Microbiome and metabolome alterations in Nrf2 knockout mice with induced gut inflammation and fed with phenethyl isothiocyanate and cranberry enriched diets

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# Abbreviations

ARE - antioxidant response element

CDCA - chenodeoxycholic acid

DCA - dichloroacetic acid

DSS – dextran sulfate sodium

GCDCA - glycochenodeoxycholic Acid

GDCA - glycodeoxycholic acid

LCA - lithocholic acid

MCA - muricholic acid

NRF2- NF-E2-related factor 2

OTU – operational taxonomic unit

PCA – principal components analysis

PEITC - phenethyl isothiocyanate

qPCR - quantitative polymerase chain reaction

**Keywords**: Nrf2, PEITC, Gut inflammation, Phenethyl isothiocyanate Cranberry

# Abstract

## Scope

Cranberries contain phytochemicals with potent antioxidant properties. Phenethyl isothiocyanate (PEITC) is abundant in crucifers and possesses anti-cancer and anti-inflammatory properties. These food additives can alter gut microbiota and improve host’s health. Microbiome and microbial metabolome interactions with the host’s cells help maintaining gastrointestinal (GI) tract homeostasis.

## Methods and results

Cranberry and PEITC enriched diets were fed to wild-type (WT) and Nrf2 knockout (KO) mice, including those challenged with dextran sulfate sodium (DSS), and their gut microbiomes and metabolomes examined. Relative abundances of *Deferribacteres*, *Epsilonbacteraeota* and *Proteobacteria* decreasedwhile *Firmicutes* and *Verrucomicrobia* increased in the DSS-challenged mice samples. These trends were reversed by PEITC and cranberry enriched diets. The diets also preserved the *Firmicutes*-to-*Bacteroidetes* ratio, an endpoint associated with gut inflammation and obesity. DSS challenge altered production of several metabolites. Nrf2 KO mice samples had lower concentrations of short-chain fatty acids (SCFA) and amino acids, and higher concentrations of secondary bile acids.

## Conclusions

Nrf2 KO mice microbiomes exhibited higher richness and diversity. PEITC and cranberry enriched diets positively affected hosts’ microbiomes and boosted several microbial metabolites. Phenotypic expression of Nrf2 impacted the microbiota and metabolic reprogramming induced by DSS-mediated inflammation and dietary supplements of cranberry and PEITC.

# 1. Introduction

Microbial communities leaving on and in the surfaces of human and animal bodies can drastically affect the host’s health. These microorganisms include archaea, bacteria and fungi that inhabit the skin and the inner lining of the gastrointestinal tract (GI). (1). They help to metabolize essential nutrients, provide energy and interact with the host’s immune system. (2, 3, 4). For example, *Butyricicoccus Pullicaecorum* and its metabolite butyrate are important for human GI homeostasis and diseases prevention (5). *Lactobacillus* strains play important role in metabolism of essential vitamins (6) and improvement of sleep quality (7). Another group of bacterial strains, *Bifidobacterium* have been suggested to influence higher cognitive functions in humans and to be associated with depression, pain and brain activity during stress. (8, 9, 10, 11, 12). Multiple studies have shown a link between composition of gut microbiomes and host’s diet containing high levels of fiber or fat, and with GI disorders such as inflammatory bowel diseases (17,13, 14, 15, 16, 18, 19, 20, 21). Host’s genotype was also found to be factor associated with microbiome composition, especially in infancy (22, 23). The combination of host genotype, gut microbiota and postnatal factors such as antibiotic usage, dietary pattern and environmental microbes have been shown to have a significant influence on human gut development and homeostasis (24, 25). Hence, the underlying mechanism of such microbiota-host crosstalk is crucial but remains poorly understood.

Cranberry have a long history of use by Native Americans as food source and medicine (26). Today, cranberry is widely consumed as fresh and dried fruit, juice and sauce. The berries are known for their high content of bioactive compounds including proanthocyanidins, flavonoids and other organic acids (27, 28). These compounds offer notable nutritional and health benefits such as reduced risk of urinary tract infections (29, 30) and inflammation (31), and improved cardiovascular health (32). Phenethyl isothiocyanate (PEITC) belongs to the isothiocyanate family of compounds which are formed when glucosinolates, a class of sulfur-containing compounds found in cruciferous vegetables, are hydrolyzed by myrosinase (33, 34). It has been shown to be associated with several biological processes such as anti-inflammatory, antioxidant and anticancer activities (35, 36).

