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Protocatechuic Acid, a Gut Bacterial Metabolite of Black Raspberries, Inhibits Adenoma Development and Alters Gut Microbiome Profiles in *Apc*$^{Min/+}$ Mice

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Administration of black raspberries (BRBs) and their anthocyanin metabolites, including protocatechuic acid (PCA), has been demonstrated to exert chemopreventive effects against colorectal cancer through alteration of innate immune cell trafficking, modulation of metabolic and inflammatory pathways, etc. Previous research has shown that the gut microbiome is important in the effectiveness of chemoprevention of colorectal cancer. This study aimed to assess the potency of PCA versus BRB dietary administration for colorectal cancer prevention using an *Apc*$^{Min/+}$ mouse model and determine how bacterial profiles change in response to PCA and BRBs. A control AIN-76A diet supplemented with 5% BRBs, 500 ppm PCA, or 1,000 ppm PCA was administered to *Apc*$^{Min/+}$ mice. Changes in incidence, polyp number, and polyp size regarding adenomas of the small intestine and colon were assessed after completion of the diet regimen. There were significant decreases in adenoma development by dietary administration of PCA and BRBs in the small intestine and the 5% BRB-supplemented diet in the colon. Pro-inflammatory bacterial profiles were replaced with anti-inflammatory bacteria in all treatments, with the greatest effects in the 5% BRB and 500 ppm PCA-supplemented diets accompanied by decreased COX-2 and prostaglandin E_2 levels in colonic mucosa. We further showed that 500 ppm PCA, but not 1,000 ppm PCA, increased IFN-γ and SMAD4 levels in primary cultured human natural killer cells. These results suggest that both BRBs and a lower dose PCA can benefit colorectal cancer patients by inhibiting the growth and proliferation of adenomas and promoting a more favorable gut microbiome condition.

Key Words Colorectal neoplasms, Genes, APC, Rubus, Gastrointestinal microbiome

INTRODUCTION

Colorectal cancer (CRC), ranked as the third leading cause of new cancer diagnoses and cancer deaths in the United States, has presented recently with increased incidence within the young adult population [1]. Despite improved screening rates, the 5-year relative survival is approximately 65% [2]. As of 2020, it is anticipated that 1.9 million new diagnoses and 935,000 deaths per year worldwide will result from CRC [3]. It is thus critical to pursue improved prophylaxis and treatment of CRC in order to reduce incidence rates and improve patient outcomes.

Current medical recommendations for the prevention of CRC include the consumption of vitamin- and nutrient-rich fruits, whole grains, and vegetables [4]. Our previous research has focused on the utilization of black raspberries (BRBs) in CRC prevention, with demonstrated efficacy in decreasing rectal polyp burden in humans through regulation of metabolic profiles and protective modulation of gene expression [5-7]. The chemopreventive effects of BRBs and

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underlying mechanisms have been further elucidated in experimentally induced esophageal cancer models. BRBs have been shown to prevent esophageal tumorigenesis in rats by virtue of chemoprotective anthocyanin (AC) components [8]. These BRB-derived increase expression of protective natural killer (NK) cell-associated cytokines, downregulate macrophage-associated cytokines to reduce macrophage accumulation, reduce neutrophil accumulation, and inhibit angiogenesis, overall decreasing the expression of dysregulated inflammatory biomarkers [8].

One compound of focus that has arisen from these prior studies is protocatechuic acid (PCA), a microbial metabolite of BRB ACs that has been demonstrated to be especially efficacious in reducing neutrophil accumulation in esophageal papilloma tissue, as well as promoting other effects such as the modification of immune cell trafficking and prevention of inflammation. PCA upregulates the expression of the pentraxin 3 (PTX3) promoter, which is silenced via hypermethylation in human esophageal cancer but when active, acts to inhibit angiogenesis and tumorigenesis [9]. Though it is less effective than BRBs in some respects, it has unique advantages as aforementioned in addition to high bioavailability and low cost [10]. However, there is a paucity of studies on the use of PCA in the management of CRC, and this application should be considered as the effects of PCA may not be limited to solely esophageal cancer.

