Gut microbiome and metabolome alterations with dietary feeding of phenethyl isothiocyanate and cranberry

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**Abstract**

Recent studies have demonstrated that diet, genotype and diseases can alter bacterial community composition which, in turn, may affect host’s wellbeing including gastrointestinal, endocrinal, and mental health. In the current study, we performed dietary intervention with phenethyl isothiocyanate (PEITC), a bioactive phytochemical found abundantly in cruciferous vegetables such as watercress, and cranberry, in wide-type (WT) C57BL/6 and Nrf2 knockout (KO) mice. In addition, some of the mice were challenged with dextran sulfate sodium (DSS) to simulate inflammatory conditions found in patients suffering from inflammatory bowel disease (IBD). Nrf2 is a master regulator of oxidative stress and inflammation and have been shown to be affected by microbial metabolites. The fecal microbiota profiles were examined by 16s ribosomal RNA sequencing. At the same time, we also isolated and analyzed microbial metabolite profiles from fecal samples to understand downstream changes in these communities.

The study showed that alpha and beta diversity differences of microbiota were associated with genotype, time progression and DSS treatment. 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 (this is NOT very clear – may be shorten it?). 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 the most abundant classes of Firmicutes (6.8% to 45.6% and 0.3% to 23.8% of the samples respectively) while the majority of *Bacteroidete* phylum was comprised of the class *Bacteroidia* (32.8% to 67.6% of the samples). In terms of individual bacterial strains, *Firmicutes Lactococcus* and *Actinobacteria Adlercreutzia* were consistently altered correlating with Nrf2 genotype irrespective of the diet.

**Need to add metabolome…**

Overall, this study showed strong association of microbial community composition alteration with Nrf2 genotype and minor association with PEITC diet.

**Keywords**

Diet; Nrf2 genotype; DSS; gut microbiota; PEITC; 16s ribosomal RNA sequencing

**1. Introduction**

Human health can be affected by environmental microorganisms including bacterial, archaea and fungi which are distributed in large quantities in human skin and other organs (Dethlefsen, McFall-Ngai, & Relman, 2007; Frank & Pace, 2008; Ley et al., 2008; Ley, Peterson, & Gordon, 2006). Among them, bacteria in the gut have been appreciated by its potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system (Maslowski & Mackay, 2011; Ramakrishna, 2013; Rowland et al., 2018). For instance, gut bacteria *butyricicoccus pullicaecorum* and *butyricicoccus pullicaecorum* produce butyrate, an essential metabolite for human GI homeostasis and disease prevention (Geirnaert et al., 2017). *Lactobacillus* strains are involved in essential vitamins metabolism (LeBlanc et al., 2013) and human sleep quality improvement (Aizawa et al., 2018). *Bifidobacterium* strains might be 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). Numerous studies have been conducted to explore gut microbiota composition responding to specific conditions such as high fat diet or inflammatory bowel disease (Butel, 2014; Cani et al., 2008; Daniel et al., 2014; Eom, Kim, Choi, Sadowsky, & Unno, 2018; Kim, Gu, Lee, Joh, & Kim, 2012; Sekirov, Russell, Antunes, & Finlay, 2010; Shim, 2013), however, some of the basic underlying molecular mechanism of gut regulation by these bacteria are poorly understood.

Systematic studies on the regulators of gut microbiome have identified that diet and host genotype play important role in host-diet-microbiome interaction. For instance, a rapid and consistent dietary response to low fat/high-plant-polysaccharide, and high fat/sugar diet on gene deficient mice has been reported that cooccurred with significant increase of relative abundance of *Firmicutes* (*Clostridiales, Lactobacillales, Turicibacterales*) and *Verrucomicrobia* (*Verrucomicrobiales*) (Carmody et al., 2015). In contrast, *Bacterioidetes* (*Bacteroidales*) significantly decreased in high-fat/sugar diet group. Additionally, *Clostridiales* and *Bacterioidales* significantly altered composition of bacterial orders during the dietary shift between low fat/high-plant-polysaccharide diet and high fat/sugar diet. Utilizing gnotobiotic mouse model with transplantation of healthy human fecal sample, the low fat/high-plant-polysaccharide diet decreased the relative abundance of *Firmicutes Erysipelotrichi*, *Firmicutes Bacilli*, and increased the relative abundance of *Bacteroidetes Bacteroidetes* compared with high fat/sugar Western diet. Twenty eight healthy subjects were 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 increased the gut bacterial diversity (Shannon’s index and Simpson’s index), and the proportion of phylum *Firmicutes*, while decreases the proportion of phylum *Bacterioidetes*. At the individual level, genus *Bacerioides* were significantly decreased by whole barley and brown rice mix diet but were not affected by either of the single ingredient diet. In addition, genus *Roseburia*, *Bifidobacterium*, *Dialister* and *Odoribacter* were significantly altered only by whole grain barley diet, and genus *Blautia* by both, mix diet and whole grain barley diet.

