

Cite this: *Food Funct.*, 2026, 17, 231

# Hemp seed mitigates colonic inflammation through macrophage polarization and microbiota-barrier axis restoration

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Hemp seed, historically considered a byproduct of the hemp fiber industry, has gained increasing attention for its nutritional and functional properties. Recent advances in hemp seed research have elucidated its benefits for gut health; however, its impact on colitis remains unclear. In this study, we demonstrate that hemp seed consumption reduced colonic inflammation and mitigated tissue injury in an experimental colitis mouse model. Notably, hemp seed reduced macrophage infiltration and promoted a phenotypic shift from pro-inflammatory M1 to anti-inflammatory M2 macrophages. It also enhanced intestinal barrier function by restoring goblet cells, upregulating tight junction proteins, and reducing systemic lipopolysaccharide translocation. Furthermore, hemp seed optimized gut microbiota composition by enriching beneficial taxa, particularly *Bifidobacterium*, while suppressing colitis-associated genera. Collectively, these findings indicate that hemp seed, as a whole-food dietary approach, confers protection against colitis by modulating immune responses, preserving barrier integrity, and reshaping gut microbiome. These results underscore the potential of hemp seed as a sustainable nutritional strategy for promoting gut health.

Received 24th September 2025,  
Accepted 21st November 2025

DOI: 10.1039/d5fo04119h

rsc.li/food-function

## 1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, is a chronic gastrointestinal disorder affecting millions of individuals globally.<sup>1</sup> Although the exact etiology of IBD remains unclear, it is widely accepted that aberrant immune responses, disrupted gut microbiota, and genetic susceptibility are key contributing factors.<sup>2</sup> Current treatment options, including aminosaliclates and corticosteroids, primarily target host inflammation. However, these therapies fail to address other critical factors in the pathogenesis of IBD, particularly their potential to exacerbate gut dysbiosis in clinical practice.<sup>3,4</sup> This limitation often results in significant side effects and variable treatment efficacy, underscoring the need for alternative or adjunctive approaches.<sup>4,5</sup> Dietary interventions, which are capable of reg-

ulating colonic inflammatory signaling, enhancing barrier function, and mitigating gut bacterial dysbiosis, are potentially promising complementary strategies for IBD prevention and treatment.

Food-grade hemp seed, historically regarded as a byproduct of the hemp fiber industry, has recently gained prominence for its health-promoting properties. Derived from industrial hemp (*Cannabis sativa* L.), hemp seed contains negligible levels of intoxicating cannabinoids and is distinguished by its rich nutritional profile.<sup>6,7</sup> Compared with other common seeds such as flax or chia, hemp seed provides greater energy density, characterized by a higher lipid content and substantial protein content, suggesting its potential as a nutrient-dense functional food.<sup>8</sup> Moreover, hemp seed is distinguished by its exceptionally high polyunsaturated fatty acid content, exceeding that of flax, chia, and most other plant seeds. Indeed, hemp seed oil contains over 75% polyunsaturated fatty acids, predominantly linoleic acid and  $\alpha$ -linolenic acid.<sup>9</sup> Additionally, hemp seed serves as an excellent source of high-quality plant-based protein, primarily composed of 11S globulin, 2S albumin, and 7S vicilin-like protein.<sup>10</sup> Hemp seed also contains potent antioxidant nutraceuticals, including tocopherols, carotenoids, and phytosterols.<sup>11–13</sup> Notably, the total polyphenol content of hemp seed ranges from 732.36 to 1457.60 mg

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gallic acid equivalents (GAE) per kg,<sup>14</sup> contributing to its antioxidant and anti-inflammatory potential.

In addition to its nutritional features and general health benefits, emerging evidence suggests that hemp seed may modulate inflammatory responses and positively influence gut microbiota composition in various animal models. For instance, hemp seed consumption improved intestinal morphology in poultry and reduced obesity-induced intestinal permeability in mice.<sup>15,16</sup> Hemp seed also mitigated antibiotic-induced dysbiosis in the caecum and jejunum,<sup>17</sup> and increased the microbiome diversity in obese mice and Angus-crossbred heifers.<sup>16,18</sup> Moreover, treatment with hemp seed-derived oil has been demonstrated to reduce inflammatory response and alleviate gut microbiota dysbiosis in an experimental colitis model.<sup>19</sup> Collectively, these findings suggest that hemp seed may be useful for improving gut health. However, the specific effects of hemp seed, as a whole-food dietary approach, on the progression and symptoms of IBD remain largely uncharacterized. Moreover, the cellular mechanisms through which hemp seed interacts with immune cells during colitis are not yet fully understood. An in-depth understanding of how hemp seed may influence immune cell regulation, gut microbial ecology, and epithelial barrier integrity is critical for developing novel dietary strategies for managing this debilitating condition.

To this end, we investigated the functional role of hemp seed in the dextran sulfate sodium (DSS)-induced colitis model in C57BL/6 mice. We demonstrate that hemp seed consumption attenuated colonic inflammation and mitigated tissue injury in DSS-treated mice. Furthermore, hemp seed supplement attenuated macrophage accumulation and polarization, enhanced intestinal barrier function, and caused beneficial changes of gut microbiota composition. These findings provide new insights into the potential of hemp seed as a sustainable and effective dietary intervention for IBD prevention and management.

## 2. Methods and materials

### 2.1. Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Purdue University (Protocol #1123002447) and were conducted in accordance with the Animal Research-Reporting of *In Vivo* Experiments (ARRIVE) and related ethical guidelines. C57BL/6 male mice (6-week-old,  $n = 6$  mice per group) were purchased from Charles River and were randomly assigned and maintained on standard AIN-93G diet or hemp seed-containing AIN-93G diet for 4 weeks. The organic hulled hemp seed is purchased from Whole Foods Market (Austin, TX, USA), ground to powder under liquid nitrogen to preserve bioactivity, and incorporated into the AIN-93G diet. The detailed composition of hemp seed used in this study is provided in Table S1. The dietary supplementation level of hemp seed powder was 3% (w/w), corresponding to a human-equivalent dose of approximately 390 mg

$\text{kg}^{-1} \text{ day}^{-1}$ , calculated from an estimated daily intake of 4 g hemp seed for a 25 g mouse and scaled to a 60 kg adult using standard mouse-to-human conversion methods.<sup>20</sup> All diets will be freshly prepared and replaced every 2–3 days to prevent lipid oxidation and preserve the stability of bioactive components. Following pretreatment with either a standard AIN-93G diet or a hemp seed-containing AIN-93G diet, mice from both groups received 2% dextran sulfate sodium (DSS; molecular weight 36–50 kDa, MP Biomedicals, Santa Ana, CA, USA) in their drinking water for 7 days to induce colitis. During the DSS treatment period, mice remained on either a standard AIN-93G diet or a hemp seed-supplemented AIN-93G diet. At the end of the experimental period, mice were sacrificed, and colonic tissues, along with blood and spleen, were harvested for analysis.