Our more recent studies have focused on the use of multiple intestinal neoplasia (*Apc*$^{Min/+}$) mice as a model for the study of CRC and its chemoprevention. The protein product encoded by the Adenomatous polyposis coli (*Apc*) gene promotes β-catenin degradation, thereby suppressing the Wnt signaling pathway. Since this pathway is involved in cellular proliferation and differentiation, and mutations are frequently implicated in human cancers, *Apc* acts as a tumor suppressor gene [11]. *Apc*$^{Min/+}$ mice are characterized by an Apc codon 850 truncation that removes the inhibition of the Wnt pathway and are hence susceptible to the rapid development of colonic and intestinal polyps [12,13].

A BRB-containing diet has been shown to reduce the proliferation of colonic polyps and adenomas within the small intestine and colon of *Apc*$^{Min/+}$ mice by modulating the metabolism of nucleotides, glutathione, lipids, and amino acids, suggesting that it affects these pathways at specific steps to restore metabolic balance [12]. A study on *Apc*$^{Min/+}$ mice fed a 5% BRB diet showed that specific phytochemicals contained in BRBs reinforce these anti-CRC effects by inhibiting the activity of DNA methyltransferases 1 and 3B, which act to disrupt tumor suppressor genes; these enzymes are overexpressed in colorectal tumors [14]. Overall, a diet containing 5% BRBs can improve survival rates and lifespan in *Apc*$^{Min/+}$ mice, likely via multiple mechanisms [15].

In addition, the role of gut bacteria is crucial in manifesting the effects of BRBs; while BRBs prevent tumorigenesis, this is no longer evident when antibiotics are simultaneously administered in *Apc*$^{Min/+}$ mice [13]. BRBs have been shown to affect the gut microbiome in F-344 rats, fortifying species that either directly exert anti-inflammatory effects or produce compounds, such as butyrate, that have anti-inflammatory properties [16,17]. Lastly, BRBs have been shown to interact with the TLR4/NF-κB/COX-2 pathway associated with CRC by lowering mRNA levels of all components [13]. Given this preliminary knowledge regarding the mechanism of BRBs as a potential chemopreventive measure in CRC, the current study was intended to further assess the efficacy of PCA specifically as a BRB metabolite in preventing the development of colonic and intestinal polyps and adenomas in *Apc*$^{Min/+}$ mice, as well as to examine how gut microbiota may change in response to BRB and PCA administration.

MATERIALS AND METHODS

Animal experiments

All protocols were carried out in accordance with institutional guidelines for animal care dictated by the Medical College of Wisconsin Animal Care and Use Committee (protocol approval number AUA2430). Eight-week-old breeding pairs of *Apc*$^{Min/+}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Four-to-six-week-old *Apc*$^{Min/+}$ mice, both sexes, were fed either the control diet or a control diet supplemented with 5% BRBs, 500 ppm PCA, or 1,000 ppm PCA for 8 weeks. Mice were euthanized by CO_2 asphyxiation. The number of colonic polyps was counted, and the size was measured as previously described [12]. Feces were collected to isolate fecal bacterial DNA for 16S gene sequencing of the V1-V3 regions as described before [16].

Diets

The control diet was an AIN-76A diet from the American Institute of Nutrition (Dyets Inc., Bethlehem, PA, USA). BRB powder was purchased from Berri Products (Corvallis, OR, USA), and stored at 4°C in vacuum-sealed plastic bags at the Medical College of Wisconsin. Water and dietary intake were recorded weekly and there were no differences among groups.

Chemicals

PCA was purchased from Millipore Sigma (St. Louis, MO, USA).

