

Gut microbiome and metabolome alterations in Nrf2 KO and DSS challenge gut inflammation mice model fed with phenethyl isothiocyanate and cranberry rich diets

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1 Introduction

Human and animal health can be affected by microorganisms including bacteria, archaea and fungi which are distributed in large quantities on surfaces throughout their bodies [1]. The role of gut bacteria is especially noted for their potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system [2-4]. To list a few examples, gut bacteria *Butyricicoccus Pullicaecorum* and *Butyricicoccus Pullicaecorum* produce butyrate, an essential metabolite for human GI homeostasis and disease prevention [5], lactobacillus strains are involved in essential vitamins metabolism [6] and human sleep quality improvement [7], and bifidobacterium strains might be able to influence human emotions like depression, reduce painful feeling, and alter brain activity during stress [8-12]. Numerous studies have been conducted to explore gut microbiota composition responding to specific conditions such as high fat diet or inflammatory bowel disease [13-19], however, some of the basic underlying molecular mechanism of gut regulation by these bacteria are poorly understood.

Systematic studies of gut microbiome regulators have shown 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 to co-occur with significant increase of relative abundance of *Firmicutes* (*Clostridiales*, *Lactobacillales*, *Turicibacterales*) and *Verrucomicrobia* (*Verrucomicrobiales*)

[20]. In contrast, *Bacterioidetes* (*Bacteroidales*) significantly decreased in high fat/sugar diet group. Additionally, *Clostridiales* and *Bacteroidales* 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 *Bacterioidetes 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 [21]. All three whole grain diets significantly increased the gut bacterial diversity (Shannon's and Simpson's indices), and the proportion of phylum *Firmicutes*, while decreases the proportion of phylum *Bacterioidetes*. At the individual level, genus *Bacterioides* 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, although opinions regarding its contribution diverge due to the potential confounding factors such as the diet [20, 22]. Simplified animal model using the same diet and living environment can help reveal the potential relationship between genotype and gut microbiota and helps remove some of the doubts. Results from a mice study conducted in 2011 [23] that used automated ribosomal intergenic spacer analysis and length-heterogeneity polymerase Chain Reaction (L-H PCR) [24] suggested that the observed gut microbiota alterations were genotype-dependent as all animals were housed at the same facility and given the same diet. Higher dissimilarities between genotypes than sexes were observed 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 [25]. 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 suggested the host-microbiota interaction by shifting bacterial composition including relative abundance of *Actinobacteria*, *Firmicutes*, and *Proteobacteria*.

Combined with diet, environmental conditions and inheritance, gut microbiome is one of the most impactful factors in maintaining human health. Gut microbiome composition determines how efficiently food is processed into metabolites such as amino acids, bile acids and short-chain fatty acids. In our study we used C57BL/6J wide type (WT) and Nrf2 gene knockout (KO) to test diets to which either cranberry or phenethyl isothiocyanate (PEITC) were added since both have been shown to boost the production of these metabolites. Possible health benefits of these food additives include cancer prevention and activation of Nrf2 pathway, a master regulator of oxidative stress and inflammation. PEITC been reported to inhibit colon inflammation [26, 27] and colon cancer [28, 29] but this effect could have been mediated by microbiome.

2. Materials and Methods

2.1 Experimental Design

C57BL/6J WT mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6J Nrf2 KO mice have been maintained in our laboratory since 2005 [30, 31]. Mice were kept in 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 given a 2-week gut microbiota equalization period during which they were fed with AIN93M control diet (Research Diets, Inc. NJ).

In the first experiment (Nov18), 6 Nrf2 KO mice were randomized into 2 treatment groups after the 2-week equalization period. One group continued receiving the control diet (AIN93M) while the second group's diet was enhanced with 0.05% PEITC. In the second experiment (May19), 10 WT mice were randomized into either the control diet group (AIN93M) or the PEITC-enhanced diet group. In the third experiment (Sep19), additional cranberry diet was introduced, and 20 mice were challenged with DSS to induce gut inflammation. Both, WT and Nrf2 KO genotypes were used, and the mice were randomized into one of four treatment groups (Naïve, DSS, DSS + PEITC, and DSS + Cranberry) within each genotype (Figure 1). Fecal samples for 16S sequencing were collected at weeks 1 and 5 in the Nov18, weeks 0 and 4 in the May19, and weeks 0, 1 and 8 in the Sep19 experiments. Samples from 3 out of 5 mice in each treatment group in Sep19 experiment were sent for sequencing. Additional samples were collected from all mice for metabolomics analysis at weeks 2 and 6 in Sep19 experiment. Since the fecal sample collection timing varied slightly between the experiments, it was realigned and labeled as baseline (end of the equalization period, i.e., Week 0), early (weeks 1 through 2) or late (weeks 4 through 8) timepoints. Fecal samples were snap frozen in liquid nitrogen and stored at -80°C for 16S ribosomal RNA (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.

2.2 16S ribosomal RNA gene 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) [32-38]. 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 Quantitative Insights Into Microbial Ecology (QIIME2) bioinformatic pipeline [39, 40] and Divisive Amplicon Denoising Algorithm 2 (DADA2 version 1.16) R package [41].

QIIME2 mapped reference at 97% similarity against representative sequences of 97% OTU in SILVA, a high quality rRNA database [42], followed by chimeric sequences removal from subsequent analyses [43]. Principle coordinates analysis (PCoA) of unweighted UniFrac analysis was performed to visualize similarity of microbial communities of the samples.

DADA2 pipeline was used to process *FastQ* sequence data files containing pair-ended reads with average length of 300 base pairs (bp) into a high-resolution 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. Sequencing depth varied between 30,008 and 422,283 reads per sample (Figure 2).

Over 94% of OTUs were identified as bacterial. 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. Finally, OTUs not mapped to any bacterial phylum were removed, and the remaining OTUs analyzed.

2.3 Microbial metabolites analysis

The concentrations of microbial metabolites (free amino acids, bile acids and SCFA) 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 index at OTU level. The index is equal to zero when there is exactly one class (a single OTU) present in a sample. Larger values of the index indicate greater number of and more evenly distributed OTUs, with the highest value of the index reaching $\ln(k)$ with k number of equally distributed OTUs. The estimates were presented as means \pm standard error of the means (SEM).

Multivariable analysis of variance (ANOVA) using genotype, diet and timepoints was performed followed by multiple comparison with false discovery rate (FDR) adjustment for the p-values.

Bacterial composition at different taxonomic levels was explored using principal components analysis (PCA) and visualized as biplots. PCA is a linear transformation that projects the data from the original n -dimensional, 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 (PC2) - the second most and orthogonal to PC1, and so on. The samples were then plotted against 2 principal components (e.g., PC1 and PC2) and labeled to check for group separation. Biplot is an extension of PCA plot that simultaneously display the labeled samples in two principal components' space as well as the direction and the magnitude of the original axes (i.e., individual taxonomic units). Multinomial regression on class (group labeling corresponding to taxonomic units) vs. principal components was performed to statistically assess the predictive power of PCA on class separation.

Metabolites' quantities were presented as heatmaps. ANOVA was used to test for group mean differences for each metabolite individually and presented as boxplots with bars and stars indicating statistically significantly different groups.

3 Results

3.1 Genotype and diet affect bacterial community richness and diversity

Nrf2 is a master regulator of anti-oxidative stress and anti-inflammatory responses to external and internal stimuli [44-48]. The impact of Nrf2 was examined by comparing the Nrf2 knockout (KO; -/-) mice vs. the control (WT) at different conditions (diet, DSS challenge, and aging). Alpha diversity analysis of the bacterial OTUs was conducted using Shannon index (Figure 3).

