control, WT PEITC, KO control and KO PEITC groups. Another 4-week dietary treatment using either AIN93M (control) or 0.05% PEITC (PEITC) diet was given as designed in **Figure 1A**. Fecal samples were collected individually per mouse at 3 timepoints: the end of equalization period (Week 0), at an early timepoint (Week 1) and at a late timepoint (Week 4), the snap froze in liquid nitrogen immediately and store at 80oC for further 16s rRNA sequencing analysis. The animal experiments were conducted under the animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers University.

**2.2 16s ribosomal RNA sequencing and analysis**

Bacterial DNA were extracted using PowerSoil DNA Isolation Kit (QIAGEN). PCR amplification of the 16S rRNA genes were carried out using PCR primers specific for the V4 region (**Table 1**) (Apprill, McNally, Parsons, & Weber, 2015; Caporaso et al., 2012; Caporaso et al., 2011; Minich et al., 2018; Parada, Needham, & Fuhrman, 2016; Quince, Lanzen, Davenport, & Turnbaugh, 2011; Walters et al., 2016). Indexed amplicons were pooled and sequenced on MiSeq (Illumina) yielding at least 8,000 300 base pair (bp) pair-ended reads. Microbial operational taxonomic units (OTUs) and their taxonomic assignments were analyzed using QIIME2 and Divisive Amplicon Denoising Algorithm 2 (DADA2 R package) tools, following mapped against representative sequencing in SILVA (Yilmaz et al., 2014), and removing chimeric sequences from subsequent analyses (Caporaso et al., 2010). Principle coordinates analysis (PCoA) of unweighted UniFrac phylogenetic or genus were performed to visualize similarity of microbial communities in each sample.

**2.3 Statistical Analyses**

The data are presented as the mean ± standard error of the mean (SEM), except as otherwise stated. Multi-variable analysis of variance (ANOVA) using genotype, diet and time points was performed followed by multiple comparison, with false discovery rate (FDR) adjustment for the p-values where necessary Principal components analysis (PCA) was performed to examine the effect of the experimental design conditions on bacterial composition on different taxonomic levels.

**3. Results**

**3.1 Gut microbiome alterations by genotype and dietary PEITC**

PEITC has been reported to inhibit colon inflammation (Cheung, Khor, Huang, & Kong, 2010; Cheung, Khor, Yu, & Kong, 2008; Liu & Dey, 2017) and colon cancer (Khor, Cheung, Prawan, Reddy, & Kong, 2008; Liu & Dey, 2017). The overall 16s sequencing depths ranges from 50 to 200 K base pair reads per sample (**Fig. 1B**). The operational taxonomic units (OTUs) were mapped to Bacteria (98.34%), Eukaryote (1.43%) and unknown (0.23%) and Archaea (less than 0.01%) kingdoms (**Fig. 1C**), and the full mapped taxonomy classes are also listed in **Table 2**. All but bacterial reads were removed from downstream analysis. Alpha diversity analysis of the bacterial OTUs was conducted using Shannon index. As shown in **Fig. 2**, more diverse microbiome and an increasing trend alpha diversity were found in Nrf2 KO group. In addition, the alpha diversity for both WT and KO group had no significant changes through time. Then, principle coordinate analysis was to further evaluate and visualize gut microbiome diversity of samples using pairwise Permo anova test (**Fig. 3**). There is a strong association between gut microbiome and host genotype at all taxonomic levels (**Fig.3 A-D**), followed by some effect of diet in the WT group on genus level (**Fig. 3C**).

**3.2 Gut microbiota profiles altered by diet**

Linear discriminant analysis effect size (LEfSe) is a computational tool to identify gut microbiota significantly changes between groups using relative abundance data (Segata et al., 2011). As shown in **Figure 4**, combing control and PEITC diet together, gut microbiome at week 0 is marked in red and gut microbiome at week 1 and week 4 is marked in green. Each taxonomy with relative abundance ≥ 0.1% and at least one specimen was included in the LEfSe analysis. In addition, the cladograms only show the phylogenetic distribution of microbial lineages associated with compared timepoints with linear discriminant analysis score ≥ 2.0. Details of all significantly altered microbiota are summarized in **Table 3**. To compare at week 0, the relative abundance of *Bacteroidetes Prevotella*, *Bacteroidetes S24\_7* were significantly decreased through the whole 4-week period under control diet. Meanwhile, other strains including *Bacteroidetes* *parabacteroides*, *Bacteroidetes* *odoribacter* and *Firmicutes* *oscillospira* were significantly altered at either week 1 or week 4. PEITC diet also significantly decreased the relative abundance of *B. Prevotella*, and *B. S24\_7* partially due to the AIN93M diet mixed up, but significantly increased the abundance of *Firmicutes Ruminococcus* for all 4-week treatment.