COX-2 and PGE2 in colonic mucosa

Colonic mucosa was used to measure COX-2 mRNA expression using primers purchased from Integrated DNA Technologies (IDT), Inc. (Coralville, IA, USA); prostaglandin E_2 (PGE_2) levels were measured using an ELISA kit (MyBioSource, San Diego, CA, USA).

Human NK cell isolation and treatments

Human NK cells were isolated from fresh peripheral blood

from healthy subjects and PBMC from healthy subjects using the NK Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol but with minor modifications, as described previously [18]. Freshly isolated NK cells were cultured in RPMI1640+10% FBS+1% P/S (RPMI, Roswell Park Memorial Institute; FBS, Fetal bovine serum; P/S, Penicillin-Streptomycin) and immediately treated with PCA at 500 ppm or 1,000 ppm or dimethylsulfoxide (DMSO) (Millipore Sigma) for 16 hours. The cells were collected for real-time PCR using the primers purchased from IDT, Inc.

Statistical analysis

GraphPad Prism was used to analyze tumor data and inflammatory markers (un-paired, two-tailed *t*-test). A *P*-value less than 0.05 was considered statistically significant. Bars: Mean. The raw microbiome sequencing data was processed by QIIME software version 1.8.0. Statistical software R with the package "phyloseq" [19] was used to analyze the microbiome data. Clustering algorithm 'uclust' was used for operational taxonomic units (OTUs) picking step with default OTU threshold set as 0.97. For each OTU, one sequence was picked as a representative. The taxonomy for each sequence was assigned using uclust algorithm and 97 percent Greengene database as reference (default). Finally, a table with 5,432 OTUs and 39 subjects was exported in biom data format. OTUs were agglomerated at the taxonomic rank of species to compare the abundance levels between different groups, and those without annotation at the species rank were filtered out. Next, we transformed the data from abundance counts to fractional abundance to normalize different library size effect across samples. Sequencing depths are shown in Table S1.

RESULTS

Adenoma development

A diet containing AIN-76A (control), 5% BRBs, 500 ppm PCA, or 1,000 ppm PCA was administered to *Apc*$^{Min/+}$ mice. Following the dietary regimen, 100% of AIN-76A diet-treated *Apc*$^{Min/+}$ mice developed adenomas of the small intestine, versus 88% of 5% BRB diet-treated mice, 100% of 500 ppm PCA diet-treated mice, and 90% of 1,000 ppm PCA diet-treated mice (Fig. 1). While none of these changes was significant, there was a marked ($P < 0.05$) decrease in the overall small intestinal polyp number and the size in all experimental treatment groups.

Twenty six percent of AIN-76A diet-treated *Apc*$^{Min/+}$ mice developed adenomas of the colon, versus 8% of 5% BRB diet-treated mice, 19% of 500 ppm PCA diet-treated mice, and 20% of 1,000 ppm PCA diet-treated mice (Fig. 2). There was a significant ($P = 0.001$) decrease in the incidence of colonic adenoma development in the 5% BRB-treated population versus the control group. However, in the colon, there was only a significant ($P < 0.05$) decrease in the size of polyps for mice given the 5% BRB-supplemented diet.

Gut microbiota

As previously noted, gut bacteria are necessary for the effects of BRBs and their metabolites to manifest [13]. Samples were taken from *Apc*$^{Min/+}$ mice administered the experimental and control diets for analysis of bacteria present. Major populations of bacteria determined included *Ruminococcus gnavus*, *Desulfovibrio C21-c20*, *Bacteroides acidifaciens*, *Akkermansia muciniphila*, *Parabacteroides distasonis*, and *Bacteroides uniformis*. *Ruminococcus gnavus* secretes a polysaccharide that induces inflammation by promoting the production of

	# of mice total	# of mice have adenoma in small intestine	Incidence (%)
Ctrl	19	19	100
5% BRBs	24	21	88
500 ppm PCA	21	21	100
1,000 ppm PCA	20	18	90

