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Black raspberries and their anthocyanin and fiber fractions alter the composition and diversity of gut microbiota in F-344 rats

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Abstract

Natural compounds can alter the diversity and composition of the gut microbiome, potentially benefiting our health. We previously demonstrated chemopreventive effects of black raspberries (BRBs) in colorectal cancer, which associates with gut dysbiosis. To investigate the effects of whole BRBs and their fractions on gut microbiota, we fed F-344 rats a control diet, 5% BRBs, the BRB anthocyanin fraction, or the BRB residue fraction for 6 weeks. Feces were collected at baseline and at weeks 3 and 6, and bacterial sequence counts were analyzed. We observed distinct patterns of microbiota from different diet groups. Beta diversity analysis suggested that all diet groups exerted time-dependent changes in the bacterial diversity. Hierarchical clustering analysis revealed that post-diet fecal microbiota was segregated from baseline fecal microbiota within each diet. It is interesting to note that fractions of BRBs induced different changes in gut bacteria compared to whole BRBs. The abundance of specific microbial species known to have anti-inflammatory effects, such as *Akkermansia* and *Desulfovibrio*, was increased by whole BRBs and their residue. Further, butyrate-producing bacteria, e.g., *Anaerostipes*, were increased by whole BRBs. Our results suggest that whole BRBs and their fractions alter the gut microbiota in ways that could significantly impact human health.

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Conflict of interest

No potential conflicts of interest were disclosed.

Keywords

Black raspberries; anthocyanins; fiber; gut microbiota

Introduction

Our body is host to trillions of microbes, which outnumber the body's cells by about 1.3 to 1. It is estimated that our gut contains up to 1000 bacterial species and 100-fold more genes than are found in the human genome (1, 2). This microbial community is commonly referred to as our hidden "metabolic organ" due to its immense impact on the host metabolism, physiology, nutrition, and immune function (3). Most bacteria are neutral to our health, but occasionally they cause illness. Scientists are using newly developed genomic techniques to investigate microbial communities and their genes, collectively known as the microbiome (3). Disruption of the microbiome can contribute to multiple disease states such as obesity (1, 4), diabetes (5), malnutrition (6), and chronic inflammatory diseases including ulcerative colitis and Crohn's disease (7).

A key factor that has been shown to influence the diversity of the microbiota in animal models and in humans is diet (1). For example, a study investigated the composition of gut microbiota in the elderly and correlated the composition of gut microbiota with diet and residence location in the community (8). Elderly residents who lived in the community were found to have a more diverse gut microbiota than residents of long-term care facilities, and the diversity was associated with the content of fat and fiber in their diet (8). Another interesting study was set to determine whether a diet change could potentially reverse the microbial profiles and perhaps even influence cancer risk (9). The incidence rates of colon cancer are much higher in African Americans (65 in 100,000) than those in rural South Africans (less than 5 in 100,000). Rural South Africans were switched from their regular diet to a high-fat, low-fiber "westernized" food regime and African Americans were switched to a low-fat, high-fiber "African diet" for 2 weeks. The abundance of specific microbial species known to promote inflammation and neoplasia progression, such as *Escherichia* and *Acinetobacter*, underwent changes in association with diet type. Thus, these results suggest that we can adapt a healthy diet to reverse the disease-prone gut microbiome.

We previously reported that black raspberry (BRB) powder, added to a synthetic diet at concentrations of 5% and 10%, resulted in 39% and 64% reductions, respectively, in the number of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in F-344 rats (10). More importantly, diets containing either 5% BRB powder, an alcohol/water-soluble extract of BRBs, or an anthocyanin-rich fraction of BRBs (all three diets contained ~ 3.8 μ mol anthocyanins/g) were found to be about equally effective in reducing NMBA tumorigenesis in the rat esophagus (11). These results indicated that the anthocyanins in BRBs contribute to a substantial portion of their chemopreventive potential. In this same study, a diet containing the alcohol/water-insoluble (residue) fraction of BRBs (containing only 0.02 μ mol anthocyanins/g) was nearly as effective as the 5% BRB powder diet and the anthocyanin diet in preventing esophageal tumorigenesis, suggesting that components other than the anthocyanins may also be chemopreventive. The residue/fiber fraction of BRBs

represents about 45% of whole BRB powder and likely contains cellulose, hemicelluloses, pectins, lignans and protein (12). Chemical analysis of the residue indicated that it also contains ellagitannins (13), which are chemopreventive in rodents.