The composition of the GI microbial communities determines the efficiency of processing food into metabolites including amino acids, bile acids and short-chain fatty acids (SCFA). This study was aimed at better understanding of the mechanisms by which cranberry and PEITC can influence the gut microbiome and microbial metabolite production, and further improve the gut health via reducing inflammation and achieving homeostasis. We utilized C57BL/6J wild type (WT) and Nrf2 knockout (KO) mice to examine the effect of diets enriched with either cranberry or PEITC. The correlations of genotype and gut microbiome were also evaluated to determine the role of Nrf2 in the inflammation and aging processes.

# 2. Experimental Section

## 2.1 Animals and study design

C57BL/6J (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Our lab has maintained C57BL/6J Nrf2 KO mice since 2005 (37, 38). Mice were kept in a controlled temperature (20-22°C) and humidity (45–55%) environment under 12-hour light/dark cycles at the Rutgers Animal Facility. Food and water were provided *ad libitum*. Three experiments were conducted for this study (shown in Figure 1). All animals were fed with AIN93M standard grain diet (Research Diets, Inc. NJ) for 2 weeks at the beginning of each experiment to equalize the microbiomes at the baseline. After the equalization period, the mice were randomized to treatment groups. In the first experiment (Exp01), 18 Nrf2 KO mice were assigned to two groups with one group continuing to receive the control diet while 0.05% of PEITC was added to the second group’s diet. Further 10 WT mice were assigned to either the control diet or the PEITC-infused diet in the second experiment (Exp02). Finally, dextran sulfate sodium (DSS) was introduced to 20 mice to induce gut inflammation, and cranberry-enriched diet was added (10% of feed by weight), in the third experiment (Exp03). WT and Nrf2 KO mice were randomly assigned to one of four treatment groups: Naïve, DSS, DSS+PEITC, and DSS+Cranberry (Figure 1). 2.5% DSS was dissolved in autoclaved water and made fresh weekly. Fecal samples were collected fresh, snap frozen in liquid nitrogen and stored at -80oC until further analysis for 16S ribosomal RNA (rRNA) sequencing and microbial metabolites. Fecal samples for 16S sequencing were collected at weeks 1 and 5 in Exp01, weeks 0 and 4 in Exp02, and weeks 0, 1 and 8 in Exp03. Separately, fecal samples were collected for metabolomic analysis at weeks 2 and 6 in Exp03. To realign the sample collection timepoints, they were labeled as baseline (end of the equalization period, i.e., Week 0), early (weeks 1 through 2) or late (weeks 4 through 8) timepoints. Animal use protocol for this study was approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC).

## 2.2 16S ribosomal RNA gene sequencing and analysis

*PowerSoil DNA Isolation Kit* (QIAGEN) was used to extract bacterial DNA from the samples. PCR primer specific to V4 variable region of 16S gene was utilized to amplify the genetic material. (Supplemental Table 1) (39, 40, 41, 42, 43, 44, 45). Sequencing indexed and pooled amplicons in *MiSeq* (Illumina) yielding more than 30,000300-base-pair (bp) pair-ended reads per sample. The data acquired by the instrument was stored in *FASTQ* files, a dedicated format for high-throughput sequencing data. Two independent workflows were used for microbial operational taxonomic unit (OTU) assignments: Quantitative Insights Into Microbial Ecology (QIIME2) bioinformatic pipeline (46, 47) and Divisive Amplicon Denoising Algorithm 2 (DADA2 version 1.16) *R* package (48).

*QIIME2* mapped the reads to reference at 97% similarity against representative sequences of 97% OTU in *SILVA* high quality ribosomal RNA (rRNA) database (49). Chimeric sequences were removed before proceeding to data analyses (50). Visualization of the microbiome similarities was performed using the results of principle coordinates analysis (PCoA) on the unweighted unique fraction metric (UniFrac).