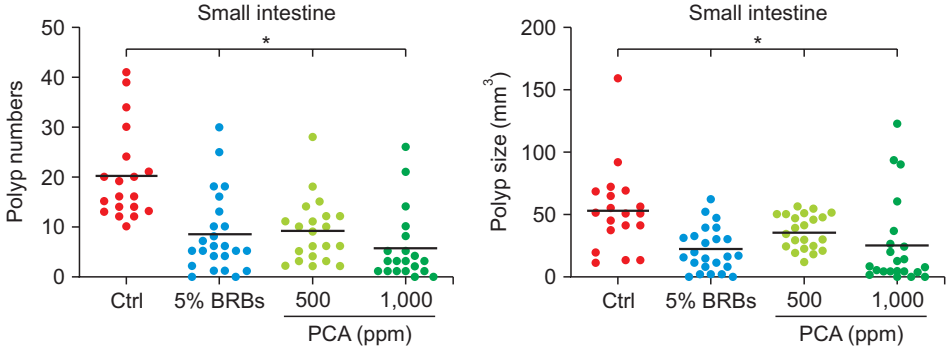

Figure 1. Administration of a diet containing 5% black raspberries (BRBs), 500 ppm protocatechuic acid (PCA), or 1,000 ppm PCA caused a significant decrease in the number and the size of polyps in the small intestine of *Apc*$^{Min/+}$ mice. Red circles represent control (ctrl) diet-treated *Apc*$^{Min/+}$ mice, blue circles represent 5% BRB diet-treated *Apc*$^{Min/+}$ mice, light green circles represent 500 ppm PCA diet-treated *Apc*$^{Min/+}$ mice, dark green circles represent 1,000 ppm PCA diet-treated *Apc*$^{Min/+}$ mice (*$P < 0.05$). Every two groups were compared. There are no differences among the three treatment groups. Bars: mean.

	# of mice total	# of mice have adenoma in small intestine	Incidence (%)
Ctrl	19	5	26
5% BRBs	24	2	8 (P = 0.001)
500 ppm PCA	21	4	19
1,000 ppm PCA	20	4	20

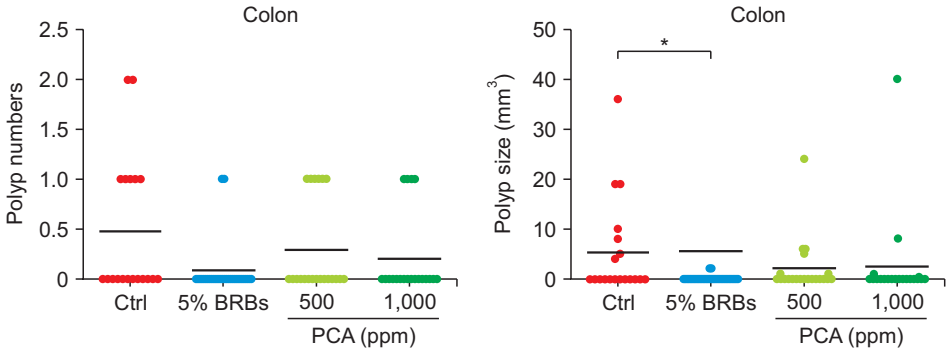

Figure 2. Administration of a diet containing 5% black raspberries (BRBs) caused a significant decrease in the polyp size in the colon of *Apc*$^{Min/+}$ mice. Red circles represent control (ctrl) diet-treated *Apc*$^{Min/+}$ mice, blue circles represent 5% BRB diet-treated *Apc*$^{Min/+}$ mice, light green circles represent 500 ppm protocatechuic acid (PCA) diet-treated *Apc*$^{Min/+}$ mice, dark green circles represent 1,000 ppm PCA diet-treated *Apc*$^{Min/+}$ mice (*P < 0.05). Every two groups were compared. There are no differences among the three treatment groups. Bars: mean.