### 2.2. Histological, immunohistochemical (IHC) and immunofluorescence analysis

Tissue fixation, paraffin embedding, sectioning, and dewaxing were performed, followed by staining with hematoxylin and eosin (H&E; Sigma-Aldrich, St Louis, MO, USA). Stained sections were visualized under a light microscope. The pathological scores were evaluated according to the parameters including crypt architecture disruption, degree of inflammatory cell infiltration, muscle thickening, and goblet cell depletion. For IHC, antigen retrieval was conducted by heating the tissue sections in 0.01 M citrate buffer (pH 6.0) at 95 °C for 10 minutes. IHC staining was performed using a horseradish peroxidase (HRP)/diaminobenzidine (DAB) Detection IHC Kit (Abcam, Cambridge, UK) following the manufacturer's protocol. To assess macrophage infiltration, colonic tissue sections were incubated with anti-F4/80 (Cell Signaling Technology, Danvers, MA, USA; catalog #70076) and anti-CD86 (Cell Signaling Technology; catalog #19589) antibodies, followed by counterstaining with hematoxylin for 1 min. The staining intensity of F4/80 and CD86 was quantified using ImageJ software (NIH, Bethesda, MD, USA) with the IHC Toolbox plugin. For immunofluorescence staining, antigen retrieval was performed as described above, and sections were incubated with an anti-E-cadherin antibody (Cell Signaling Technology; catalog #3195), followed by an Alexa Fluor® 555-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology; catalog #4413). Nuclei were counterstained using DAPI-containing Fluoromount-G™ Mounting Medium (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence intensity was quantified using ImageJ. Periodic acid-Schiff (PAS) staining was performed to visualize mucin-producing goblet cells. Dewaxed sections were treated with periodic acid (Thermo Scientific Chemicals) for 10 minutes, followed by incubation with Schiff's reagent (Thermo Scientific Chemicals) for 5 minutes. PAS-positive cells were visualized by light microscopy and quantified using ImageJ by calculating the number of PAS<sup>+</sup> cells per crypt. Histological scoring was performed by a blind investigator to minimize bias.



### 2.3. Total RNA isolation and quantitative polymerase chain reaction (qPCR) analysis

Total RNA was isolated from the colon tissues or HCT8 cells using TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality and concentration were assessed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Then, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). qRT-PCR was performed in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using PowerUp SYBR Master Mix (Thermo Fisher Scientific). All the mouse- or human-specific primers were purchased from Thermo Fisher Scientific. Primer sequences were obtained from the PrimerBank database, and detailed information is provided in Table S2. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed relative to the control mice or cells using the  $2^{-\Delta\Delta C_t}$  method.

### 2.4. Fecal DNA extraction and 16S rRNA gene amplicon sequencing

Following a 4-week pretreatment with either a standard AIN-93G diet or a hemp seed-containing AIN-93G diet, fecal samples were collected prior to DSS treatment. Total genomic DNA was extracted from fecal samples using Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research Operations, Tustin, CA, USA) following the manufacturer's instructions. Amplicon library preparation and 16S rRNA gene amplicon sequencing using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) were performed as previously described.<sup>21,22</sup> Briefly, the V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified by PCR using region-specific primers, which were subjected to paired-end sequencing using  $2 \times 250$  cycles at the Purdue Genomics and Genome Editing Facility.

Raw sequencing data were processed using the Quantitative Insights into Microbial Ecology 2 pipeline (QIIME2, version 2024.10), including demultiplexing and denoising. A feature table was constructed, and features were taxonomically assigned using a pretrained naïve Bayes classifier (SILVA database, release 138; 99% identity). A tree was generated using the q2-phylogeny plugin in QIIME2. Alpha diversity metrics, including Shannon index, Pielou's evenness, and Chao1 richness, were calculated. Principal-coordinate analysis based on the Bray–Curtis dissimilarity matrix, as well as Unweighted and Weighted UniFrac was plotted using the *phyloseq* package in R (version 2024.12.1). Data visualization and statistical analyses were performed in R using the *phyloseq*, *vegan*, *qiime2R*, *ggplot2*, *tidyverse*, and *ggpubr* packages. Microbial taxa with a relative abundance below 0.005% across all samples were excluded from downstream visualizations. Statistical comparisons of  $\alpha$ -diversity will be performed using the Student's *t* test or Mann–Whitney *U* test, while permutational multivariate analysis of variance (PERMANOVA, 999 permutations) will be applied to assess differences in  $\beta$ -diversity.

### 2.5. Cell assays

Human colonic epithelial HCT-8 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; catalog #10013CV, Corning Inc., Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS). All cultures were maintained at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. To determine the effect of hemp seed oil on the gene expression of tight junction proteins, HCT-8 cells were treated with lipopolysaccharide (LPS; 10  $\mu\text{g mL}^{-1}$ , Sigma-Aldrich, St Louis, MO, USA), with or without co-treatment with hemp seed oil (3  $\mu\text{g mL}^{-1}$ ), for 24 hours. The hemp seed oil used in this study was directly extracted from hemp seed through a cold-press process, and therefore closely reflects the endogenous lipid composition in the original hemp seed. Following treatment, total RNA was extracted, and quantitative polymerase chain reaction (qPCR) analysis was performed as described in Section 2.3.

### 2.6. Lipopolysaccharide (LPS) determination in plasma

Quantification of plasma LPS levels was performed using an ELISA kit (MBS261904, MyBiosource, San Diego, CA) in accordance with the manufacturer's instructions.