Host genotype may also influence the human gut microbiota, however there may be some controversial opinions regarding its contribution due to the potential confounding factors such as the diet (Carmody et al., 2015; Ussar et al., 2015). Simplified animal model using the same diet and living environment can help reveal the potential role of genotype in regulating the gut microbiota and helps removing some of the doubts. For instance, an eight-mouse line study reveals that 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). There is also a higher similarity between genotype than sex, suggesting that genotype is a stronger factor than gender in regulating gut microbiota. Another evidence of gut microbiota determined by genotype comes from a genetic defect of toll-like receptor 2 (TLR2)-deficient mouse study (Albert, Sommerfeld, Gophna, Marshall, & Gophna, 2009). The genus level of *Helicobacter* was significantly elevated in TLR2 knock-out mice compared to the wide-type. Moreover, some genetic defect such as NOD2 and ATG16L1 were linked to inflammatory bowel diseases and showed the host-microbiota interaction by shifting bacterial composition including relative abundance of *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Combined with diet, environmental intervention and inheritance, gut microbiome is considered one of the most complicated systems in maintaining human health and its role in human diseases. Since isothiocyanates such as phenethyl isothiocyanate (PEITC) has been shown to possess many healthy beneficial effects including cancer prevention and Nrf2, a master regulator of oxidative stress and inflammation, the effect of PEITC and Nrf2 genotype might affect gut microbiome but remains unknown. In the current study, C57BL6/J wide-type (WT) and Nrf2 gene knockout (KO) mice were treated with PEITC for a 4-week period. Fecal samples at week 0, week 1 and week 4 were collected and analyzed using 16s ribosomal RNA (rRNA) sequencing technology to examine the alterations of gut microbiota between dietary treatment and genotype.

**2. Materials and Methods**

**2.1 Experimental design, animals and diet**

C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6J knock-out (KO) mice have been maintained in our laboratory since 2005 (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*. The study was stacked into three experiments.

All mice were firstly given a 2-week gut microbiota equalization period during which they fed with AIN93M control diet (Research Diets, Inc. NJ).

In *Experiment 1*, 10 Nrf2 KO mice were randomized after the 2-week equalization period into two group: one group continued receiving the control diet (AIN93M) while the second group’s diet was enhanced with 0.05% PEITC. *Experiment 2* used 18 WT mice that were randomized into either the control diet group (AIN93M) or the enhanced PEITC-enhanced diet group. In *Experiment 3* we introduced additional cranberry diet and challenged the animals with DSS to induce gut inflammation, and both genotypes were tested (WT and Nrf2 KO). The assignment by into 1 of four treatment groups (Naïve, DSS, DSS + PEITC, and DSS + Cranberry) was done within each genotype (**Figure 1**).

Mice fecal samples were collected individually at 3 timepoints – at the end of equalization period (Week 0), at an early timepoint (Week 1) and at a late timepoint (Week 4), snap frozen in liquid nitrogen and stored at 80oC for 16s rRNA sequencing and microbial metabolites analysis. All animal experiments were conducted under the animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers University. In total

**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. QIIME2 mapped reference at 97% similarity against representative sequences of 97% OTU in SILVA(Yilmaz et al., 2014), following by chimeric sequences removed 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.

