frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 April 2021
doi: 10.3389/fphar.2021.665376

Atractylodin Attenuates Dextran Sulfate Sodium-Induced Colitis by Alleviating Gut Microbiota Dysbiosis and Inhibiting Inflammatory Response Through the MAPK Pathway

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Specialt section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 08 February 2021
Accepted: 14 April 2021
Published: 29 April 2021

Citation:
Qu L, Lin X, Liu C, Ke C, Zhou Z, Xu K, Cao G and Liu Y (2021) Atractylodin Attenuates Dextran Sulfate Sodium-Induced Colitis by Alleviating Gut Microbiota Dysbiosis and Inhibiting Inflammatory Response Through the MAPK Pathway.
Front. Pharmacol. 12:665376.
doi: 10.3389/fphar.2021.665376

In this study, we investigated the therapeutic effects and mechanism of atractylodin (ATL) on dextran sulfate sodium (DSS)-induced ulcerative colitis in mice. We found that atractylodin could significantly reverse the effects of DSS-induced ulcerative colitis, such as weight loss, disease activity index score; shorten the colon length, and reverse the pathological changes in the colon of mice. Atractylodin could inhibit the activation of colonic macrophages by inhibiting the MAPK pathway and alleviate intestinal inflammation in the mouse model of ulcerative colitis. Moreover, it could protect the intestinal barrier by inhibiting the decrease of the tight junction proteins, ZO-1, occludin, and MUC2. Additionally, atractylodin could decrease the abundance of harmful bacteria and increase that of beneficial bacteria in the intestinal tract of mice, effectively improving the intestinal microecology. In an LPS-induced macrophage model, atractylodin could inhibit the MAPK pathway and expression of the inflammatory factors of macrophages. Atractylodin could also inhibit the production of lactate, which is the end product of glycolysis; inhibit the activity of GAPDH, which is an important rate-limiting enzyme in glycolysis; inhibit the malonylation of GAPDH, and, thus, inhibit the translation of TNF-α. Therefore, ours is the first study to highlight the potential of atractylodin in the treatment of ulcerative colitis and reveal its possible mechanism.

Keywords: ulcerative colitis, malonylation, atractylodin, MAPK pathway, tight junctions

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease of unknown etiology that affects the colon and rectum. It is one of the two forms of inflammatory bowel disease (IBD). The characteristic of UC is that mucositis begins at the rectum and extends progressively to the proximal colon (Kobayashi et al., 2020). Of late, the global incidence of UC is steadily increasing. The incidence of UC in western Canada is the highest (16.7 per 100,000 individuals), while in Europe, it is 1.6–11.9 per 100,000 individuals (Kaur and Goggolidou, 2020). Currently, the pharmacotherapy of UC is based mainly on the following four basic drug categories: 5-aminosalicylates (5-ASA), steroids, immunosuppressants, and biological drugs (Panés et al., 2017). Local mesalazine continues to be

the first-line treatment for patients with mild or moderate active UC limited to the rectum, whereas oral and local 5-ASA are the main treatment approaches for patients with left or extensive colitis (Cohen and Dalal, 2015). A study reports that only 60% of patients with mild to moderate UC achieved remission with mesalazine (Ford et al., 2011). Anti-TNF drugs may increase the risk of severe infection and cancer (Fellermann, 2013). Therefore, there is an urgent need for the effective management of ulcerative colitis; consequently, an increasing number of studies aimed at discovering novel and effective natural products are being conducted.

In patients with UC, several pro-inflammatory factors, such as tumor necrosis factor (TNF), interleukin-6, and interleukin-1β are produced in the intestinal mucosa (Tatiya-aphiradee et al., 2018). Intestinal goblet cells secrete MUC2 may be the key factor in determining the susceptibility of an individual to UC (Morampudi et al., 2016). The mucous layer of the colon in patients with UC is characterized by a decreased synthesis of mucoprotein 2 (Van Klinken et al., 1999). The barrier function of the intestinal epithelium is partly maintained by tight junctions (TJs) present between adjacent epithelial cells. TJs constitute a complex functional compound that is mainly composed of TJ proteins. Abnormal expression of TJ proteins (ZO-1 and occludin) in intestinal tissues promotes intestinal permeability and pathogen infiltration, and induces immune dysfunction and IBD (Zeisel et al., 2018). Macrophages are the primary immune cells that play a key role in the development, progression, and reversal of inflammation (Fujiwara and Kobayashi, 2005). There is a causal relationship between the regression of intestinal inflammation and the differentiation of monocyte macrophages in patients with IBD. Macrophages are now considered a potential target in the development of novel therapeutic methods (Na et al., 2019). The activation of macrophages is closely related to the MAPK pathway (Smith et al., 2014). The expression of phosphorylated p38 MAPK in the nucleus of immune effector cells in the UC mucosal crypt is significantly increased (Dahan et al., 2008). There is evidence that the intestinal microflora of patients with IBD is significantly different from that of healthy individuals. Although clinical data are limited, they support the efficacy of treatment strategies for altering the microbiota of patients with IBD (Plichta et al., 2019).

Several natural products isolated from vegetables, fruits, and herbs have been reported to be effective in treating dextran sulfate sodium (DSS)-induced ulcerative colitis in mice. For example, 2-O-6-D-glucosyl-L-ascorbic acid from *Lycium barbarum* can increase the expression of the intestinal tight junction proteins, ZO-1, and occludin, and regulate the diversity of intestinal short-chain fatty acids and intestinal flora, thereby being effective in the treatment of DSS-induced colitis (Dong et al., 2020). Parthenolide can regulate the balance of intestinal flora and suitably control the levels of short-chain fatty acids and Treg/Th17 in the intestinal mucosa to prevent UC (Liu et al., 2020). Paeoniflorin can reduce the infiltration of Gram-positive bacteria in the intestinal tract and inhibit the MDP-NOD2 pathway, which is dependent on Gram-positive bacteria, effectively reducing colitis in mice (Luo et al., 2021). Physalin B significantly improves the clinical symptoms and signs of DSS-induced UC mice, and reduces the loss of body weight as well as the shortening of colon length (Zhang et al., 2020).

