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 *Firmicutes* and *Verrucomicrobia* increased while *Proteobacteria*, *Deferribacteres* and *Epsilonbacteraeota* decreased in the DSS-challenged groups. These trends were reversed by PEITC and cranberry enriched diets. The diets also preserved the *Firmicutes*-to-*Bacteroidetes* ratio, an indirect indicator of gut inflammation and obesity. DSS challenge altered the 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 are potentially involved in essential vitamins’ metabolism (6) and improvement of human 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). Numerous studies have been conducted to explore gut microbiota composition responding to specific conditions such as high fat or high fiber diet, or inflammatory bowel diseases (17,13, 14, 15, 16, 18, 19, 20, 21). Host’s genotype may also influence the human gut microbiota, especially in infancy (22, 23). The combination of host genotype, gut microbiota and postnatal factors such as antibiotic usage, dietary pattern and environmental microbes shows 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 have a wide range of biological activities including anticancer, antioxidant, and anti-inflammatory effects (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). The aim of this study was to better understand 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 test 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*. The study consisted of three experiments (shown in Figure 1). All animals were fed with AIN93M standard grain diet (Research Diets, Inc. NJ) for the first 2 weeks of the experiments 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 randomized into 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. Additional samples were collected from all the mice for metabolomic analysis at weeks 2 and 6 in Exp03. Since the fecal sample collection timing varied slightly between 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. 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

*PowerSoil DNA Isolation Kit* (QIAGEN) was used to extract bacterial DNA from the samples. PCR amplification of the 16S rRNA genes were carried out using PCR primers specific for the V4 region (Supplemental Table 1) (39, 40, 41, 42, 43, 44, 45). 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 (46, 47) and Divisive Amplicon Denoising Algorithm 2 (DADA2 version 1.16) *R* package (48).

*QIIME2* mapped reference at 97% similarity against representative sequences of 97% OTU in SILVA, a high quality rRNA database (49), followed by chimeric sequences removal from subsequent analyses (50). Visualization of the microbiome similarities was performed using the results of principle coordinates analysis (PCoA) on the unweighted unique fraction metric (UniFrac).

*DADA2* pipeline was used to process *FastQ* sequence data files containing pair-ended reads with average length of 300 bp into a high-resolution OTU table (i.e., amplicon sequencing variants). The reads were sorted, and the quality scores were examined. This resulted in the 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. Chimeric OTUs were identified and removed. Taxonomy was assigned to the OTUs by exact matching (100% identity) to the SILVA reference database.

The OTUs mapped to the *Eukaryota* and *Archaea* Kingdoms, and the OTUs that could not be mapped to a Kingdom, were removed. Additionally, bacterial OTUs belonging to phylum *Cyanobacteria* were removed as contamination from food. Finally, OTUs that were not mapped to any bacterial phylum were removed, and the remaining OTUs analyzed.

## 2.3 Microbial metabolites analysis

Microbial metabolites that included bile acids, free amino acids, and SCFA were quantified 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 linear transformation that projects the data from the original *n*-dimensional, correlated space (here, each taxonomic unit represented a dimension) onto a new, orthogonal *n*-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. The results of the PCA analysis were visualized with biplots by plotting the data against the first two principal components and color-coding the points for genotype, diet or DSS challenge. Simultaneously, the biplots displayed the direction and the magnitude of the original axes (i.e., individual taxonomic units). 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 and stars indicating statistical significance between different groups.

# 3 Results

## 3.1 Data acquisition

The depth of sequencing varied between 30,008 and 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 identified in the three experiments, respectively (Table 1).

## 3.2 Diet, genotype and inflammation affect bacterial community 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 a 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 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) and increased as the mice aged. 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 even 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, p-value < 0.01). The results are shown in Figure 2B and suggest that the two additives had protective effects on the microbiome richness and diversity. The transformation also removed the aging effect.

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

Next, OTU counts were aggregated at the *Phylum* level. In total, 22 phyla were identified, top 10 of which accounted for >99.96% of all the hits. Since deeper sequencing could increase the chances of identifying rare organisms (Supplemental Figure 3), and the samples varied quite greatly by sequencing depth (Supplemental Figure 1), rare phyla were excluded from downstream analysis. Data from the three experiments was combined for the 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.

The top 10 most abundant phyla were used in the next step of PCA analysis. The results showed a strong effect of diet on the microbial composition. 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). Exp03 data was reanalyzed separately to remove a potential study effect in examining the effect of Nrf2 KO (Figure 5).