**3.3 Gut microbiota profiles altered between Nrf2 KO and WT genotypes**

Nrf2 is a master regulator of anti-oxidative stress and anti-inflammatory responses to external and internal stimuli(Huang, Li, Su, & Kong, 2015; Mitsuishi, Motohashi, & Yamamoto, 2012; Osburn & Kensler, 2008; Taguchi & Yamamoto, 2017; D. D. Zhang, 2006). As described previously in dietary intervention, gut microbiome alteration is also highly correlated with genotype (**Fig. 3**). As shown in **Figure 5** and **6**, as well as listed in **Table 3** and **4**, the relative abundance of *Actinobacteria adlercreutzia*, *Cyanobacteria YS2*, *Tenericutes mycoplasmataceae* and *Firmicutes lactococcus* were significantly altered in Nrf2 KO group at week 1. At week 4, the relative abundance of *A. Adlercreutzia*, *Bacteroidetes Rikenella*, *C. YS2*, *F. Ruminococcus* and *Actinobacteria Olsenella*, *B. Rikenellaceae*, *F. Lactobacillus*, *Firmicutes Lachnospiraceae*, *Tenericutes Mollicutes* were significantly increased and decreased in Nrf2 KO group. Moreover, some strains showed PEITC dietary specificity including *Firmicutes coprococcus*, *F. ruminococcus*, *A. olsenella*, *B. S24\_7*, *Firmicutes clostridium*, *Firmicutes dehalobacterium*, *Firmicutes* *allobaculum*, *Proteobacteria sutterella*, and *Proteobacteria* *desulfovibrioaceae* at week 1 and *P. sutterella*, *Tenericutes anaeroplasma* at week 4 on Nrf2 KO groups.

**3.4 *Firmicutes* to *Bacteroidetes* ratio altering by genotype and time**

The ratio of *Firmicutes* to *Bacteroidetes* shows correlation with aging and body mass index changes (Koliada et al., 2017; Mariat et al., 2009). In our study, we observed a strong impact on the ratio (*Bacteroidetes*/*Firmicutes*) by genotype (**Fig. 7A**). Nrf2 KO group has more abundance of *Bacteroidetes* and less abundance of *Firmicutes* proportionally. In **Figure 7B**, the ratio was lower at week 0 and “equalized” to ~1.0 at week 1 and 4. In addition, there was no correlation between the *Firmicutes* to *Bacteroidetes* ratio and diet (**Fig. 7C**). The average ratio of *Bacteroidetes*/*Firmicutes* on WT group deceased from around 1.7 to 1.0 at week 1 and remained low at 1.1 at week 4, however, KO groups decreased from 2.15 to nearly 1.5 with significant higher value than the ratio of WT groups (**Fig. 7D**). These trends have shown less or no impact by diet. To sum up, the *Bacteroidetes* to *Firmicutes* ratio are showing correlations with genotype and aging, suggesting potential biomarker characteristics in some circumstance.

**4. Discussion**

In this study, we treated WT and Nrf2 KO mice with 0.05% PEITC and control diet or 4 weeks and compared the gut microbiome alterations by genotype, diet and time. Genotype is more dominant than diet and time in regulating the gut microbiome alterations. *Firmicutes*, *Bacterodidetes* and *Proteobacteria* are the major phyla altered by Nrf2 KO genotype and partially PEITC diet. Individual bacterial strains like *Firmicutes Ruminococcus* is altered by both Nrf2 KO genotype and 0.05% PEITC diet. All other strains altered by genotype, diet and time are summarized in **Table 3**, **4** and **5**.

