Moringa oleifera Lam. Peptide Remodels Intestinal Mucosal Barrier by Inhibiting JAK-STAT Activation and Modulating Gut Microbiota in Colitis

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Ulcerative colitis is a chronic inflammatory bowel disease (IBD), but progress in exploring its pathogenesis and finding effective drugs for its prevention and treatment has stalled in recent years. The seeds of Moringa oleifera Lam. are rich in proteins known to have multiple physiological activities. In our earlier work, we had isolated and purified a peptide (MOP) having the sequence KETTTIVR, from M. oleifera seeds; however, its anti-inflammatory activity and mechanism in vivo were unclear. Here we used the dextran sulfate sodium (DSS)-induced colitis model to study the anti-inflammatory activity and mechanism of this MOP. Our results are the first to show that MOP can ameliorate the pathological phenotype, inflammation, and intestinal barrier disruption in mice with colitis. Furthermore, RNA sequencing revealed that MOP inhibits the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway activation. Next, by using 16s rRNA gene sequencing, we found that MOP can ameliorate DSS-induced gut microbiota dysbiosis. In addition, an untargeted metabolomics analysis suggested that MOP is able to regulate the level of lipid and amino acid metabolites in IBD-stricken mice. Altogether, these results indicate that MOP ameliorates colitis by remodeling intestinal mucosal barrier by inhibiting JAK-STAT pathway’s activation and regulating gut microbiota and its metabolites, thus providing a basis for further processing and design of bioactive foods from M. oleifera seeds.

Keywords: Moringa oleifera seed, peptide, colitis, intestinal mucosal barrier, gut microbiota, JAK-STAT pathway
INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestines, including Crohn’s disease (CD) and ulcerative colitis (UC) (1, 2). Worryingly, in recent years the prevalence and incidence of IBD have both increased annually (1, 3). In addition, patients who have had IBD for many years face higher risks of developing related cancers, which can adversely affect their body and mind (4, 5). Traditional drugs for treating IBD, including 5-aminosalicylic acid (5-ASA), glucocorticoids or immunomodulators, can relieve its progression but these drugs may have significant side effects (6). Therefore, it is imperative to explore natural products and their derivatives as support for the treatment and management of IBD.

The intestinal mucosal barrier consists of mechanical barrier, chemical barrier, microbial barrier and immune barrier (7). Notably, the integrity of physical barriers in the gut is regulated in part by cytokines produced by gut-resident cell (8, 9). Those cytokines that play key roles in IBD exert biological effects by inducing activation of Janus kinase/signal transducer and activator of transcription (JAK-STAT) by binding to cytokine receptor (10, 11). Moreover, there is growing evidence suggests that an imbalance between cytokines and excessive activation of the JAK-STAT pathway leads to gut barrier disruption, disease perpetuation and tissue destruction (8, 12). Therefore, inhibiting activation of the JAK-STAT pathway may be an effective way to impair intestinal inflammation and improve the intestinal barrier.

Gut microbiota is one of the major components of the intestinal mucosal barrier (13, 14). A large body of evidence suggests that changes in the composition and function of gut microbiota are key factors directly contributing to the onset and progression of IBD (15), and therefore targeting the gut microbiota may be a novel strategy for the treatment of IBD. Furthermore, bioactive metabolites derived from host and gut microbiota, such as short-chain fatty acids, amino acids, choline derivatives, and indole derivatives, are important molecules that orchestrate the interaction between gut microbiota and host medium (16, 17). Notably, these metabolites have been reported to play an indirect role in remodeling the intestinal barrier by modulating signal transduction and immune responses (18).