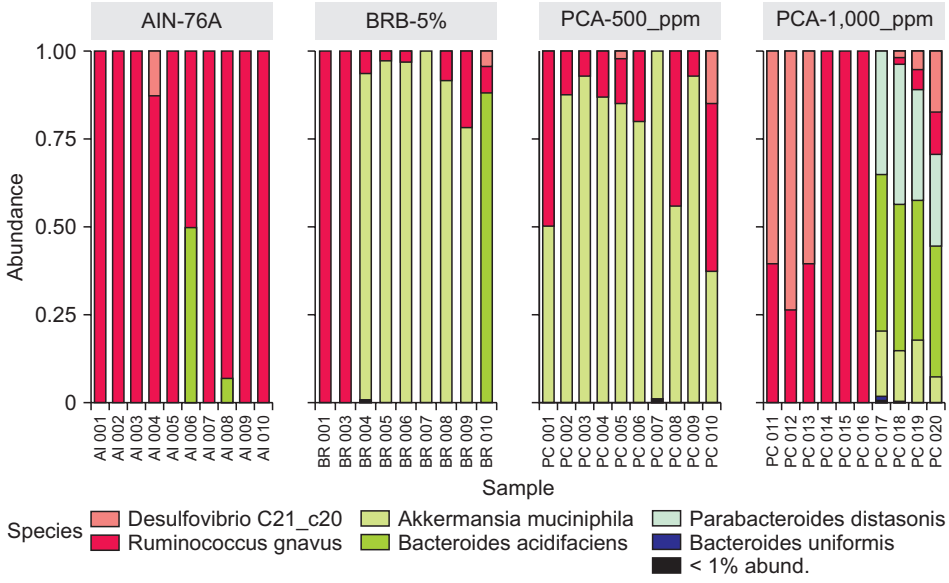

Figure 3. Abundance of bacterial species drawn from samples of *Apc*$^{Min/+}$ mice fed the AIN-76A, 5% black raspberries (BRB), 500 ppm protocatechuic acid (PCA), and 1,000 ppm PCA supplemented diets, from left to right. AIN-76A samples were dominated by *Ruminococcus gnavus* with minor appearances of *Desulfovibrio C21-c20* and *Bacteroides acidifaciens*. *Akkermansia muciniphila* became more prevalent in the 5% BRB and 500 ppm PCA samples, and to some extent the 1,000 ppm PCA samples. *Ruminococcus gnavus* and *Desulfovibrio C21-c20* persisted in all samples but did so most notably in the 1,000 ppm PCA group. *Bacteroides acidifaciens* appeared in one sample of the 5% BRB group and in several of the 1,000 ppm PCA samples. *Parabacteroides distasonis* and *Bacteroides uniformis* appeared only in the 1,000 ppm PCA samples.

tumor necrosis factor alpha (TNF-α) via an NF-κB-mediated pathway [20]. *Desulfovibrio C21-c20* produces lipopolysaccharide (LPS) that activates the NF-κB inflammatory cascade [21]. *Akkermansia muciniphila* degrades mucin and may have a role in the maintenance of gut barrier function and control of inflammation [22,23]. *Parabacteroides distasonis* has been shown to promote colitis in mouse models [24].

Mice given the AIN-76A control diet demonstrated a prevalence of *Ruminococcus gnavus* with less frequent occurrences of *Desulfovibrio C21-c20* and *Bacteroides acidifaciens* (Fig. 3). The abundance of *Ruminococcus gnavus* gave way to a domination by *Akkermansia muciniphila* in the mice fed 5% BRB and 500 ppm PCA, and to a lesser degree in the 1,000 ppm PCA fed group. *Ruminococcus gnavus* and *Desulfovibrio C21-c20* nonetheless continued to be present in all treatment groups. *Bacteroides acidifaciens* appeared