In parallel to *QIIME2*, *FASTQ* files were also processed in *DADA2* pipeline. DADA2 aligned sequencing against SILVA reference database by exact match (100% identity). It produced a high-resolution OTU table containing amplicon sequencing variants. The reads were sorted and filtered using quality scores. This resulted in the truncation of forward reads to 280 bp and reverse reads to 220 bp. After the assembly, chimeric OTUs were removed.

OTUs that could not be mapped to a Kingdom or Phylum as well as those mapped to the *Eukaryota* or *Archaea* Kingdoms were removed. OTUs belonging to phylum *Cyanobacteria* were also removed as these resulted from food contamination.

## 2.3 Analysis of microbial metabolites

Concentrations of microbial metabolites that included bile acids, free amino acids, and SCFA were estimated in fecal samples collected at weeks 2 and 6. Liquid chromatography mass spectrometry (LC-MS)-based targeted analysis was performed to estimate the metabolite concentrations in the samples (51).

## 2.4 Statistical analyses

Shannon index was used to assess the alpha diversity in the samples at OTU level. This index can range from zero, which corresponds to having a single class (i.e., a single OTU) in the sample, to *ln(k)* with *k* equally distributed OTUs. The larger values of the index, therefore, represent microbial communities with greater number of and more equally distributed classes of OTUs. The estimates were presented as means +/- standard error of the means (SEM). Multivariable analysis of variance (ANOVA) was used to estimate the effects of genotype, diet and aging, followed by multiple comparison with false discovery rate (FDR) adjustment for the p-values. Principal components analysis (PCA) was utilized to investigate the bacterial composition of the samples at different taxonomic levels. PCA is a technique that projects the data from the original *p*-dimensional, correlated space onto a new, orthogonal *p*-dimensional space such that the first principal component (PC1) will be in the direction that explains most of variability in the data, the second (PC2) - the second most and orthogonal to PC1, and so on. In this case, each taxonomic unit at each level of analysis represented a single dimension. The results of the PCA analysis were visualized with biplots by plotting the data against the first two principal components and marking the points for genotype, diet or DSS challenge. Simultaneously, the biplots displayed the direction and the magnitude of the original axes relative to each other. To assess the predictive power of PCA, multinomial regression on group labels (corresponding to taxonomic units) vs. principal components was performed.

Heatmaps were used to visualize concentrations of metabolites in the samples. The group mean differences were estimated and tested using analysis of variance (ANOVA) for each metabolite individually and shown as boxplots with bars corresponding to SEM and and stars indicating statistical significance between the groups.

# 3 Results

## 3.1 Data acquisition

The depth of sequencing varied from 30,008 to 422,283 reads per sample (Supplemental Figure 1). Over 94% of the OTUs were identified as bacterial. In total, 10,197 (94.78% of total OTUs), 7,994 (98.34%) and 7,558 (96.07%) bacterial OTUs were detected in the three experiments, respectively (Table 1).

## 3.2 Diet, genotype and inflammation altermicrobiome richness and diversity

The effect of Nrf2 KO was examined because Nrf2 is a master regulator of anti-oxidative stress and anti-inflammatory responses to external and internal stimuli (52, 53, 54, 55, 56). The effect was evaluated by comparing Nrf2 KO (-/-) mice with the WT mice conditioned on diet, DSS challenge and aging. Shannon index was used to estimate alpha diversity of the samples at the out level. The results are presented in Figure 2A. Shannon index averages from Exp03 were compared using mixed-effects linear regression models. The index average was significantly higher in the Nrf2 KO group compared to WT (p-value < 0.01). The index averages at both, the early and the late time points were significantly higher than at the baseline (both p-values < 0.01). Alpha diversity was lower in DSS-challenged groups when they received the dietary additives of cranberry or PEITC (both p-values < 0.01). Sequencing depth affects Shannon index as higher number of reads increases the probability of observing less common OTUs (Supplemental Figure 2A). Therefore, a sensitivity analysis was conducted to investigate Shannon index inflation due to sequencing depth differences by adding 1 to all OTU counts. This removed Shannon index correlation with sequencing depth (Supplemental Figure 2B). Repeating the analysis of the transformed count data showed that the genotype effect remained significant, with Nrf2 KO samples having higher mean Shannon index compared to WT (p-value = 0.02). However, the differences of DSS+Cranberry or DSS+PEITC with the unchallenged group became non-significant, with only the DSS+AIN93M group being significantly lower as compared to the control no-DSS+AIN93M group (p-value < 0.01). The results are shown in Figure 2B.

