Dietary Pterostilbene Inhibited Colonic Inflammation in Dextran-Sodium-Sulfate-Treated Mice: A Perspective of Gut Microbiota

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Abstract
Dietary interventions based on the use of bioactive nutraceuticals might offer an effective adjuvant therapeutic and preventive method for inflammatory bowel disease by reshaping colitis-associated bacterial dysbiosis. The current study aimed to determine the anti-inflammatory effect of pterostilbene (PTE, a methylated derivative of resveratrol) and its potential modulatory roles in gut microbiota in a dextran sodium sulfate (DSS)-induced colitis mouse model. Our results supported our hypothesis that dietary PTE exerted protective effects against colonic inflammation; evidenced by the reduced colonic tissue damage, decreased disease activity index, and lowered production of pro-inflammatory cytokines such as interferon gamma, interleukin (IL)-2, IL-4, and IL-6 in the colon of DSS-treated mice. Moreover, α-diversity analysis indicated that dietary PTE significantly improved gut microbial evenness and diversity. Noteworthy, PTE modified gut microbiota composition toward a healthier profile by boosting the richness of Bilophila and decreasing the distribution of pathogenic Bilophila and Rc4-4. Pearson correlation analysis also revealed strong associations between the shifting of gut microbiota and expression of inflammatory cytokines in the colon. Overall, our study demonstrated that dietary PTE alleviated the severity of colitis in DSS-treated mice and gut microbiota may play an indispensable role in this process mechanistically.

Keywords: pterostilbene; anti-inflammation; gut microbiota

Introduction
Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is a chronic inflammatory gastrointestinal disease, which largely increases the risk of colon cancer. The notion that gut microbiota plays critical roles in the development of intestinal inflammation has been supported by a mass of animal studies. Dietary components, as the only environmental resource of input for gut microbiota, legitimately exhibit a large influence on the composition of the gut microbiota. Therefore, alleviating gut microbiome dysbiosis by administration of bioactive components may be a possible adjuvant therapeutic method for IBD. Supportively, previous studies reported that polyphenols enriched diet could significantly improve IBD by modulating gut microbiota1,2.

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene, PTE) is an abundant stilbenoid in blueberries. It is a demethylated analog of resveratrol, which is one of the most studied phenolic compounds considering its broad health benefits. Compared with resveratrol, PTE exhibits higher bioavailability,3 suggesting higher absorption of bioavailable PTE and/or its metabolites in the gastrointestinal tract. Previous studies documented the cardioprotective,4 anti-oxidative,5 anti-aging,6 anti-diabetic,7 anti-breast cancer8 properties of PTE. In comparison with resveratrol, PTE was more potent in preventing colon tumorigenesis and Alzheimer disease.9,10 Our previous study found that dietary resveratrol could significantly improve dextran sodium sulfate (DSS)-induced chronic colitis by preventing body weight loss, attenuating tissue damage, and rebalancing inflammatory cytokines.11 One study reported that pterostilbene 4'-β-Glucoside, a derivate of PTE, protected against DSS-induced colitis.12 However, the anti-colitic effects of PTE per se, which we investigated in the current study, have not been studied yet.

A number of studies revealed that dietary supplementation of resveratrol could regulate gut microbiota diversity and composition, which is heavily involved in the prevention of atherosclerosis and obesity.13-15 Our previous study also showed that dietary resveratrol attenuated colitis via restoring gut microbiota dysbiosis in DSS-treated mice.11 Noteworthy, recent couple studies claimed that dietary PTE might be able to create a favorable intestinal microenvironment for certain bacteria and modified gut microbiota composition toward a healthier
Therefore, our current study determined the anti-inflammatory effects of PTE in DSS-treated mice and elucidated the potential mechanisms from the perspective of gut microbiota.

Results

**PTE supplementation alleviated DSS-induced colitis**

The chronic inflammation induced by DSS lowered the body weight gain in the DSS group compared to mice fed a regular control diet (Figure 1A). The final body weight of mice in the DSS group was significantly lower compared with the control group (Figure 1B). As the diet and DSS treatments went on, body weight gain of mice in the pterostilbene-containing diet (PCD) and PCD-DSS groups were consistently higher than that of mice in the DSS group (Figure 1A). The final body weight of mice in the PCD-DSS group was significantly higher than that of mice in the DSS group ($P = 0.045$) (Figure 1B).

