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Gut bacteria are required for the benefits of black raspberries in *Apc^{Min/+}* mice

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Abstract

BACKGROUND: The gut microbiota plays a pivotal role in the development of inflammatory bowel disease and colorectal cancer.

OBJECTIVE: To determine whether the gut microbiota is required for the chemoprotective effects of black raspberries (BRBs) in *Apc^{Min/+}* mice.

METHODS: *Apc^{Min/+}* mice were given (a) a control diet for 8 weeks, or (b) the control diet for 4 weeks and then a 5% BRB diet for additional 4 weeks, or (c) the control diet and antibiotics for 4 weeks followed by the 5% BRB diet and antibiotics for the next 4 weeks. At the end of the study, all the mice were euthanized, and colonic and intestinal polyps were counted. mRNA expression levels of *TLR4*, *NF-κB1*, and *COX2* were determined in colon and small intestine of these *Apc^{Min/+}* mice by quantitative real-time PCR.

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Authors' contribution

Study design and acquisition, analysis, and interpretation of data: Pan Pan, Kiyoko Oshima, Yi-Wen Huang, Martha Yearsley, Jianying Zhang, Mark Arnold, Jianhua Yu, and Li-Shu Wang.

Manuscript writing and revising: Pan Pan and Li-Shu Wang.

Final approval: Pan Pan and Li-Shu Wang.

Competing interests

No potential competing interest was disclosed.

RESULTS: 5% BRBs significantly suppressed intestinal and colonic polyp development in the *Apc^{Min/+}* mice, whereas antibiotics significantly abolished BRBs' chemoprotective effects. BRBs decreased mRNA levels of *TLR4*, *NF-κB1*, and *COX2* in colon, whereas significantly enhanced mRNA levels of *TLR4* and *NF-κB1* were observed in small intestine of BRB-treated *Apc^{Min/+}* mice fed antibiotics.

CONCLUSIONS: The gut microbiota is required for BRBs' chemoprotection against polyp development in *Apc^{Min/+}* mice.

Keywords

Black raspberries; *Apc^{Min/+}*; antibiotics; TLR4; NF-κB; COX2

1. Introduction

The human gastrointestinal tract harbors trillions of microbes [1,2], and accumulating evidence suggests a pivotal role for the gut microbiota in the development of inflammatory bowel disease (IBD) [3] and colorectal cancer (CRC) [4–8]. Thus, CRC patients host a distinct microbiota compared with healthy subjects [9]. Also, the gut microbiota gradually changes with disease progression across stages of CRC [10]. In addition, fecal bacteria from CRC patients can promote intestinal carcinogenesis in mice [11]. In light of these findings, alterations in microbial composition have the potential to become noninvasive diagnostic and prognostic tools for CRC in humans [12–14].

Pathobiotic bacteria can infiltrate through the gut epithelial surface barrier, invade adjacent normal tissues, generate toxic products, and further promote colonic inflammation and tumorigenesis [15]. Animal studies have reported that mice treated with antibiotics were less susceptible to colitis-associated colon cancer (CAC) induced by the azoxymethane (AOM)/dextran sodium sulfate (DSS) [16,17]. In these studies, antibiotics suppressed colon cancer development by inhibiting bacteria-induced colonic inflammation. However, it is important to note that majority of sporadic CRC patients show no history of IBD [6, 8], and large epidemiologic studies suggest a positive association between frequent use of antibiotics and a higher risk of developing CRC [18–21]. For instance, the Nurses' Health Study in the United States showed that women who had taken antibiotics for more than 2 months between ages 20 and 39 years or between 40 and 59 years had respective multivariable odds ratios (OR) of 1.36 (95% confidence interval (CI): 1.03–1.79) and 1.69 (95% CI: 1.24–2.31) of developing CRC after age 60 [18]. Therefore, maintaining a healthy and balanced gut microbiota is essential for preventing CRC.