Among these compounds, we were more interested in atractylodin, a natural compound obtained from Atractylodes, which is known for its anti-inflammatory effects. Intraperitoneal injection of atractylodin can significantly reduce the severity of disease progression in mice with rheumatoid arthritis, which is manifested by the reduction of paw swelling, clinical arthritis score, and histopathological changes in the joint (Chuang et al., 2019). Atractylodin not only significantly reduces the histopathological changes during lipopolysaccharide (LPS)-induced acute lung injury but also decreases myeloperoxidase activity, wet/dry weight ratio of the lung, protein leakage, and inflammatory cell infiltration (Tang et al., 2018). However, whether atractylodin can improve DSS-induced ulcerative colitis in mice and whether it can regulate the abundance of intestinal microorganisms have not been reported. It is also unclear whether the anti-inflammatory effect of atractylodin is related to the inhibition of macrophage activation. Therefore, we performed *in vivo* and *in vitro* studies by using a DSS-induced mouse model of UC and LPS-stimulated RAW264.7 cells, respectively, investigated the effects of atractylodin on macrophage activation, and evaluated if it could be a potential target for UC treatment.

MATERIALS AND METHODS

Reagents and Chemicals

Atractylodin (purity > 98%) was purchased from Chengdu Push Biotechnology Co., Ltd (Chengdu, China). Sulfasalazine was procured from Shanghai Sine Tianping Pharmaceutical Co., Ltd (Shanghai, China). DSS (36,000–50,000 Da) was obtained from MP Biomedicals (Solon, OH, United States). Dimethyl sulfoxide and LPS O55:B5 were purchased from Merck (Darmstadt, Germany). Mouse TNF-α ELISA kit was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Lactate assay kit was obtained from Nanjing Jiancheng Bioengineering Institute. GAPDH assay kit was purchased from GENMED Biotechnology Co., Ltd. (Shanghai, China). Pierce co-immunoprecipitation kit was purchased from Thermo Scientific (Waltham, MA, United States). Antibodies against P38 (#9212S), P-P38 (#4511S), P44/42 MAPK (ERK 1/2) (#4695S), P-P44/42 MAPK (T202/Y204) (#9101S), SAPK/JNK (#9252S), and P-SAPK/JNK (T183/Y185) (#4668S) were purchased from CST (Boston, MA, United States). Antibodies against GAPDH (#60004-1-Ig), IL-1β (#16806-1-AP), and IL-6 (#66146-1-Ig) were purchased from Proteintech (Wuhan, China). Antibodies against β-actin (#K200058^{M}) was purchased from Solarbio (Beijing, China). MUC2 antibodies (#ab97386) were purchased from Abcam Inc. (Cambridge, MA, United States). Antibodies against TNF-α (#AF7014) were purchased from Affinity (Liyang, China). Anti-Kmal antibody (#PTM-901) was purchased from PTM Biotechnology Co., Ltd. (Hangzhou, China).

Animal Experiments

Specific-pathogen-free male BALB/c mice (6–8-weeks old, 18–22 g) were purchased from the Experimental Animal Center of China Three Gorges University (Yichang, China)

(animal license No. SYXK (E) 2017–0,067). BALB/c mice were housed in SPF animal room with temperature of 24 ± 1°C and humidity of 50–70%. They were subjected to a 12 h:12 h light: dark cycle and allowed to acclimatize to their surroundings for one week. Forty mice were randomized into the following five groups: normal, model, atractylodin-10 mg/kg, atractylodin-20 mg/kg, and Sulfasalazine (SASP), with eight mice per group. From the first to the eighth day, the mice in the normal group were given free access to pure drinking water, whereas those in the other groups were provided access to a 3.5% DSS solution. The pure water and DSS solution were changed

2°h, incubated with the primary antibody overnight, and then incubated with the secondary antibody at room temperature for 2 h. An ECL chemiluminescence detection kit was used to detect the protein bands.

Enzyme-Linked Immunosorbent Assay

The cell-culture medium was collected and centrifuged at 1,000 x*g* for 20 min at 4°C. The supernatant was used to detect the concentration of TNF-α according to the manufacturer's instructions provided in the ELISA kit (Linghang et al., 2020).

Co-Immunoprecipitation

The cell protein lysates were collected, 3 μM trichostatin A (GLPBIO, Montclair, United States) and 50 mM nicotinamide (Merck, Darmstadt, Germany) were added for protein extraction. First, the GAPDH antibody was immobilized on the coupling resin and then the control agarose resin was used to pretreat the cell lysate. Next, the GAPDH-immobilized antibody was used for immunoprecipitation. After elution, the malonylated antibody was used for SDS-PAGE detection.

Determination of Lactate

The supernatants of RAW264.7 cells treated for 24 h and untreated cells were collected and centrifuged at 1,000 x*g* for 20 min at 4°