Mixed-effects regression analysis showed that the alpha diversity was higher in Nrf2 KO compared to the WT genotypes (p-value < 0.01), went up as the study progressed (both, the p-values for the early and the late timepoints vs. the baseline < 0.01), and was lower in the DSS+PEITC and DSS+Cranberry diet groups compared to the group that was not challenged with DSS (both p-values < 0.01).

However, Shannon index (as well as other indices measuring inequalities in the samples) is biased by the sample's sequencing depth. Specifically, deeper sequencing results in identification of more, rare OTUs, therefore inflating the index (Figure 4A). To remediate for this effect, a sensitivity analysis was conducted by, first, adding 1 to all counts in the combined OTU table. Even though the zeros in the table could represent either complete absence of an OTU from a sample or very low abundance, the zeros were treated similarly here. This remediation removed the Shannon index/sequencing depth correlation (Figure 4B). After repeating the analysis on the corrected Shannon index, genotype **differences remained statistically significant (higher alpha diversity in the Nrf2 KO group compared to WT, p-value = 0.02)** but aging effect disappeared and only the DSS+AIN93M group's alpha diversity remained significantly lower compared to the group not challenged with DSS (p-value < 0.01). **These results suggest that PEITC and cranberry-rich diets had protective effect on the hosts' microbiome diversity.** The averages of the corrected Shannon indices are presented in Figure 5.

3.2 Principal components analyses reveal association of microbiome composition at Phylum and Class taxonomic levels with genotype and diet

OTU counts were aggregated at the **Phylum level**. In total, 17 phyla were identified, top 10 of which accounted for >99.96% of all hits. Since deeper sequencing increases chances of identifying rare organisms (Figure 6), and the samples varied greatly by sequencing depth (Figure 2), rare phyla were not included in the downstream analysis. PCA was conducted on the combined data from the 3 experiments, but scores and loadings were graphed in separate panels by genotype and experiment to highlight the differences (Figure 7). The biplot showed large between-experiment variability, specifically, higher relative abundance of *Bacteroidetes*, and lower relative abundance of *Verrucomicrobia* in the first two experiments (Nov18 and May 19) compared to the third one (Sep19). Relative abundances of *Firmicutes* and *Actinobacteria* were higher in the WT DSS-treated mice in the Sep19 experiment compared to all other groups, while *Epsilonbacteraeota* were more abundant in all Nrf2 KO and WT control (AIN93M) groups

compared to the rest. Additionally, DSS+PEITC group samples showed trend reversal from the positive control (DSS+AIN93M) group in WT, suggesting protective effect of PEITC on microbiome of DSS-treated mice.

To remove study effect, Sep19 data was separated and reanalyzed. The top 10 most abundant Phylum were used for the PCA (Figure 8). The analysis revealed strong diet effect on the microbial composition. Specifically, relative abundance of *Firmicutes* and *Verrucomicrobia* increased while relative abundance of *Proteobacteria*, *Deferribacteres* and *Epsilonbacteraeota* decreased in all WT DSS-treated groups compared to the control (AIN93M).

Class-level aggregation yield 31 classes, with top 17 adding up to >99.99% of the total hits. The PCA showed strong negative effect of Nrf2 KO on *Bacilli* class (phylum (p.) *Firmicutes*) that was consistent in all 3 experiments (Figure 9). Separate analysis of Sep19 data identified 18 out of the 31 classes, with 2 of them at a very low level, hence, only 16 classes were used in this analysis. The biplot (Figure 10) showed clear separation by genotype. Relative abundance of *Clostridia* (p. *Firmicutes*) was higher while *Betaproteobacteria*, *Epsilonproteobacteria* and *Deltaproteobacteria* (p. *Proteobacteria*), as well as *Campylobacteria* (p. *Epsilonbacteraeota*), *Brachyspirae* (p. *Spirochaetes*), and *Deferribacteres* (p. *Deferribacteres*) were lower in the all three DSS-treated groups. *Verrucomicrobiae* (p. *Verrucomicrobia*) and *Gammaproteobacteria* (p. *Proteobacteria*) had higher relative abundance in the DSS+AIN93M and DSS+Cranberry groups.

3.3 Firmicutes/Bacteroidetes ratio

Firmicutes to Bacteroidetes ratio (F/B) have been linked to biological activity including aging [49] and body mass index change [50] and maintaining intestinal homeostasis. Increased F/B ratio was associated with obesity while the ratio decreased was correlated positively with inflammatory bowel disease [51]. In this study, the F/B ratios were calculated within each sample and compared across the experiments, genotype, diet and timepoints (Figures 11 and 12). Samples from the first two experiments (Nov18 and May19) contained equal or lower abundance of Firmicutes compared to Bacteroidetes but the F/B ratios in the WT mice samples were higher than in the Nrf2 KO samples in all 3 experiments. Formal analysis using a mixed-effects linear regression models was conducted on Sep19 data. A model with no interaction terms showed significant decrease of F/B ratio in DSS+PEITC and DSS+Cranberry groups ($\log_2[F/b] = -0.51$ and -0.46 , and p-values <0.01 and $=0.01$, respectively) as well as decrease in the Nrf2 KO group compared to WT ($\log_2[F/B] = -1.02$, p-value <0.01). The control diet group (AIN93M, no DSS challenge) average F/B ratio difference with the DSS control (DSS+AIN93M) as well as difference between early or late vs. timepoints vs. baseline were not statistically significant. Results from a model containing an interaction term for the genotype and diet confirmed significant association of F/B ratio with genotype ($\log_2[F/B] = -1.40$, p-value <0.01), as well as with PEITC and Cranberry diets ($\log_2[F/b] = -0.60$ and -0.71 , respectively, with both p-values <0.01). Additionally, the F/B ratio of the AIN93M group was significantly lower than the DSS+AIN93M ($\log_2[F/B] = -1.40$, p-value <0.01). These results suggest that PEITC and Cranberry supplements to regular grain diet reversed the effect of DSS challenge on the balance of Firmicutes and Bacteroidetes in the hosts' intestines.

3.4 Linear Discriminant Analysis of aging and dietary effects

To further examine the potential differences of the microbiota between control AIN93M diet versus PEITC diet, a parallel analysis in QIIME2 was conducted using Linear discriminant analysis Effect Size (LEfSe).

Firstly, the potential aging effect on the microbiota was examined by comparing the Control samples at baseline (shown in red) with the early (Week 1) and late (Week 4) timepoints shown in green). Taxa with relative abundance $\geq 0.1\%$ 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 (Figure 13). *Bacteroidetes Prevotella*, *Bacteroidetes Parabacteroides*, *Bacteroidetes*, and *Bacteroidetes S24_7* relative abundance decreased, while *Bacteroidetes Bacteroidales*, *Firmicutes Clostridiales*, *Firmicutes Oscillospira*, *Proteobacteria Desulfovibrionaceae*, and *Tenericutes Anaeroplasma* increased over time.

Next, the impact of PEITC-supplemented diet was examined. Figures 14 show the impact of PEITC diet by comparing the microbiota for control diet at baseline (Week 0, shown in red) and at the later timepoints (Weeks 1 or 4, shown in green). Relative abundance of *Firmicutes Ruminococcus* significantly increased and *Bacteroidetes S24_7* significantly decreased at both at the later timepoints compared to baseline. Some bacteria were uniquely correlated with diet. *Bacteroidetes Odoribacter*, *Tenericutes Mycoplasmataceae* and *Proteobacteria Desulfovibrionaceae* significantly higher relative abundance in the control (AIN93M) group while *Firmicutes Clostridiales*, *Firmicutes Ruminococcus* and *Acidobacteria Ellin 6075* were found in significantly higher abundance in the PEITC group.

