Cranberry and phenethyl isothiocyanate alters gut microbiome and metabolome in Nrf2 knockout mice under inflammation

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**Abstract**

**Scope**

Cranberries are enriched with antioxidants and can help prevent bacterial infections, while phenethyl isothiocyanate (PEITC) found in cruciferous vegetables has anti-cancer and anti-inflammatory properties. Incorporating these into diet may have potential health benefits for human gut. Microbes and metabolites interactions play crucial roles in maintaining gastrointestinal tract balance.

**Methods and results**

In this study, we focusd on the alteration of cranberry and PEITC on both wide-type (WT) and Nrf2 knockout (KO) mice gut microbiome and metabolites, and its potentials in reducing the risk of inflammation. We found out that Nrf2 KO mice had higher alpha diversity than WTs. Cranberry and PEITC reserved the inflammatory effect of dextran sulfate sodium through increasing the diversity of mice gut microbiota. XXXX

*cranberry and PEITC alters Nrf2 KO mice and DSS induced ones differently. XXX XXX are signififantly chagned by diet atleration. Top changes in metabolites including xxxxxx.*

**Conclusions**

These results suggest that inflammation induced microbiome changes partically reversed by cranberry and PEITC diet. In future, the correlation of inflammatory indicators would be measrued to correalte with microbiome/metabolite signature in order to further elucidate the relatioship between microbe, metabolites and health status.

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# **1 Introduction**

Cranberry has been consumed by humans for years, and native Americans are believed to have been the first to use cranberries as food and medicine[1, 2]. Nowadays, they are widely used in the food industry such as juice, sauces, and dried fruit. Cranberries are known for their high content of proanthocyanidins, flavonoids, anthocyanins, and other organic acids[3-7]. They been found to have health benefits including reducing the risk of urinary tract infections[8, 9], improving cardiovascular health[10, 11], and reducing inflammation[12]. Phenethyl isothiocyanate, or PEITC, is a member of the isothiocyanate family of compounds, which are formed when glucosinolates, a class of sulfur-containing compounds found in cruciferous vegetables, are hydrolyzed by enzymes[13, 14]. PEITC has been found to have a wide range of biological activities, including anticancer, anti-inflammatory, and antioxidant effects[15-17]. These properties have been attributed to the ability of PEITC to modulate various signaling pathways involved in cell growth, differentiation, and apoptosis[18]. Currently, a variety of small molecules found in plants exhibit strong beneficial effects to human health and incorporating them adequately into our diet offers a natural way to promote overall health and potentially reduce the risk of chronic diseases.

Human health can also be affected by microorganisms including bacteria, archaea and fungi which are distributed in large quantities on surfaces throughout their bodies [19]. The role of gut bacteria is especially noted for their potential beneficial effects in metabolizing essential nutrients, providing energy, and enhancing immune system [20-22]. For example, *Butyricicoccus Pullicaecorum* and *Butyricicoccus Pullicaecorum* produce butyrate, an essential metabolite for human GI homeostasis and disease prevention [23]. Lactobacillus strains are involved in essential vitamins metabolism [24] and human sleep quality improvement [25]. *Bifidobacterium* strains influence human emotions like depression, reduce painful feeling, and alter brain activity during stress [26-30]. Numerous studies have been conducted to explore gut microbiota composition responding to specific dietary patterns, such as high fat diet, inflammatory bowel disease [31-37], and high fibers[38, 39]. In addition, research suggest that host genotype may influence the human gut microbiota, especially the infant period[40, 41]. The combination of host genotype, gut microbiota, as well as postnatal factors such as antibiotic usage, dietary pattern and environmental microbes shows significant influence on human gut development and homeostasis[42, 43]. Hence, the underlying mechanism of such microbiota-host crosstalk is crucial yet remains poorly understood.