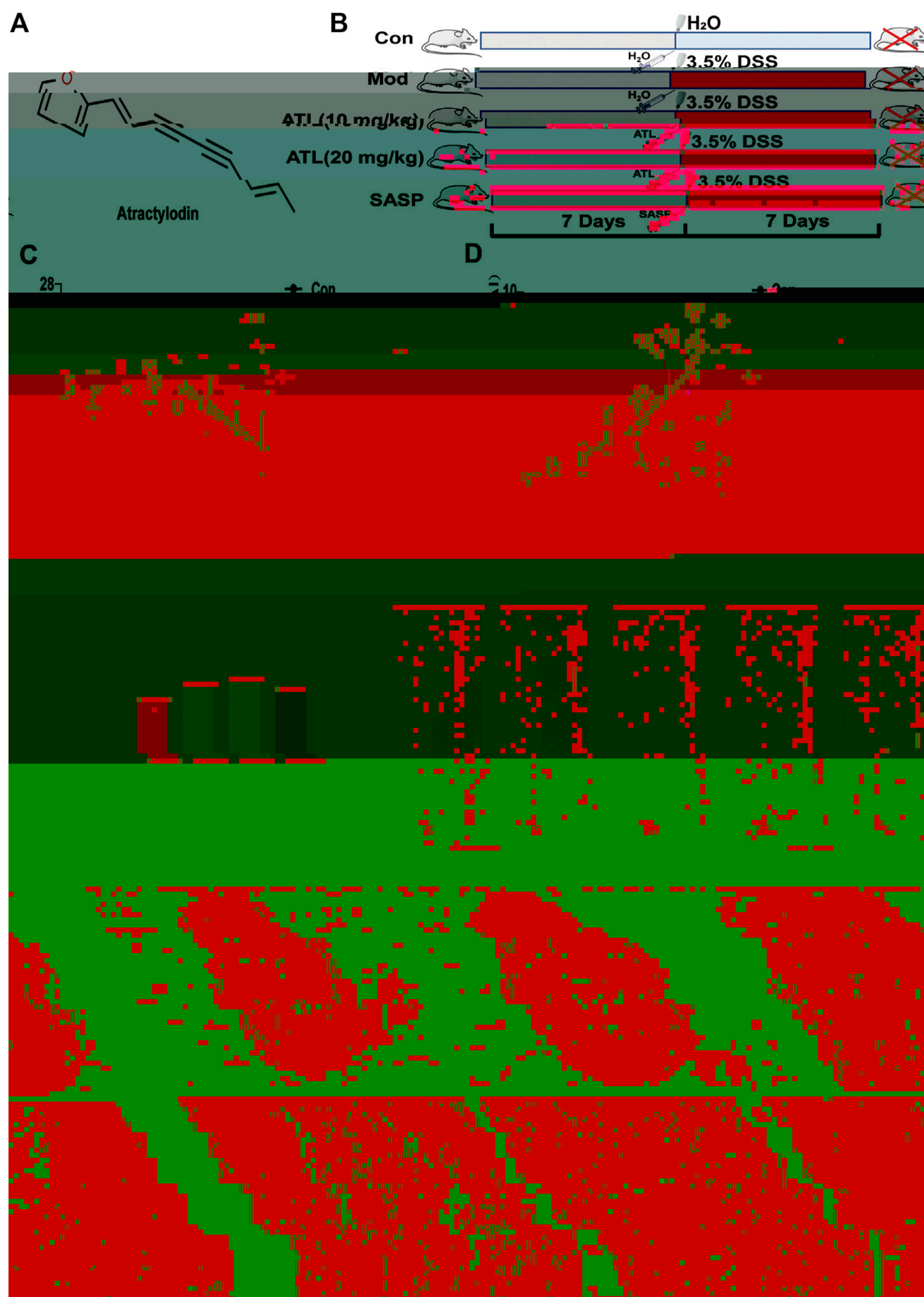

FIGURE 1 | Atractylodin (ATL) attenuates symptoms of dextran sulfate sodium-induced colitis in mice **(A)** Structure of ATL **(B)** Schematic diagram of animal experimental design. Control (CON), model (MOD), atractylodin (ATL), sulfasalazine (SASP) **(C)** Daily body weights of mice **(D)** Calculated disease activity index scores **(E,F)** Images of the intestine and statistics of colon length in each group **(G)** Representative HE-stained images of colon sections (× 400 magnification). Data are expressed as the mean ± SEM, $n = 8$. Data were analyzed using one-way ANOVA. #$p < 0.05$, ##$p < 0.01$ compared to the control group. *$p < 0.05$, **$p < 0.01$ compared to the model group.

group. After treatment with ATL (20 mg/kg), the number of colonic goblet cells increased significantly (**Figure 2A**). The expression of MUC2 in the colons of DSS-induced mice was found to be significantly decreased. After atractylodin (20 mg/kg) treatment, the expression of MUC2 in the colon increased significantly (**Figure 2B**). The expression of ZO-1 and occludin in the colons of DSS-induced mice decreased significantly. After treatment with ATL (20 mg/kg), the expression of tight junction proteins ZO-1 and occludin increased significantly (**Figures 2C,D**).