In the present study, changes in the diversity of gut microbiota were investigated in F-344 rats in response to the dietary administration of whole BRB powder, BRB-derived anthocyanins or the BRB-derived residue fraction. Our results suggested that all three dietary treatments produced distinct changes in the composition of the gut microbiota.

Material and Methods

Black raspberries and preparation of BRB-derived anthocyanins and residues

Black raspberries (*Rubus occidentalis*) of the Munger variety were obtained from BerriProducts LLC (Corvallis, OR). The process for picking, washing and freeze-drying the berries and grinding them into a powder has been previously described in detail (14). BRB powder was shipped to the laboratory of Dr. Stephen Hecht for preparation of BRB anthocyanins and residues. The scheme for preparing BRB-derived anthocyanins and the residue fraction was described previously (11).

Animal bioassay

Male F-344 rats, 4-to-5 weeks old, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed and maintained according to the recommendations of the American Association of Laboratory Animal Care at the Medical College of Wisconsin. Briefly, rats were housed two or three per cage under standard conditions ($20 \pm 2^\circ\text{C}$, $50 \pm 10\%$ relative humidity, and 12-hour light/dark cycles). AIN-76A synthetic diet and water were available *ad libitum*. Hygienic conditions were maintained by twice-weekly cage changes. Two weeks after arrival in the animal facility, the rats were randomly assigned into 4 groups of 8 animals each and fecal material was collected from each rat for bacterial DNA extraction. Rats were then placed on an AIN-76A control diet or the AIN-76A diet containing either 5% whole BRB powder, 0.2% BRB anthocyanins or 2.25% of the residue fraction for 6 weeks. The amount of each fraction added to the diet was based on the weight percent contribution of each fraction to 5% BRB powder (11). Body weight and food intake data was recorded weekly after administration of the various diets. Fecal material was collected again from each rat at weeks 3 and 6. Bacterial DNA in feces was extracted within 1 hour after the feces were collected using PowerBead Tubes (MO BIO Laboratories Inc., Carlsbad, CA) and following the manufacturer's instructions. Bacterial sequence counts were analyzed using Roche 454 sequencing.

Roche 454 sequencing

The V1–V3 regions of the bacterial 16S gene were amplified from fecal samples using bar-coded primers as described (15, 16). Equal quantities of the amplified bar-coded samples were pooled and sequenced using Roche 454 pyrosequencing.

Data analysis

Sequencing data was processed using Qiime and a table of sequencing count for each Operational taxonomic unit (OTU) was exported for further analysis. Samples were separated by four different dietary groups: control diet, whole BRBs, anthocyanins, or residue. Twenty-four samples were developed from each diet and the dataset was analyzed using R 3.3.2 software, with Vegan 2.4–2 Ecodist 1.2.3 packages, to calculate Bray-Curtis dissimilarity index, hierarchical clustering, Non-metric Multi-dimensional Scaling (NMDS) ordination, and Adonis statistical testing. For Adonis tests, a p -value of <0.05 was considered significant. For genera tests between groups, we present full p -values for Kruskal-Wallis analysis of variance followed by the Games Howell post hoc test (using Userfriendlyscience 0.6–0 package in R). Heatmaps were generated by normalizing the percent abundance of each bacterial Species within each sample to the average number of sequences for 24 samples in each dietary group. White-to-black shading indicates relatively lower-to-higher number of sequences.