In this study, we used C57BL/6J wide type (WT) and Nrf2 gene knockout (KO) and treated cranberry and PEITC diets. Both the gut microbiome signature and associated metabolites will be measured. Since Nrf2 gene is highly associated with inflammation, the KO group would increase the risk of chronic inflammation. This will help us to understand how host genotype would affect the gut microbiota and metabolites. The aim of this study was to understand the mechanism of how cranberry and PEITC change the gut microbiome and metabolites, and further improve the gut health and reduce the inflammatory levels.

**2 Experimental Section**

## Animals and study 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 [44, 45]. 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. All mice were given a 2-week gut microbiota equalization period during which they were fed with AIN93M control diet (Research Diets, Inc. NJ).

The experimental design is summarized in **Figure 1**. In stage 1, both WT and KO mice were randomized into 2 groups and treated with either control diet (AIN93M) or control diet with 0.05% PEITC after 2-week equalization period. In stage 2, WT and KO mice were randomized into 4 groups and treated with control diet, dextran sulfate sodium (DSS) at 2.5%, DSS with 0.05% PEITC, and DSS with cranberry diet (xx% in AIN93M). DSS was dissolved in autoclaved water and made freshly weekly. Fecal samples were collected freshly, snap frozen in liquid nitrogen and stored at -80oC for further study. Samples from stage 1 week 0 and week 4 were, stage 2 week 0, 1 and 8 were selected and sent for 16S ribosomal RNA (rRNA) sequencing and fecal metabolites analysis. All animal experiments were conducted under the animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers University.

## 16S ribosomal RNA sequencing and analysis

Sample DNA was 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) [46-52]. 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 [53, 54] and Divisive Amplicon Denoising Algorithm 2 (DADA2 version 1.16) R package [55].

QIIME2 mapped reference at 97% similarity against representative sequences of 97% OTU in SILVA, a high quality rRNA database [56], followed by chimeric sequences removal from subsequent analyses [57]. 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.

Microbial metabolites analysis

The fecal metabolites, including free amino acids, bile acids and short-chain fatty acid were measured from samples collected at stage 2, week 2 and week 6. Both liquid chromatography mass spectrometry and gas chromatography mass spectrometry were used for untargeted analysis. (Need more info from the Professor I forget his name.) instrument, mobile phase, time and standard compound etc.

## Statistical Analyses

## Alpha diversity was assessed using Shannon’s index at OTU level. The estimates were presented as means +/- 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. Metabolites analysis was performed by ANOVA 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**

## Overall raw datasets

Sequencing depth varied between 30,008 and 422,283 reads per sample (**Supplemental Figure 1**). Over 94% of 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 KO/PEITC, WT/PEITC, and DSS associated groups respectively (**Supplemental Table 1**). Additionally, bacterial OTUs belonging to *phylum Cyanobacteria* were removed as contamination from diet. Finally, OTUs not mapped to any bacterial *phylum* were removed, and the remaining OTUs analyzed.

## Diet, genotype and DSS affect bacterial community richness and diversity

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 stud 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 (**Supplemental 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 (**Supplemental 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 suggested 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**.

## Principal components analyses reveal association of microbiome composition with diet and genotype

At the Phylum level, OTU counts were aggregated. 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 phylum 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 the 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).

At the Class level, its aggregation yields 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 31classes, 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.

## Firmicutes/Bacteroidetes (F/B) ratio

In this study, the F/B ratios were calculated within each sample and compared across the experiments, diet, genotype 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 (log2[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 (log2[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 (log2[F/B] = -1.40, p-value <0.01), as well as with PEITC and Cranberry diets (log2[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 (log2[F/B] = -1.40, p-value <0.01). These results suggest that PEITC and cranberry to regular grain diet reversed the effect of DSS challenge on the balance of Firmicutes and Bacteroidetes in the hosts’ intestines.

## Linear Discriminant Analysis of 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 eliminated 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 ≥ 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 timpoints compared to baseeline. 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.