Gut bacteria have been appreciated in metabolizing essential nutrients, providing energy and enhancing immune system (Maslowski & Mackay, 2011; Ramakrishna, 2013; Rowland et al., 2018). For instance, *B. pullicaecorum* produce butyrate, an essential metabolite for human homeostasis and disease prevention{(Geirnaert et al., 2017) while *Lactobacillus* strains are involved in essential vitamins metabolism (LeBlanc et al., 2013). *Ruminococcus* are anaerobic, gram-positive bacteria and the phylum of *Firmicutes*. Eleven *Ruminococcus* species have been identified so far (La Reau & Suen, 2018; Rainey & Janssen, 1995). Previous studies show that *Ruminococcus* spp. degrade and fermente cellulosic biomass into short-chain fatty acid (SCFA) for herbivorous ruminants (Flint, Bayer, Rincon, Lamed, & White, 2008; Leschine, 1995; Qin et al., 2010). *R. torques* was reported abundantly in the irritable bowel syndrome subjects in the double blind study (Lyra et al., 2010). Multiple probiotic interventions were able to reduce *R. torques* significantly, suggesting that *R. torques* may be used as biomarker in evaluating probiotic activity. As a part of normal flora in gastrointestinal tract, another *Ruminococcus* *gnavus* showed a high abundance at inflammatory bowel diseases (IBD) patients, with increased level of oxidative stress in the gut (Hall et al., 2017), potentially caused by cytokine production such as TNF-a (Henke et al., 2019). *R.* *gnavus* was also been reported to be overpopulated in infants who developed respiratory and skin allergic diseases (Chua et al., 2018). Mice orally garaged by *R. gnavus* developed airway inflammation by secreting cytokine interleukin 25, interleukin 33 and others. In this study, we observed a significant increase in the abundance of *Firmcutes* *Ruminoccus* in all fecal samples of Week 4 but not in Week 1 samples (**Table 3**). It was firstly considered as aging effect to interrupt the gut microbiome balance. Furthermore, we divided into WT and Nrf2 KO group and found out the increased level of *F. Ruminoccus* only occurred in Nrf2 KO group but not in the WT group (**Table 4** and **5**). Since Nrf2 is a master regulator of anti-oxidative stress and anti-inflammation, Nrf2 KO could somehow link to the increased “pathogenic” strains such as *F. Ruminoccus*. We also found *B. Rikenella* and *Cyanobacteria* phylum were significantly elevated in Nrf2 KO groups, which both of them were involved in gut dysregulations (Carmichael, 1992, 1994; Couturier-Maillard et al., 2013; Johnson, Heaver, Walters, & Ley, 2017). Therefore, Nrf2 is linking to certain gut microbiome alterations which could lead to gut diseases.

Being the most phylum presented in human gut, the *Firmicutes* to *Bacteroidetes* ratio shows strong connection with external factors. The ratio changes between 0.4 to 0.6 among infant (3 weeks to 10 months) and seniors (70 to 90 years old), and significantly increased to 10.9 in adults (25 to 45 years old) (Mariat et al., 2009). The ratio is increased in response to dietary fiber, which reduce the risk of diarrhea induced by *E. coli* (Molist, Manzanilla, Perez, & Nyachoti, 2012). Total parenteral nutrition diet, normally treating critically ill patients, decrease the ratio after 14-day treatment on male Sprague-Dawley rat model (Hodin et al., 2012). Some clinical studies attempt to use the abundance of *Firmicutes* and *Bacteroidetes* phyla and species, and the *Firmicutes* to *Bacteroidetes* ratio as indicator for obesity (Armougom & Raoult, 2008; Indiani et al., 2018). In our study, the *Firmicutes* to *Bacteroidetes* ratio changes significantly by folds in a genotype and time dependent manner, but not by PEITC. It is highly possible that single phytochemical PEITC addition at 0.05% w/w in diet is not strong enough to interfere the *Firmicutes* to *Bacteroidetes* ratio.

In our study, genotype shows strong impact on the gut microbiome and diversity. In addition, 0.05% PEITC in the diet can alter the relative abundance of several bacterial strains, yet not entire gut microbiome or diversity. It is important to point out that studying an ecosystem like gut microbiome should consider more parameters in a comprehensive environment. For instance, research has demonstrated that cage and internal individual effects contribute up to 32% and 46% of gut microbiota variances during identifying the bacterial composition (Hildebrand et al., 2013). Several methods are used to eliminate the background noise interfering and reveal a more actual gut microbiome, including giving control diet to all animals for a while to normalize the gut microbiota baseline or choosing gnotobiotic (germ-free) mice and given the same fecal samples to all animals(Roopchand et al., 2015; Turnbaugh et al., 2009; L. Zhang et al., 2018). In our study, we have caged all the mice with the same genotype together before randomly assigned into different diet groups and given them AIN93M control diet for a 2-week period of gut microbiome “equalization” procedure. Even through, we still able to improve the future study for establishing baseline microbiome probably using antibiotic treatments and germ-free mice model (Lundberg, Toft, August, Hansen, & Hansen, 2016).