Fecal microbial transplantation (FMT) is a dramatic method of replacing bacteria in dysbiosis patients with more beneficial bacteria. Many clinical trials have shown clinical improvements in some recipients with active ulcerative colitis (UC) [22–27] and Crohn's disease [28]. However, FMT may not be suitable for preventing or treating CRC, which usually develops gradually over 10 or more years. On the other hand, consuming prebiotics in the diet could be an elegant way to promote a healthy gut microbiota and modulate dysbiosis [29]. Prebiotics—typically dietary fibers and their fermented metabolites, short-chain fatty acids (SCFAs)—are drawing enormous attention due to their overall health

benefits [30–32]. Meta-analyses have shown that high intake of dietary fiber, especially fiber from whole grains and cereals, negatively associates with the risk of developing CRC [33].

Our laboratory focuses on dietary constituents, such as black raspberries (BRBs), that have chemoprotective effects against colon cancer. BRBs are enriched in dietary fiber and many chemopreventive compounds such as anthocyanins and ellagitannins. We previously showed that BRBs were beneficial to patients with CRC [34, 35] or familial adenomatous polyposis (FAP) [36]. 5% BRBs in the diet, containing ~3.8 μmol anthocyanins/g and 2.25% fiber, suppressed colorectal carcinogenesis and modulated immune cells in mouse models of CRC [37–39]. BRBs changed the gut microbiota of rats in ways that might contribute to their chemopreventive effects [40]. Importantly, we demonstrated that functional free fatty acid receptor 2 (FFAR2)—the receptor for SCFAs—is required for BRB-mediated effects [39], suggesting that bacterial fermentation of BRBs and their components is a critical mechanism. In addition, toll-like receptors (TLRs), as well as its downstream nuclear factor kappa B (NF- κ B) and cyclooxygenase 2 (COX2), has been shown to associate with human CRC [41–44]. We previously demonstrated that 5% BRBs suppressed the protein levels of NF- κ B and COX2 in DSS-induced UC in mice [45], an inflammatory colonic injury that can dramatically increase the risk of CRC. Therefore, the current study aimed to directly determine whether BRBs need the gut microbiota to exert their protective effects in *Apc^{Min/+}* mice, as well as to examine BRBs' effects on The TLR4/NF- κ B/COX2 pathway.

2. Materials and methods

2.1. Animals and BRBs

All animal study protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee under the animal protocol AUA00002430 “Identification of specific berry types and berry components that exhibit anti-inflammatory and anti-cancer activities in different animal cancer models.” Breeding pairs of *Apc^{Min/+}* mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

A synthetic diet from the American Institute of Nutrition (AIN-76A; Dyets Inc., Bethlehem, PA) was used as the control diet. BRB powder was purchased from Berri Products LLC (Corvallis, OR) and stored at 4°C. The sugar and starch content of the BRB diet was adjusted to create an isocaloric diet [37–40, 46].

2.2. Animal experiments

Four- to five-week-old *Apc^{Min/+}* mice were randomly assigned to three study groups. The mice in groups 1 and 2 (G1 and G2) were fed regular drinking water and the control AIN-76A diet for 4 weeks. Then the mice in G1 continued on the control diet, while the mice in G2 changed to 5% BRB diet. The mice in group 3 (G3) were first given the control diet and antibiotics in the drinking water (1 g/L ampicillin, 1 g/L neomycin, 1g/L metronidazole, and 0.5 g/L vancomycin) for 4 weeks. For the next 4 weeks, they were fed 5% BRBs along with the antibiotic treatment (Fig. 1A). At the end of the study, all the mice were euthanized by CO₂ asphyxiation, and the number of colonic and intestinal polyps was determined. Whole tissues of the colon and small intestine of all the mice were collected,

fixed in formalin, and embedded in paraffin (FFPE). Hematoxylin and eosin (H&E)-stained tissue sections were evaluated histopathologically by our pathologists.