## PEITC and cranberry feeding partially reverse the DSS-induced changes in fecal metabolite

Metabolite profiles of DSS, DSS + PEITC, and DSS + Cranberry treatment group were analyzed using the samples from week 2 and week 6. The concentrations of free amino acids, bile acids and short-chain fatty acids (SCFA) were quantified. The overall levels of all amino acids were elevated in the cranberry diet group (Figure 15A) except taurine. 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 slow 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**

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*) [42]. In contrast, *Bacterioidetes* (*Bacteroidales*) significantly decreased in high fat/sugar diet group. Additionally, *Clostridiales* and *Bacterioidales* significantly altered composition of bacterial orders during the dietary shift between low fat/high plant polysaccharide diet and high fat/sugar diet. Utilizing gnotobiotic mouse model with transplantation of healthy human fecal sample, the low fat/high plant polysaccharide diet decreased the relative abundance of *Firmicutes* *Erysipelotrichi*, *Firmicutes Bacilli*, and increased the relative abundance of *Bacteroidetes Bacteroidetes* compared with high fat/sugar Western diet. Twenty-eight healthy subjects were given 60 g of whole grain barley, brown rice or equal mixture of two ingredients every day for 4 weeks [58]. 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 *Bacerioides* were significantly decreased by whole barley and brown rice mix diet but were not affected by either of the single ingredient diet. In addition, genus *Roseburia*, *Bifidobacterium*, *Dialister* and *Odoribacter* were significantly altered only by whole grain barley diet, and genus *Blautia* by both, mix diet and whole grain barley diet. Gut microbiome and genotype’s relationship? Examples and results.

Gut microbiome and the metabolites

Gut bacteria have been appreciated for many years with its potential beneficial effects in metabolizing essential nutrients, providing energy and enhancing immune system [20-22]. For instance, gut bacteria *Butyricicoccus Pullicaecorum* and *Butyricicoccus Pullicaecorum* produce butyrate, an essential metabolite for human homeostasis and disease prevention [23] while Lactobacillus strains are involved in essential vitamins metabolism [24]. 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* [59, 60]. Previous studies show that *Ruminococcus* degraded and fermented cellulosic biomass into short-chain fatty acid (SCFA) for herbivorous ruminants [61-63]. Recently, *Ruminococcus Torques* was reported to be abundant in the irritable bowel syndrome subjects in a placebo control double blind study [64]. 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 [65], potentially caused by cytokine production such as TNF-a [66]. *Firmicutes* has also been reported to be overpopulated in infants who developed respiratory and skin allergic diseases [67]. Mice orally garaged 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 *Firmcutes Ruminoccus* 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 *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* 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 [68-71]. 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.

Gut inflammation, microbiome, and metabolites

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Future work

In this study, we conclude that mice genotype is strongly associated with gut microbiome richness and diversity and compositional changes. However, there are more factors which should be considered. Research has demonstrated that cage and internal individual effects are contributing up to 32% and 46% of gut microbiota variability, respectively [72]. 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 [73-75]. 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 [76]. Germ-free mice is an alterative approach, yet the high maintenance of environment and immune deficiency is the disadvantages. Hence, a universal method should be developed to minimize the error of animal experiment and keep data consistence and comparable.

In this study, we performed both 16s rRNA sequencing and metabolite analysis on fecal samples collected from either PEITC, cranberry, DSS or their combination treated mice. Results XXXX. There conclusion provide insigsht about how small molecules from vegetables and fruist contribute to human health through GI tract system, and will lead future e

~~although opinions regarding its contribution diverge due to the potential confounding factors such as the diet.~~ ~~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 [77] that used automated ribosomal intergenic spacer analysis and length-heterogeneity polymerase Chain Reaction (L-H PCR) [78] 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 [79]. 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~~*~~.~~

~~Firmicutes to Bacteroidetes ratio (F/B) have been linked to biological activity including aging [80] and body mass index change [81] and maintaining intestinal homeostasis. Increased F/B ratio was associated with obesity while the ratio decreased was correlated positively with inflammatory bowel disease [82].~~

**Acknowledgements**

**Conflict of Interest**

The authors declare no conflicts of interest.