DADA2 1.16 pipeline was used to process FastQ file containing pair-ended reads with average length of 300 base pairs (bp) into a high resolution operational taxonomic unit (OTU) table (i.e. amplicon sequencing variants). The reads were sorted, and quality scores examined, resulting in truncation of forward reads to 280 bp and reverse reads to 220 bp based on the quality score profiles. The reads were then merged and aggregated. Additionally, chimeric OTUs were identified and removed. Taxonomy was assigned to the OTUs by exact matching (100% identity) to Silva reference database (Yilmaz et al., 2014). Sequencing depth varied roughly from 25,000 to 400,000 reads per sample (**Figure 2**). Majority (over 94%) of OTUs were identified as bacterial, with OTUs mapped to *Eukaryota* and *Archaea* Kingdoms, as well as OTUs that could not be mapped to a Kingdom, were removed. In total, 10,197 (94.78% of total OTUs), 7,994 (98.34%) and 7,558 (96.07%) bacterial OTUs were identified in the 3 experiments respectively (**Table 2**). Additionally, bacterial OTUs belonging to phylum Cyanobacteria were removed as they most likely originated in the food and were not a part of the gut microbiomes. OTUs not mapped to any bacterial phylum were removed as well and the remaining OTUs analyzed.

**2.3 Microbial metabolites analysis**

The concentrations of microbial metabolites (short-chain fatty acids (SCFA), bile acids, and free amino acids) were quantified in fecal samples collected at weeks 2 and 6 using liquid chromatography mass spectrometry (LC-MS)-based targeted and untargeted analysis.

**2.4 Statistical analyses**

Alpha diversity was assessed using Shannon’s diversity index. The index is equal to zero when there is exactly one class (OTU)present in a sample. Larger values of the index indicate greater diversity and more evenly distributed OTUs. The estimates were presented as means +/- standard error of the means (SEM) (**Figure 3**). 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.

Bacterial composition at different taxonomic levels were explored using principal components analysis (PCA) and visualized as biplots (Figure 3). PCA is a linear transformation that projects the data from the original *n*-dimensional, possibly correlated space (here, each taxonomic unit was viewed as a dimension) onto a new, orthogonal *n*-dimensional space such that the first principal component (PC1) is in the direction that explains most of variability in the data, second - the second most (and is orthogonal to PC1), and so on. The samples are then plotted against 2 principle components (e.g. PC1 and PC2) and labeled to check for group separation. Biplots is an extension of PCA plot that simultaneously display the labeled samples in two principle components’ space as well as the direction and the magnitude of the original axes (i.e. individual taxonomic units). Multivariate analysis of class (group labeling corresponding to taxonomic units) vs. principal components was performed to statistically assess the predictive power of PCA on class separation. Compositional Omics Model-Based Integration framework was used to combine and visualize microbial count and microbial metabolite data (Hawinkle 2020).

**3. Results**

**3.1 Dietary PEITC induces gut microbiota changes: bacterial diversity**

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 (95.92%), Eukaryote (3.44%) and Archaea (0.02%) kingdoms, with 0.62% reads unmapped (**Fig. 1C**). All but bacterial reads were removed from downstream analysis. Alpha diversity analysis of the bacterial OTUs was conducted using Shannon index (**Fig. 2**). There is a significant more diverse microbiome and increasing alpha diversity trend in Nrf2 KO group compared to WT group. In addition, microbiome diversity between Nrf2KO and WT groups has no significant difference. Principle coordinates analysis was used to predict and visualize the genetic distance and relativity of gut microbiome from collected fecal samples using pairwise Permoanova 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 Dietary PEITC induced gut microbiota changes: bacterial identification**

To further examine the potential differences of the microbiota between control AIN93M diet versus PEITC diet, QIIME2 and Linear discriminant analysis Effect Size (LEfSe) were used and the results are shown in **Fig. 4**. Firstly, we examined the potential aging effect on the microbiota by comparing the microbiota for control diet at base line Week 0 shown in red versus Week 1 and Week 4 in green as analyzed by LEfSe, and Taxa with relative abundance ≥ 0.1% present in at least one specimen were included. In addition, the cladograms showing the phylogenetic distribution of the microbial lineages associated with different time points, using lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 were displayed (**Fig 4**). *Bacteroidetes Prevotella, Bacteroidetes Parabacteroides, Bacteroidetes, and Bacteroidetes S24\_7* were decreased, while *Bacteroidetes Bacteroidales, Firmicutes Clostridiales, Firmicutes Oscillospira, Proteobacteria Desulfovibrionaceae, and Tenericutes Anaeroplasma* were increased. **Fig. 4 C, D** show the impact of PEITC diet by comparing the microbiota for control diet at base line Week 0 shown in red and at Week 1 or Week 4 shown in green and **Table 2** summarizes the results for the combined Nrf2 WT and KO. *Firmicutes Ruminococcus* and *Bacteroidetes* *S24\_7* were significantly increased and decreased at both Week 1 and Week 4. Some microbiotas were uniquely correlated with diet. *Bacteroidetes Odoribacter*, *Tenericutes Mycoplasmataceae*, *Proteobacteria Desulfovibrionaceae* were significantly increased with control AIN93M diet, while *Firmicutes Clostridiales*, *Firmicutes Ruminococcus* and *Acidobacteria Ellin 6075* were significantly increased by PEITC diet at either Week 1 or Week 4.