Moringa oleifera Lam., in the plant family Moringaceae, has various pharmacological potential and health benefits (19, 20). In particular, studies have shown that M. oleifera seeds are rich in protein and harbor a variety of therapeutic properties, namely antibacterial, anti-oxidative stress, anti-inflammatory, and anti-cancer activities (21–23). Although much research has investigated the biological activity of M. oleifera seeds, far fewer studies have considered the proteins in these seeds, and even less is known about their peptides. According to many studies, peptides can have immunomodulatory effects and may ameliorate colitis injury by reducing inflammatory cell infiltration, improving intestinal barrier, and modulating host gut microbiota (24, 25). Currently, it is unclear whether M. oleifera seed-derived peptides are also anti-inflammatory, and if so, whether they modulate gut microbiota and regulate metabolism in vivo to exert their anti-inflammatory effects in vivo, especially vis-à-vis acute colitis. In earlier work, we had identified the ultrafiltration peptide components (i.e., < 3 kDa) of M. oleifera seeds protein hydrolysates, finding a highly active α-glucosidase inhibitory peptide with the amino acid sequence KETTITVR that also exhibited anti-inflammatory activity in vitro (26). Yet whether it has anti-UC activity in vivo and its mechanism of action remain unclear.

In this study, we demonstrate, for the first time to our best knowledge, the beneficial effects of an active peptide (KETTITIVR, MOP) identified by our team from M. oleifera seeds in mice with ulcerative colitis and explored the mechanisms by it ameliorates colitis.

RESULTS

MOP Improved the Pathological Phenotype of DSS-Induced Colitis

To investigate the effect of the MOP on dextran sulfate sodium (DSS)-induced colitis, colitis was induced in mice by continuous administration of 3% DSS in water for 10 days, with different doses of MOP supplemented to them during DSS induction (Figure 1A). Compared with the DSS group, DSS-induced colitis was significantly reduced in DSS + H MOP group, as evinced by their significant weight loss (Figure 1B), reduced DAI scores (Figure 1C), and remission of colonic shortening (Figure 1D). Moreover, an enlarged spleen was observed in colitis mice whereas the MOP treatment was capable of significantly relieving that splenomegaly condition, particularly in the high dosage group (Figure 1E). We next examined the level of myeloperoxidase (MPO) in serum, finding it was significantly higher in DSS-induced colitis mice than the control group, whereas the high dosage of MOP intervention reversed this abnormal change (Figure 1F). Histological analysis uncovered colonic mucosal damage in colitis mice. Importantly, the high dosage of MOP intervention significantly reduced such mucosal damage (including the greater crypt depth) and inflammatory cell infiltration, resulting in lower histological scores (Figures 1G–I). These results suggested the MOP treatment significantly improved DSS-induced colitis.

MOP Improved the Colon Inflammation and Gut Barrier Disruption of DSS-Induced Colitis

To further estimate the influence of MOP upon inflammation in colitis, we detected the levels of TNF-α, IL-1β, IL-6, and IL-10 in the serum from the control, DSS, and DSS + H MOP groups (Figures 2A–D). Higher levels of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 and lower levels of anti-inflammatory cytokine IL-10 were found in colitis mice when compared with the control group. By contrast, MOP reversed these changes to cytokines in serum. Consistently, we found that MOP decreased the expression level of TNF-α, IFN-γ, IL-1β, and IL-6 but increased that of IL-10 in colon tissue (Supplementary Figures 2A–E).
To assess how MOP may affect the DSS-induced colonic inflammatory infiltration, colon tissue sections were stained for specific markers of macrophages (F4/80) and neutrophils (LY6G). We found that the DSS group displayed macrophage and neutrophil infiltration, as evinced by its significantly increased counts of F4/80 and Ly6G-positive cells. Conversely, MOP intervention evidently reduced the infiltration of macrophages and neutrophils (Figures 2E–H).

Furthermore, alisin blue staining results revealed fewer mucus-secreting goblet cells in the DSS group than the control group. In stark contrast, the MOP intervention reversed this reduction of goblet cells as well as damage to the colon crypt (Figures 2I, J). In line with these findings, the expression level for the gene encoding mucin-2 (Muc-2) in colon tissue showed the same trend (Supplementary Figure 2F). To evaluate the expression of antibacterial peptides in colitis, we examined the expression levels of antimicrobial peptide genes in colon tissue, finding that the MOP treatment reversed the DSS-induced decrease in mRNA expression levels of both Reg3b and Reg3g (Supplementary Figures 2G, H).