## 3.3 Principal components analysis shows association of microbiome composition with diet and genotype

Phylum level PCA analysis was conducted on aggregated OTU counts. Out of 22 phyla that were identified, top 10 accounted for >99.96% of all the hits. Since deeper sequencing could increase the chances of identifying uncommon taxonomic units (Supplemental Figure 3) and the samples varied significantly by sequencing depth (Supplemental Figure 1), rare phyla were excluded from downstream analysis. Data from the three experiments was combined for this analysis; however, each group of samples was visualized separately to highlight the differences between experimental conditions (Figure 4). The PCA suggested relatively high inter-experiment variability, specifically, the relatively lower abundance of *Bacteroidetes,* and the relatively higher abundance of *Verrucomicrobia* in Exp03 as compared to Exp01 and Exp02. Relative abundances of *Firmicutes* and *Actinobacteria* were higher in the WT DSS-treated mice in Exp03 compared to all the other groups, while *Epsilonbacteraeota* were more abundant in all Nrf2 KO and WT control (no-DSS+AIN93M) groups compared to the rest. DSS+PEITC samples grouped between the negative (no-DSS+AIN93M) and the positive (DSS+AIN93M) controls, suggesting the potential protective effect of PEITC on microbiome of DSS-challenged mice. To remove any potential confounding effect of experiments, Exp03 data was analyzed separately (Figure 5). The results showed a strong effect of DSS challenge and diet on microbiome composition at phylum level in WT mice. Specifically, relative abundance of *Firmicutes* and *Verrucomicrobia* increased, while relative abundance of *Proteobacteria*, *Deferribacteres* and *Epsilonbacteraeota* decreased in all the WT DSS-treated groups as compared to the control diet groups (no-DSS+AIN93M).

In total, 31 *classes* of bacteria were identified across the three experiments. The top 17 classes accounted for more than 99.99% of the total hits. PCA showed strong negative correlation of Nrf2 KO with *Bacilli* class (phylum (p.) *Firmicutes*) that was observed in all three experiments (Figure 6). Separately, Exp03 data was reanalyzed, with 18 classes being identified in the samples out of which 16 classes contained almost all the hits and were used in the analysis. shows The samples were visibly separated by genotype (Figure 7). The relative abundance of *Clostridia* (p*.* *Firmicutes*) was higher while *Betaproteobacteria*, *Deltaproteobacteria* (p. *Proteobacteria*), and *Epsilonproteobacteria*, as well as *Brachyspirae* (p. *Spirochaetes*), *Campylobacteria* (p*.* *Epsilonbacteraeota*), and *Deferribacteres* (p. *Deferribacteres*) were lower in all three DSS-treated groups. *Gammaproteobacteria* (p. *Proteobacteria*) and *Verrucomicrobiae* (p. *Verrucomicrobia*) had higher relative abundance in the DSS+AIN93M and DSS+Cranberry groups.