3.5 PEITC and cranberry feeding partially reverse the DSS-induced changes in fecal metabolome

Metabolomics profiles of DSS, DSS + PEITC, and DSS + Cranberry treatment group fecal samples collected at weeks 2 and 6 were analyzed and the concentrations of free amino acids, bile acids and short-chain fatty acids (SCFA) were quantified (Experiment 3 only).

Principal components analysis showed that overall levels of all but one (taurine) amino acids were elevated in the Cranberry diet group (Figure 15A). However, for bile acids genotype rather than diet played a bigger role, with higher production of all bile acids in the Nrf KO and especially increase of LCA, DCA, MCA, CDCA, GDCA and GCDCA driving the separation between the two genotypes (Figure 15B).

Multinomial regression models were fitted to predict sample treatment and diet or genotype with principal components as predictors. The model with the first 3 principal components accurately classified 29 out of 48 samples (60.4%) by treatment/diet and the predictive power increased slowly by adding more PC (Table 3). However, the model predicting genotype correctly classified 34 out of 48 samples (70.8%) with just the first principal component (Table 4) suggesting stronger separation of the samples by genotype.

Examination of the metabolites individually showed that DSS treatment altered the production of several of them while PEITC and cranberry feeding reversed the changes (Figure 16 A). For example, DSS decreased the concentrations of many amino acids such as glutamate, phenylalanine, and proline, but PEITC and cranberry cotreatments prevented these decreases (Figure 16 B-D). Furthermore, PEITC and cranberry cotreatments reversed the DSS-induced increases of secondary bile acids, mainly deoxycholic acid (DCA), lithocholic acid (LCA), and muricholic acid (MCA) (Figure 16 E-G). In contrast, PEITC and cranberry cotreatments had limited effects on the DSS-induced changes in SCFA (Figure 16 H-J). Overall, these data indicated that PEITC and cranberry (rich in anthocyanins) are capable of modulating the metabolic responses to DSS treatment in the colorectal tract, potentially through their effects on the microbiome.

In addition, the concentrations of fecal metabolites were compared between WT and Nrf2 KO mice. Interestingly, compared to WT, Nrf2 KO mice had lower levels of amino acids (shown by glutamate, phenylalanine, and proline) and SCFA, and higher levels of secondary bile acids (shown by DCA, LCA, and MCA) than WT mice (Figure 617A-I), which were similar to the metabolite profile of DSS-treated WT mice.

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 [2-4]. For instance, gut bacteria *Butyricicoccus Pullicaecorum* and *Butyricicoccus Pullicaecorum* produce butyrate, an essential metabolite for human homeostasis and disease prevention [5] while *Lactobacillus* strains are involved in essential vitamins metabolism [6]. The current study demonstrated that host genotype and diet may alter gut microbiota. Both bacterial diversity and individual bacterial strains changed significantly based on different genotype and diet, and Nrf2 KO genotype shows stronger effects on the bacterial diversity than diet. *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, the most abundant bacterial phyla, have been altered by both, diet and Nrf2 KO. Individual bacteria at different taxonomic levels showed a pattern of being consistently affected by both, genotype and diet. For instance, *Firmicutes Ruminococcus* was observed to be in higher relative abundance in the PEITC-supplemented groups and in Nrf2 KO mice.

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* [52, 53]. Previous studies show that *Ruminococcus* degraded and fermented cellulosic biomass into short-chain fatty acid (SCFA) for herbivorous ruminants [54-56]. Recently, *Ruminococcus Torques* was reported to be abundant in the irritable bowel syndrome subjects in a placebo control double blind study [57]. Multiple probiotic interventions were able to reduce *Ruminococcus Torques* abundance 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* species, *Gnavus* showed to be in high abundance in the IBD patients, with increased level of oxidative stress in the gut [58],

potentially caused by cytokine production such as TNF- α [59]. *Firmicutes* has also been reported to be overpopulated in infants who developed respiratory and skin allergic diseases [60]. Mice orally gavage by purified *Ruminococcus Gnavus* also developed airway inflammation by cytokine secretion such as interleukin 25, 33 and others. In this study, we observed a significant increase in the abundance of *Firmicutes Ruminococcus* in fecal samples at the late but not at the early timepoints irrespective of diet and genotype. Accumulation of harmful inflammatory bacteria in the guts is considered has been linked to aging. However, we found that the increased level of *Firmicutes Ruminococcus* was mainly associated with Nrf2 KO suggesting that Nrf2 KO accelerates the increase of *Firmicutes Ruminococcus*'s relative abundance. This suggests that Nrf2 might play an important role in regulating the gut microbiota profile and suppress pathogenic species such as *Firmicutes Ruminococcus* as the animal age.

Interestingly, we also observed that the phylum *Ruminococcus* were also elevated at the early timepoint in the PEITC groups. *Bacteroidetes Rikenella* was also found significantly elevated in Nrf2 KO groups, suggesting that it may contribute or prevent gut diseases [61-64]. 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 the future.

In this study, we conclude that mice genotype is strongly associated with gut microbiome richness and diversity and compositional changes. However, many more factors contribute to difference. Research has demonstrated that cage and individual effects are contributing up to 32% and 46% of gut microbiota variability, respectively [65]. Several methods are used to eliminate the background noise that include feeding the animals with a control diet for several weeks to equalize microbiomes at baseline or using gnotobiotic (germ-free) mice implanted with homogenized fecal samples [66-68]. In this study, we employed the former, but it still produced moderate level of variability at the baseline. However, gnotobiotic models are not without complications as they require germ-free facilities and the animals' immune system may be affected by the lack of microbiome at the early stages of their lives. A middle ground can be reached by pretreating the animals with wide-spectrum antibiotics and providing them with high fiber content food before implanting them with homogenized fecal samples [69].

5 Figures and Tables

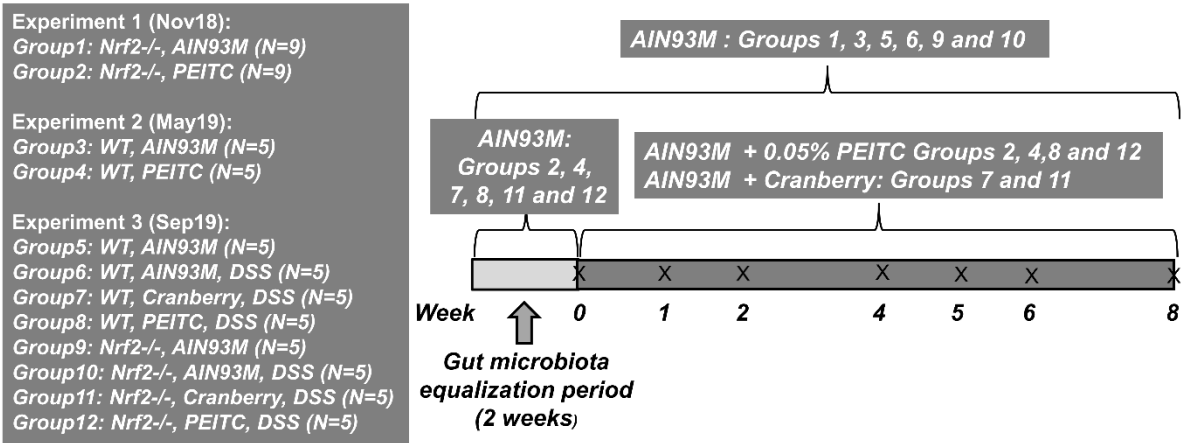


Figure 1: Experimental designs and 16s rRNA metagenomics summary. A. Experimental design. B. Sequence depth. C. OTUs annotated based on reference genome.