Results

A total of 6593, 8995, 8281, and 7886 OTUs were found in fecal samples from rats fed the control diet, the whole BRBs, the anthocyanin fraction, and the residue fraction, respectively. The number of sequences per sample in different dietary groups ranged from 5058 to 18180 and can be classified into 26 distinct Orders. NMDS ordination of the Bray-Curtis dissimilarities showed that the post-diet fecal microbiota at 3 and 6 weeks were distant from the baseline fecal microbiota at week 0, suggesting time-dependent changes in bacterial diversity within each diet (Figure 1). In the figures the relative physical distances between the data points in the figure reflects relative dissimilarities in microbiome compositions. The p -values between the groups using multivariate analysis of variance (Adonis) are 0.003 or less. Analyses of bacterial genera that drive these differences are discussed below. Hierarchical clustering (using the Ward method) of the data revealed the segregation of the baseline fecal microbiota, from the post-diet fecal microbiota within each dietary group (Figure 2). The description of all sample IDs used in the current study were shown in Supplementary Table 1.

Heatmap visualization at the Genus level suggested distinct changes associated with different dietary groups. The control diet associated with an increased abundance of *Asaccharobacter*, a decreased abundance of *Clostridium* and *Acetanaerobacterium* at weeks 3 and 6, and a transiently increased abundance of *Lactococcus*, *Veillonella* and *Sarcina* at week 3 (Figure 3A). The whole BRBs associated with an increased abundance of *Anaerostipes*, *Ruminococcus*, *Akkermansia* and *Coprobacillus*, a decreased abundance of *Acetivibrio* at weeks 3 and 6, a transiently increased abundance of *Allobaculum*, and a transiently decreased abundance of *Anaerotruncus* at week 3 (Figure 3B). The BRB-derived anthocyanin fraction associated with an increased abundance of *Anaerovorax* and *Dorea*, a decreased the abundance of *Bifidobacterium* and *Lactococcus* at weeks 3 and 6, a transiently increased abundance of *Asaccharobacter*, and a transiently decreased abundance of *Prabacteroides* at week 3 (Figure 3C). The BRB-derived residue fraction associated with an increased abundance of *Anaerotruncus*, *Coprobacillus*, *Desulfovibrio*, *Vectivallis* and

Mucispirillum, a decreased abundance of *Streptococcus*, *Turicibacter* and *Acetivibrio* at weeks 3 and 6, a transiently increased abundance of *Ethanoligenens*, and a transiently decreased abundance of *Bifidobacterium* at week 3 (Figure 3 D). The sequence counts and *p*-values for Kruskal-Wallis analysis of variance for each bacterial species followed by the Games Howell *post hoc* tests for select species were shown in Supplementary Table 2. The descriptive values of the expression for each bacterial species in Figure 3 were shown in Supplementary Table 3.

Discussion

Our gut microbiota comprises about 500–1000 different bacterial species and the composition is plastic, depending on the age, diet, nutrition status, and other factors (17). This dynamic microbiome evolves in a mutualistic relationship with our body (18). Gut microbiota plays many important roles to maintain the host health, including fermentation of indigestible dietary compounds, synthesis of vitamins and other beneficial metabolites, and controlling the balance of our immune system (17, 18). Dysbiosis defines a situation in which the composition and/or activities of the gut microbiota are altered due to a deleterious state of our body (19), and has been shown to associate with many diseases, especially obesity (20, 21), diabetes (5, 22), metabolic syndrome (23), cardiovascular disease (24), inflammatory bowel disease (25) and colorectal cancer (26).

Natural compounds and foodstuff can alter the composition and diversity of the gut microbiome. For example, 6-week consumption of a wild blueberry powder drink increases *Bifidobacterium* in the gut of healthy human volunteers (27). Four-week feeding of green kiwifruit diet to healthy rats increased cecal *Lachnospiraceae* (28). Our previous studies demonstrated beneficial effects of BRBs against colorectal cancer in humans (29–31) and mice (32–36). We also showed that BRBs suppressed NMBA-induced esophageal squamous cell carcinoma in rats. The current study investigated if BRBs could change the composition of gut microbiota in healthy rats. We fed the F-344 rats with the AIN-76A control diet, or the control diet containing either 5% whole BRB powder, 0.2% BRB anthocyanins or 2.25% of the BRB-derived residue fraction for 6 weeks. We observed time-dependent alterations within each dietary group: all diets produced changes in bacterial diversity and post-treatment samples were segregated from the baseline samples.