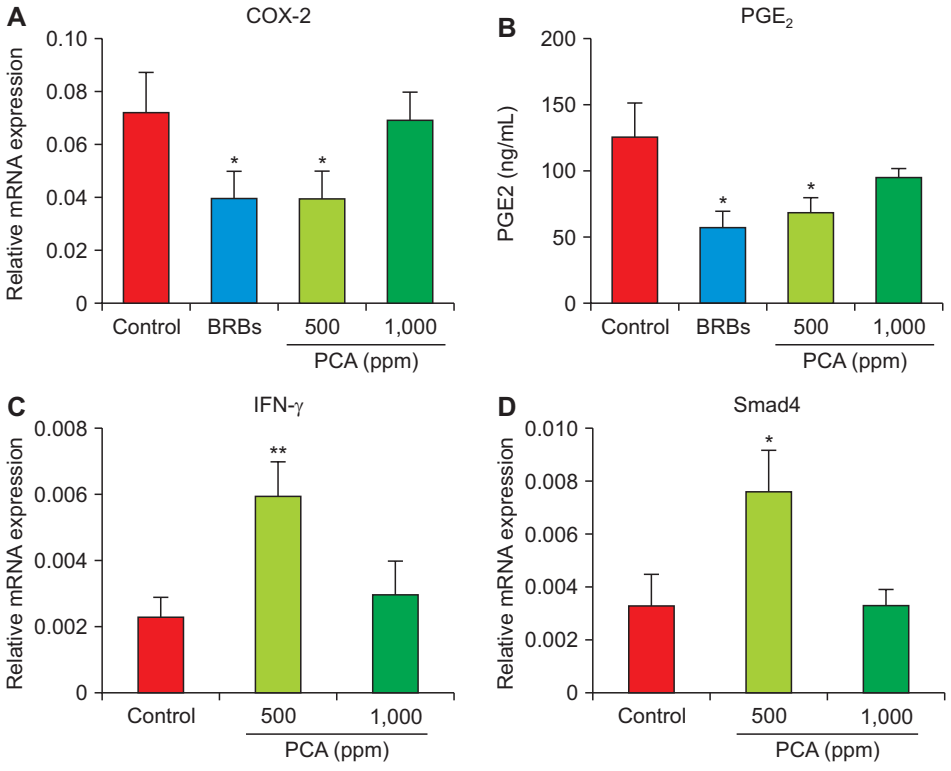

Figure 4. Black raspberries (BRBs) and 500 ppm protocatechuic acid (PCA) are anti-inflammatory. (A, B) BRBs and 500 ppm PCA decreased COX-2 and prostaglandin E_2 (PGE_2) levels in colonic mucosa (n = 5 mice). (C, D) Five hundred ppm PCA increased IFN-γ and SMAD4 levels in primary cultured human natural killer cells (n = 5 human subjects). *P < 0.05, **P < 0.01.

infrequently in the 5% BRB and 1,000 ppm PCA groups. *Parabacteroides distasonis* and *Bacteroides uniformis* appeared solely in the 1,000 ppm PCA samples.

Inflammation

In order to better define the anti-inflammatory effects of BRBs and PCA, further analysis was conducted on specific inflammatory and immune-mediating markers. The enzyme COX-2 responds to inflammatory stimuli by promoting the synthesis of pro-inflammatory prostaglandins including PGE_2 [25]. Both COX-2 and PGE_2 are related to colorectal tumorigenesis [26]. The consumption of BRBs and 500 ppm PCA significantly (P < 0.05) decreased the levels of COX-2 mRNA expression in colonic mucosa (Fig. 4). Levels of PGE_2 were also significantly (P < 0.05) reduced in the colonic mucosa of mice given a BRB or 500 ppm PCA diet (Fig. 4A and 4B).