**Author Contributions**

# **References**

[1] Lowe, F. C., Fagelman, E., Cranberry juice and urinary tract infections: what is the evidence? *Urology* 2001, *57*, 407-413.

[2] Reid, G., The role of cranberry and probiotics in intestinal and urogenital tract health. *Crit Rev Food Sci Nutr* 2002, *42*, 293-300.

[3] Sun, J., Hai Liu, R., Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Lett* 2006, *241*, 124-134.

[4] He, X., Liu, R. H., Cranberry phytochemicals: Isolation, structure elucidation, and their antiproliferative and antioxidant activities. *J Agric Food Chem* 2006, *54*, 7069-7074.

[5] Yin, R., Kuo, H. C., Hudlikar, R., Sargsyan, D.*, et al.*, Gut microbiota, dietary phytochemicals and benefits to human health. *Curr Pharmacol Rep* 2019, *5*, 332-344.

[6] Feghali, K., Feldman, M., La, V. D., Santos, J., Grenier, D., Cranberry proanthocyanidins: natural weapons against periodontal diseases. *J Agric Food Chem* 2012, *60*, 5728-5735.

[7] Wang, Y., Johnson-Cicalese, J., Singh, A. P., Vorsa, N., Characterization and quantification of flavonoids and organic acids over fruit development in American cranberry (Vaccinium macrocarpon) cultivars using HPLC and APCI-MS/MS. *Plant Sci* 2017, *262*, 91-102.

[8] Jepson, R. G., Williams, G., Craig, J. C., Cranberries for preventing urinary tract infections. *Cochrane Database Syst Rev* 2012, *10*, CD001321.

[9] Howell, A. B., Bioactive compounds in cranberries and their role in prevention of urinary tract infections. *Mol Nutr Food Res* 2007, *51*, 732-737.

[10] Sun, J., Chu, Y. F., Wu, X., Liu, R. H., Antioxidant and antiproliferative activities of common fruits. *J Agric Food Chem* 2002, *50*, 7449-7454.

[11] Reed, J., Cranberry flavonoids, atherosclerosis and cardiovascular health. *Crit Rev Food Sci Nutr* 2002, *42*, 301-316.

[12] Cai, X., Han, Y., Gu, M., Song, M.*, et al.*, Dietary cranberry suppressed colonic inflammation and alleviated gut microbiota dysbiosis in dextran sodium sulfate-treated mice. *Food Funct* 2019, *10*, 6331-6341.

[13] Johnson, I. T., Glucosinolates: bioavailability and importance to health. *Int J Vitam Nutr Res* 2002, *72*, 26-31.

[14] Dayalan Naidu, S., Suzuki, T., Yamamoto, M., Fahey, J. W., Dinkova-Kostova, A. T., Phenethyl Isothiocyanate, a Dual Activator of Transcription Factors NRF2 and HSF1. *Mol Nutr Food Res* 2018, *62*, e1700908.

[15] Gupta, P., Wright, S. E., Kim, S. H., Srivastava, S. K., Phenethyl isothiocyanate: a comprehensive review of anti-cancer mechanisms. *Biochim Biophys Acta* 2014, *1846*, 405-424.

[16] Keum, Y. S., Owuor, E. D., Kim, B. R., Hu, R., Kong, A. N., Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC). *Pharm Res* 2003, *20*, 1351-1356.

[17] Ramirez, C. N., Li, W., Zhang, C., Wu, R.*, et al.*, In Vitro-In Vivo Dose Response of Ursolic Acid, Sulforaphane, PEITC, and Curcumin in Cancer Prevention. *AAPS J* 2017, *20*, 19.

[18] Hwang, E. S., Lee, H. J., Effects of phenylethyl isothiocyanate and its metabolite on cell-cycle arrest and apoptosis in LNCaP human prostate cancer cells. *Int J Food Sci Nutr* 2010, *61*, 324-336.