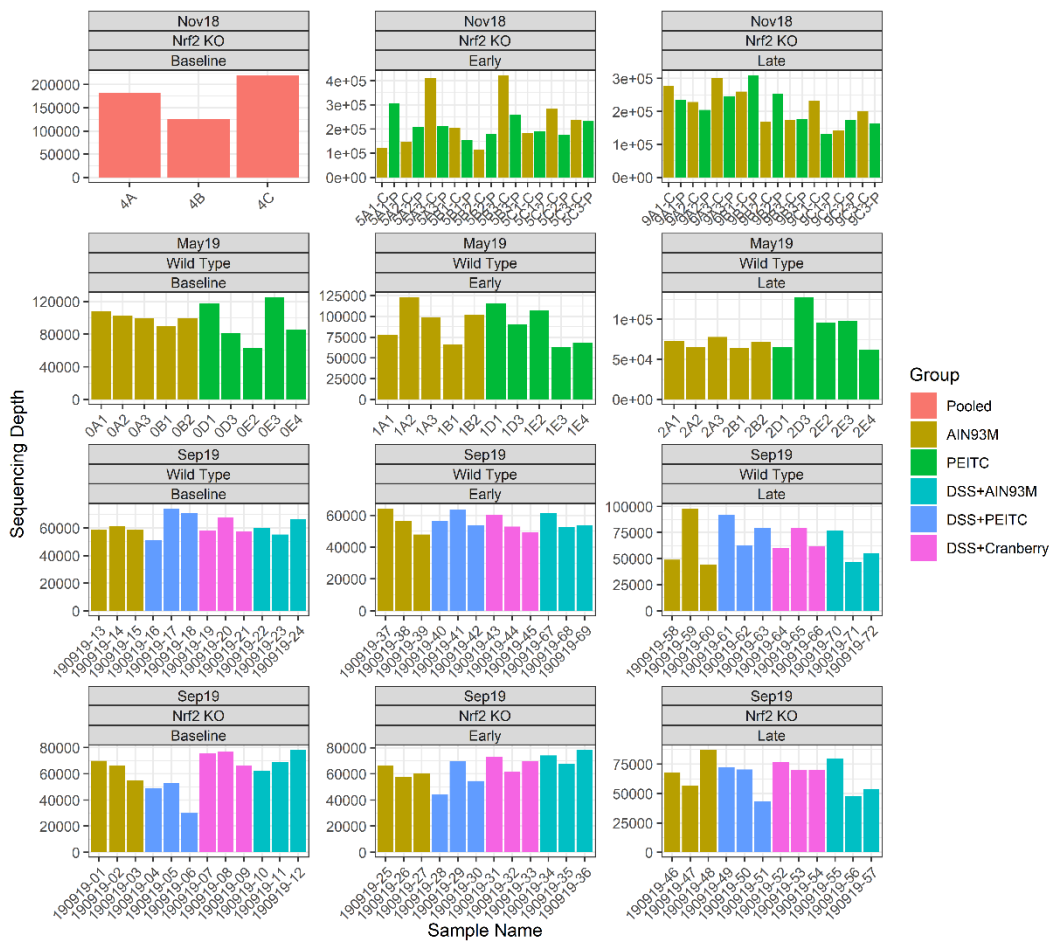


Figure 2: 16S sequencing depth

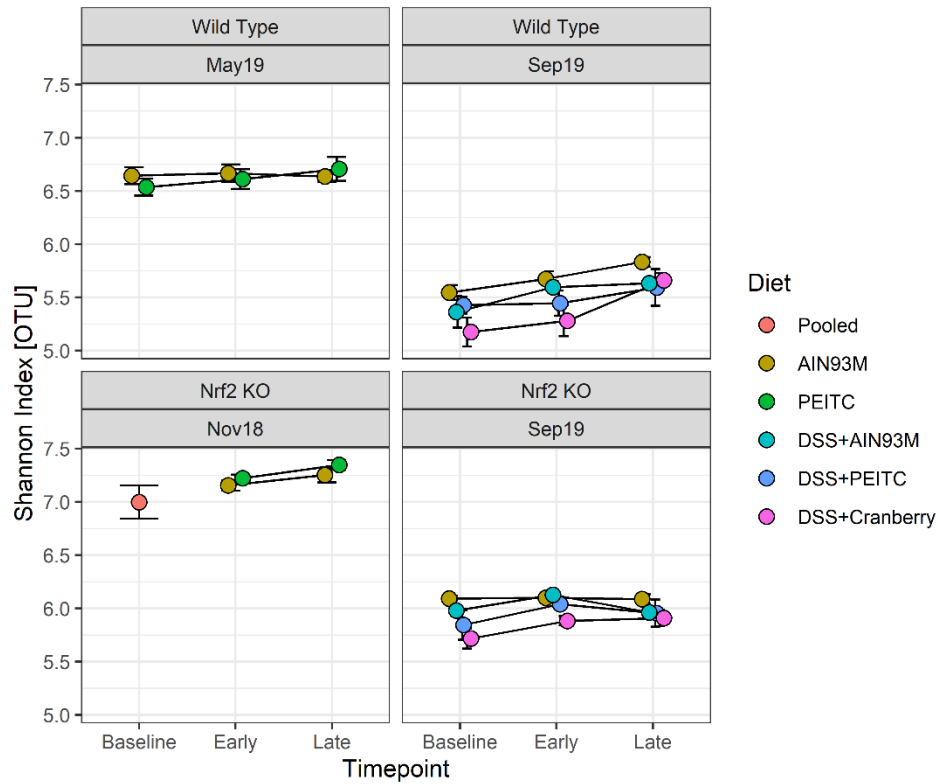


Figure 3: Shannon's index (Alpha diversity)