Despite the evidence that supports the correlation between dysbiosis and human diseases, the underlying mechanisms and the causal relationship still remain unknown. It is largely controversial whether the diseases cause dysbiosis, or vice versa. In addition, many other factors may influence the findings regarding the abundance of particular species in the gut microbiome, such as the stage of disease, the status of the individual human host, the condition of experiments and the technique used to analyze the results. For example, one study showed increased levels of *Bacteroides* in colorectal cancer patients compared to healthy human subjects using real-time quantitative polymerase chain reaction technique (37), whereas another group found lower proportions of *Bacteroides* in patients with colorectal adenoma compared to healthy analyzed by 16S rRNA genes sequencing (38). Therefore, results from different studies need to be carefully interpreted particularly when single species of bacteria are compared.

Though our understanding of the functions of specific gut bacteria has grown rapidly, functions for majority of gut bacteria remain unclear. Decreased levels of *Akkermansia* have been observed in patients with inflammatory bowel diseases, such as ulcerative colitis, and metabolic disorders, such as obesity and diabetes, suggesting its potential anti-inflammatory effects (39). Supplementation of *Akkermansia* (40) or feeding certain natural compounds that can increase the abundance of *Akkermansia*, such as red pitaya (*Hylocereus polyrhizus*) betacyanins (41), could attenuate the inflammation in mice, suggesting an anti-inflammatory role of *Akkermansia*. In addition, decreased abundance of *Desulfovibrio* has been reported in the high-fat diet-induced obese mice, which were associated with elevated inflammation and the activation of the Wnt pathway (42). Our current study observed significantly increased abundance of *Akkermansia* by BRB diet and increased abundance of *Desulfovibrio* by the BRB residue fraction in rats. Previously, we demonstrated that whole BRBs, anthocyanins and the residue fraction decreased cellular proliferation marker Ki67 and inflammatory biomarkers, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nuclear factor (NF)- κ B, soluble epoxide hydrolase (sEH), pentraxin-3 (PTX3), and prostaglandin E₂ (PGE₂), in the NMBA-induced esophageal cancer in rats (11, 43, 44), suggesting that all three diets can suppress tumorigenesis by inhibiting inflammation. In the current study, rats fed whole BRBs or the residue fraction had increased abundance of *Akkermansia* and *Desulfovibrio* suggesting that they might contribute to the anti-inflammatory effects of whole BRBs and the residue fraction. Also, *Anaerostipes* is one of the major butyrate-producing bacteria identified in human gut (45). Our current study observed significantly increased abundance of *Anaerostipes* by BRBs, suggesting that BRBs may enhance the population of butyrate-producing bacteria. Interestingly, the residue fraction did not significantly change the abundance of *Anaerostipes*. It is possible that other components in BRBs influenced the butyrate-producing bacteria, which in turn ferment fiber into short-chain fatty acids (SCFAs) including butyrate. Furthermore, one group found that six-week consumption of a wild blueberry drink, which contained polyphenols, in particular anthocyanins and dietary fiber, significantly increased the abundance of *Bifidobacterium* in healthy human volunteers (27). Another group observed that one month-consumption of psyllium seed husk (*Plantago ovata*) (7.0 g daily) increased the abundance of *Bifidobacteria* in the healthy women who had relatively low level of *Bifidobacteria* before the treatment (46). By contrast, our study observed significantly reduced abundance of *Bifidobacterium* after six-week feeding of the anthocyanin-rich fraction of 5% BRBs in rats but it was neither changed by whole BRBs nor BRB residue. These results suggest that different food sources and species may lead to different changes in the gut microbiome. We previously found equal effectiveness in reducing NMBA-induced esophagus tumorigenesis in rats from either 5% BRBs, the anthocyanin-rich fraction of 5% BRBs or the residue fraction of 5% BRBs (11). Interestingly, we observed distinct effects of these three diets on the gut microbiota in healthy rats, suggesting that changes induced by a specific food and its components in microbiota does not follow a simple formula, and suggesting that complicated interactions between different food components and bacteria occur in the gut. Different fractions of BRBs might select and stimulate different bacterial species, which could in turn lead to further changes of the interactions between bacterial species that leads to a healthy gut bacteria community. Therefore, the entire gut microbiota benefits our health rather than the