DSS treatment resulted in colon damage, which was associated with typical phenotypes including rectal bleeding and diarrhea. As shown in Figure 1C, the highest disease activity index (DAI) score in the DSS group revealed the most severe colon damage, whereas PTE supplementation significantly alleviated colon damage, as indicated by the lower DAI score. Moreover, the DSS group revealed shorter colon length compared with the control group, which is a typical indicator of colitis (Figure 1D). However, PTE administration remarkably increased the colon length in the PDC-DSS group, compared with the DSS group (Figure 1D). Collectively, these data indicate that PTE supplementation shows the potential of preventing inflammation induced by DSS in the colon from mice.

**PTE administration ameliorated colitis and regulated inflammatory cytokines**

As shown in Figure 2A, the loss of crypts, epithelium disruption, goblet cell reduction, and the infiltration of immune cells marked the severity of colitis in the DSS group, whereas the colonic histological morphology in the control and PCD groups displayed a healthy status. Although DSS induced abominable colonic inflammation, PTE consumption partially countered colitis, as indicated by the recovery of copious crypts, elevation of goblet cells, and less immune cell infiltration relative to what was observed for mice in the DSS group (Figures 2A and 2B).

The elevated presence of immune cells and reduced goblet cells in colon tissue are pertinent to the abnormal deposition of cytokines related to inflammation. Enzyme-linked immunosorbent assay analysis revealed that DSS treatment markedly
raised the production of pro-inflammatory cytokines, including interferon gamma (IFNγ), interleukin (IL)-2 and IL-6, and suppressed the secretion of anti-inflammatory cytokine IL-4 compared with control mice (Figure 3). Anti-inflammatory cytokine IL-10 was upregulated in the DSS group to counteract inflammation, as indicative of more severe colitis in the DSS group. However, PTE administration strikingly diminished the overproduction of pro-inflammatory cytokines, such as IFNγ, IL-2, and IL-6, and boosted the production of anti-inflammatory IL-4. Moreover, IL-10 levels were lower after the supplementation of PTE in colitic mice, suggesting the amelioration of colonic inflammation with the presence of PTE. Taken together, all these data indicated the protective effect of PTE against colonic inflammation induced by DSS in mice.

**PTE supplementation improved diversity of gut microbiota**

Using 16S rRNA gene sequencing, we determined the fecal bacterial profiles in four groups. Higher number of operational taxonomic units (OTUs) were observed in the PCD group, whereas the control, DSS and PCD-DSS groups showed lower OTUs (Figure 4A). Consistently, the PCD group showed higher Shannon index and Inverse Simpson, alpha diversity indices accounted for evenness and diversity, than DSS and PCD-DSS groups (Figures 4B and 4C), suggesting the potential prebiotic effects of PTE. To assess overall group-specific clustering of 16S rRNA sequences, UniFrac distances, which can identify dimensions that better explain variance that plot on principal coordinate (PCoA), were determined. PCoA analysis on UniFrac distance revealed DSS-induced colitis-driven clustering across different groups. As shown in Figure 4D, weighted PCoA displayed a significant group clustering by 16S rRNA sequence, and 56.2% of the variation was explained by PCoA1, which mainly accounted for the differences among the control, PCD, DSS, and PCD-DSS groups (control vs DSS, $P=0.0109$; control vs PCD-DSS, $P=0.005$; DSS vs PCD, $P=0.003$; DSS vs PCD-DSS, $P=0.048$; PCD vs PCD-DSS, $P=0.0089$). PCoA2 and PCoA3 explained 17.83% and 10.02% of observed variations among the four groups, respectively.

**Dysbiosis was partially restored by PTE in colitic mice**

Results at phylum level exhibited a pronounced deposition of Firmicutes and Bacteroidetes, followed by Actinobacteria in the control group (Figure 4E). The PCD group revealed a higher presence of Actinobacteria as compared with the control group ($P=0.048$), which might explain significantly higher OTUs in the PCD group. The DSS group was marked by a tendency towards higher abundance of Verrucomicrobia ($P=0.001$) and Proteobacteria ($P=0.002$) and lower abundance of Actinobacteria ($P=0.048$) and Firmicutes ($P=0.004$) as compared with the control group. However, PTE administration significantly lowered the relative abundance of Proteobacteria ($P=0.039$) and raised the relative abundance of Actinobacteria ($P=0.035$) as compared with colitic mice. Overall, these results revealed the dysbiosis in the DSS group, and amelioration of dysbiosis by PTE supplementation in colitic mice.