2.3. Quantitative real-time PCR

RNA was isolated from FFPE samples of colon and small intestine according to the manufacturer's instructions (Recover All Total Nucleic Acid Isolation Kit for formalin-fixed and paraffin-embedded tissues, Ambion, Grand Island, NY). Quantitative PCR was performed to measure the relative expression levels of *TLR4* (Mm.PT.58.41780308.g), *NF- κ B1* (Mm.PT.58.30400172), and *COX2* (Mm.PT.58.17730756). Respective primers were purchased from Integrated Device Technology (San Jose, CA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal reference gene. *Ct*-method was performed to analyze the relative expression levels of the respective genes.

2.4. Measurements of fecal microbial populations by quantitative real-time PCR

Fecal specimens were collected every week for 4 weeks from the antibiotic-treated *Apc^{Min/+}* mice. Fecal DNA was isolated according to the manufacturer's instructions (PowerSoil[®] DNA Isolation Kit, MO Bio laboratories, Carlsbad, CA). Universal primers were designed to measure the overall populations of bacteria (Forward: ACTCCTACGGGAGGCAGCAGT; Reverse: ATTACCGCGGCTGCTGGC) as previously described [47–51].

2.5. Statistical analysis

One-way ANOVA and *post-hoc* analysis were performed using SigmaPlot (Systat Software, San Jose, CA) to analyze polyp number and relative gene expression. A *p* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Gut bacteria are required for BRBs' anti-tumor effects in *Apc^{Min/+}* mice

The Adenomatous polyposis coli (*Apc*) gene, a tumor suppressor, functions to induce β -catenin degradation and suppress the Wnt signaling pathway, and mutations in the *Apc* gene contribute strongly to CRC [52, 53]. Moreover, multiple intestinal neoplasia mice (*Apc^{Min/+}*), which carry a truncating mutation at codon 850 of the *Apc* gene, develop multiple colonic and intestinal polyps, making them a widely used model of human CRC [53]. We fed *Apc^{Min/+}* mice with either a control diet (G1) or 5% BRBs from week 4 to week 8 (G2). Another group of *Apc^{Min/+}* mice were first given the control diet and antibiotics in the drinking water for 4 weeks. Starting at week 4, the mice received the 5% BRB diet along with antibiotics for 4 weeks (G3) (Fig. 1A). After 1 week of the antibiotic treatment, there was a substantial decrease in the overall fecal bacterial population (Fig. 1B). At the end of study, we examined the number of colonic and intestinal polyps. The results confirmed those of our previous studies [38,39]: that 5% BRBs in the diet significantly suppress colonic (Fig. 2A) and intestinal (Fig. 2B) tumor development in *Apc^{Min/+}* mice. However, antibiotics completely abolished the anti-tumor effects of the BRB diet in small intestine (Fig. 2), suggesting that gut microbiota is required for BRBs' beneficial effects in *Apc^{Min/+}* mice.

Although large epidemiologic studies have suggested a positive association between frequent use of antibiotics and risk of CRC in humans [18–21], this conclusion is controversial in animal models as it is dependent on the model. In one study, antibiotics promoted intestinal polyp development in *Apc^{Min/+}* mice [54], while in another study that used the CAC model, antibiotics suppressed tumor growth induced by AOM/DSS [55]. The underlying mechanism of the latter study suggests that antibiotics might inhibit bacteria-induced inflammation, thereby suppressing tumor development. Other studies have reported similar results: severe colonic inflammation exacerbated polyposis and antibiotics rescued that symptom [56, 57]. Interestingly, one study observed that antibiotics depleted the gut microbiota but induced mild inflammation in the gastrointestinal track of wild-type mice [58], suggesting that colonic inflammation and dysbiosis cannot be cured simply by depleting gut bacteria.