activities of individual bacteria. More studies are needed to investigate how BRBs and their fractions affect the gut microbiome in healthy human subjects and patients with cancer. In addition, difference of inter-species (laboratory animals and humans) in the gut microbiota should also be taken into consideration. Many factors, including diet, health status, and living environment, could influence the interactions between gut bacteria and the host system. Therefore, when comparisons are made between studies conducted in different species, special caution is required on interpretation of the different and/or same results.

Interestingly, one group found that animal-based diet induced stronger changes in gut microbiota compared to plant-based diet in healthy human volunteers, and these changes were reverted back to their original composition just 2 days after the animal-based diet discontinued (47). Whether BRBs' effects on gut microbiota are only transient needs further investigation. In addition, fiber has been reported to promote healthy gastrointestinal (GI) function acting as laxatives, e.g., reducing transit time and increasing stool volume. In human colon cancer patients, BRB intervention improved bowel movement in 75% of patients who consumed BRBs (29). Furthermore, diet rich in fiber has been shown to inhibit the growth of pathogenic bacteria, such as *Enterobacteriaceae* (*Shigella* and *Escherichia*), at least partially, through the mildly acidic pH environment induced by high content of SCFAs (48, 49). BRBs contain abundant soluble fiber that can be metabolized to SCFAs in the gut that may contribute to BRB-mediated effects of inhibiting the growth of pathogenic bacteria and stimulating the growth of beneficial bacteria, which may benefit our health.

In summary, the current study demonstrated that BRBs and their fractions were able to alter the composition and the diversity of gut microbiome in healthy rats in a time-dependent manner. Distinct patterns were observed between different diets. These changed bacterial species might be beneficial organisms for the prevention of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BRB	Black raspberry
COX-2	Cyclooxygenase-2
FFAR2	Fatty acid receptor 2
GI	Gastrointestinal
iNOS	Inducible nitric oxide synthase
NF-κB	Nuclear factor- κ B

NMBA	<i>N</i> -nitrosomethylbenzylamine
NMDS	Non-metric multi-dimensional scaling
OTU	Operational taxonomic unit
PGE₂	Prostaglandin E ₂
PTX3	Pentraxin-3
SCFAs	Short-chain fatty acids
sEH	Soluble epoxide hydrolase

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A

B

Figure 1:

Non-metric multi-dimensional scaling (NMDS) ordination analysis showed that the post-diet fecal microbiota (at weeks 3 and 6) were distant from the baseline fecal microbiota (at week 0) of the control diet (**A**), the whole BRB diet (**B**), the BRB-derived anthocyanin fraction (**C**) and the BRB-derived residue fraction (**D**). Adonis analysis (multivariate analysis of variance) results in p-values of 0.003 for (**A**), and 0.001 for (**B-D**).

A

Control

B

BRBs

C

D

Figure 2:

Hierarchical clustering analysis showed that the post-diet fecal microbiota (at weeks 3 and 6) were segregated from the baseline fecal microbiota (at week 0) of the control diet (**A**), the whole BRB diet (**B**), the BRB-derived anthocyanin fraction (**C**) and the BRB-derived residue fraction (**D**). Adonis analysis (multivariate analysis of variance) results in p-values of 0.003 for (**A**), and 0.001 for (**B-D**).

A

Control

B

BRBs

Figure 3: Heatmap analyses of the fecal microbiota in rats treated with the control diet (A), the whole BRB diet (B), the BRB-derived anthocyanin fraction (C) and the BRB-derived residue fraction (D) for 6 weeks. White-to-black shading indicates relatively lower-to-higher number of counts.