We next assessed the relative abundance of bacteria at genus level across the different groups. *Bifidobacterium*, belonging to the phylum Actinobacteria, confers lots of health benefits to the host, including anti-inflammation. Intriguingly, the DSS group displayed lower relative abundance of *Bifidobacterium* than did the control group (Figure 5A). However, PTE supplementation remarkably mounted the presence of *Bifidobacterium* in colitic mice. *Bilophila* was the predominant genus in the DSS group compared with the control group (Figure 5B), which was consistent with our previous reports and the fact that human stool-derived *Bilophila* induced inflammation in mice. However, PTE supplementation significantly reduced the relative abundance of *Bilophila* in colitic mice. Furthermore, *Rc4-4* showed a higher deposition in the DSS group as compared with the control group (Figure 5C). The higher proportion of *Rc4-4*
positively correlated with inflammation. But dominance of Rc4-4 was abolished by PTE administration, suggesting protective effects of PTE against the colonic inflammation by partially reducing potential harmful bacteria in the colon. Overall, these data indicated amelioration of dysbiosis by PTE in colitic mice.

Linear discriminant analysis effect size was applied to determine the taxa that most likely discriminated the differences among groups. As shown in Figure 5D and 5E, linear discriminant analysis effect size analysis revealed that different groups displayed distinct bacterial signatures. The DSS group showed higher presence of Bilophila, Akkermansia, Sutterella, and Rc4-4, but Lactobacillaceae, Streptococcaceae, and Coriobacteriaceae displayed higher distribution in the control group (Figure 5D and 5E). The PCD group was dominated by Bifidobacterium, and PTE supplementation distinctly diminished abundance of bacteria that was higher in the DSS group, including Bilophila and Rc4-4. Collectively, these data revealed that PTE supplementation could partially improve gut microbiota dysbiosis in colitic mice.

**Gut microbiota dysbiosis strongly associated with the levels of inflammatory cytokines**

Pearson correlation analysis was performed by using SPSS 17.0 to visualize the association between inflammatory cytokines and gut microbiota. As shown in Figure 6, we observed strong positive correlation between proinflammatory cytokines and pathogenic gut microbiota. For example, Sutterella was significantly positively correlated with GM-CSF, IFN-γ, IL-1, IL-2, and KC/GRO. Furthermore, it was also negatively correlated with anti-inflammatory cytokine IL-4. Similarly, pathogenic Dorea also significantly correlated with many pro-inflammatory cytokines, like GM-CSF, IL-1, KC/GRO, and TNF-α. Similarly, it was also negatively correlated with IL-4. More interestingly, the well-known probiotic Lactobacillus was negatively, though not statistically significant, correlated with all the pro-inflammatory cytokines and positively correlated with IL-4. Since aberrantly high levels of IL-10, an anti-inflammatory cytokine, were observed in the DSS group, the correlated relationship between IL-10 and inflammatory cytokines behaved more like pro-inflammatory cytokine.

**Discussion**

Pterostilbene is a natural compound that was first identified in blueberries. Owing to its high bioactivity, PTE has drawn much attention in lowering or inhibiting various diseases. Collective evidence revealed the potentials of PTE in lowering the risk of multiple human diseases, including cardiovascular disease, metabolic disorder, inflammation and diabetes, due to its anti-
oxidant capacity.22 Moreover, PTE was shown to be a promising anti-cancer agent in multiple human cancers according to preclinical trials.23 The striking beneficial effects of PTE may be ascribed to its higher bioavailability than resveratrol, which shares similar chemical structures. The presence of two methoxy groups in PTE elevates the lipophilicity,3 which results in higher permeability, slower metabolism and higher absorption, and finally higher bioavailability. Yet, the relationship between oral consumption of PTE and colon health has not been reported. Therefore, we investigated the function of PTE in the prevention of colonic inflammation induced by DSS in mice.