**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), so we next examine the impact of Nrf2 genotypes Nrf2 wildtype (WT) versus Nrf2 knockout (KO; -/-) in control diet and in PEITC diet. To reveal individual microbiota altered by this genotype difference, we compared KO with WT group at bacteria phylum levels at different time points and visualized (**Fig. 5**, **6**) and summarized them (**Table 3**, **4**). In details, relative abundance of *Actinobacteria Adlercreutzia*, *Cyanobacteria YS2*, *Tenericutes Mycoplasmataceae* and *Firmicutes Lactococcus* were significantly increased and decreased in Nrf2 KO group at week 1, irrespective of the diet. While at Week4, the relative abundance of *Actinobacteria Adlercreutzia*, *Bacteroidetes Rikenella*, *Cyanobacteria YS2*, *Firmicutes Ruminococcus* and *Actinobacteria Olsenella*, *Bacteroidetes Rikenellaceae*, *Firmicutes Lactobacillus*, *Firmicutes Lachnospiraceae*, *Tenericutes Mollicutes* were significantly increased and decreased in Nrf2 KO group at week 4, irrespective of the diet. Interestingly, *Firmicutes Coprococcus/Ruminococcus* and *Actinobacteria Olsenella*, *Bacteroidetes S24\_7*, *Firmicutes Clostridium/Dehalobacterium/Allobaculum*, *Proteobacteria Sutterella/Desulfovibrioaceae* were significantly increased and decreased respectively in relative abundance by PEITC diet in Nrf2 KO mice at week 1 but only a few bacteria such as *Proteobacteria Sutterella*, *Tenericutes Anaeroplasma* were significantly decreased by PEITC diet in Nrf2 KO mice at week 4.

**3.4 *Bacteroidetes*/*Firmicutes* ratio altering by genotype, time and dietary treatment**

The ratio of Bacteroidetes versus Firmicutes shows association with certain biological activity including 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 host genotype (**Fig. 7 A**). In addition, we also observed a slight association between the ratio and aging process from week 0 to week 4 (**Fig. 7B**), and no association between the ratio and animal 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. Patterns of *Bacteroidetes*/*Firmicutes* ratio are showing correlations with genotypes and aging, suggesting potential utilization of gut microbiome indicator in future clinicals.

**Metabolome results – Chi Chen…**

**4. Discussion**

Gut bacteria have been appreciated for many years with its potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system (Maslowski & Mackay, 2011; Ramakrishna, 2013; Rowland et al., 2018). For instance, gut bacteria *butyricicoccus pullicaecorum* and *butyricicoccus 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). Our current study demonstrates that host genotype and PEITC diet alter gut microbiota. Both bacterial diversity and individual bacterial strains change significantly based on different genotype and PEITC diet, and Nrf2 genotype shows stronger effects on the bacterial diversity than PEITC diet. *Firmicutes*, *Bacterodidetes* and *Proteobacteria* are the major bacterial phyla been altered by both PEITC diet and Nrf2 KO genotype. Individual bacteria at different taxonomic levels shows consistently affected pattern by both genotype and diet. For instance, *Firmicutes Ruminococcus* is observed increased by 0.05% PEITC treatment, and in Nrf2 KO genotype mice. All other altered microbiota changed significantly by either dietary PEITC or genotype are summarized in **Table 3**-**5**.