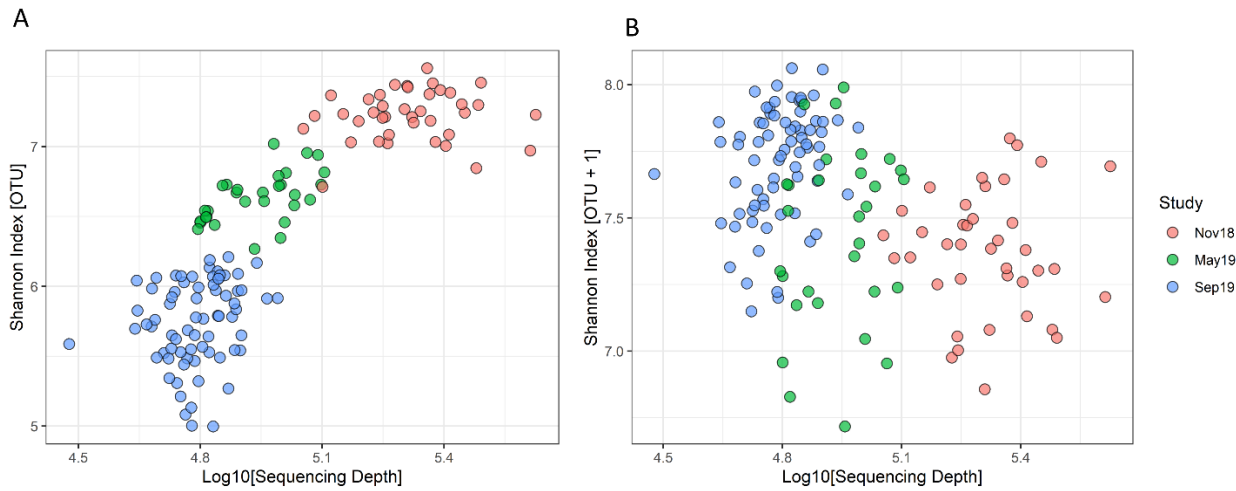


Figure 4: Shannon index vs. sequencing depth. (A) original and (B) OTU+1

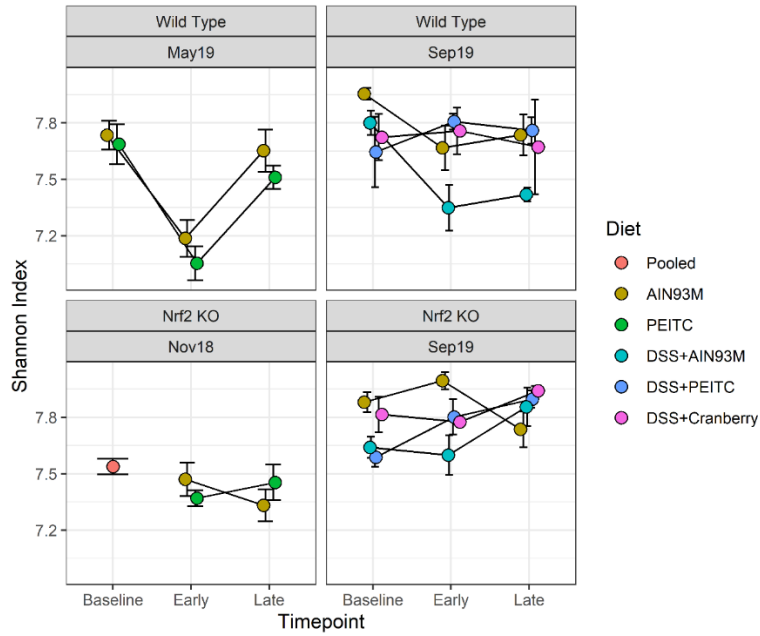


Figure 5: average corrected Shannon index (OTU+1)