To further study MOP’s effect on the gut barrier, we examined the mRNA expression levels of zonula occludens 1 (ZO-1), occludin, and claudin-1 in mice colon tissue. Their expression levels were significantly lower in the colitis group than the control group. But the MOP administration significantly reversed the DSS-induced downregulation in the levels of...
FIGURE 2 | MOP ameliorates colonic inflammation and intestinal barrier disruption in DSS-induced colitis. (A–D) Expression levels of TNF-α, IL-6, IL-1β, and IL-10 in serum determined by ELISA (n = 8); (E, F) Representative immunohistochemical images of macrophages (F4/80) in mouse colon sections (scale bar = 50 μm, n=8) and the number of positive cells (n = 23); (G, H) Representative images of immunohistochemistry of neutrophils (Ly6G) in mouse colon sections (scale bar = 50 μm, n=8) and the number of positive cells (n = 20); (I, J) Representative images of Alcian blue-stained colon sections (scale bar = 50 μm, n=8) and the number of mucus-producing cupped cells (n = 40). (K) Representative images of ZO-1 and occluding, and representative protein blot images of claudin-1, with their associated protein expression normalized to β-tubulin. Quantification of immunoblots, performed in Image J software (n = 3). Data are the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical analysis was performed using Student’s t-test.
occludin, ZO-1, and claudin-1 (Supplementary Figures 2I–K). To confirm this pattern in gene expression, we used western blotting to assess the protein expression of ZO-1, occludin, and claudin-1; these results were consistent with those of their genes’ mRNA expression (Figure 2K). Collectively, these results suggested that MOP treatment suppressed DSS-induced colonic inflammation and attenuated the DSS-impaired functioning of the intestinal mucosal barrier.

MOP Ameliorates Colitis by Inhibiting Activation of the JAK-STAT Pathway

To determine the underlying biological processes and pathways by which MOP ameliorates DSS-induced colitis in mice, RNA-Seq analyses were conducted. Significant differences in transcriptional profiles were detected among control, DSS, and DSS+H MOP mice groups (Figures 3A, B). Specifically, relative to the control group, DSS caused the upregulation of 2274 genes and the downregulation of 1280 genes in the mouse colon (Supplementary Figures 3A, B). In addition, a total of 621 and 1873 genes were respectively upregulated and downregulated in the DSS+H MOP group compared with the DSS group (Supplementary Figure 3C). The follow-up GO enrichment analysis indicated that MOP modulates the immune response of DSS-induced colitis (Figure 3C). Interestingly, the KEGG pathway analysis of the differentially expressed genes (DEGs) indicated that the JAK-STAT pathway was a highly enriched functional pathway (Figure 3D). Heatmap results showed that DSS induced the upregulation of JAK-STAT pathway-related genes’ expression in mice with colitis, but the MOP administration significantly downregulated it (Figure 3E). This suggested the MOP intervention could have exerted its anti-inflammatory effects by inhibiting the JAK-STAT signaling pathway.

To further confirm whether or not MOP inhibits the JAK-STAT pathway, RT-qPCR was used to check the expression levels of key genes operating in this pathway (Figure 3F). The results revealed augmented levels of their expression in the DSS group vis-à-vis the control group. As expected, in the DSS+H MOP group the increasing expression of these genes involved in the JAK-STAT pathway was reversed. Consistent with that, the western blotting results showed that phosphorylation levels of JAK2 and STAT3 were significantly increased in DSS-induced colitis mice, and MOP administration reversed this trend (Figure 3G). Overall, MOP suppressed inflammatory responses by participating in DSS-induced immunomodulation and inhibiting the JAK-STAT pathway in mice with colitis.