### 2.7. *In vitro* culture of bifidobacterium pseudolongum

*Bifidobacterium pseudolongum* (ATCC 25526) was cultured in de Man, Rogosa, and Sharpe (MRS) broth (BD Difco, Detroit, MI, USA) at 37 °C within an anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA), which was maintained under an atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub>. The culture was inoculated (1 : 100, v/v) into fresh MRS broth supplemented with or without hemp seed powder at final concentrations of 0.03% or 0.3% (w/v) and incubated at 37 °C for 24 h under anaerobic conditions. Bacterial growth was determined by measuring optical density at 600 nm (OD<sub>600</sub>) using a spectrophotometer.

### 2.8. Statistical analysis

All data are presented as the mean  $\pm$  standard error of the mean (SEM). Shapiro–Wilk test was used to assess the normality of data distributions, and Levene's test was applied to evaluate equal variance of data before statistical analysis. Statistical comparisons between two groups were performed using either Student's *t* test or Wilcoxon–Mann–Whitney test (when the normality test fails). For statistical comparisons among the three groups, one-way ANOVA followed by Tukey's or Fisher's *post hoc* test was used. When data did not meet the normality assumptions, Kruskal–Wallis test on Ranks was applied, followed by appropriate *post hoc* analyses. All of these data analyses were performed by using SigmaPlot software (Grafiti LLC, Palo Alto, CA, USA). *P* values less than 0.05 are reported as statistically significant.



### 3. Results

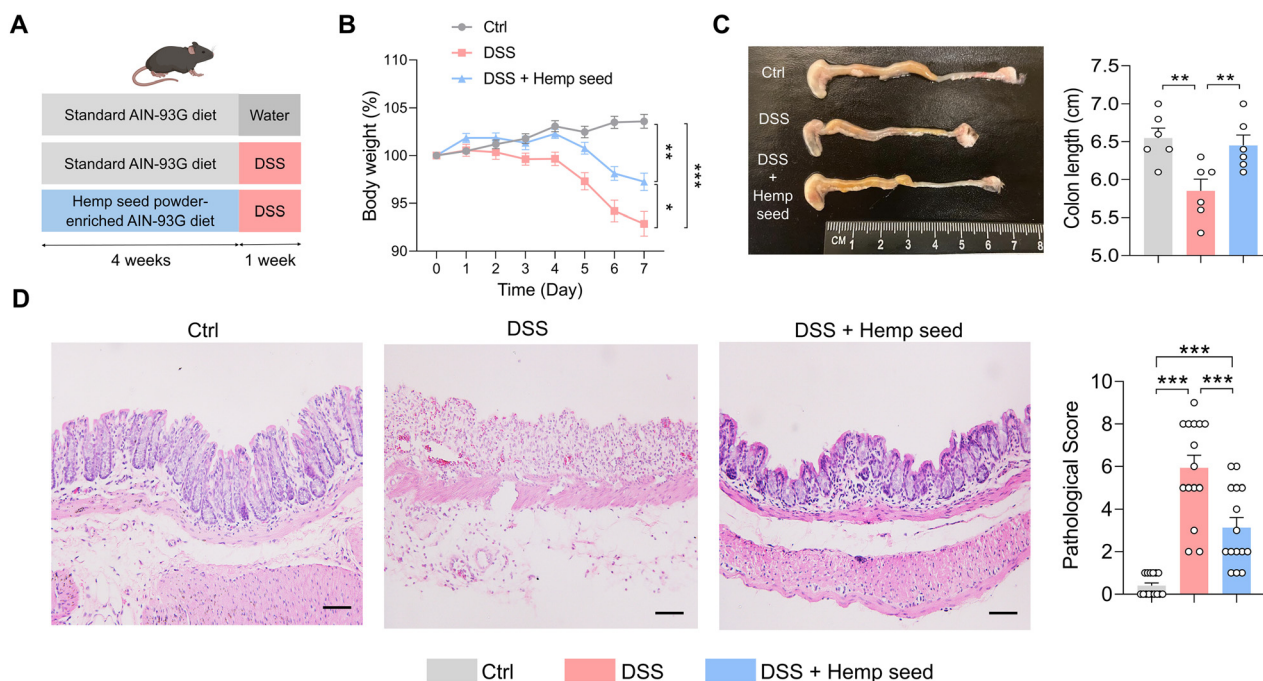
#### 3.1. Hemp seed consumption alleviated DSS-induced colitis in mice

To investigate the effects of hemp seed consumption on colitis, we employed the DSS-induced IBD model in C57BL/6 mice. Mice were pre-treated with either a standard AIN-93G diet or an AIN-93G diet supplemented with hemp seed powder for four weeks, followed by one week of DSS administration to induce colitis (Fig. 1A). The dietary supplementation level of hemp seed powder was 3% (w/w), corresponding to a human-equivalent dose of approximately  $390 \text{ mg kg}^{-1} \text{ day}^{-1}$ . This value was calculated based on an estimated daily intake of 4 g hemp seed for a 25 g mouse and subsequently scaled to an average adult body weight of 60 kg using standard mouse-to-human dose conversion methods.<sup>20</sup> Hemp seed supplementation did not affect body weight during the pretreatment phase under basal conditions, indicating no obvious toxic or adverse effects (Fig. S1). Importantly, hemp seed consumption alleviated DSS-induced weight loss, indicating its protective effects against colitis-related disease progression (Fig. 1B). Moreover, the colon shortening, a hallmark of colonic inflammation, was attenuated in the hemp seed-fed colitis mice, compared with their standard diet-fed counterparts (Fig. 1C). Histological evaluation of colonic tissues using hematoxylin and eosin (H&E) staining revealed that DSS treatment caused

colon epithelial damage and inflammation, which was attenuated by hemp seed (Fig. 1D). Together, these results demonstrate that hemp seed consumption alleviated DSS-induced colitis in mice.