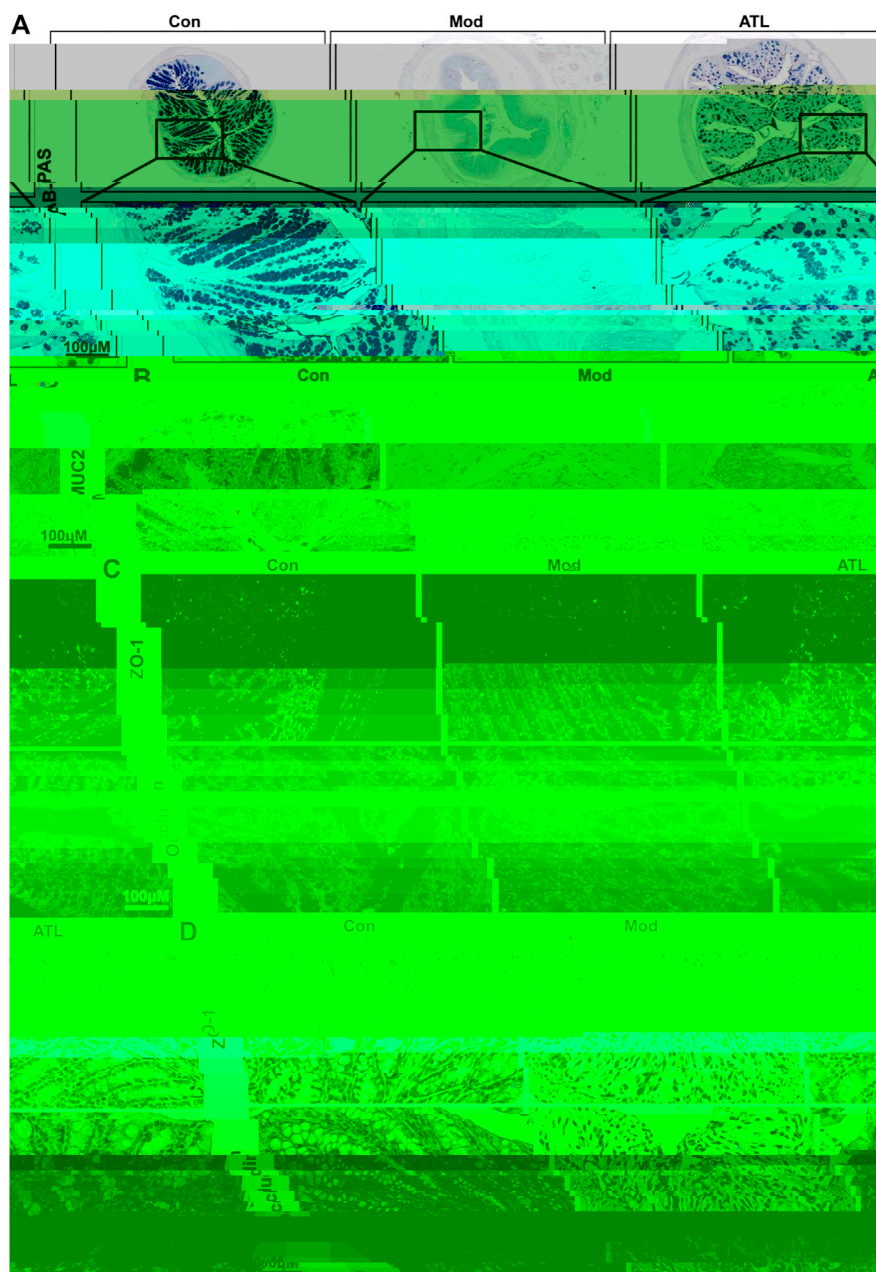

FIGURE 2 | ATL attenuates intestinal barrier of DSS-induced colitis in mice **(A)** Representative Alcian Blue-Periodic Acid Schiff (AB-PAS) images of colon sections **(B)** Representative immunostaining images of colon sections stained for Mucin-2 (MUC2) **(C)** Protein expression of zona occludens-1 (ZO-1) and occludin from colon tissue **(D)** Representative immunostaining images of colon sections stained for ZO-1 and occludin.

Atractylodin can Improve the Intestinal Inflammation of Mice With UC

The levels of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, were significantly increased in mice that consumed DSS-infused water. After treatment with ATL (20 mg/kg), the levels of the pro-inflammatory cytokines were found to be significantly decreased (**Figure 3A**). Moreover, in DSS-induced mice, F480 and iNOS were determined to be significantly increased, whereas these levels decreased significantly after atractylodin treatment (**Figure 3B**). In DSS-induced mice, expression of the MAPK pathway proteins, such as phosphorylated p38, phosphorylated JNK, and phosphorylated ERK, was significantly upregulated in the colon tissue (**Figure 3C**). After treatment with ATL (20 mg/kg), phosphorylated p38 (**Figure 3D**), phosphorylated JNK (**Figure 3E**), and phosphorylated ERK levels (**Figure 3F**) in the colon tissue were significantly downregulated.