[19] Dethlefsen, L., McFall-Ngai, M., Relman, D. A., An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 2007, *449*, 811-818.

[20] Ramakrishna, B. S., Role of the gut microbiota in human nutrition and metabolism. *J Gastroen Hepatol* 2013, *28*, 9-17.

[21] Rowland, I., Gibson, G., Heinken, A., Scott, K.*, et al.*, Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr* 2018, *57*, 1-24.

[22] Maslowski, K. M., Mackay, C. R., Diet, gut microbiota and immune responses. *Nat Immunol* 2011, *12*, 5-9.

[23] Geirnaert, A., Calatayud, M., Grootaert, C., Laukens, D.*, et al.*, Butyrate-producing bacteria supplemented in vitro to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Sci Rep-Uk* 2017, *7*.

[24] LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F.*, et al.*, Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr Opin Biotech* 2013, *24*, 160-168.

[25] Aizawa, E., Tsuji, H., Asahara, T., Takahashi, T.*, et al.*, Bifidobacterium and Lactobacillus Counts in the Gut Microbiota of Patients With Bipolar Disorder and Healthy Controls. *Front Psychiatry* 2018, *9*, 730.

[26] Desbonnet, L., Garrett, L., Clarke, G., Kiely, B.*, et al.*, Effects of the Probiotic Bifidobacterium Infantis in the Maternal Separation Model of Depression. *Neuroscience* 2010, *170*, 1179-1188.

[27] Schmidt, C., Mental health: thinking from the gut. *Nature* 2015, *518*, S12-15.

[28] Tillisch, K., Labus, J. S., Ebrat, B., Stains, J.*, et al.*, Modulation of the Brain-Gut Axis After 4-Week Intervention With a Probiotic Fermented Dairy Product. *Gastroenterology* 2012, *142*, S115-S115.

[29] Cryan, J. F., Dinan, T. G., Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 2012, *13*, 701-712.

[30] McKernan, D. P., Fitzgerald, P., Dinan, T. G., Cryan, J. F., The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. *Neurogastroent Motil* 2010, *22*, 1029-+.

[31] Cani, P. D., Bibiloni, R., Knauf, C., Waget, A.*, et al.*, Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008, *57*, 1470-1481.

[32] Kim, K. A., Gu, W., Lee, I. A., Joh, E. H., Kim, D. H., High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One* 2012, *7*, e47713.

[33] Daniel, H., Gholami, A. M., Berry, D., Desmarchelier, C.*, et al.*, High-fat diet alters gut microbiota physiology in mice. *ISME J* 2014, *8*, 295-308.

[34] Shim, J. O., Gut microbiota in inflammatory bowel disease. *Pediatr Gastroenterol Hepatol Nutr* 2013, *16*, 17-21.

[35] Eom, T., Kim, Y. S., Choi, C. H., Sadowsky, M. J., Unno, T., Current understanding of microbiota- and dietary-therapies for treating inflammatory bowel disease. *J Microbiol* 2018, *56*, 189-198.

[36] Butel, M. J., Probiotics, gut microbiota and health. *Med Maladies Infect* 2014, *44*, 1-8.

[37] Sekirov, I., Russell, S. L., Antunes, L. C. M., Finlay, B. B., Gut Microbiota in Health and Disease. *Physiol Rev* 2010, *90*, 859-904.

[38] Chen, L., Liu, B., Ren, L., Du, H.*, et al.*, High-fiber diet ameliorates gut microbiota, serum metabolism and emotional mood in type 2 diabetes patients. *Front Cell Infect Microbiol* 2023, *13*, 1069954.

[39] Heinritz, S. N., Weiss, E., Eklund, M., Aumiller, T.*, et al.*, Intestinal Microbiota and Microbial Metabolites Are Changed in a Pig Model Fed a High-Fat/Low-Fiber or a Low-Fat/High-Fiber Diet. *PLoS One* 2016, *11*, e0154329.