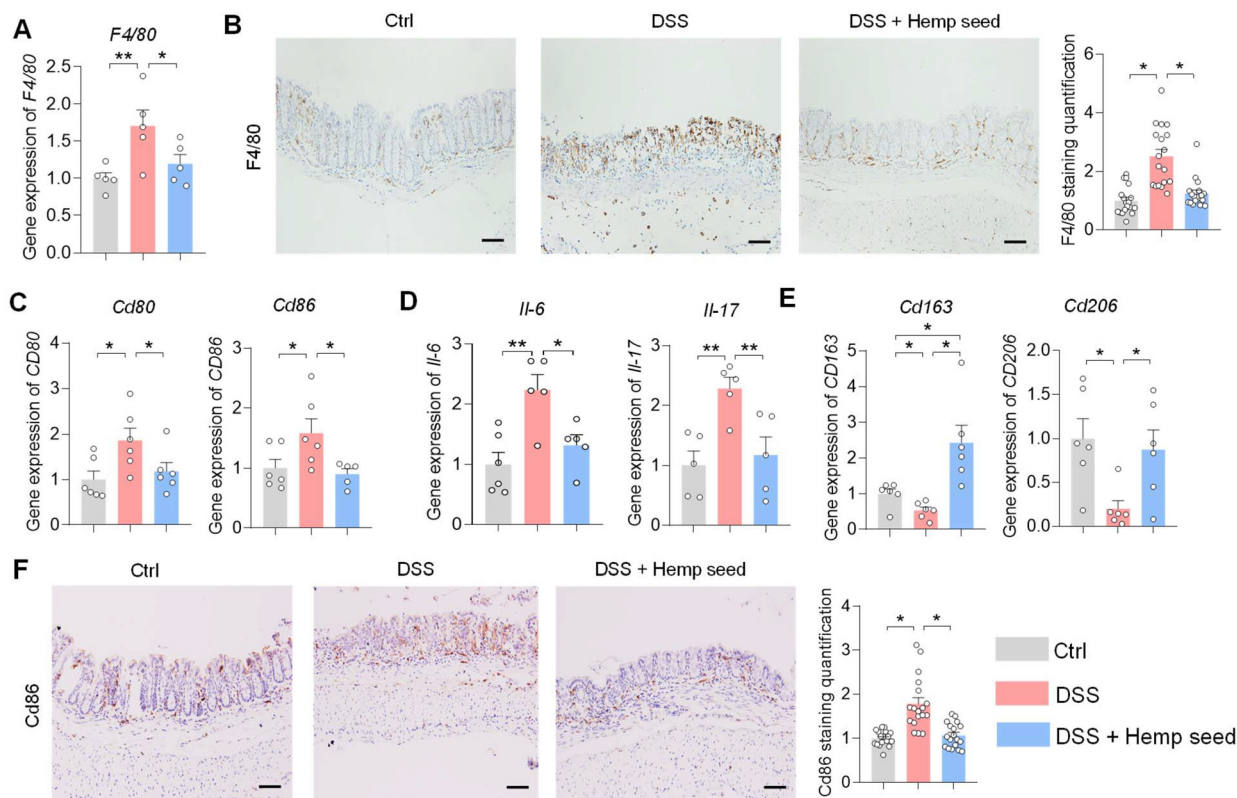
#### 3.2. Hemp seed consumption modulated macrophage accumulation and polarization during colitis

Given the critical role of macrophages in modulating the pathogenesis of colitis,<sup>23</sup> we investigated the effects of hemp seed consumption on macrophage accumulation and polarization in the DSS-induced colitis model. The quantitative PCR (qPCR) analysis revealed an increase in macrophage marker (F4/80) gene expression in colitis mice (Fig. 2A). Consistently, immunohistochemical staining confirmed the increased accumulation of F4/80<sup>+</sup> macrophages in colonic tissues of colitis mice (Fig. 2B). Notably, hemp seed consumption reduced the accumulation of total macrophages in DSS-treated mice, indicating a regulatory effect of hemp seed on macrophage recruitment and infiltration during colitis (Fig. 2A and B). We further analyzed macrophage polarization markers and found that hemp seed treatment reduced the expression of M1 macrophage markers (*Cd80*, *Cd86*) and associated pro-inflammatory cytokines, especially *Il-6* and *Il-17* expression (Fig. 2C and D). In contrast, hemp seed enhanced the expression of M2 macrophage markers (*Cd163*, *Cd206*), indicating a shift toward an anti-inflammatory macrophage pheno-



**Fig. 1** Hemp seed consumption decreased dextran sulfate sodium (DSS)-induced colitis. (A) Scheme of animal experiment. C57BL/6 mice were fed either a standard diet or a hemp seed powder-enriched diet (3% w/w) for 4 weeks, followed by 7 days of DSS (2% w/v) treatment. During the DSS treatment period, mice remained on either a standard AIN-93G diet or a hemp seed-supplemented AIN-93G diet. Mice that did not receive DSS treatment served as healthy controls. (B) Body weight of mice during the DSS treatment period. (C) Colon length of mice. (D) Hematoxylin and eosin (H&E) staining of distal colon tissue of mice (magnification 200 $\times$ , scale bars: 50  $\mu\text{m}$ ) and pathological score analysis ( $n = 15$  random fields per group). The results are expressed as mean  $\pm$  SEM.  $n = 6$  mice per group. Statistical significance was determined using one-way ANOVA or Kruskal–Wallis test on Ranks. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .





**Fig. 2** Hemp seed consumption modulated macrophage accumulation and polarization during colitis. (A) Gene expression of macrophage marker *F4/80* in colon. (B) Immunohistochemical staining of *F4/80* (magnification 200 $\times$ , scale bars: 50  $\mu$ m) and quantification of *F4/80* staining intensity in colon ( $n = 18$  random fields per group). (C) Gene expression of M1 macrophage markers *Cd80* and *Cd86* in colon. (D) Gene expression of pro-inflammatory cytokines *Il-6* and *Il-17* in colon. (E) Gene expression of M2 macrophage markers *Cd163* and *Cd206* in colon. (F) Immunohistochemical staining of *Cd86* (magnification 200 $\times$ , scale bars: 50  $\mu$ m) and quantification of *Cd86* staining intensity in colon ( $n = 18$  random fields per group). The results are expressed as mean  $\pm$  SEM.  $n = 5$ –6 mice per group. Gene expressions were normalized to *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* and expressed relative to the control mice using the  $2^{-\Delta\Delta Ct}$  method. Statistical significance was determined using one-way ANOVA or Kruskal–Wallis test on Ranks. \* $P < 0.05$ , \*\* $P < 0.01$ .

type (Fig. 2E). Moreover, immunohistochemical staining confirmed the elevated *Cd86* signal in the colonic tissues of DSS-induced colitis mice, whereas hemp seed supplementation reduced *Cd86*<sup>+</sup> macrophage staining intensity (Fig. 2F). Together, these findings demonstrate that hemp seed consumption modulated macrophage accumulation and polarization in mice during colitis.