**Acknowledgements**

We thank Dr. Min Tu and Dr. Dibyendu Kumar from Waksman Institute of Microbiology, Rutgers University for the help of 16s rRNA sample preparation and sequencing. We also thank all members of Dr. Kong’s laboratory for their invaluable support and technical assistance. This work was supported in part by institutional funds and by R01 AT009152 from the National Center for Complementary and Integrative Health (NCCIH) and R01 CA200129, from the National Cancer Institute (NCI).

**Conflict of Interest States**

The authors declare no conflict of interest.

**Data availability statement**

The datasets used and/or analyzed during the current study are available from the corresponding author.

**Legend**

**Figures**

**Figure 1.** Experimental designs and 16s rRNA metagenomics summary. **A**. Experimental design. **B**. Sequence depth. **C**. OTUs annotated based on reference genome.

**Figure 2**. The Shannon index of all samples as measurement of alpha diversity with the means and SEM are plotted. Samples are separated by genotype.

**Figure 3**. Biplots of samples by PCA results using relative abundance of different bacterial classes. The arrows represent direction of increase of the top 5 bacteria. Circles represent samples from week 0, squares represent samples from week 1, and diamonds represent samples from week 4 for both WT and KO groups. A. Phylum. B. Classes. C. Genus. D. Species.

**Figure 4**.Comparison of microbiome differences between before (Week 0 in red) and after treatment (week 1 or week 4 in green) in the control and PEITC group, analyzed by Linear discriminant analysis effect size (LEfSe). Taxa with relative abundance ≥ 0.1% present in at least one specimen were included. The cladograms indicate the phylogenetic distribution of the microbial lineages associated with time points, with lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 displayed. Significance differences for each group of the most abundant class are indicated by color, red indicating before treatment (week 0), green after treatment (Week 1 or Week 4), yellow non-significant. Each node’s diameter is proportional to the taxon’s abundance. Nodes represent phylogenetic levels from domain to genus (from inside out) (week 0: n=3, Week 1: n=9; Week 4: n=9). A. Week 1 gut microbiome alterations on AIN93M group compared with week 0. B. Week 4 gut microbiome alterations on AIN93M group compared with week 0. C. Week 1 gut microbiome alterations on PEITC group compared with week 0. D. Week 4 gut microbiome alterations on PEITC group compared with week 0.

**Figure 5**. Comparison of microbiome differences between genotype at week 1, analyzed by LEfSE. Taxa with relative abundance ≥ 0.1% present in at least one specimen were included. The cladograms indicate the phylogenetic distribution of the microbial lineages associated with different experiment, with lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 displayed. Significance differences for each group of the most abundant class are indicated by color. Green dots represent bacterial from Nrf2 KO group, blue dots represent bacterial group WT group, and yellow dots represent non-significant. Each node’s diameter is proportional to the taxon abundance. Nodes represent phylogenetic levels from domain to genus (from inside out). A. AIN93M group. B. PEITC group.

**Figure 6**. Comparison of microbiome differences between different mouse strains at week 4, analyzed by LEfSE. Taxa with relative abundance ≥ 0.1% present in at least one specimen were included. The cladograms indicate the phylogenetic distribution of the microbial lineages associated with different experiment, with lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 displayed. Significance differences for each group of the most abundant class are indicated by color. Green dots represent bacterial from Nrf2 KO group, blue dots represent bacterial group WT group, and yellow dots represent non-significant. Each node’s diameter is proportional to the taxon abundance. Nodes represent phylogenetic levels from domain to genus (from inside out). A. AIN93M group. B. PEITC group.

**Figure 7**. Log2 ratio of Bacteroidetes to Firmicutes on different genotypes, timepoints and diets. A. Log2 ratio distinguished by genotype. B. Log2 ratio distinguished by time. C. Log2 ratio distinguished by diet. D. Ratio Bacteroidetes to Firmicutes over time and genotype.

**Tables**

**Table 1** V4 primer sequence used for 16s RNA sequencing library preparation

**Table 2** 16s sequencing results mapped bacterial communityat difference taxonomy classes with top associated bacteria

**Table 3** List ofGut microbiota significantly changed by either AIN93M or PEITC diet at week 1 and week 4 for WT and KO C57BL/6J mice.

**Table 4** List ofGut microbiota significantly changed due to genotype at week 1.

**Table 5** List ofGut microbiota significantly changed due to genotype at week 4.

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