The dynamic interactions between a host and its gut microbiota and between diet and the microbiota play critical roles in the prevention and treatment of CRC [59–61]. A healthy dietary pattern has been shown to associate with a lower risk of CRC [33]. Microbiome studies have identified several “good” bacterial species, such as *Akkermansia muciniphila*, and some “bad” bacterial species, including *Escherichia coli* and *Fusobacterium spp* [7]. Therefore, maintaining a balanced and healthy gut microbiota by adopting a healthy lifestyle would help lower the risk of developing CRC [6, 32, 62].

3.2. BRBs suppress the TLR4/NF- κ B/COX2 pathway in *Apc^{Min/+}* mice

TLRs are important regulators of intestinal epithelial homeostasis. They belong to the interleukin (IL)-1 superfamily of transmembrane receptors that recognize pathogen-associated molecular patterns (PAMPs). In particular, lipopolysaccharide (LPS) fragments from degraded outer membrane of gram-negative bacteria interact with TLR4, leading to the recruitment of downstream adaptor molecules, such as myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6) [63]. The complex then phosphorylates the inhibitor of NF- κ B kinases (IKKs), activating NF- κ B. NF- κ B signaling plays an essential role in inflammation and cell survival. One of its downstream targets is COX2 [63]. Therefore, we examined the expression of TLR4/NF- κ B/COX2 pathway in BRB-treated *Apc^{Min/+}* mice.

We found that 5% BRBs in the diet significantly decreased mRNA levels of *TLR4* (Fig. 3A), *NF- κ B* (Fig. 4A), and *COX2* (Fig. 5A) in colon of the *Apc^{Min/+}* mice. However, the TLR4/NF- κ B/COX2 pathway was not significantly changed by BRBs (Figs. 3B, 4B, and 5B) in small intestine, suggesting that other mechanisms contributed to the BRBs’ anti-tumor effects in small intestine. Intriguingly, significantly enhanced mRNA levels of *TLR4* (Fig. 3B) and *NF- κ B* (Fig. 4B) were observed in small intestine of the BRB-treated *Apc^{Min/+}* mice fed antibiotics. Our results agree with those of Grasa L *et al.*, who reported that antibiotics induced mild inflammation and TLR4 expression in the gastrointestinal tract of wild-type mice [58]. It is likely that the antibiotic treatment eliminated majority of the gut bacteria, whereas some other (or unknown) bacteria were selected and able to grow and induce a mild inflammatory response. Further investigations need to examine the mechanisms underlying the increased expression of TLR4 and NF- κ B promoted by

antibiotics in the small intestine, as well as whether this enhancement contributes to antibiotics' ability to counter the beneficial effects of BRBs.

The TLR4/NF- κ B/COX2 pathway has been examined in CRC patients and animal models, but the results have been surprisingly inconsistent. For example, studies showed that human intestinal epithelial cells normally express low levels of TLR4 [63], while specimens from UC [41] or CRC patients [42,43], as well as from the CRC mouse model [41], showed increased expression of TLR4. In addition, high expression of TLR4 and MyD88 has been shown to associate with liver metastasis and poor prognosis in patients with CRC [44]. In contrast, another study demonstrated that normal colon epithelium and CRC cell lines were positive for TLR4, and specimens from metastatic CRC patients showed either loss of expression or strong downregulation of TLR4 in comparison with normal tissue and non-metastatic tumors [64]. Interestingly, one group found that TLR4 expression by tumor cells associated with a lower rate of tumor recurrence, whereas TLR4 expression by fibroblasts associated with a high rate of tumor recurrence [65]. Therefore, the TLR4/NF- κ B/COX2 pathway might play different roles in different compartments of the tumor microenvironment.