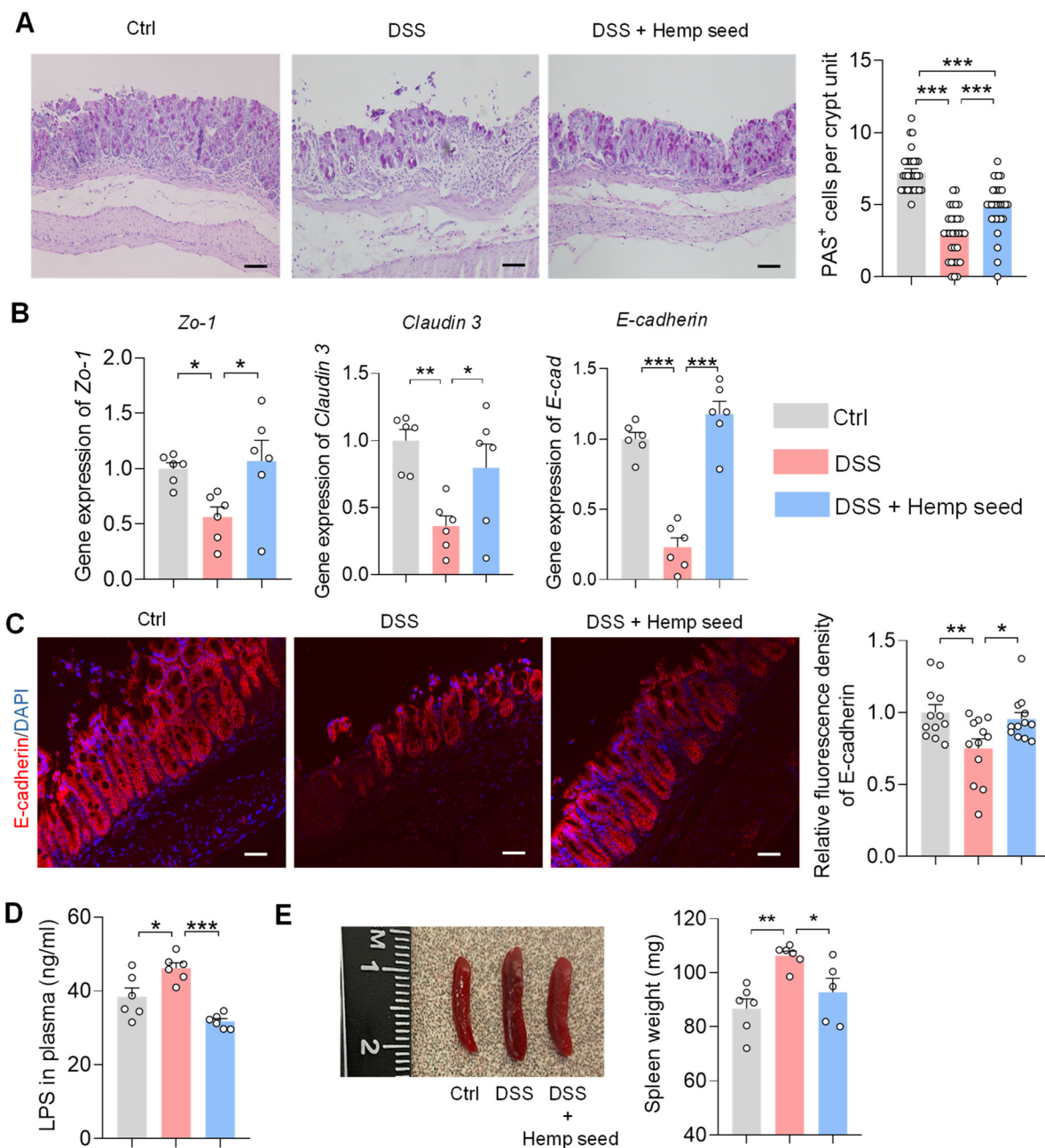
### 3.3. Hemp seed alleviated intestinal barrier dysfunction and reduced systemic inflammation

Intestinal barrier disruption is a hallmark of colitis, often characterized by the loss of mucin-producing goblet cells and compromised epithelial junctions.<sup>24</sup> To evaluate goblet cell status, periodic acid-Schiff (PAS) staining was performed. DSS-treated colitis mice exhibited a significant reduction in PAS<sup>+</sup> goblet cells, whereas hemp seed supplementation restored their presence in the intestinal epithelium, indicating a protective effect on mucus production and goblet cell integrity under colitis conditions (Fig. 3A). We next assessed the expression of key tight junction and adherens junction proteins, which are

essential for maintaining epithelial integrity. DSS treatment significantly downregulated the colonic expression of *Zonula occludens-1 (Zo-1)*, *claudin-3*, and *E-cadherin*. Remarkably, hemp seed supplementation restored the transcription of these genes, suggesting improved epithelial structural integrity (Fig. 3B). Immunofluorescence staining further confirmed the elevated E-cadherin protein level in the colonic epithelium (Fig. 3C). Consistent with impaired barrier function, plasma levels of lipopolysaccharide (LPS), which is a surrogate marker of gut permeability and endotoxemia, were elevated in DSS-treated mice (Fig. 3D). Hemp seed intervention reduced circulating LPS levels, further supporting its role in maintaining barrier function and limiting microbial product translocation (Fig. 3D). Finally, we observed that hemp seed supplementation attenuated colitis-associated splenomegaly, a hallmark of systemic immune activation (Fig. 3E). This finding suggests that, in addition to reinforcing intestinal barrier integrity, hemp seed may also dampen systemic inflammation elicited during colitis.