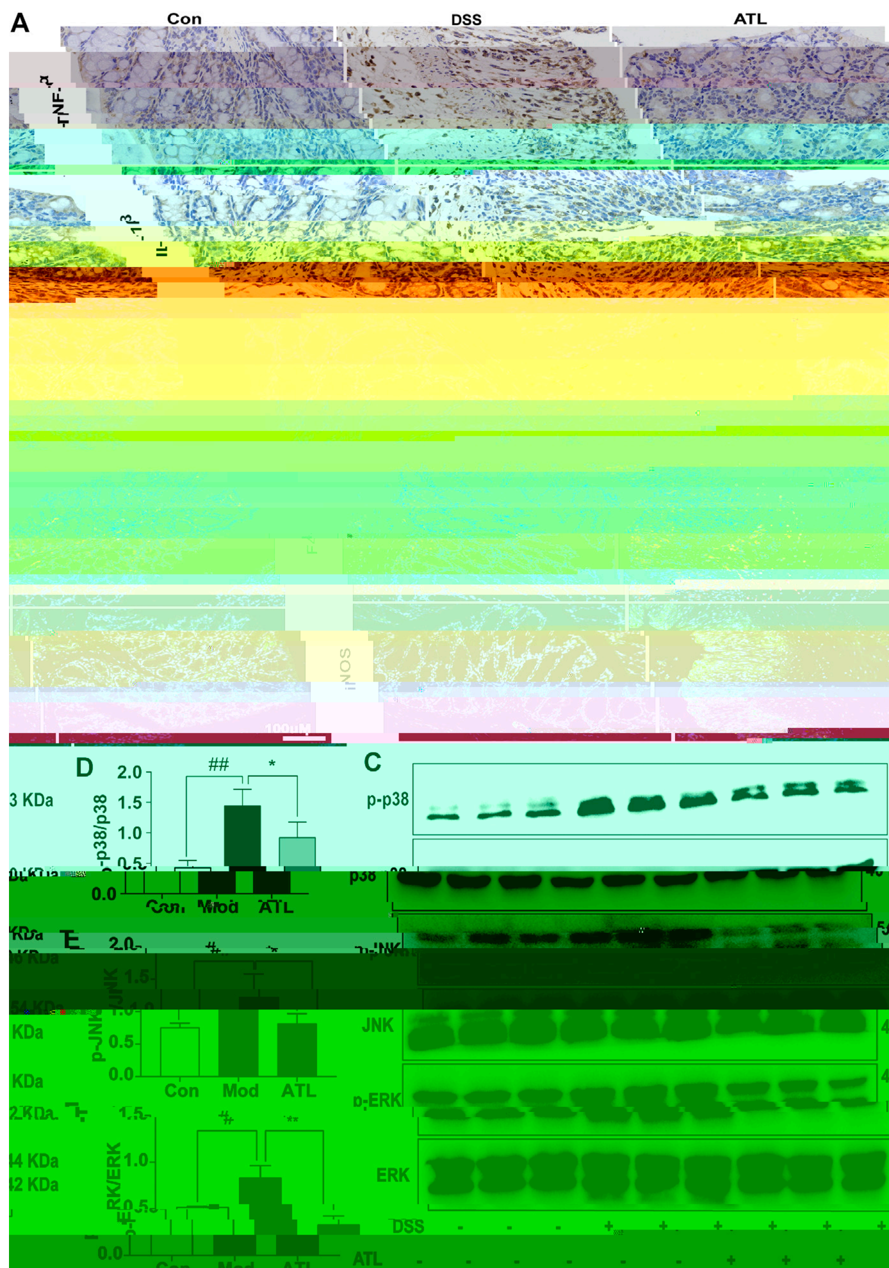

FIGURE 3 | ATL attenuates intestinal inflammation of DSS-induced colitis in mice **(A)** Immunohistochemistry of inflammatory cytokines (TNF-α, IL-6, and IL-1β) **(B)** Immunofluorescent staining of macrophages (F4/80, iNOS) **(C)** Representative western blots of p-p38, p38, *p*-JNK, JNK, *p*-ERK, and ERK in colon tissue **(D–F)** Relative density of each signaling band was calculated. Data are expressed as the mean ± SEM, $n = 3$. Data were analyzed using one-way ANOVA. #$p < 0.05$, ##$p < 0.01$ compared to the control group. *$p < 0.05$, **$p < 0.01$ compared to the model group.

Atractylodin can Inhibit the MAPK Pathway and the Expression of Inflammatory Factors in Macrophages

Cell viability assays showed that atractylodin was not cytotoxic to RAW264.7 cells at a concentration of 0–40 μM (**Figure 4A**). The mRNA levels of TNF-α, IL-1β, IL-6, and iNOS in RAW264.7 cells stimulated by LPS (100 ng/ml) for 24 h were significantly increased. The expression of IL-1β, IL-6, and iNOS decreased by varying degrees after 24 h of atractylodin intervention (**Figures 4B,C–E**). The MAPK pathway proteins, including phosphorylated p38, phosphorylated JNK, and phosphorylated ERK, in the LPS-induced macrophages were upregulated (**Figure 4F**). After treatment with different concentrations of atractylodin, phosphorylated p38 (**Figure 4G**), phosphorylated JNK (**Figure 4H**), and phosphorylated ERK (**Figure 4I**) in the macrophages were downregulated.

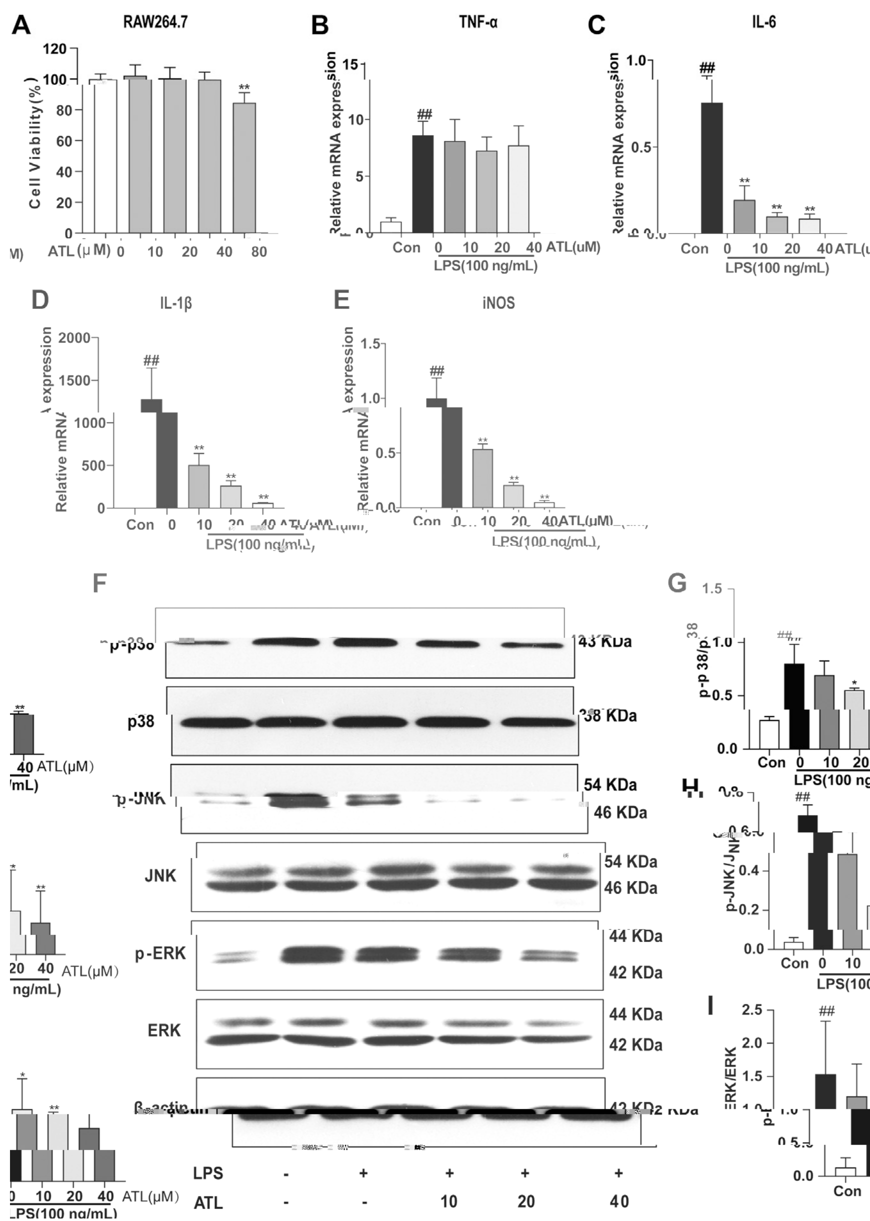

FIGURE 4 | ATL can inhibit the MAPK pathway and expression of inflammatory factors in macrophages **(A)** Cell viability of RAW264.7 cells treated without or with different concentrations of ATL (B–E) mRNA levels of tumor necrosis factor alpha (TNF-α) **(B)** interleukin 6 (IL-6) **(C)**, interleukin-1*β* (IL-1*β*) **(D)** and inducible nitric oxide synthase (iNOS) **(E)** in RAW264.7 cells subjected to LPS (lipopolysaccharide) (100 ng/ml) stimulation and ATL treatment **(F)** Representative western blots of p-p38, p38, *p*-JNK, JNK, *p*-ERK, and ERK in RAW264.7 cells **(G–I)** Relative density of each signaling band was calculated. Data were obtained from three independent experiments and are presented as the mean ± SEM. Data were analyzed using one-way ANOVA, $n = 3$. #$p < 0.05$, ##$p < 0.01$ compared to the control group. *$p < 0.05$, **$p < 0.01$ compared to the LPS (100 ng/ml) + 0 μM ATL group.