MOP Could Alleviate Gut Microbiota Dysbiosis in Colitis Induced by DSS

To investigate whether MOP can alleviate DSS-induced gut microbiota dysbiosis, we used 16S rRNA gene amplicon sequencing to profile the composition of mouse gut microbiota. These results showed that MOP ameliorated the DSS-induced reduction in gut microbiota richness and diversity in mice with colitis, as seen in the changed Chao, Shannon, and Simpson indices (Figures 4A–C). The Bray-Curtis-based PCoA (principal coordinates analysis) revealed a significant difference between the control and DSS groups ($R_2 = 0.393, P = 0.001$, Figure 4D). Further, gut microbiota structures were also segregated between the DSS+H MOP and control groups ($R_2 = 0.3693, P = 0.001$) and likewise for the DSS and DSS+H MOP groups ($R_2 = 0.2453, P = 0.001$). Next, we performed a LEfSe analysis of the differential gut microbiota found between the three groups at the taxonomic level, from the phylum to genus (Supplementary Figure 4). These results indicated that Escherichia-Shigella (from the Proteobacteria phylum to genus), Bacteroides (from the Bacteroidetes family to genus), and Clostridium_sensu_stricto_1 (from the Clostridium phylum to genus) were the dominant microbial taxa in the DSS group, while Blautia and Oscillibacter (from Firmicutes phylum to genus) were relatively enriched in the DSS+H MOP group.

To further investigate the possible impact of MOP on the gut microbiota of mice with colitis, we analyzed different taxa in the three experimental groups. At the phylum level, Firmicutes, Bacteroidetes, Verrucomicrobia, and Proteobacteria were the major phyla dwelling in the cecal contents microbiota (Figure 4E). At the family level, in the DSS group the relative abundance of Enterobacteriaceae increased while that of Lachnospiraceae as well as Oscillospiraceae decreased, being characteristic of gut dysbiosis that could be reversed by the MOP treatment (Figure 4F). The changed microbiota at the genus level are depicted in Figure 4G. Compared with the control group, the relative abundances of Bacteroides, Escherichia-Shigella, and Enterococcus increases in colitis mice. However, MOP treatment restored the relative abundances of these various bacteria. It is crucial to note that MOP caused an enrichment of Lachnospiraceae_NK4A136_group, norank_f: Lachnospiraceae, Blautia, Ruminococcus, and unclassified_f: Ruminococcaceae, these belonging to Lachnospiraceae and Ruminococcaceae, respectively.

To understand the associations between differentially enriched microbes and inflammatory factors, intestinal barrier, and antimicrobial peptides, Spearman correlations were performed (Figures 4H, I). These results showed that Eubacterium_fissicatenan_group, Enterococcus, Eubacterium_nodatum_group, Bacteroides, Escherichia-Shigella, and Clostridium_sensu_stricto_1 each had a strong positive correlation with the TNF-$\alpha$, IL-1$\beta$, IL-6, and MPO levels in serum, as well as the TNF-$\alpha$ level in colon. However, those six taxa had negative correlations with the mRNA IL-10 levels ($P < 0.05$) in serum, and likewise with colonic barrier proteins ZO-1, occludin ($P < 0.01$), claudin-1 ($P < 0.001$), mucin Muc-2, and antimicrobial peptides Reg3g, Reg3b ($P < 0.01$) levels in the colon, respectively. In addition, Lachnospiraceae_NK4A136_group, Ruminococcus, norank_f: Lachnospiraceae, unclassified_f: Ruminococcaceae, and Family_XIII_UCG-001, were negatively correlated with TNF-$\alpha$, IL-1$\beta$, IL-6, and MPO levels in the serum, yet positively correlated with IL-10 in the serum, ZO-1, occludin and claudin-1, Muc-2, and Reg3g, Reg3b in the colon. Altogether, these results suggested that MOP can alleviate DSS-induced gut microbiota dysbiosis and increased the relative abundance of Lachnospiraceae and Ruminococcaceae members in particular.
MOP Alters Metabolic Profiles and Regulates the Metabolism of Lipids and Amino Acids