To further determine whether hemp seed oil recapitulates the barrier-supporting effects of whole hemp seed, we investi-





**Fig. 3** Hemp seed alleviated intestinal barrier dysfunction and reduced systemic inflammation. (A) Periodic acid–Schiff (PAS) staining in colon (magnification 200 $\times$ , scale bars: 50  $\mu$ m) and quantification of PAS<sup>+</sup> cells per crypt unit ( $n = 30$  random crypt units per group). (B) Gene expression of tight junction protein *Zonula occludens-1* (*Zo-1*), *Claudin-3*, and *E-cadherin* in colon. (C) Immunofluorescence staining of E-cadherin (magnification 200 $\times$ , scale bars: 50  $\mu$ m) and quantification of E-cadherin staining intensity in colon ( $n = 12$  random fields per group). (D) Plasma concentration of lipopolysaccharide (LPS) in mice. (E) Spleen weight of mice. The results are expressed as mean  $\pm$  SEM.  $n = 5$ –6 mice per group. Gene expressions were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) and expressed relative to the control mice using the  $2^{-\Delta\Delta Ct}$  method. Statistical significance was determined using one-way ANOVA or Kruskal–Wallis test on Ranks. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

gated its impact on human colonic epithelial HCT-8 cells under inflammatory stress. Treatment with hemp seed oil upregulated the expression of tight junction-related genes, including *ZO-1* and *CLAUDIN-5*, in LPS-challenged cells, indicating enhanced epithelial barrier function (Fig. S2). Together, these findings demonstrate that hemp seed alleviated DSS-induced intestinal barrier dysfunction by restoring goblet cell

populations, enhancing tight junction protein expression, and reducing systemic LPS translocation.

#### 3.4. Hemp seed consumption modulated the composition of gut microbiota

Gut microbiota plays a critical role in maintaining intestinal homeostasis and modulating host immune responses.<sup>25</sup> To



evaluate the effect of hemp seed consumption on the gut microbiota under normal conditions, we analyzed the fecal microbial communities of mice fed either a standard diet or hemp seed diet prior to DSS treatment using 16S rRNA sequencing. Hemp seed consumption, despite showing no effects on alpha diversity, led to marked alterations in  $\beta$  diversity, indicating shifts in microbial community structure (Fig. 4A and B). Moreover, hemp seed intake induced significant changes in gut microbial taxonomic composition (Fig. 4C–E). Notably, mice fed the hemp seed diet exhibited increased relative abundances of beneficial gut bacterial genera, particularly *Bifidobacterium*, and decreased abundances of colitis-associated genera such as *Staphylococcus* and *Enterococcus*, compared to those fed a standard AIN-93G diet (Fig. 4E and Table S3). To validate the 16S rRNA sequencing results, we conducted quantitative PCR analysis, which confirmed a similar pattern of increased *Bifidobacterium* abundance (Fig. S3). Furthermore, *in vitro* bacterial culture assay demonstrated that hemp seed powder promoted the growth of *Bifidobacterium pseudolongum* under anaerobic conditions compared with the control medium (Fig. S4). Together, these findings suggest that hemp seed can precondition and optimize gut microbial composition before disease onset, potentially contributing to its later protective effects during DSS-induced inflammation.

## 4. Discussion

IBD is a growing global health concern, driven by complex interactions between the microbiota and the immune system.<sup>2</sup> Recent advances have shown that hemp seed-derived bioactive components, including hemp seed oil, alleviated colitis symptoms and restored gut microbiota dysbiosis;<sup>19</sup> however, the effects of whole hemp seed consumption remain less well understood. In this study, we demonstrated that hemp seed consumption, as a whole-food dietary approach, reduced colonic inflammation and mitigated tissue damage in a DSS-induced colitis mouse model. Moreover, hemp seed intake attenuated macrophage accumulation and modulated polarization from proinflammatory M1 to anti-inflammatory M2, as well as protected intestinal barrier integrity. Importantly, hemp seed positively influenced gut microbial ecology by enriching beneficial taxa, particularly *Bifidobacterium*, both *in vivo* and *in vitro*. Together, these findings support hemp seed as a promising dietary intervention for mitigating IBD symptoms and improving gut health.

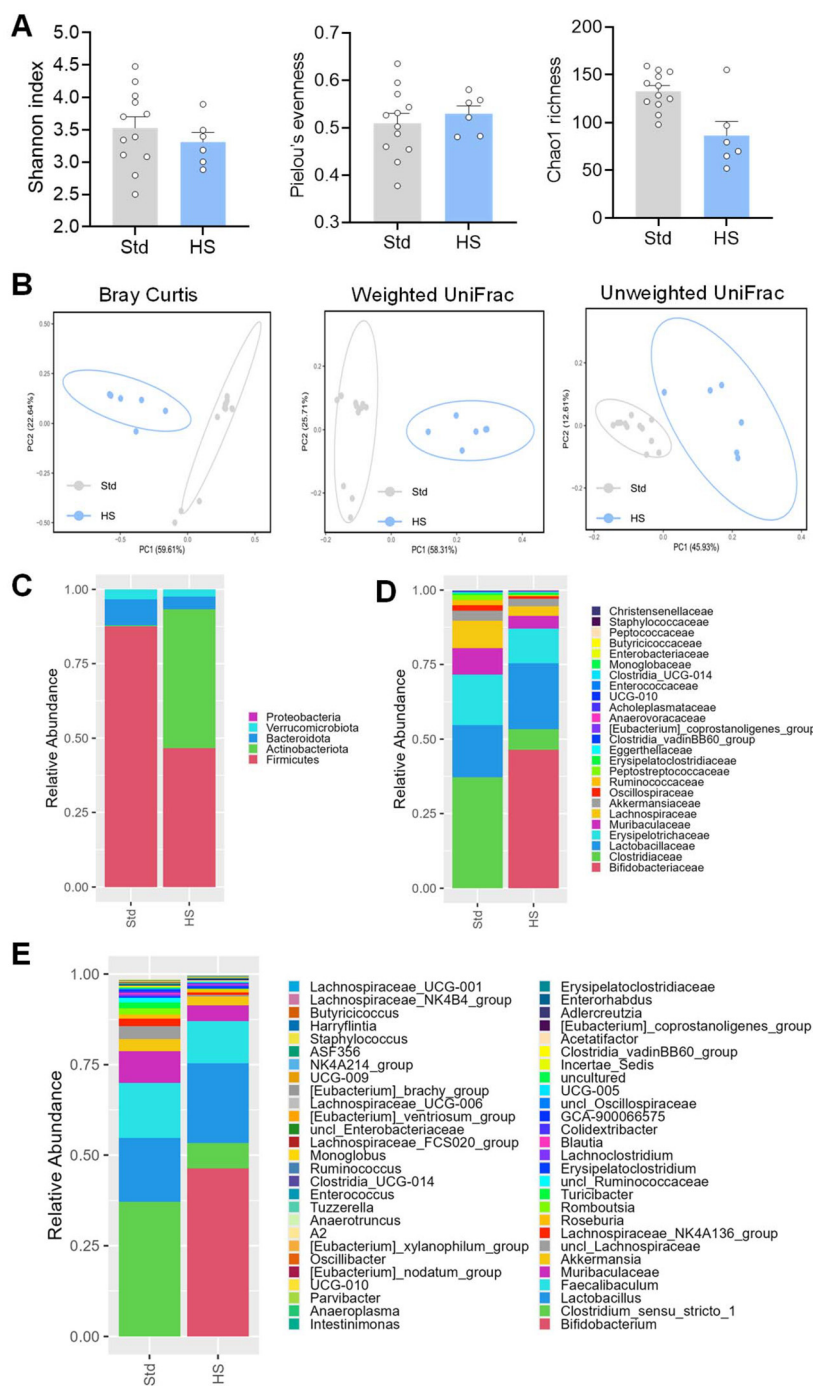
Hemp seed emerges as a novel multifunctional dietary seed with distinct nutritional and bioactive properties. Hemp seed is notable for its lipid composition, with over 75% of its total fatty acids consisting of polyunsaturated fatty acids, including substantial amounts of  $\alpha$ -linolenic acid (C18:3, omega-3),<sup>26</sup> which is known for its anti-inflammatory and antioxidative actions. In addition, hemp seed provides proteins rich in arginine, glutamine, and branched-chain amino acids (BCAAs).<sup>27,28</sup> These amino acids play critical roles in protein synthesis, immune regulation, and tissue repair,<sup>29,30</sup> thereby