[40] Spor, A., Koren, O., Ley, R., Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol* 2011, *9*, 279-290.

[41] Olivares, M., Laparra, J. M., Sanz, Y., Host genotype, intestinal microbiota and inflammatory disorders. *Br J Nutr* 2013, *109 Suppl 2*, S76-80.

[42] Carmody, R. N., Gerber, G. K., Luevano, J. M., Gatti, D. M.*, et al.*, Diet Dominates Host Genotype in Shaping the Murine Gut Microbiota. *Cell Host Microbe* 2015, *17*, 72-84.

[43] Ussar, S., Griffin, N. W., Bezy, O., Fujisaka, S.*, et al.*, Interactions between Gut Microbiota, Host Genetics and Diet Modulate the Predisposition to Obesity and Metabolic Syndrome. *Cell Metab* 2015, *22*, 516-530.

[44] Shen, G., Xu, C., Hu, R., Jain, M. R.*, et al.*, Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin. *Mol Cancer Ther* 2006, *5*, 39-51.

[45] Lin, W., Wu, R. T., Wu, T. Y., Khor, T. O.*, et al.*, Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. *Biochem Pharmacol* 2008, *76*, 967-973.

[46] Apprill, A., McNally, S., Parsons, R., Weber, L., Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol* 2015, *75*, 129-137.

[47] Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D.*, et al.*, Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *P Natl Acad Sci USA* 2011, *108*, 4516-4522.

[48] Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D.*, et al.*, Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme J* 2012, *6*, 1621-1624.

[49] Minich, J. J., Humphrey, G., Benitez, R. A. S., Sanders, J.*, et al.*, High-Throughput Miniaturized 16S rRNA Amplicon Library Preparation Reduces Costs while Preserving Microbiome Integrity. *Msystems* 2018, *3*.

[50] Parada, A. E., Needham, D. M., Fuhrman, J. A., Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016, *18*, 1403-1414.

[51] Quince, C., Lanzen, A., Davenport, R. J., Turnbaugh, P. J., Removing Noise From Pyrosequenced Amplicons. *Bmc Bioinformatics* 2011, *12*.

[52] Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G.*, et al.*, Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *Msystems* 2016, *1*.

[53] Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A.*, et al.*, Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019, *37*, 852-857.

[54] Estaki, M., Jiang, L., Bokulich, N. A., McDonald, D.*, et al.*, QIIME 2 Enables Comprehensive End-to-End Analysis of Diverse Microbiome Data and Comparative Studies with Publicly Available Data. *Curr Protoc Bioinformatics* 2020, *70*, e100.

[55] Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W.*, et al.*, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016, *13*, 581-583.

[56] Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J.*, et al.*, The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Research* 2014, *42*, D643-D648.

[57] Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K.*, et al.*, QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010, *7*, 335-336.

[58] Martinez, I., Lattimer, J. M., Hubach, K. L., Case, J. A.*, et al.*, Gut microbiome composition is linked to whole grain-induced immunological improvements. *Isme J* 2013, *7*, 269-280.

[59] La Reau, A. J., Suen, G., The Ruminococci: key symbionts of the gut ecosystem. *J Microbiol* 2018, *56*, 199-208.

[60] Rainey, F. A., Janssen, P. H., Phylogenetic analysis by 16S ribosomal DNA sequence comparison reveals two unrelated groups of species within the genus Ruminococcus. *FEMS Microbiol Lett* 1995, *129*, 69-73.

[61] Qin, J., Li, R., Raes, J., Arumugam, M.*, et al.*, A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010, *464*, 59-65.

[62] Leschine, S. B., Cellulose degradation in anaerobic environments. *Annu Rev Microbiol* 1995, *49*, 399-426.

[63] Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R., White, B. A., Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol* 2008, *6*, 121-131.