Primary cultured human natural killer cells were treated with 500 ppm and 1,000 ppm PCA. Only 500 ppm PCA, but not 1,000 ppm PCA, significantly increased both IFN-γ (P < 0.01) and SMAD4 (P < 0.05) levels (Fig. 4C and 4D). IFN-γ is produced by NK cells and factors in immunoregulation as well as the inhibition of tumorigenesis [27]. SMAD4 is a tumor suppressor gene that downregulates the TGF-β pathway, which is frequently implicated in inflammation [28]. This gene is frequently inactivated in CRC and thereby associated with a number of pathological characteristics seen in CRC [29].

DISCUSSION

In our previous studies, it was determined that BRBs and PCA exert protective effects against both esophageal and colorectal cancer by modulating innate inflammatory and metabolic pathways. Using *Apc*$^{Min/+}$ mice as a CRC model, we observed in this study that an AIN-76A diet with the addition of 5% BRBs significantly decreased the number and the size of polyps in the small intestine and decreases the incidence and the size of polyps in the colon. This corroborates a similar result from a previous study, in which a 5% BRB treatment administered for eight weeks reduced the incidence and the number of colonic polyps [12]. In combination, these results further reinforce that a diet supplemented with 5% BRBs has the potential to prevent adenoma development in both the small intestine and colon. It is likely, based on prior discussed studies, that this adenoma suppression is due to the metabolic and genetic modulation that BRBs have been shown to exert [12,14]. Future studies dedicated to further examination of this modulation may yield more definitive cause-effect relationships. Since studies conducted with BRBs previously have demonstrated a wide range of possible activities of BRBs and their components, it would be helpful to delineate which mechanisms precisely are operative in the suppression of adenomas, or to assess relative activity levels of these mechanisms. It is certainly plausible that several modulations contributed to reduced adenoma formation in the current study.

According to a pharmacokinetic study in humans, about

70% of ingested cyanidin-glucosides are converted to PCA in the human gut [30]. Therefore, for comparative purposes, we fed mice an amount of PCA (500 ppm in the diet) equivalent to about 70% of the anthocyanin content in a 5% BRB diet. According to our results, PCA was effective at this dose, suggesting that it could be responsible for part of the chemopreventive activity of whole BRBs. PCA supplementations at both 500 and 1,000 ppm had similar effects, albeit in the small intestine only. PCA did not have significant activity in the colon. This may be reflective of determinations made in previous research where PCA had lesser anti-inflammatory effects than whole BRBs [9]. This discrepancy may also be due to the route of administration. However, it is important to note that while it was hypothesized that direct administration of PCA, as opposed to metabolism of whole BRBs into PCA, would improve PCA bioavailability, whole BRBs still had an overall greater breadth of effects. Thus, another option for future consideration may be examining adenoma response to different percentage supplementations of whole BRBs.

This study also aimed to examine the changes in gut microbiota that result from BRB and PCA administration, based on the knowledge that gut bacteria play a vital role in the effectiveness of BRBs [13]. The inflammatory qualities of bacteria in the colon and intestine are critical to consider in the context of their role in CRC development as chronic inflammation is a significant risk factor for tumorigenesis; it can mediate DNA damage via the release of reactive oxygen species and allow invasion of malignant bacteria through the weakened gut barrier [31]. It is therefore pertinent to examine changes in the gut microbiome that might reduce the capacity for inflammation and thus tumorigenic damage.

In *Apc*$^{Min/+}$ mice given the AIN-76A control diet, gut samples predominantly contained *Ruminococcus gnavus*, a pro-inflammatory bacterial species that could contribute to the chronic inflammation that characterizes CRC. *Desulfovibrio C21-c20* was also present, and it is of interest to note its interaction with the TLR/NF-κB pathway, as we previously remarked that BRBs act to decrease mRNA levels in the TLR4/NF-κB/COX-2 pathway. While COX-2 production is not noted in literature as being relevant to *Desulfovibrio C21-c20*, the base mechanism of bacterial LPS binding to a toll-like receptor to activate NF-κB, in association with the impact of BRBs, may merit further exploration.