*Ruminococcus* are anaerobic, gram-positive bacteria and belong to the phylum of *Firmicutes*. So far, eleven *Ruminococcus* species have been identified and fall into bacterial family *Ruminococcaceae* and *Lachnospiraceae* (La Reau & Suen, 2018; Rainey & Janssen, 1995). Previous studies show that *Ruminococcus* spp. degraded and fermented cellulosic biomass into short-chain fatty acid (SCFA) for herbivorous ruminants (Flint, Bayer, Rincon, Lamed, & White, 2008; Leschine, 1995; Qin et al., 2010). Recently, *Ruminococcus Torques* was reported abundantly in the irritable bowel syndrome subjects in a placebo control double blind study (Lyra et al., 2010). Multiple probiotic interventions were able to reduce *Ruminococcus Torques* significantly based on results obtained from quantitative real-time polymerase chain reaction (qPCR), suggesting that *Ruminococcus Torques* may be used as biomarker in evaluating probiotic activity. As a part of normal flora in gastrointestinal tract, another *Ruminococcus* specie *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). This *Firmcutes* specie was also been reported to be overpopulated in infants who developed respiratory and skin allergic diseases (Chua et al., 2018). Mice orally garaged by purified *Ruminococcus Gnavus* also developed airway inflammation by cytokine secretion such as interleukin 25, 33 and others. In our current 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 irrespective of diet and Nrf2 genotype (**Table 3**). It is considered as an aging effect of gut microbiome to accumulate potential harmful inflammatory bacteria in the gut. However, when we divided the samples into WT group and Nrf2 KO group and found that the increased level of *Firmucutes Ruminoccus* were all from Nrf2 KO group but not in the Nrf2 WT group (**Table 4**, **5**), suggesting Nrf2 KO accelerates the increase of *Firmucutes Ruminoccus*. Since Nrf2 is a master regulator of anti-oxidative stress and anti-inflammation, suggesting that Nrf2 would play an important role in regulating the gut microbiota profile and suppressed certain “pathogenic” species such as *Firmucutes Ruminoccus* as the animal ages.

Interestingly, we also observed that the phylum *Ruminoccus* were also elevated on week 1 PEITC groups (Table 3). It may be caused by the disruption of 0.05% PEITC dietary intervention on the composition at early phase of gut microbiome. Other gut microbiota including *Bacteroidetes Rikenella* and *Cyanobacteria* were also found significantly elevated in Nrf2 KO groups, and both of them were involved in contributing or preventing gut diseases(Carmichael, 1992, 1994; Couturier-Maillard et al., 2013; Johnson, Heaver, Walters, & Ley, 2017). Overall, genetic KO (mutation) has a strong impact on the host microbiota profile over time and should be considered as one biomarker when developing probiotic or microbiota intervention therapy in future.

**Add discussion on Chi Chen metabolome…**

In this study, we conclude that mice genotype is strongly tightened up with gut microbiota alteration and diversity changes. Meanwhile, 0.05% PEITC dietary intervention also induces bacterial profiles changes by increasing and decreasing certain bacterial species. However, the study of gut microbiome is exploring an ecosystem containing more than just these two factors, but a more comprehensive and global environment. Research has demonstrated that cage and internal individual effects are contributing up to 32% and 46% of gut microbiota variances using 16s rRNA sequencing technology to identify the bacterial composition(Hildebrand et al., 2013). Several methods are used to eliminate the background noise interfering the 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). Our study showed no significant cage effect among all fecal samples, otherwise we would observe significantly clusters from **Fig. 3A, B**. Meanwhile, we observed samples significantly clustered based on mice genotype (**Fig. 6 A-D)**. Indeed, we discovered bacterial composition variance at the beginning of experiments after 2-week microbiome stabilization phase. These findings provide evidence to improve future microbiome study to set up baseline microbiota using antibiotic treatments and germ-free mice model(Lundberg, Toft, August, Hansen, & Hansen, 2016).

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**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. The means and SEM are plotted here.

**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 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 (Baseline), 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) (Baseline: n=3, Week 1: n=9; Week 4: n=9). A. Week 1 gut microbiome alterations on AIN93M group compared with baseline. B. Week 4 gut microbiome alterations on AIN93M group compared with baseline. C. Week 1 gut microbiome alterations on PEITC group compared with baseline. D. Week 4 gut microbiome alterations on PEITC group compared with baseline.

**Figure 5**. Comparison of microbiome differences between different mouse strains 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 Bacteroidetes and Firmicutes ratio over genotype, time and diet. A. Ratio distinguished by genotype. B. Ratio distinguished by time. C. Ratio distinguished by diet. D. Ratio 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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