## 3.4 *Firmicutes*/*Bacteroidetes* ratio

Biological activities such as aging, change of body mass index, and maintaining intestinal homeostasis have been linked to *Firmicutes* to *Bacteroidetes* ratio (F/B) (57, 58). Increased F/B has been associated with obesity while decreased ratio has correlated positively with inflammatory bowel disease (IBD) (59). Therefore, the F/B ratio was used as an endpoint in this study to examine differences in microbiome composition across genotypes, diets and timepoints. (Figures 8A). The abundance of *Firmicute*s was lower or equal to the abundance of *Bacteroidetes* in Exp01 and Exp02 samples but the F/B ratios in the WT mice samples were higher than in the Nrf2-KO samples in all 3 experiments. To avoid possible confounding effect of experiments, F/B ratios form Exp03 were analyzed and visualized separately (Figures 8). Mixed-effects linear regression models were used to estimate F/B differences in Exp03. First, a mixed-effects model with no interaction terms was fitted. The ratio averages were significantly lower in DSS+PEITC and DSS+Cranberry groups compared to the DSS+AIN93M control group (log2[F/B] = -0.51 and -0.46, and *p*-values <0.01 and =0.01, respectively). The average ratio was also lower in the Nrf2-KO group as compared to WT (log2[F/B] = -1.02, *p*-value <0.01). The control group’s average F/B difference with the DSS control (i.e., no-DSS+AIN93M versus DSS+AIN93M) as well as the differences between early or late timepoints vs. baseline were not statistically significant. Adding a genotype-diet interaction term to the model confirmed the significant association of F/B with genotype (log2[F/B] = -1.40, *p*-value <0.01), and the Cranberry or PEITC diets (log2[F/B] = -0.60 and -0.71, respectively, with both *p*-values <0.01). Furthermore, the ratio in the no-DSS+AIN93M group was significantly lower than that of the DSS+AIN93M group (log2[F/B] = -0.75, *p*-value <0.01). These results suggest that PEITC or Cranberry dietary supplements preserved the balance of *Firmicute*s and *Bacteroidete*s in the intestines of the hosts treated with an inflammatory agent DSS.

## 3.4 Dietary and aging effects examined via linear discriminant analysis

Linear discriminant analysis Effect Size (*LEfSe*) was conducted with *QIIME2* to further examine the effects of diet and aging. Microbiome compositional changes s over time were examined by comparing the control samples at baseline (shown in Figure 9A and B in red) with the early (Week 1, Figure 9A in green) and late (Week 4, Figure 9B in green) timepoints. Taxa with relative abundance of ≥ 0.1% present in at least one specimen were included. The cladograms used lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 and showed phylogenetic distribution of the microbial lineages at different time points. The analysis showed that relative abundances of *Bacteroidetes Parabacteroides*, *Bacteroidetes Prevotella*, *Bacteroidetes S24\_7,* and *Bacteroidetes* decreased while *Bacteroidetes Bacteroidales*, *Firmicutes Clostridiales*, *Firmicutes Oscillospira*, *Proteobacteria Desulfovibrionaceae*, and *Tenericutes Anaeroplasma* increased over time.

Separately, the effect of PEITC addition to the diet was examined and presented in Figures 9C and D. The figures show the impact of PEITC diet by comparing the microbiota in the 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 the later time points compared to baseline. Several bacterial taxa were shown to be correlated with diet. Specifically, *Bacteroidetes Odoribacter*, *Tenericutes Mycoplasmataceae* and *Proteobacteria Desulfovibrionaceae* were found in significantly higher abundance while *Firmicutes Clostridiales*, *Firmicutes Ruminococcus* and *Acidobacteria Ellin 6075* abundances were significantly lower in the control diet group compared to the PEITC group.

## 3.5 Cranberry and PEITC additives partially preserved metabolomic profiles in the DSS-treated mice

Fecal samples of the unchallenged (no DSS), DSS, DSS+Cranberry and DSS+PEITC treated WT and Nrf2 KO mice from Exp03 were collected at weeks 2 and 6 and analyzed for the concentrations of free amino acids, SFCA and bile acids.

Univariable analysis of metabolite concentrations showed that DSS challenge altered production of several of them while PEITC and cranberry infused diets protected against the changes (Figure 10A). Specifically, DSS challenge decreased the concentrations of amino acids such as glutamate, phenylalanine, and proline but PEITC and cranberry fed mouse samples retained the levels found in the controls (Figure 10B-D). PEITC and cranberry cotreatments also reversed the DSS-induced increases of secondary bile acids, mainly deoxycholic acid (DCA), lithocholic acid (LCA), and muricholic acid (MCA) (Figure 10E-G). In contrast, the diet additives had little to no effect on SCFA in DSS-challenged mice (Figure 10H-J). These results suggest that PEITC and cranberry can modulate the metabolic responses to DSS challange in the colorectal tract, possibly mediated through microbiome. The fecal metabolite concentrations were also compared between Nrf2 KO and WT mice samples. Interestingly, the Nrf2 KO mice had lower concentrations of amino acids (glutamate, phenylalanine, and proline) and SCFA, and higher concentrations of secondary bile acids (DCA, LCA, and MCA) compared to the WT mice (Figure 11A-I). These trends mirrored the metabolic profile difference between the DSS-challenged and unchallenged WT mice.