enhancing the nutritional and functional value of hemp seed. The whole hemp seed also contains approximately 27.6% total dietary fiber, comprising 5.4% soluble fiber and 22.2% insoluble fiber.<sup>31</sup> Notably, sulfated polysaccharides derived from hemp seed have been shown to reduce oxidative damage.<sup>32,33</sup> Finally, hemp seed contains abundant polyphenolic and flavonoid compounds that exhibit antioxidant and free-radical scavenging activities, which can attenuate oxidative stress-induced inflammatory signaling cascades and contribute to alleviating mucosal damage and inflammatory responses during colitis.<sup>14,34,35</sup> Together, all the nutritional components within hemp seed could act synergistically to support intestinal homeostasis, modulate inflammation, and enhance host-microbiota interactions, ultimately leading to improved gut barrier integrity and reduced susceptibility to inflammation-associated disorders.

Macrophage infiltration is a hallmark of intestinal inflammation in colitis.<sup>23</sup> Our findings showed that hemp seed consumption reduced colitis-associated macrophage accumulation in the colon, suggesting a mechanism underlying its immunomodulatory activity. Beyond infiltration, macrophage polarization plays a crucial role in shaping inflammatory responses. Pro-inflammatory M1 macrophages exacerbate colitis and impair barrier integrity, while anti-inflammatory M2 macrophages promote resolution and tissue repair.<sup>23,36,37</sup> Notably, hemp seed intake downregulated M1 markers while enhancing M2 markers, underscoring its role in modulating macrophage polarization and promoting inflammatory resolution. In parallel, we observed reduced *IL-6* expression following hemp seed treatment. *IL-6* is a key pro-inflammatory cytokine secreted by M1 macrophages known to drive Th17 differentiation and chronic intestinal inflammation.<sup>38,39</sup> This suppression of *IL-6* may contribute to attenuating Th17-mediated responses and enhancing inflammatory resolution. Consistent with our findings, a recent study showed that hemp seed oil and its major bioactive component, phytol, promoted M2 macrophage polarization and decreased *IL-6* production in human monocyte-derived macrophages.<sup>40</sup> Together, these results underscore the immunomodulatory potential of hemp seed-derived compounds. Future studies are needed to define the specific bioactive constituents in hemp seed responsible for modulating macrophage polarization and to explore their therapeutic applications in other inflammatory disorders.

The intestinal barrier comprises epithelial cells, tight junction complexes, and a protective mucus layer, all essential for preventing pathogen and antigen translocation.<sup>41</sup> Here, we found that hemp seed consumption restored mucin-producing goblet cells, upregulated tight junction protein expression, and reduced intestinal permeability in colitis mice. These findings are consistent with previous studies showing that hemp seed-derived products enhance gut barrier function. Indeed, hemp seed cake supplementation was reported to improve intestinal morphology with increased villus height, surface area, and the villus/crypt ratio in poultry,<sup>15</sup> while hemp seed-derived nanovesicles upregulated tight junction proteins (*ZO-1*, *Claudin-4*, and *Occludin*) in a colitis mouse model.<sup>42</sup> Additionally, we





**Fig. 4** Hemp seed modulated the gut microbiota composition. (A) Effect of hemp seed on  $\alpha$  diversity of fecal microbiota, assessed by Shannon index, Pielou's evenness, and Chao1 richness. (B) Effect of hemp seed on  $\beta$  diversity of fecal microbiota, assessed using Bray–Curtis dissimilarity, unweighted and weighted UniFrac distances. (C–E) Effect of hemp seed on composition of the microbiota at (C) phylum, (D) family, and (E) genus levels. The results are expressed as mean  $\pm$  SEM.  $n = 12$  mice in the standard diet (Std) group and  $n = 6$  mice in hemp seed diet (HS) group. Statistical significance was determined using Student's  $t$ -test or Wilcoxon–Mann–Whitney test.

observed a significant attenuation of colitis-associated increase of circulating LPS levels following hemp seed intake, indicating restored barrier integrity and reduced systemic leakage. Similar effects were reported in obese mice, where hemp seed reduced gut-to-circulation leakage.<sup>16</sup> These findings support

the role of hemp seed in maintaining epithelial integrity under inflammatory conditions.