Our results demonstrated that *Ruminococcus gnavus* and *Desulfovibrio C21-c20* levels were dramatically decreased in mice given a 5% BRB or 500 ppm PCA-supplemented diet; the decrease in pro-inflammatory bacteria likely indicates a lower level of inflammation in the small intestine and colon. The gut bacterial composition of the 5% BRB and 500 ppm PCA fed mice were overall similar, with *Akkermansia muciniphila* being the predominant species. This reflects a more anti-inflammatory bacterial profile and, in combination with previously discussed results, indicates that a 5% BRB or 500 ppm PCA diet can effectively exert protective effects against CRC by both inhibiting adenoma development and promoting a less inflammatory microbiome. However, 5% BRBs showed better performance in preventing and decreasing the size of colonic adenomas, and this is a consideration to take into account in making a choice of treatment. The increase in *Akkermansia muciniphila* corresponds with a previously discussed study in F-344 rats fed BRBs and anthocyanins, but the decrease in *Desulfovibrio* in the current study contradicts the previous results [16]. It is possible that this inconsistency may be due in part to animal species selection (rats versus mice), but further exploration may be warranted to specifically examine the response of *Desulfovibrio* to BRBs.

However, *Ruminococcus gnavus* and *Desulfovibrio C21-c20* returned to higher levels in the 1,000 ppm PCA diet group, with the latter becoming even more prevalent than in the control group. While the 1,000 ppm PCA diet had similar effectiveness in inhibiting adenoma development to the other experimental treatments, the gut microbiota appeared to retain a moderately inflammatory profile in half of the samples. *Akkermansia muciniphila*, *Bacteroides acidifaciens*, *Parabacteroides distasonis* and *Bacteroides uniformis* were also present in the 1,000 ppm PCA samples, creating largely anti-inflammatory bacterial profiles in approximately the other half of the group. This variability may be due to the comparatively small sample size as only 20 mice were given this diet, or it may suggest that a higher dose of PCA is excessive and may disrupt the gut microbiome.

Although both 5% and 10% BRBs suppressed rat esophageal tumor development, the cancer-inhibiting effects of 5% BRBs were better than those exerted by 10% BRBs, suggesting that more is not always better [32]. Likewise, the current study suggests that 1,000 ppm PCA is not more effective than 500 ppm PCA. One thousand ppm PCA did not offer a significant advantage over the other experimental diets in prevention of adenoma development, so further investigation may be needed in order to determine the optimal dosage of PCA for maximal benefit.

BRBs suppressed adenoma development partly via alteration of gut microbiome profiles, particularly by replacing pro-inflammatory bacteria with anti-inflammatory bacteria. We further demonstrated that whole BRBs and 500 ppm PCA decreased inflammatory markers such as COX-2 and PGE_2. In addition, 500 ppm PCA, but not 1,000 ppm PCA, enhanced IFN-γ and SMAD4 expression in primary cultured natural killer cells; both IFN-γ and SMAD4 are involved in crucial anti-inflammatory signaling in suppressing colorectal cancer development [18,33,34]. Our results suggest that an enhanced anti-inflammatory gut microbiome by virtue of whole BRBs and 500 ppm PCA could result in an anti-inflammatory tumor microenvironment and thereby suppress tumor development.

Overall, this study confirms the effectiveness of 5% BRB and 500 ppm PCA dietary supplementation in reducing the extent of small intestinal and colonic adenomas in a murine CRC model, and suggests that these regimens have the

potential to modify the gut bacterial profile in beneficial ways that enhance the proportion of anti-inflammatory species over pro-inflammatory ones. The results of this study suggest BRBs and PCA as alternative chemopreventive treatments for CRC and possibly other malignancies, and support previous literature regarding the effectiveness of BRBs.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2022.27.1.50.

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