4. Conclusions

In the current study, we investigated the relationship between the gut microbiota and BRB-mediated chemo-protective effects against CRC. We demonstrated that antibiotic treatment abolished BRBs' anti-tumor effects, indicating that the gut microbiota is required for BRBs' beneficial effects in *Apc*^{Min/+} mice. In addition, BRBs suppressed the TLR4/NF- κ B/COX2 pathway in colon of *Apc*^{Min/+} mice, whereas antibiotic treatment increased the expression of TLR4 and NF- κ B in small intestine of the BRB-treated *Apc*^{Min/+} mice.

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Abbreviations

Apc	Adenomatous polyposis coli
AOM	azoxymethane
BRBs	black raspberries
CAC	colitis-associated colon cancer
CI	confidence interval
COX2	cyclooxygenase 2
CRC	colorectal cancer

DSS	dextran sodium sulfate
FAP	familial adenomatous polyposis
FFAR2	free fatty acid receptor 2
FMT	fecal microbial transplant
IBD	inflammatory bowel disease
IKKs	inhibitor of nuclear factor kappa B kinases
IL-1	interleukin-1
IRAK	IL-1 receptor-associated kinase
LPS	lipopolysaccharide
MyD88	myeloid differentiation factor 88
NF-κb	nuclear factor kappa B
OR	odds ratio
PAMPs	pathogen-associated molecular patterns
SCFAs	short-chain fatty acids
TLRs	Toll-like receptors
TRAF6	tumor necrosis factor receptor-associated factor 6
UC	ulcerative colitis

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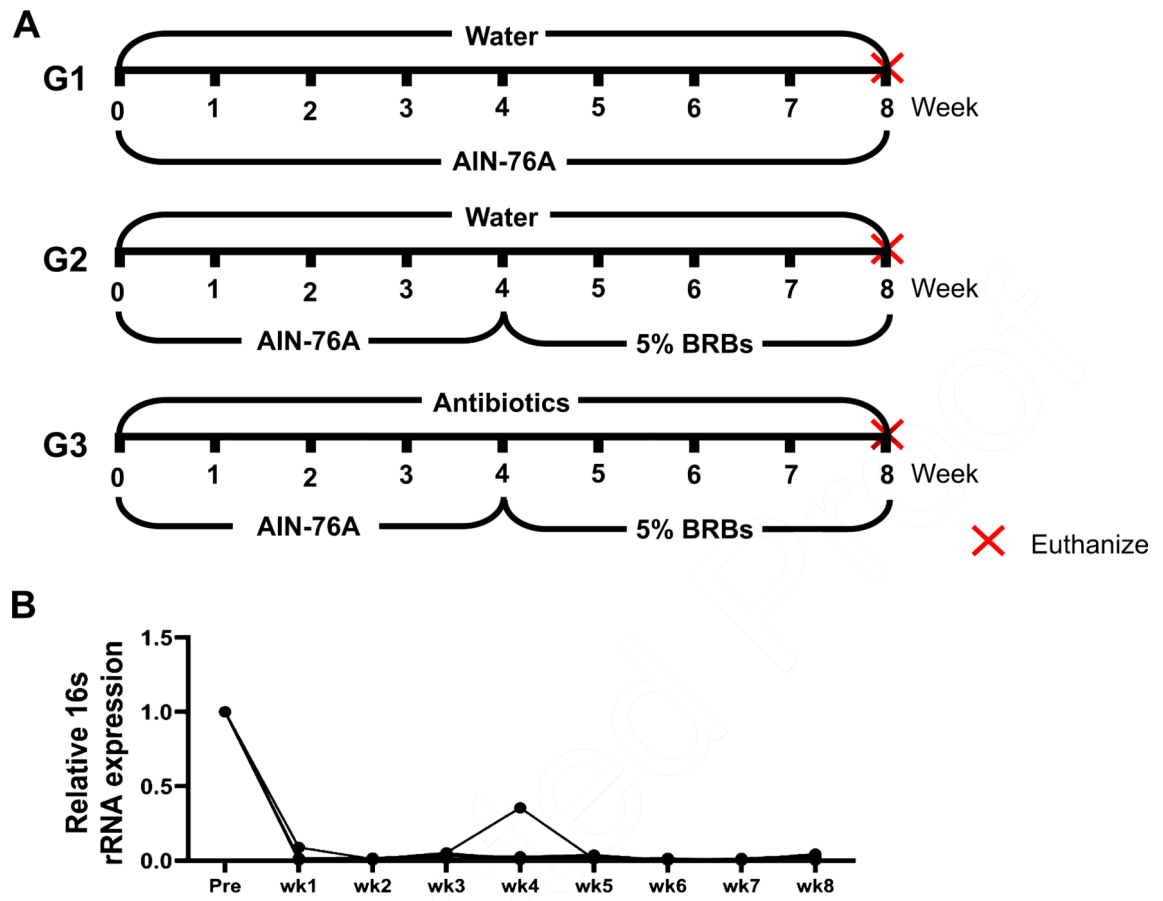