Atractylodin Can Target GAPDH and Inhibit the Inflammatory Process

TNF-α was found to be highly expressed in the supernatant and lysate of LPS-treated macrophages; however, its levels were decreased by varying degrees after treatment with different concentrations of atractylodin. The expression of TNF-α protein significantly decreased after treatment with 20 μM ($p < 0.05$) atractylodin and significantly decreased at 40 μM ($p < 0.01$) (**Figures 5A,B**) of atractylodin. Lactate levels in the supernatant of the LPS-treated macrophages were found to be significantly increased, whereas treatment with different concentrations of atractylodin led to a decrease. Lactate levels were significantly decreased after treatment with 20 μM ($p < 0.05$) of atractylodin and extremely significantly decreased upon treatment with 40 μM ($p < 0.01$) of the compound (**Figure 5C**). Molecular docking experiments with atractylodin and GAPDH revealed binding free energy of 5.42 kcal/mol (**Figure 5D**)

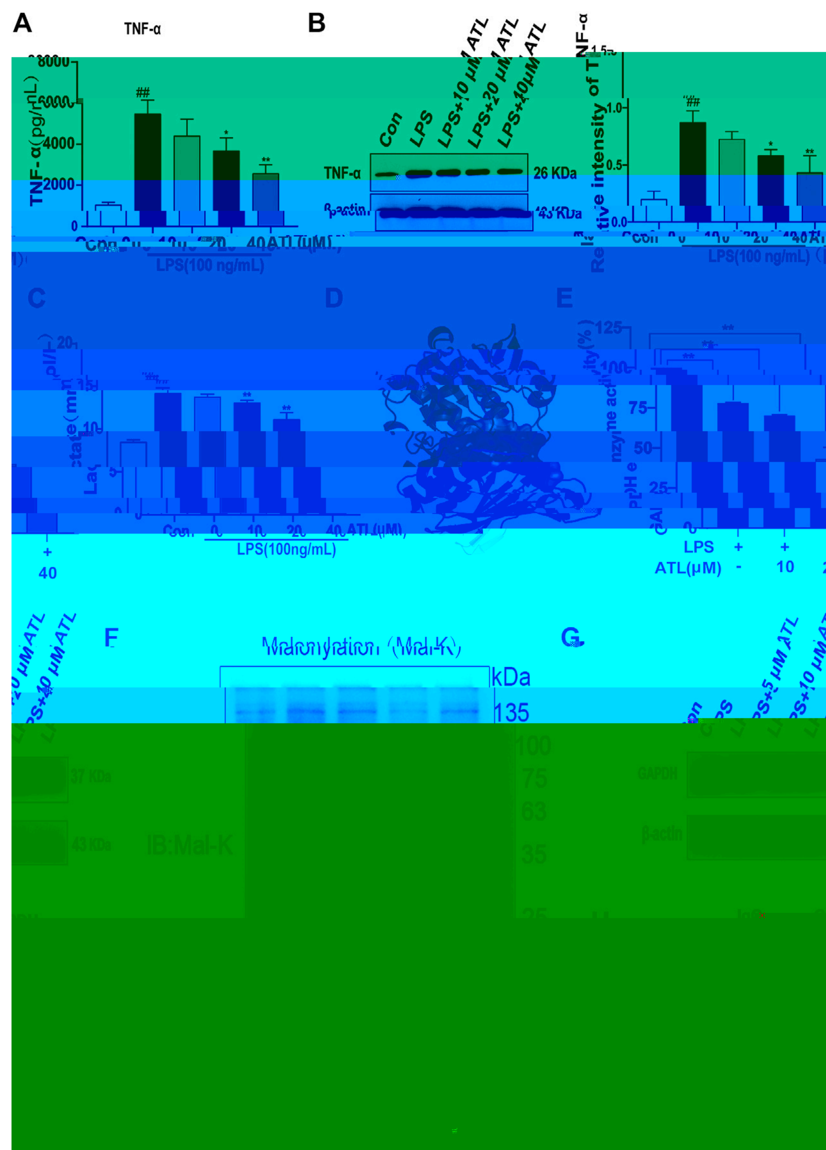

FIGURE 5 | ATL targets GAPDH to inhibit the inflammatory process **(A)** TNF-α levels in the supernatant of RAW264.7 cells were measured using ELISA (enzyme linked immunosorbent assay) **(B)** TNF-α levels in the lysate of RAW264.7 cells were measured using western blotting **(C)** Lactate levels in the supernatant of RAW264.7 cells were measured using a lactate assay kit **(D)** Molecular docking of ATL with GAPDH **(E)** GAPDH enzyme activity in the lysate of RAW264.7 cells was measured using a GAPDH enzyme activity kit **(F)** Western blot analysis to determine lysine malonylation (mal-K) in the lysates of RAW264.7 treated with LPS (100 ng/ml) for 24 h **(G)** GAPDH expression in LPS-treated RAW264.7 cells was analyzed using western blotting **(H)** Immunoprecipitated GAPDH from the control, LPS-treated (100 ng/ml), and LPS (100 ng/ml) + ATL (20 μM)-treated RAW264.7 cells and samples probed with an anti-mal-K antibody (lower panel). GAPDH expression in the immunoprecipitated (upper panel) samples was also examined. Data were obtained from three independent experiments and are presented as the mean ± SEM. Data were analyzed using one-way ANOVA, $n = 3$. #$p < 0.05$, ##$p < 0.01$ compared to the control group. *$p < 0.05$, **$p < 0.01$ compared to the LPS (100 ng/ml) + 0 μM ATL group.

and suggested that GAPDH was likely an anti-inflammatory target of atractylodin. We investigated the effects of atractylodin on GAPDH activity and found that its activity decreased gradually with an increase in atractylodin concentration (**Figure 5E**). However, different concentrations of atractylodin did not affect the expression of the GAPDH protein (**Figure 5G**). In LPS-treated macrophages, malonylation of the total protein was significantly enhanced, and we found that atractylodin could inhibit this process (**Figure 5F**). Results from the immunoprecipitation assay revealed that atractylodin could significantly inhibit malonylation of the GAPDH protein (**Figure 5H**).