[64] Lyra, A., Krogius-Kurikka, L., Nikkila, J., Malinen, E.*, et al.*, Effect of a multispecies probiotic supplement on quantity of irritable bowel syndrome-related intestinal microbial phylotypes. *BMC Gastroenterol* 2010, *10*, 110.

[65] Hall, A. B., Yassour, M., Sauk, J., Garner, A.*, et al.*, A novel Ruminococcus gnavus clade enriched in inflammatory bowel disease patients. *Genome Med* 2017, *9*, 103.

[66] Henke, M. T., Kenny, D. J., Cassilly, C. D., Vlamakis, H.*, et al.*, Ruminococcus gnavus, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc Natl Acad Sci U S A* 2019, *116*, 12672-12677.

[67] Chua, H. H., Chou, H. C., Tung, Y. L., Chiang, B. L.*, et al.*, Intestinal Dysbiosis Featuring Abundance of Ruminococcus gnavus Associates With Allergic Diseases in Infants. *Gastroenterology* 2018, *154*, 154-167.

[68] Johnson, E. L., Heaver, S. L., Walters, W. A., Ley, R. E., Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. *J Mol Med (Berl)* 2017, *95*, 1-8.

[69] Couturier-Maillard, A., Secher, T., Rehman, A., Normand, S.*, et al.*, NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J Clin Invest* 2013, *123*, 700-711.

[70] Carmichael, W. W., Cyanobacteria secondary metabolites--the cyanotoxins. *J Appl Bacteriol* 1992, *72*, 445-459.

[71] Carmichael, W. W., The toxins of cyanobacteria. *Sci Am* 1994, *270*, 78-86.

[72] Hildebrand, F., Nguyen, T. L., Brinkman, B., Yunta, R. G.*, et al.*, Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol* 2013, *14*, R4.

[73] Roopchand, D. E., Carmody, R. N., Kuhn, P., Moskal, K.*, et al.*, Dietary Polyphenols Promote Growth of the Gut Bacterium Akkermansia muciniphila and Attenuate High-Fat Diet-Induced Metabolic Syndrome. *Diabetes* 2015, *64*, 2847-2858.

[74] Zhang, L., Carmody, R. N., Kalariya, H. M., Duran, R. M.*, et al.*, Grape proanthocyanidin-induced intestinal bloom of Akkermansia muciniphila is dependent on its baseline abundance and precedes activation of host genes related to metabolic health. *J Nutr Biochem* 2018, *56*, 142-151.

[75] Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E.*, et al.*, The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 2009, *1*, 6ra14.

[76] Lundberg, R., Toft, M. F., August, B., Hansen, A. K., Hansen, C. H., Antibiotic-treated versus germ-free rodents for microbiota transplantation studies. *Gut Microbes* 2016, *7*, 68-74.

[77] Kovacs, A., Ben-Jacob, N., Tayem, H., Halperin, E.*, et al.*, Genotype Is a Stronger Determinant than Sex of the Mouse Gut Microbiota. *Microb Ecol* 2011, *61*, 423-428.

[78] Ritchie, N. J., Schutter, M. E., Dick, R. P., Myrold, D. D., Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. *Appl Environ Microbiol* 2000, *66*, 1668-1675.

[79] Albert, E. J., Sommerfeld, K., Gophna, S., Marshall, J. S., Gophna, U., The gut microbiota of toll-like receptor 2-deficient mice exhibits lineage-specific modifications. *Environ Microbiol Rep* 2009, *1*, 65-70.

[80] Mariat, D., Firmesse, O., Levenez, F., Guimaraes, V.*, et al.*, The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* 2009, *9*, 123.

[81] Koliada, A., Syzenko, G., Moseiko, V., Budovska, L.*, et al.*, Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol* 2017, *17*, 120.

[82] Stojanov, S., Berlec, A., Strukelj, B., The Influence of Probiotics on the Firmicutes/Bacteroidetes Ratio in the Treatment of Obesity and Inflammatory Bowel disease. *Microorganisms* 2020, *8*.