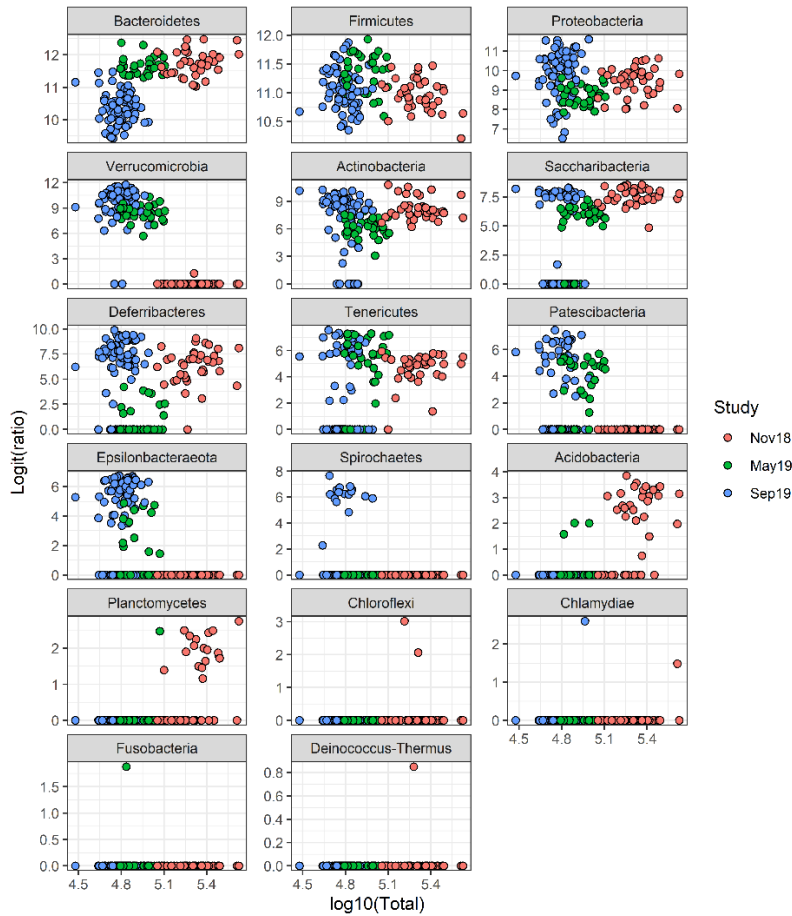


Figure 6: logit relative abundance of Phylum vs. sequencing depth

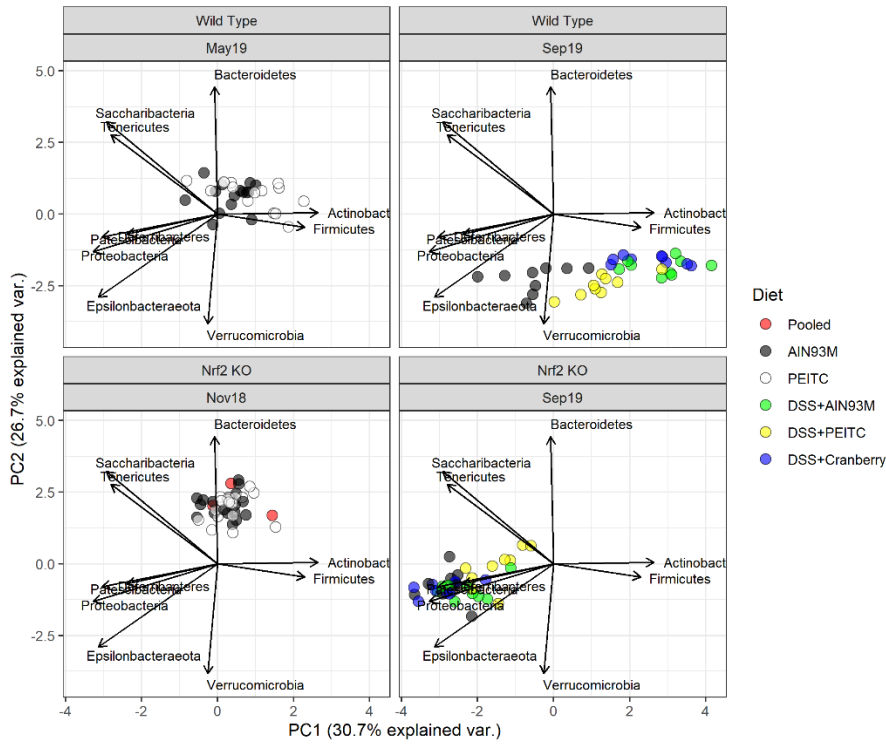


Figure 7: biplot of logit relative abundance of Phylum

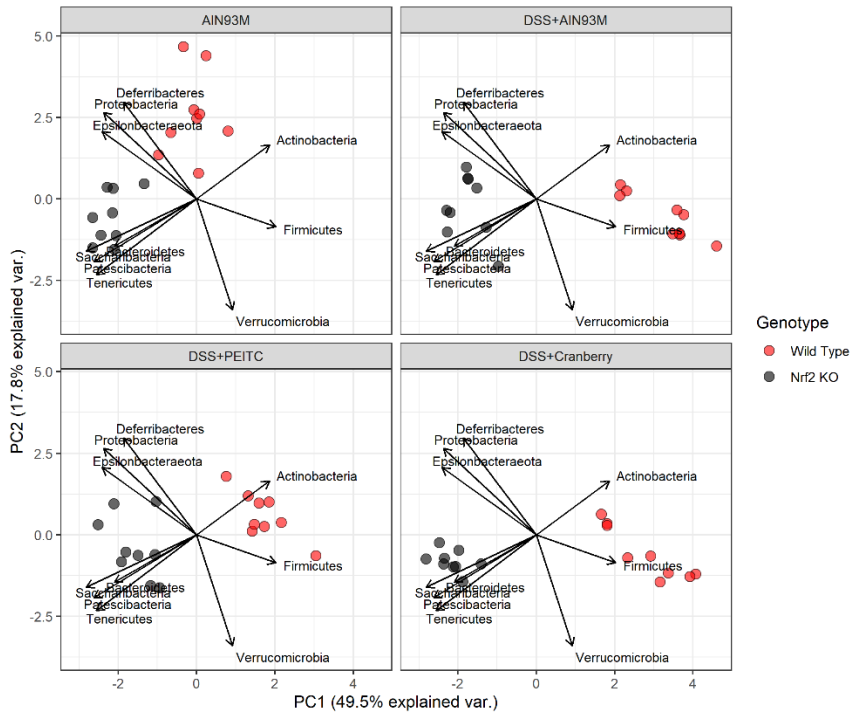


Figure 8: biplot of logit relative abundance of Phylum Sep19 experiment only

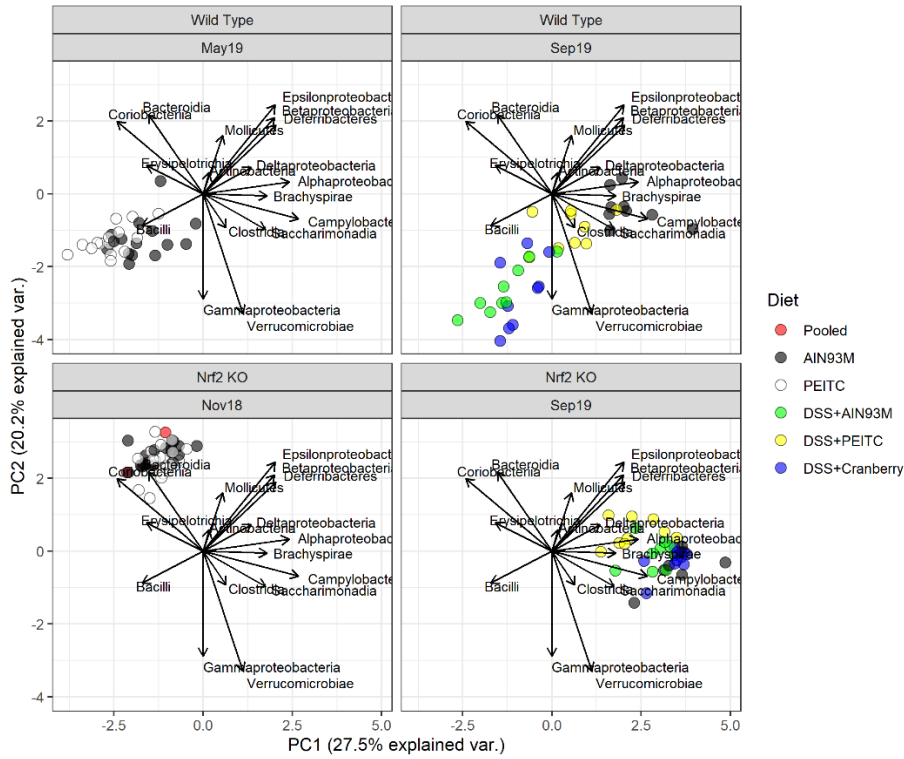


Figure 9: biplot of logit relative abundance of Classes

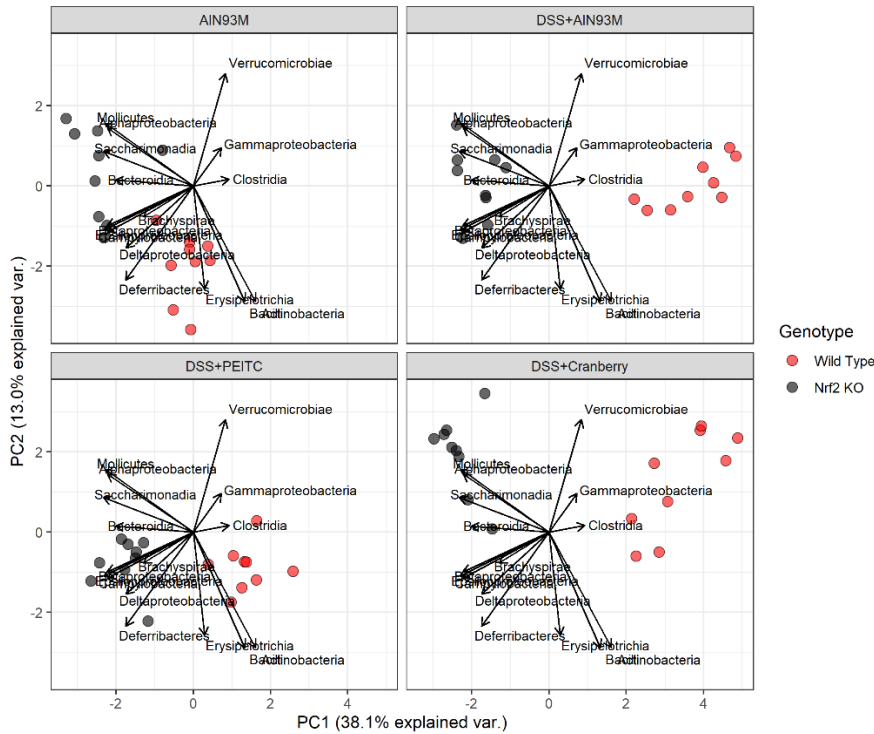


Figure 10: biplot of logit relative abundance of Classes in Sep19 experiment only

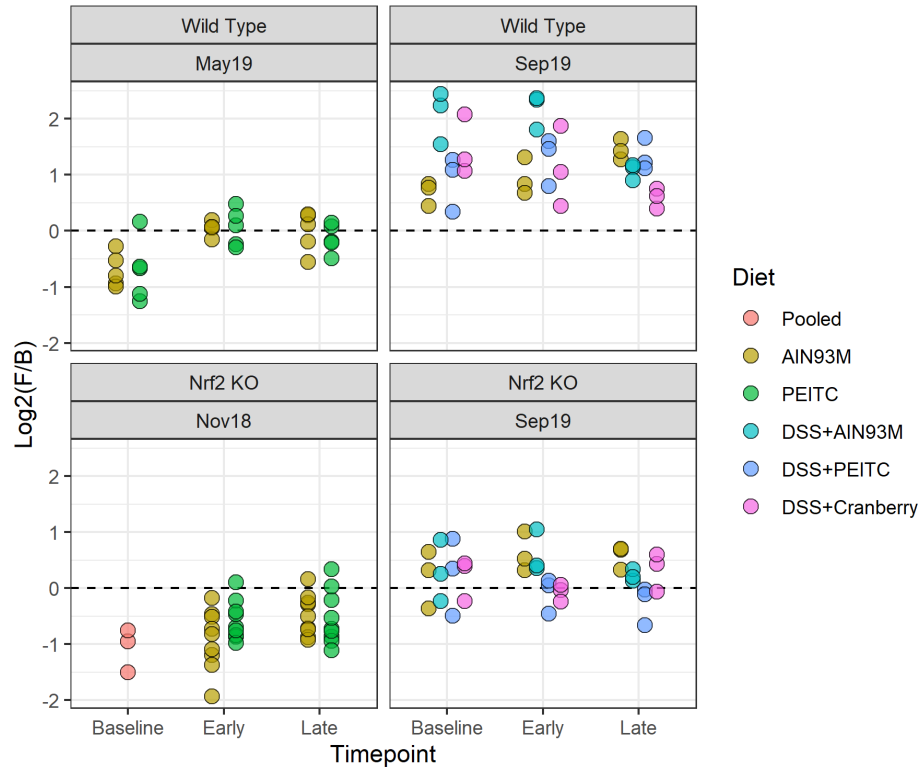


Figure 11: \log_2 of Firmicutes/Bacteroidetes ratios by experiment, genotype and diet over time