PCA revealed that all amino acids except for taurine were at higher concentrations in the DSS+Cranberry group compared to the rest (Figure 12A). PCA on SCFA also showed that adding cranberry and, to a lesser extent, PEITC to a diet increased production of all measured analytes (Figure 12B). Analysis of bile acid concentrations showed that genotype played a bigger role, with higher concentrations of all bile acids, specifically, LCA, DCA, MCA, CDCA, GDCA and GCDCA detected in the Nrf2 KO compared to WT (Figure 12C). Lastly, the three types of metabolites were pulled together and a PCA on the combined data conducted. The biplot reveiled that the concentrations of bile acids were negatively correlated with PA, AA, BA and VA SCFAs (Figure 13). Principal components from the combined model were used as explanatory variables in multinomial regression models to classify the samples by diet, DSS challenge and genotype. The model with the first 5 principal components accurately classified 48 out of 80 samples (60.0%) by diet and DSS challenge, with the predictive power increasing slightly with the addition of PCs (Table 2). However, the same model was able to correctly identified 61 out of 80 samples (76.3%) by genotype suggesting stronger separation of the samples by that predictor (Table 3).

# 4 Discussion

The important role that diet and genotype play in shaping of microbial communities inhabiting the host’s GI has been systematically reported in the literature. For example, significant increase of relative abundance of *Firmicutes* (*Clostridiales*, *Lactobacillales*, *Turicibacterales*) and *Verrucomicrobia* (*Verrucomicrobiales*) has been shown to co-occur with a rapid and consistent dietary response to low fat/high plant polysaccharide, and high fat/high sugar diets in gene deficient mice. At the same time, *Bacterioidetes* (*Bacteroidales*) have been shown to decrease significantly in high fat/high sugar diets. Additionally, dietary shift from low fat/high plant to high fat/high sugar diets significantly altered relative abundances of *Clostridiales* and *Bacteroidales* bacterial orders. In gnotobiotic mouse model with transplanted fecal samples from a healthy human donor, the low fat/high plant polysaccharide diet also decreased the relative abundance of *Firmicutes* *Erysipelotrichi*, *Firmicutes Bacilli*, and increased the relative abundance of *Bacteroidetes* compared with high fat/high sugar Western diet. In a four-week crossover trial, twenty-eight healthy subjects were given 60 g of whole grain barley, brown rice or equal mixture of two ingredients daily (60). Microbial community diversity, measured by Shannon and Simpson indices, significantly increased in all three diets over time. They also showed increased relative abundance of phylum *Firmicutes*, while the abundance of *Bacterioidetes* decreased over time. Genus *Bacteroides* was significantly decreased by whole barley and brown rice mix diet but was not affected by either of the single ingredient diet. Additionally, genera *Roseburia*, *Bifidobacterium*, *Dialister* and *Odoribacter* were significantly altered only by the whole grain barley diet, and genus *Blautia* by both, mix diet and whole grain barley diet.

Opinions regarding the contribution genotype to human gut microbiota vary due to the potential confounding factors such as diet and cultural differences. Simplified animal models in a controlled environment can eliminate the confounding effects. An in vivo study (61) used automated ribosomal intergenic spacer analysis and length-heterogeneity polymerase chain reaction (L-H PCR) (62) to show that alterations in microbiome composition were genotype-dependent as all animals were under identical conditions and fed the same diet. Observed dissimilarities between genotypes were larger compared to dissimilarities between sexes suggesting that impact of genotype was higher on microbial composition. Another in vivo study showed evidence of gut microbiota association with genetic defect of toll-like receptor 2 (TLR2) (63). TLR2 knock-out mice had significantly higher relative abundance of genus *Helicobacter* compared to the wild type. Genetic defects of NOD2 and ATG16L1 were associated with inflammatory bowel diseases and hinted at the host-microbiota interaction by altering bacterial composition including relative abundance of *Actinobacteria*, *Firmicutes*, and *Proteobacteria*.