In line with the anti-inflammatory action of PTE in colon cancer cells,24 oral consumption of PTE strikingly inhibited colonic inflammation induced by DSS in mice, as indicated by the lower DAI scores, less infiltration of immune cells, reduced production of pro-inflammatory cytokines, and presence of more goblet cells and crypts in colon tissues as compared to colitic mice (Figure 2). Our previous research revealed the anti-inflammatory effects of resveratrol in colitic mice.11 PTE, as an analog of resveratrol, also inhibited colitis in mice, but by regulating different inflammatory cytokines, which suggested the involvement of different signal pathways. For example, different from resveratrol, dietary PTE re-boosted the production of IL-4 in DSS-treated mice, which was a critical T helper type 2-skewing cytokine that depended on the activation of signal transducer and activator of transcription 6.25 The biological mechanisms, besides gut microbiota involvement, underlying anti-colitic effects of PTE will be further investigated in the future.

Both PTE and resveratrol reduced the weaning-induced intestine damage and redox imbalance.26 PTE displayed higher efficiency than resveratrol, which may be attributed to higher bioavailability of PTE than of resveratrol.26 It is worthy to note that PTE could be demethylated to a bioactive metabolite pinostilbene by gut microbiota in the colon.27 It is reasonable to hypothesize that the anti-inflammatory effects of PTE may be attributed to PTE and its metabolites. Comparison of PTE, resveratrol, and their derivatives against colitis in mice will be furtherly investigated.

Gut microbiota is one of the most critical elements in maintaining colon health. Disruption of the balance of gut microbiota leads to colonic inflammation.28 Elevated abundance of specific bacteria, such as Escherichia coli, was associated with colonic inflammation.29 In contrast, higher presence of beneficial bacteria, such as Bifidobacteria, displayed protective effect against colitis.30 Emerging evidence revealed the potentials of bioactive compounds in inhibiting colitis through modulating composition of the gut microbiota. As shown in Figure 4D and 4E, DSS treatment induced dysbiosis, and PCoA showed significant clustering between the DSS and control groups. However, PTE supplementation remarkably changed the beta-

Figure 4. Pterostilbene (PTE) administration partially reversed alpha-diversity of gut microbiota in colitic mice including (A) Number of operational taxonomic units (OTUs), (B) Shannon index, and (C) inverse Simpson index. (D) Gut microbiota clustering based on treatments presented in principal coordinates analysis plot (beta-diversity). Relative abundance of bacteria was assessed at phylum level (E). Significant differences are indicated as: *, P < 0.05; **, P < 0.01, n = 5.
diversity in colitic mice (PCD vs DSS-PCD, \( P = 0.048 \)). At phylum level, DSS treatment induced higher abundance of Proteobacteria and lower presence of Actinobacteria and Firmicutes compared with the control group (Figure 4E). However, PTE administration significantly elevated the presence of Actinobacteria and decreased the deposition of Proteobacteria.

The DSS group displayed higher distribution of Bilophila in colon tissues (Figure 5B), but PTE supplementation markedly lowered relative abundance of Bilophila. Our research corroborates the reports that Bilophila wadsworthia induced systemic inflammation, as well as the aggravation of metabolic dysfunction in mice.\textsuperscript{15,31} Interestingly, resveratrol also lowered the relative abundance of Bilophila in DSS-induced mice and high-fat-fed mice.\textsuperscript{11,32} These results suggested the regulatory effects of stilbenoids on Bilophila, which may act as protective agents of the Bilophila-driven diseases. In line with the elevated abundance of Rc4-4 in colitic mice induced by 2,4,6-trinitrobenzenesulfonic acid,\textsuperscript{33} DSS treatment showed higher abundance of Rc4-4. However, PTE significantly reduced the presence of Rc4-4 in colitic mice. Moreover, PTE consumption modulated gut
microbiota through upregulation of beneficial bacteria, such as *Bifidobacterium* (Figure 5A). *Bifidobacterium* supplementation in humans revealed reduction of colitis,28 and increased relative abundance of *Bifidobacterium* by PTE administration may be partially responsible for the amelioration of colitis in mice. Overall, PTE potentially reduced colonic inflammation through modulating the composition of gut microbiota, which may be a novel agent to reduce colitis.