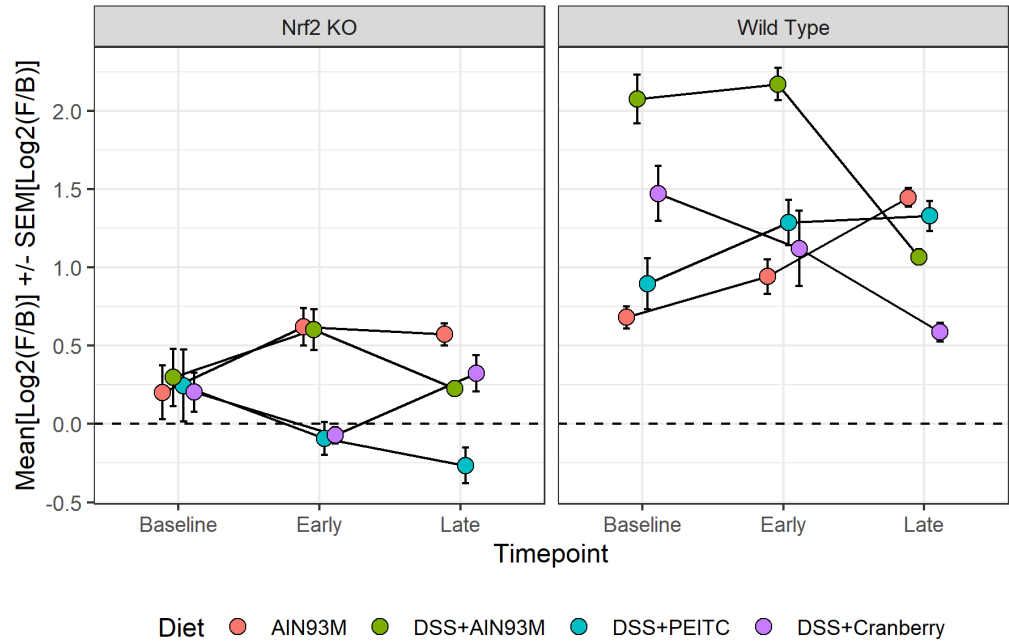


Figure 12: means of \log_2 F/B ratios by genotype and diet over time. The bars represent standard errors of \log_2 (F/B) ratios.

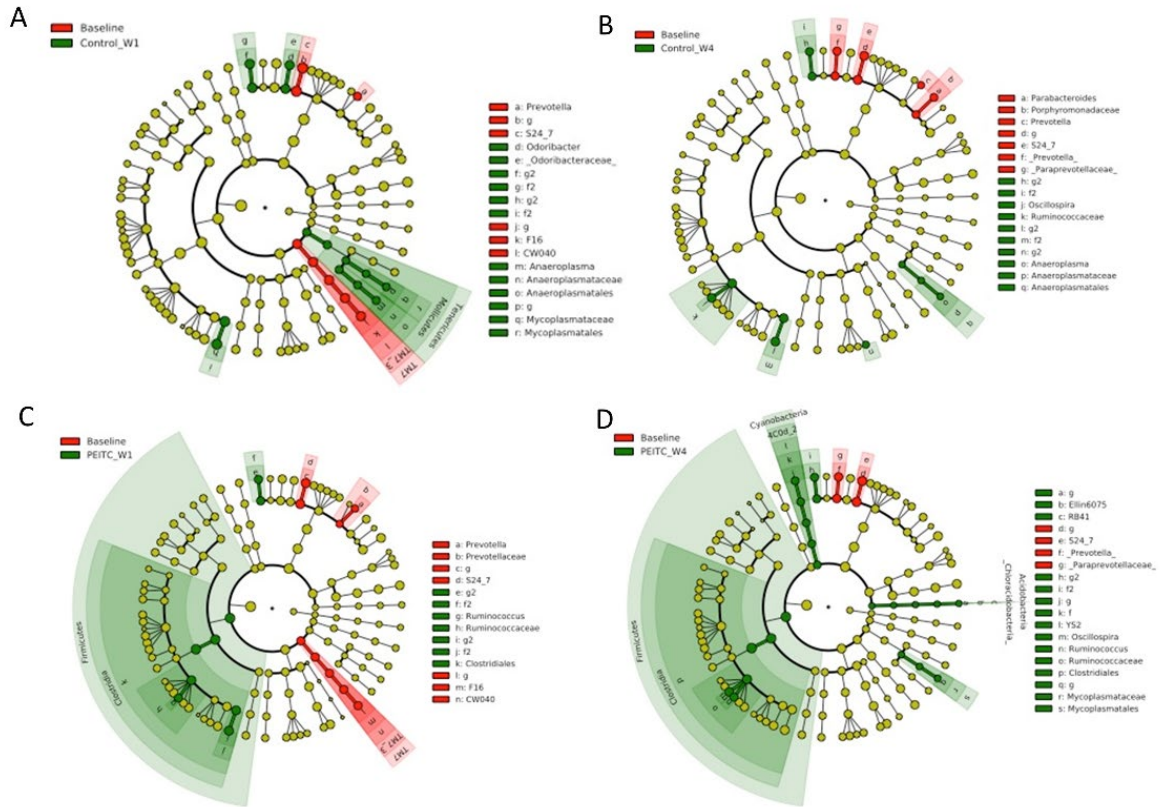


Figure 13: Linear discriminant analysis Effect Size (LEfSe) analysis of aging effect.

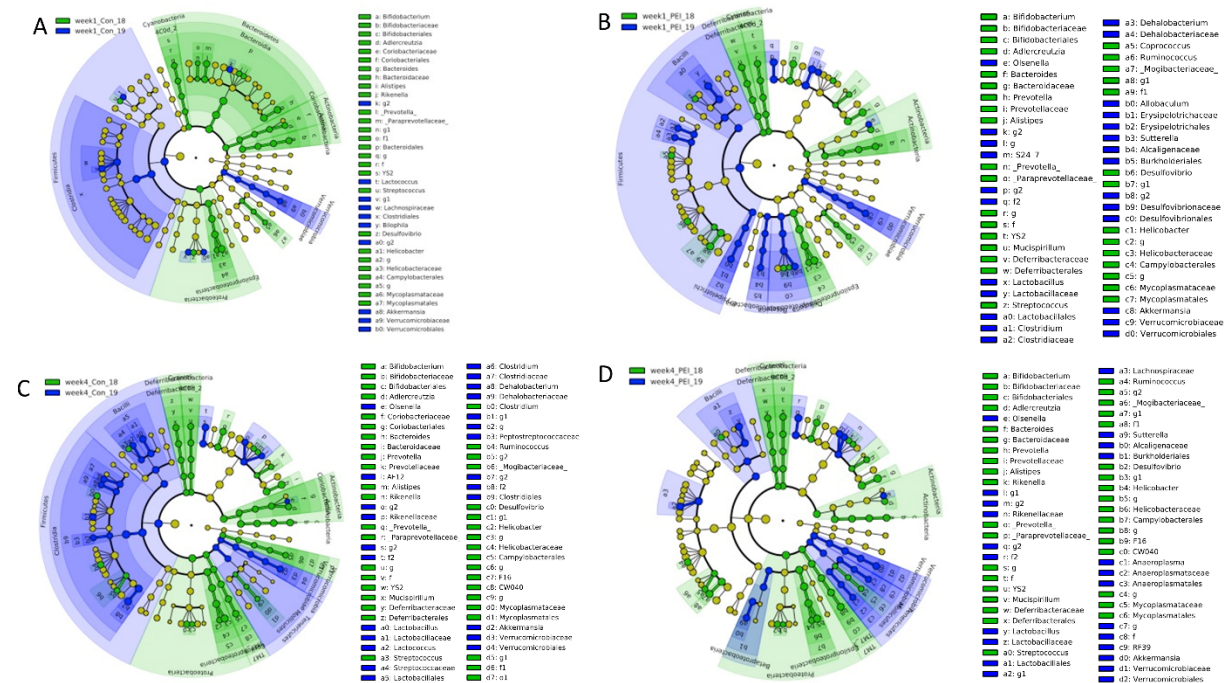


Figure 14: Linear discriminant analysis Effect Size (LEfSe) analysis of diet effect.