Atractylodin Can Improve the Intestinal Microbiota of Mice With DSS-Induced Ulcerative Colitis

The cumulative species curve revealed that nearly 5,000 species of intestinal microorganisms were present in the three samples, which

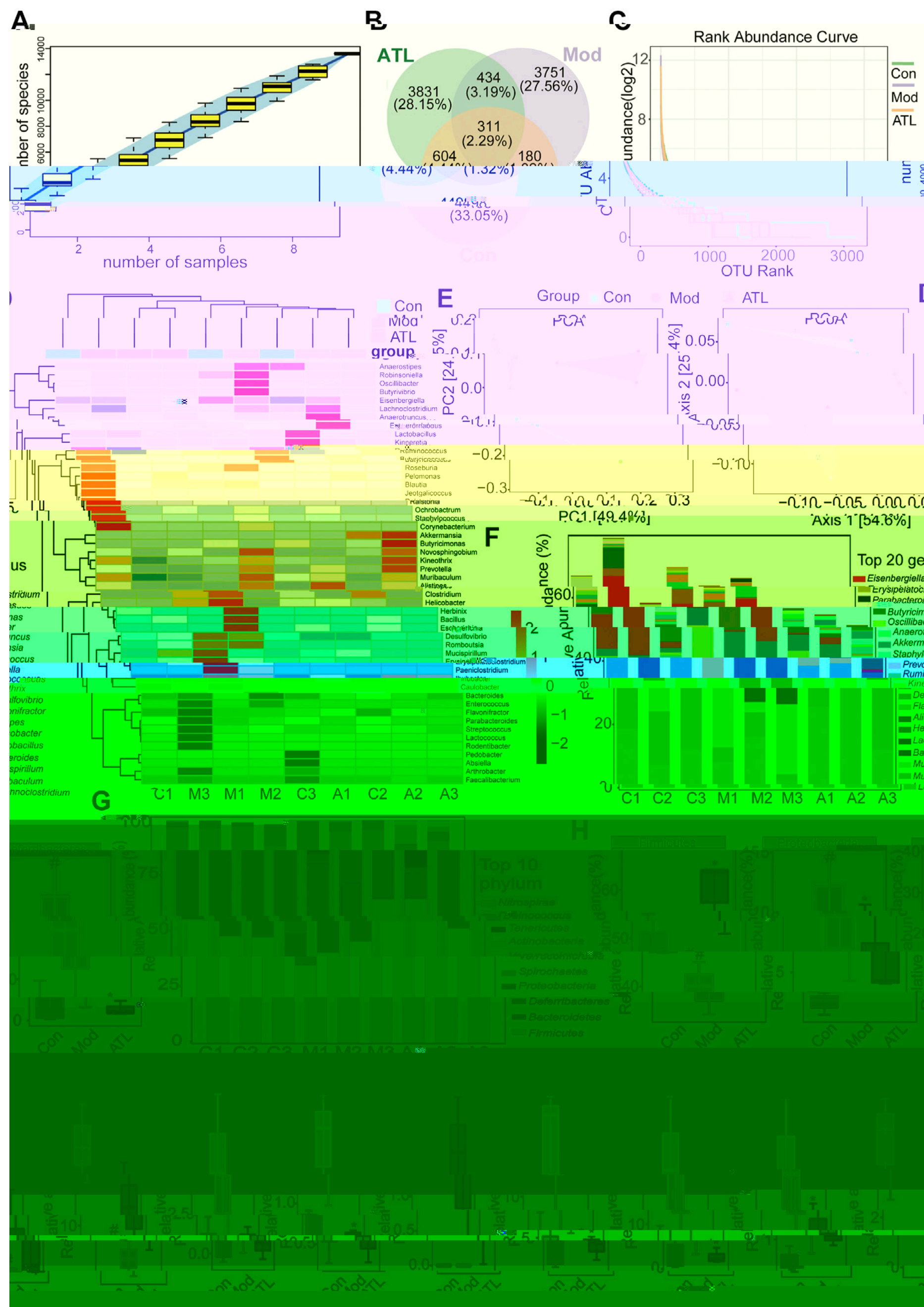

FIGURE 6 | ATL can improve the intestinal microbiota of DSS-induced colitis in mice **(A)** Sample number and species richness were estimated from a species accumulation boxplot **(B)** Venn diagram of species **(C)** Abundance grade curve of species level **(D)** Heatmap demonstrating the relative abundance of intestinal bacteria at the genus level **(E)** PCA (principal component analysis) and PCoA (principal coordinates analysis) demonstrated distinct structural changes in the overall bacterial community for each group **(F)** Microbial community bar plot at the genus level **(G)** Microbial community bar plot at the phylum level **(H)** Relative abundance of different microbial flora at the phylum level and the genus level **(I)** Data were obtained from three independent experiments and are presented as the mean

could essentially cover the common species (**Figure 6A**). The number of common intestinal bacteria in the normal and atractylodin (20 mg/kg) group was significantly higher than that in the normal and model group (**Figure 6B**). The grade curve at the species level showed that the abundance of the intestinal microflora was the most in the normal group and the least in the model group. The abundance of intestinal microflora in the atractylodin (20 mg/kg) group was midway between the normal and model groups (**Figure 6C**). Cluster analysis revealed that the microflora composition of the normal and model groups was significantly separated, whereas that of the

atractylodin (20 mg/kg)-treated groups was close to the composition of the normal group (**Figure 6D**). Principal component analysis and principal coordinate analysis showed that the distribution of intestinal flora in the normal and model groups was well separated, whereas the atractylodin (20 mg/kg) and normal groups were closely clustered (**Figure 6E**). The distribution maps of the top 20 genera and top 20 phyla with the highest abundance among different groups of mice in this study are shown in **Figures 6F,G**, respectively. The abundance of *Firmicutes* decreased significantly after DSS treatment but increased significantly in the atractylodin (20 mg/kg) group. After DSS treatment, the abundance of Proteobacteria and Deferribacteres increased significantly, whereas treatment with atractylodin (20 mg/kg) resulted in a significant decrease in their abundance (**Figure 6H**). After DSS modeling, the abundance of pathogenic bacteria, including *Helicobacter, Desulfovibrio, Bacteroides, Flavonifractor, and Mucispirillum* increased significantly, while that of beneficial bacteria, including *Alistipes and Muribaculum,* decreased significantly. Atractylodin (20 mg/kg) treatment not only decreased the abundance of these pathogenic bacteria but also increased the abundance of beneficial bacteria including *Akkermansia* (**Figure 6I**).