Hemp seed is notable for its high content of  $\alpha$ -linolenic acid, a major plant-derived omega-3 polyunsaturated fatty acid, which contributes to its functional lipid profile. In our



study, hemp seed oil upregulated the expression of tight junction-related genes in LPS-challenged human colonic epithelial cells, highlighting its role in restoring tight junction protein expression and enhancing epithelial barrier integrity under inflammatory stress. The LPS-stimulated colonic cell model complements the DSS-induced colitis mouse model by enabling mechanistic evaluation of the epithelial anti-inflammatory and barrier-protective effects of hemp seed oil. In DSS-induced colitis, epithelial damage allows bacterial components such as LPS to penetrate the mucosa and activate epithelial TLR4 signaling, leading to cytokine production and tight junction disruption.<sup>43</sup> This *in vitro* model thus provides a controlled system to investigate how hemp seed oil mitigates LPS-induced epithelial inflammation and function. However, the specific lipid species or bioactive molecules responsible for these protective effects remain to be identified. Previous research has shown that  $\alpha$ -linolenic acid-derived lipid mediators, such as 13-(S)-hydroperoxyoctadecatrienoic acid [13-(S)-HPOTrE] and 13-(S)-hydroxyoctadecatrienoic acid [13-(S)-HOTrE], can modulate the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome and autophagy signaling pathways, thereby suppressing inflammatory responses and attenuating LPS-induced septic shock in mice.<sup>44</sup> Future investigations employing targeted lipidomic approaches are warranted to identify bioactive lipid mediators derived from hemp seed oil that are involved in the regulation of inflammatory signaling. Finally, given that the *in vivo* benefits were observed with whole-seed intake in the mouse model, future studies should aim to differentiate the functional contributions of other non-lipid components in hemp seed, particularly the protein, fiber, or polyphenol fractions, in maintaining gut barrier integrity and mucosal homeostasis. It is also important to elucidate how these components interact with or act synergistically with the lipid fraction to enhance the overall gut-protective effects of hemp seed.

Dysbiosis is both a hallmark and a driver of IBD pathogenesis.<sup>45</sup> Modulating the gut microbiome through dietary interventions offers a promising avenue for addressing disease progression at its origin.<sup>46–49</sup> Our study demonstrated that fecal microbiota of hemp seed-fed mice exhibited increased abundances of beneficial bacterial genera, particularly *Bifidobacterium*, which are known for their anti-colitis and gut-protective properties.<sup>50,51</sup> In contrast, the relative abundances of colitis-associated and potentially pathogenic genera, including *Staphylococcus*<sup>52,53</sup> and *Enterococcus*,<sup>54,55</sup> were significantly reduced compared to mice fed a standard diet. These shifts in microbial composition suggest that hemp seed consumption may contribute to gut health by promoting beneficial microbial populations while suppressing opportunistic or inflammation-associated taxa. These results are consistent with prior findings showing that hemp seed consumption optimizes the gut microbiome composition in multiple animal models. In particular, in a high-fat diet-induced obesity mouse model, treatment with hemp seed has been shown to increase the abundance of beneficial gut bacteria.<sup>16</sup> Furthermore, dietary supplementation with hemp seed cake has been found

to increase rumen microbiome diversity in Angus-crossbred heifers, indicating a positive impact on microbial populations in the rumen.<sup>18</sup> Beyond animal models, hemp seed bran protein extract has been shown to have prebiotic-like effects in an *in vitro* colon gut model, promoting the growth of beneficial bacteria such as *Bifidobacterium bifidum* and *Bacteroides fragilis*, while reducing opportunistic bacteria like *Bilophila wadsworthia* and *Desulfovibrio*.<sup>56</sup> Collectively, these results support the potential of hemp seed to beneficially modulate gut microbial ecology. However, in the present study, microbiota sequencing was conducted on fecal samples collected prior to DSS treatment. Thus, the observed microbial changes reflect the baseline preventive effects of hemp seed supplementation under normal physiological conditions, rather than microbial shifts occurring during active colitis. Future investigations are needed to include microbiota analyses under inflammatory conditions to fully elucidate how hemp seed influences microbial dynamics during disease progression. Moreover, further studies are warranted to determine the functional roles of specific hemp seed components, particularly dietary fiber, in regulating microbial growth, diversity, and metabolic interactions.

## 5. Conclusion

The present study demonstrates that dietary hemp seed reduced colonic inflammation and alleviated tissue injury in the DSS-induced colitis mouse model. These protective effects are associated with modulation of macrophage dynamics, enhancement of intestinal barrier integrity, and beneficial shifts in gut microbiota composition. Further research is warranted to evaluate the efficacy of hemp seed and its bioactive components using additional colitis models and human clinical trials. While the DSS-induced colitis model effectively mimics acute epithelial injury and innate immune activation, it does not fully capture the chronic, relapsing nature or the adaptive immune involvement characteristic of human IBD. Employing complementary models, especially T cell transfer-induced colitis or genetically susceptible IL-10 knockout mice, could help delineate the broader immunomodulatory and mucosal healing effects of hemp seed under diverse pathological contexts. In addition, this study was limited to findings derived from a mouse model, which may not fully represent the complex physiological and immune responses in humans. Therefore, future human studies are warranted to validate translational relevance and confirm the potential of hemp seed as a functional food for intestinal health. Moreover, mechanistic studies exploring the pathways through which hemp seed influences immune regulation, microbiota diversity, and epithelial function will be essential for translating these findings into dietary interventions. Collectively, our study provides insights into the role of hemp seed in gut health and reinforces its potential as a promising and sustainable dietary intervention for IBD management.



## Author contributions

Conceptualization: W. W., S. S., and T. J.; funding acquisition: W. W.; investigation: J. W., D. C., G. S., E. A. L., and K. L. W.; supervision: W. W., and T. J.; writing – original draft: W. W., J. W., and D. C.; writing – review and editing: W. W., T. J., S. S., Q. J., and L. R. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

## Data availability

All data generated during this study are included in the published article and its supplementary information (SI). Supplementary information: primer sequences used in the study, as well as the raw data of bacterial 16S sequencing results. See DOI: <https://doi.org/10.1039/d5fo04119h>.

Additional datasets analyzed during the current study are available from the corresponding author upon reasonable request.

## Acknowledgements

This work was supported by the USDA/NIFA Agriculture and Food Research Initiative (AFRI) Grant No. 2025-67017-44988 (W. W.); the Showalter Trust Young Investigator Award No. 41001324 (W. W.); and the Purdue University AG-HHS One Health Collaborative Seed Grant (W. W. and Q. J.). We would like to thank Prof. Kee-Hong Kim from the Department of Food Science at Purdue University for assistance with Western Blotting analysis, and Prof. Arun Bhunia from the Department of Food Science at Purdue University for assistance with RNA quantification and analysis. Tissue processing and analysis were conducted at the Histology Research Laboratory at Purdue University. 16S rRNA sequencing was conducted in the Purdue Genomics and Genome Editing Facility. The hemp seed oil was kindly provided by Down on the Farm, LLC.

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