Fig. 1.

(A) Animal experimental protocol of the current study. *Apc^{Min/+}* mice were randomly assigned to three study groups. The mice in G1 were fed regular drinking water and the control diet for 8 weeks. The mice in G2 were fed regular drinking water and the control diet for 4 weeks, and then change to 5% BRBs for additional 4 weeks. The mice in G3 were first given the control diet and antibiotics (1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole, and 0.5 g/L vancomycin) in the drinking water for 4 weeks. For the next 4 weeks, they were fed 5% BRBs along with the antibiotic treatment. (B) Antibiotics in the drinking water substantially decreased gut bacterial populations in *Apc^{Min/+}* mice. wk: week.

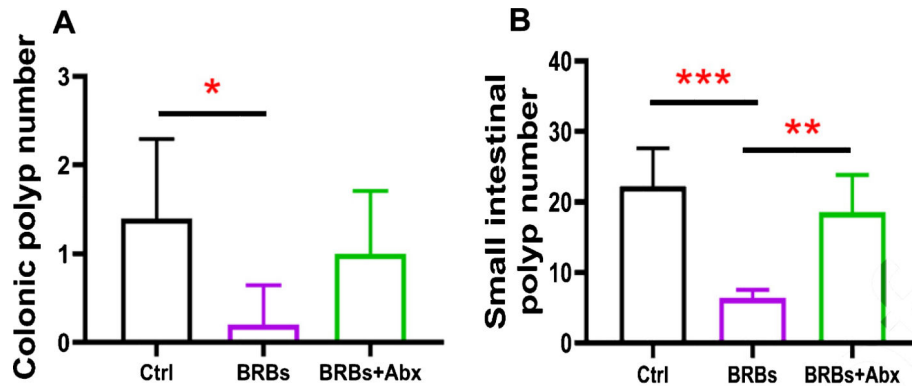


Fig. 2.

Gut bacteria are required for the benefits of BRBs in *Apc^{Min/+}* mice. BRBs significantly decreased the number of polyps in colon (A) and small intestine (B) of *Apc^{Min/+}* mice, but antibiotics abolished those BRB-mediated chemoprotective effects. Ctrl: control diet; Abx: antibiotics. $n = 5$ per group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