DISCUSSION

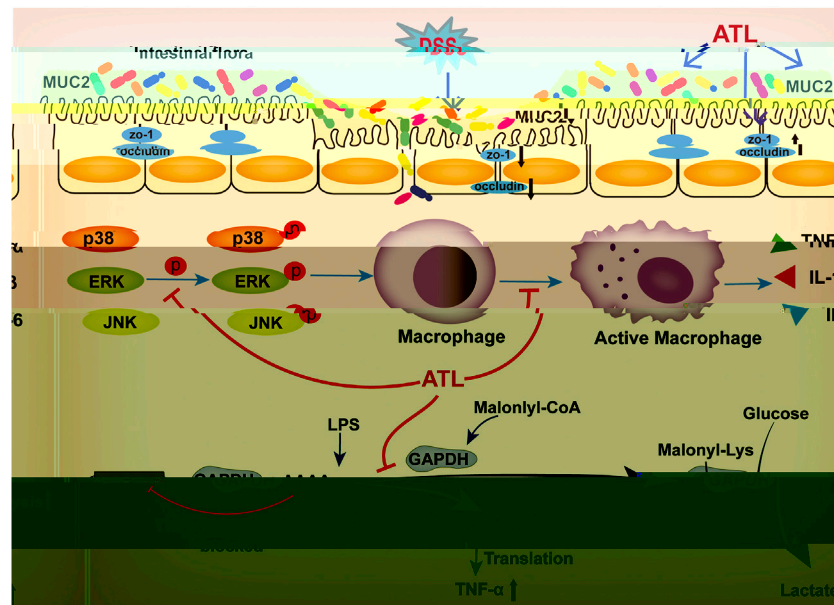

FIGURE 7 | Atractylodin attenuates dextran sulfate sodium-induced colitis by alleviating gut microbiota dysbiosis and inhibiting inflammatory response through the MAPK pathway. The mechanism could likely be attributed to the effect of atractylodin in inhibiting the MAPK pathway; inhibiting the activation of macrophages in colon tissue; inhibiting the decrease in tight junction proteins, ZO-1, occludin, and MUC2 to protect the intestinal barrier; decreasing the abundance of harmful bacteria and increasing that of beneficial bacteria, thereby improving the overall intestinal microflora. *In vitro* experiments revealed that atractylodin could inhibit the MAPK pathway and macrophage activation. Atractylodin could not inhibit the transcription of TNF-α but could inhibit its translation, which might be related to its effect of inhibiting the malonylation of the GAPDH protein.

UC in patients, as increased microbial groups of Firmicutes are mostly related to healthy intestines (Schierová et al., 2020). Proteobacteria is one of the larger bacterial communities, which includes several pathogenic bacteria, such as *Escherichia coli*, *Salmonella*, *Vibrio cholerae*, and *Helicobacter pylori* among others. The proportion of Proteobacteria in patients with ulcerative colitis is known to increase significantly (Xu et al., 2021). Adhesively invasive *E. coli* increased the abundance of Proteobacteria and Deferrobacter in a porcine model of experimental colitis and these findings were similar to that observed in patients with IBD (Munyaka et al., 2016). The results of our study indicated that atractylodin could significantly increase the abundance of Firmicutes and significantly reduce that of *Proteus* and *Deferrobacteria*. At the genus level, atractylodin could significantly inhibit the abundance of pathogenic bacteria, such as *Helicobacter*, *Vibrio desulfuricus*, *Bacteroides*, *Flavobacterium, and Mucispirillum*, and increase that of beneficial bacteria including *Alistipes*, *Muribaculum*, and *Akkermansia*.

To summarize, atractylodin could significantly improve the progression of DSS-induced ulcerative colitis. The mechanism could likely be attributed to the effect of atractylodin in inhibiting the MAPK pathway; inhibiting the activation of macrophages in colon tissue; inhibiting the decrease in tight junction proteins, ZO-1, occludin, and MUC2 to protect the intestinal barrier; and decreasing the abundance of harmful bacteria and increasing that of beneficial bacteria, thereby improving the overall intestinal microflora. *In vitro* experiments revealed that atractylodin could inhibit the MAPK pathway and macrophage activation. Moreover, we found that atractylodin could not inhibit the transcription of TNF-α but could inhibit its translation, which might be related to its effect of inhibiting the malonylation of the GAPDH protein (**Figure 7**). Overall, the findings of our study indicated that atractylodin may be a potentially effective compound in the management of ulcerative colitis and, therefore, warrants further exploration.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Sequence Read Archive (SRA) 417 database of NCBI repository, accession number PRJNA715347.

ETHICS STATEMENT

The animal study was reviewed and approved by the Hubei University of Chinese Medicine Animal Ethics Committee with respect to ethical issues and scientific care.

AUTHOR CONTRIBUTIONS

LQ, GC, and YL designed the experiments. XL, LQ, and CL performed animal studies. LQ, XL, CK, and GC performed molecular biology experiments. LQ, KX, and ZZ analyzed the

16S rRNA gene-sequencing data. LQ, GC, and YL prepared the manuscript and were primarily responsible for the final content. All authors read and approved the final manuscript.

FUNDING

This research was funded by National Natural Science Foundation of China (No. 82074018) and Major Special Projects of Science and Technology of Hubei Province (No. 2020ACA007).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.665376/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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