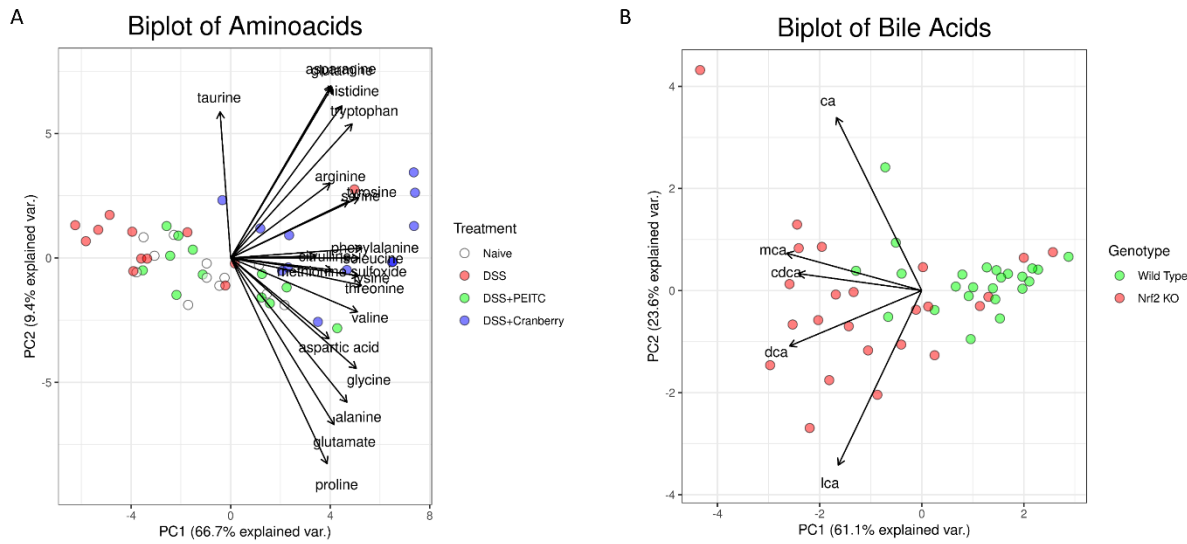


Figure 15: biplots of amino acids by diet and bile acids by genotype.

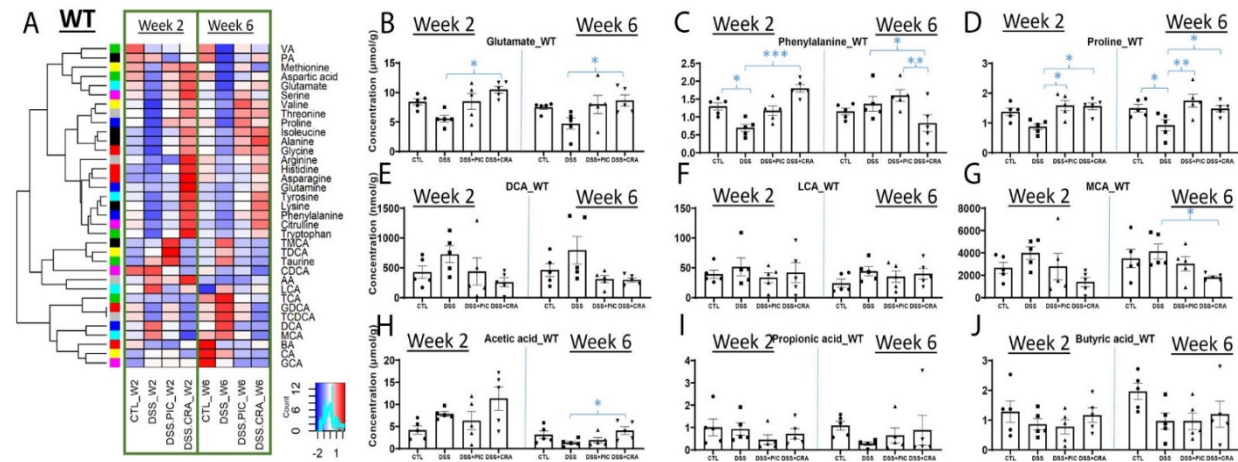


Figure 16: Effects of DSS, PEITC and cranberry cotreatments on fecal metabolome of WT mice. Fecal samples collected at week 2 and 6 of 4 treatments, including control (CTL), DSS, DSS+PEITC (DSS+PIC), and DSS+cranberry (DSS+CRA), were analyzed by 4 LC-MS methods (143). The concentrations of amino acids, bile acids, and SCFA were quantified. (A) A heatmap on the distribution of amino acids, bile acids and SCFA in fecal samples from 4 treatments. (B-D) Concentrations of major amino acids, including glutamate, phenylalanine, and proline. (E-G) Concentrations of major bile acids, including DCA, LCA, and MCA. (H-J) Concentrations of major SCFA, including acetic acid (AA), propionic acid (PA), and butyric acid (BA).

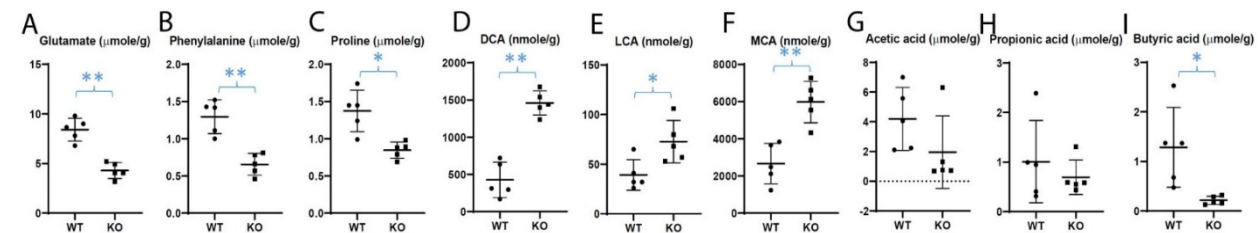


Figure 17: Differences in fecal metabolite profile between WT and Nrf2-null (KO) mice. The concentrations of amino acids, bile acids, and SCFA were quantified in the fecal samples from untreated WT and KO mice (143). (A-C) Concentrations of glutamate, phenylalanine, and proline. (D-F) Concentrations of major bile acids. (G-I) Concentrations of major SCFA.

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

Forward Primer	Reverse Primer
515F (Parada)	806R (Apprill)
GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT

Table 2: OTUs mapping to Kingdoms. Number of detected OTUs (% total)

Kingdom	Experiment 1: Nrf2 KO Mice	Experiment 2: WT Mice	Experiment 3: WT and Nrf2 KO	Combined
Bacteria	10,197 (94.78%)	7,994 (98.34%)	7,558 (96.07%)	22,251 (95.73%)
Eukaryota	472 (4.39%)	116 (1.43%)	232 (2.95%)	812 (3.49%)
Archaea	4 (0.04%)	0 (0%)	2 (0.03%)	6 (0.03%)
Unknown	86 (0.80%)	19 (0.23%)	75 (0.95%)	175 (0.75%)

Table 3: multinomial regression predictions of treatment groups by microbial metabolite PCA

Predicted treatment/diet PC1+PC2+PC3	Actual treatment/diet			
	DSS	Naïve	DSS + PEITC	DSS + Cranberry
DSS	8	4	4	0
Naïve	2	4	1	0
DSS + PEITC	1	3	6	1
DSS + Cranberry	1	1	1	11

Table 4: multinomial regression predictions of genotype by microbial metabolite PCA

Predicted genotype PC1	Actual genotype	
	Wild Type	Nrf2 KO
Wild Type	18	6
Nrf2 KO	8	16

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