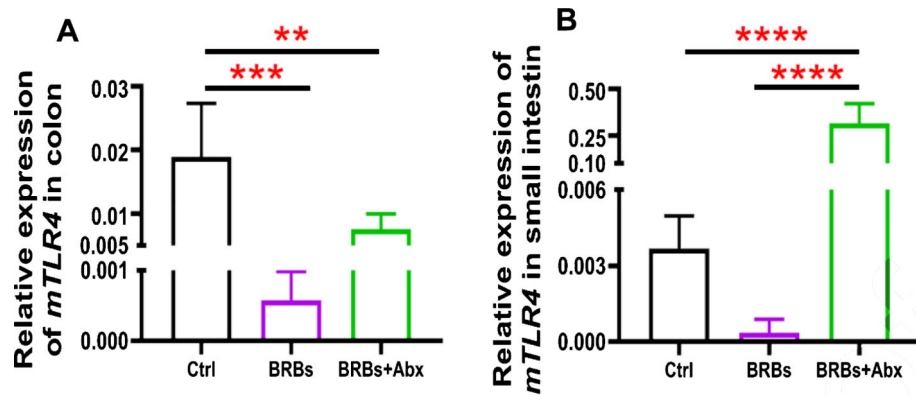


Fig. 3. mRNA expression levels of *TLR4* in colon (A) and small intestine (B) of *Apc^{Min/+}* mice. Ctrl: control diet; Abx: antibiotics. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

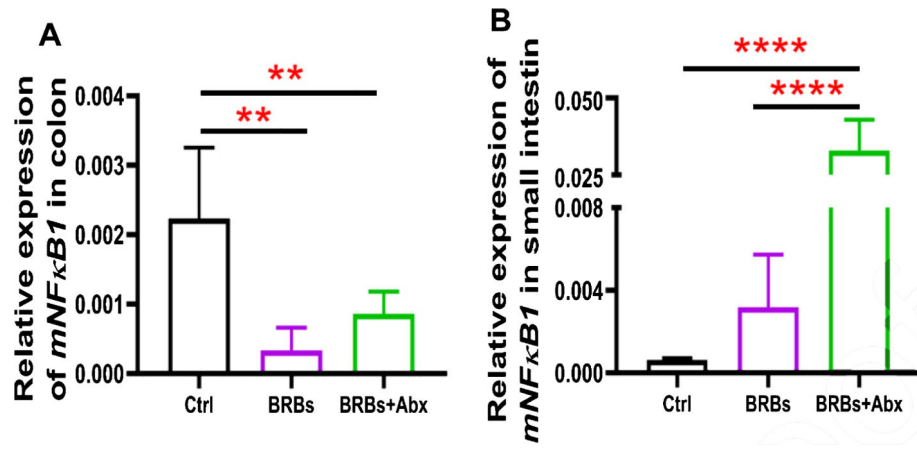


Fig. 4. mRNA expression levels of *NF-κB1* in colon (A) and small intestine (B) of *Apc^{Min/+}* mice. Ctrl: control diet; Abx: antibiotics. ** $p < 0.01$; **** $p < 0.0001$.

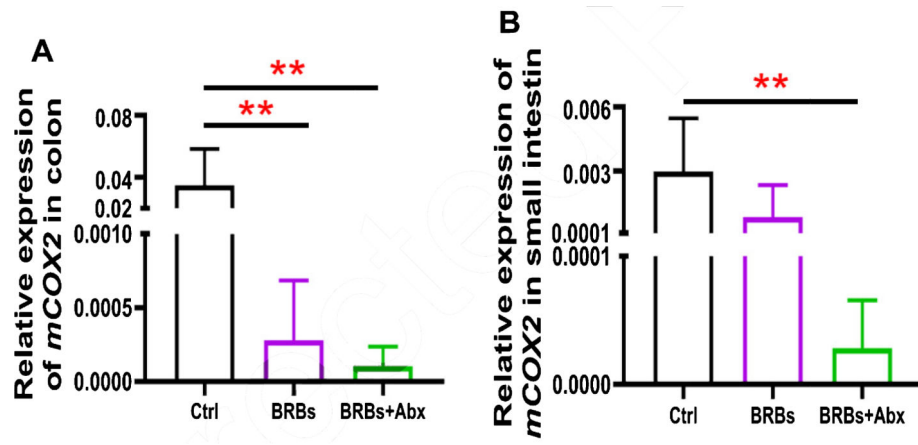


Fig. 5. mRNA expression levels of *COX2* in colon (A) and small intestine (B) of *Apc^{Min/+}* mice. Ctrl: control diet; Abx: antibiotics. ** $p < 0.01$.