The role of gut microbiome has been a focal point of many studies over the past several decades, with its potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system (2, 3, 4). For example, *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). Our study showed that the genotype of the host and diet may was linked with microbiome composition. Both bacterial diversity and individual bacterial strain relative abundances changed significantly based on genotype and diet. Nrf2 KO and diet affected bacterial phylum that were found in high abundance such as *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. Nrf2 KO mice and mice fed with PEITC-enriched diet also had higher relative abundance of *Firmicutes Ruminococcus*.

*Ruminococcus* are anaerobic, gram-positive bacteria and belong to the phylum of *Firmicutes*. To date, eleven *Ruminococcus* species have been identified. These species belong to bacterial family *Ruminococcaceae* (64, 65). Previous studies have shown that *Ruminococcus* produced SCFA by fermenting and degrading cellulose in herbivorous ruminants (66, 67, 68). Samples collected from IBD subjects in a placebo controlled double-blind study contained *Ruminococcus Torques* in high abundance (69). The abundance of this bacteria was significantly reduced by multiple probiotic interventions as measured via quantitative real-time polymerase chain reaction (qPCR). These results suggest potential usefulness of *Ruminococcus Torques* as a biomarker for probiotic efficacy. As a part of normal flora in gastrointestinal tract, another *Gnavu,* another *Ruminococcus* species, was detected in larger quantities in IBD patients experiencing an increase of oxidative stress level in the guts. (70) that was positively correlated with an increase of pro-inflammatory cytokine production such as TNF-a (71). Infants who developed respiratory and skin allergic illnesses have been reported to have elevated levels of *Firmicutes* (72). *Ruminococcus Gnavus* caused airway inflammation via secretion of interleukins 25 and 33 in mice after oral gavage of the purified bacteria. The study found *Firmcutes Ruminoccus* in higher abundance in fecal samples at the late timepoint independent of diet and genotype but the same was not observed at the early timepoint. Aging has been linked to the accumulation of harmful inflammatory bacteria in the guts. However, in the current study higher relative abundance of *Firmucutes Ruminoccus* had a stronger association with Nrf2 KO genotype. This suggests that Nrf2 might be increasingly suppressing pathogenic species such as *Firmucutes Ruminoccus* as the animal age.

Phylum *Ruminoccus* was elevated at the early timepoint of the current study in the PEITC groups. *Bacteroidetes Rikenella* was also found in higher relative abundance in Nrf2 KO mice which suggest a possible link of the bacteria to inflammatory diseases of GI tract (73, 74, 75, 76). Overall, host’s genome interaction with microbiome makes its profiling a prime candidate for developing novel biomarkers that can be utilized in development of new therapies and in future clinical trials.

The results of this study suggest a strong association between mice genotype and gut microbiome richness, diversity and composition. However, several factors might have contributed to some of the observed variability. Prior research has attributed a significant proportion of this variability to factors such as the "cage effect," which reflects the influence of housing arrangements (up to 32%), and individual mouse-to-mouse differences (up to 46%) (77). These findings underscore the need of methodological refinements in experimental designs to reduce intersubject variability by controlling experimental conditions. This can be achieved by equalization of microbiome via co-housing and feeding the animals a control diet pre-baseline or using gnotobiotic (germ-free) mice implanted with homogenized fecal samples (78, 79, 80). In this study, the former approach was adapted, yet variability at the baseline was still noteworthy, illustrating the complexities of achieving uniformity. Gnotobiotic models, while effective in creating a more homogeneous microbiomes, require specialized germ-free facilities and can potentially affect immune system development in the early stages due to the absence of normal microbiota. An alternative approach involves pretreating animals with broad-spectrum antibiotics and providing high-fiber feed before inoculating them with homogenized fecal samples (81). This method strikes a balance between practicality and efficacy, potentially reducing the unwanted variability without the logistical and biological complexities of gnotobiotic systems. These considerations are valuable for enhancing the robustness and reproducibility of microbiome research, ensuring more reliable outcomes.

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