In conclusion, this study correlated gut microbiota diversity and composition to long-term exposure of dietary PTE in a DSS-induced colitis mouse model. Our results revealed the anti-inflammatory effects of dietary PTE (at a human equivalent dose of 2.3 mg/kg/day)35 in a DSS-induced colitis mouse model and provided a potential mechanistic perspective that PTE strikingly modulates the composition of gut microbiota, which may be a novel agent to reduce colitis.

**Materials and methods**

**Animals and experimental design**

The animal experimental design and protocol were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. Forty male CD-1 mice (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were housed in a temperature-controlled environment (22 ± 2°C) with 65% relative humidity and fixed 12 h light/dark cycle with free access to water and basal diet (AIN-93G). After one week acclimation, mice were randomly assigned to four groups as follows: the control group was fed with standard AIN-93G diet (Dyets Inc., Bethlehem, PA, USA) and regular water; the PCD group was fed with 0.025% PTE (w/w, >99% purity) (Quality Phytochemicals, East Brunswick, NJ, USA) and regular water; the DSS group was treated with standard diet and 1.5% (w/v) DSS water (International Lab, Chicago, IL, USA); and the DSS-PCD group received 0.025% PTE (w/w) supplemented diet and 1.5% DSS drinking water (w/v). The mice received DSS water ad libitum for 4 days to induce colonic inflammation, followed by 7 days of regular water for recovery. After the fourth DSS treatment, all mice were sacrificed with CO2 asphyxiation.

**DAI and histological evaluation**

DAI was scored by the body weight loss: 0 (0% loss), 1 (1%–5% loss), 2 (5%–10% loss), 3 (10%–20% loss); stool consistency: 0 (normal), 1 (soft but formed), 2 (loose and formless), 3 (diarrhea); and rectal bleeding: 0 (none), 1 (hemoccult positive), 2 (blood), 3 (gross bleeding).11

Colon tissues were fixed by 10% formalin and embedded in paraffin to perform hematoxylin and eosin staining as we described previously.2 Histological assessment was scored as follows. Epithelium: 0, normal; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration: 0, none; 1, infiltrate around crypt basis; 2, infiltrate reaching to mucosa; 3, extensive infiltration to mucosa; 4, infiltration of submucosa.36

**Assessment of inflammatory cytokines in colonic mucosa**

Colonic mucosa were homogenized in MSD Tris Lysis buffer (Rockville, Maryland, USA) containing protease inhibitor cocktail and phosphatase inhibitor cocktail as we described previously.2,11 IFNγ, IL-2, IL-6, IL-10, and IL-4 were measured by U-PLEX Enzyme-linked immunosorbent assay kit (Meso Scale Discovery, Rockville, Maryland, USA) according to the manufacturer’s protocol.

**Fecal microbiota analysis by 16S rRNA sequencing**

Fecal bacterial DNA was extracted by Power Fecal DNA isolation kit (MoBio Laboratories Inc, Carlsbad, CA, USA) following the manufacturer’s protocol. The primer sequences used to amplify V3-V4 regions of 16S rRNA gene were: Forward Primer 5’-TGCTCGGCAGGGCAGCTGTATATAGTGATATAGCTACGGGNGGCWGGCGAGCGGTGCTCACAGCTGTATATAAGAGACAGCTACGGGNGGCWGGCAG and Reverse Primer 5’-GTCTCGTGGGCTCAGATGTGTATATAGCGAGTCCTCGAGATGTGTATATAAGAGACAGCTACGGGNGGCWGGCAG. The second PCR was conducted to attach dual indices and Illumina sequencing adapters using Nextera XT Index Kit (Illumina, San Diego, CA, USA). PCR products were quantified using Qubit dsDNA BR Assay kit (Life Technology, Carlsbad, CA, USA), and the size of PCR products was verified by DNA analysis ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, USA). PCR final products were pooled in equimolar concentration and diluted to 4 nM and denatured by NaOH. After combining the ampiclon library and PhiX control, the samples were loaded onto the 600-cycle MiSeq Reagent kit v3 cartridge and sequenced on an Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA).

**Statistical analysis**

Statistical significance was evaluated by one-way ANOVA followed by Tukey post hoc test. Nonparametric ANOVA
(Kruskal-Wallis test) followed by Dunn post hoc test was applied for the data that do not pass normal distribution test.

References


[18] Li et al., Infectious Microbes & Diseases (2021) 3:1