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 consistent 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. Figure 7 biplot shows clear separation by genotype. 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 8). 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. Mixed-effects linear regression models were next used to estimate these differences in Exp03. First, a mixed-effects model with no interaction terms was fitted to the F/B. The ratio averages were significantly lower in DSS+PEITC and DSS+Cranberry groups compared to the no-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] = -1.40, *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 Linear discriminant analysis of aging and dietary effects

Linear discriminant analysis Effect Size (LEfSe) was conducted with QIIME2 to further examine the effects of diet and aging. The composition changes in the microbiomes 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. In addition, the cladograms using lineages with Linear Discriminant Analysis (LDA) score ≥ 2.0 and showing the phylogenetic distribution of the microbial lineages at different time points were drawn. 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 DSS, DSS+Cranberry and DSS+PEITC treated mice from Exp03 were collected at weeks 2 and 6 and analyzed for the concentrations of free amino acids, bile acids and SCFA. Principal components analysis showed that concentrations of all but one (taurine) amino acids were higher in the DSS+Cranberry group (Figure 10A). However, for bile acids genotype rather than diet 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 10B). Firstly, a few principal components were used as explanatory variables in multinomial regression models to classify the samples by diet, DSS challenge and genotype. The model with the first 3 principal components accurately classified 29 out of 48 samples (60.4%) by diet and DSS challenge, with the predictive power increasing slightly with the addition of PCs (Table 2). To classify the samples by genotype, however, a multinomial model with just PC1 was sufficient. It correctly identified 34 out of 48 samples (70.8%) suggesting stronger separation of the samples by genotype (Table 3). 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 11A). 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 11B-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 11E-G). In contrast, the diet additives had little to no effect on SCFA in DSS-challenged mice (Figure 11H-J). These results suggest 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. Lastly, the fecal metabolite concentrations from Nrf2 KO and WT mice samples were compared. 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 12A-I). These trends mirrored the metabolic profile difference between the DSS-challenged and unchallenged WT mice.

# 4 Discussion

The important role of diet and genotype on the composition of microbes 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 between the genotype and gut microbiota. 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. Higher dissimilarities between genotypes than sexes were observed suggesting that genotype was a stronger factor than gender in regulating gut microbiota. Another in vivo study showed evidence of gut microbiota association with genetic defect of toll-like receptor 2 (TLR2) (63). Genus *Helicobacter* was significantly elevated in TLR2 knock-out mice compared to the wild 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*.

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 demonstrated that host genotype and diet may alter gut microbiota. Both bacterial diversity and individual bacterial strain relative abundances changed significantly based on diet and genotype, and Nrf2 KO genotype showed 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 genotype and diet. For instance, *Firmicutes Ruminococcus* was found in higher relative abundance in the PEITC-added diet 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* (64, 65). Previous studies showed that *Ruminococcus* degraded and fermented cellulosic biomass into short-chain fatty acid (SCFA) for herbivorous ruminants (66, 67, 68). *Ruminococcus Torques* was reported to be abundant in the irritable bowel syndrome subjects in a placebo control double blind study (69). 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 (70), potentially caused by cytokine production such as TNF-a (71). *Firmicutes* has also been reported to be overpopulated in infants who developed respiratory and skin allergic diseases (72). Mice orally garaged by purified *Ruminococcus Gnavus* also developed airway inflammation by cytokine secretion such as interleukins 25 and 33. This study showed a significant increase in the abundance of *Firmcutes Ruminoccus* in fecal samples at the late but not at the early timepoints irrespective of diet and genotype. Aging has been linked to the accumulation of harmful inflammatory bacteria in the guts but in this study, we found that the increased level of *Firmucutes Ruminoccus* was mainly associated with Nrf2 KO suggesting that Nrf2 KO accelerates the increase of *Firmucutes Ruminoccus*’s relative abundance. This suggests that Nrf2 might play an important role in regulating the gut microbiota profile and suppress pathogenic species such as *Firmucutes Ruminoccus* as the animal age.

Interestingly, we also observed that the phylum *Ruminoccus* was elevated at the early timepoint in the PEITC groups. *Bacteroidetes Rikenella* was also found significantly elevated in Nrf2 KO groups, suggesting that it may correlate with gut diseases (73, 74, 75, 76). Overall, genetic KO (mutation) has a strong impact on the host microbiota profile over time and should be considered as a biomarker when developing probiotic or microbiota intervention therapy in the future.

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 the background noise by feeding the animals with a control diet for several weeks or using gnotobiotic (germ-free) mice implanted with homogenized fecal samples (78, 79, 80). In this study, we employed the former approach, 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 the microbiota. An alternative approach involves pretreating animals with broad-spectrum antibiotics and providing high-fiber food before inoculating them with homogenized fecal samples (81). This method strikes a balance between practicality and efficacy, potentially reducing 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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