To investigate the effect of MOP on the metabolic profile of colitis mice, we analyzed plasma samples via untargeted metabolomics. The resulting PLS_Da plot showed that the metabolic profiles of DSS-induced colitis mice and the control group differed significantly, in terms of both negative and positive ion mode, while the MOP intervention group was also significantly different from the DSS group (Figures 5A, B). The resulting PCA plot also indicated a satisfactory classification among the three groups (Supplementary Figures 5A, B). We
FIGURE 4 | MOP modified the dysbiosis of intestinal microbiota in DSS-induced colitis. (A–C) Alpha diversity index analysis of intestinal microbiota abundance (Chao index) and diversity (Shannon and Simpson indexes). (D) Principal coordinate analysis (PCoA) plots of Bray–Curtis distance matrix for the composition of gut microbiota of different groups at the ASV level. Community structure composition of different microbial groups, at the taxonomic level of phylum (E) and family (F). (G) Comparison of relative abundances at the genus level among the three experimental groups (n = 7). (H) Spearman correlations between gut microbiota and inflammatory factors and MPO enzyme activity in mice serum parameters (n = 7). (I) Spearman correlations between intestinal microbiota and parameters of inflammatory genes, intestinal barrier genes, and antimicrobial peptide genes in mouse colon tissue (n = 6). The red coloring indicates positive correlations, while the blue coloring indicates negative correlations. The intensity of the color is proportional to value (strength) of the Spearman correlation coefficient. Data are the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
detected 325 different metabolites between the three groups of mice (P < 0.05, \textit{Supplementary Table S1}). According to the heatmap analysis of significantly changed differential metabolites (\textit{Figure 5C}), phosphatidylcholine (PC; 18:1/22:6, 14:0/0:0, 16:0/18:3, 16:1/22:6), lysophosphatidylcholine (LPC; 18:2, 16:0), and LysoPC (15:0, 16:1/0:0) in the DSS group—as previously reported by Tefas et al. (27)—being significantly lower than the control group; however, the MOP treatment significantly reversed the trend of decreasing these metabolites, which is striking. Using the KEGG database, the differential metabolites

\textbf{FIGURE 5} | MOP alters metabolic profiles and regulates the metabolism of lipids and amino acids. (A, B) Linear discriminant analysis of metabolomic profiles in mouse serum in the positive ion (A) and negative ion (B) mode (n = 5). (C) Heatmap of significantly altered metabolites. (D) KEGG pathway enrichment analysis of differential metabolites.
were then subjected to pathway enrichment analysis; 15 significantly enriched metabolic pathways (P < 0.05) were identified, these mainly involving amino acid and lipid metabolism (Figure 5D). To explore the relationship of metabolites to inflammatory factors and gut barrier and antimicrobial peptides, the top-100 differential metabolites in terms of their relative abundance underwent Spearman correlations (Supplementary Figure 5C). These metabolites had strong correlations with inflammatory factors (TNF-α, IL-1β, IL-6, IL-10, IFN-γ) and MPO levels in serum, and intestinal barrier (ZO-1, occludin, claudin-1), mucin (Muc-2) and antimicrobial peptides (Reg3b, Reg3g) mRNA levels in colon. We used Spearman correlation analysis to identify potential relationships between alterations in gut microbes and metabolites (Supplementary Figure 5D). Our findings reveal a significant correlation between gut microbes and metabolites.

According to the KEGG database/pathway enrichment results, the significantly enriched plasma metabolites and metabolic pathways were interconnected and formed a discernible metabolic network (Figure 6). The metabolic pathways involved include sphingolipid metabolism, sphingolipid metabolism, glycine, serine and threonine metabolism, tyrosine metabolism, tryptophan metabolism, and phenylalanine metabolism. This suggested that MOP has ameliorated DSS-induced colitis not only by modulating a single metabolic pathway, but also indirectly via interrelationships between certain metabolites, thereby modulating a complex metabolic network.

**DISCUSSION**

Recent epidemiological statistics indicate that the incidence of UC continues to increase worldwide, now affecting 0.3% of the global population (28). Therefore, it is particularly urgent to develop natural medicines with low side effects and proven safety. Many studies have shown that peptides have various physiological effects such as anti-inflammatory ones (29). Yet whether MOP is capable of alleviating colitis and its mechanism of action both remain unclear. In our study, we found that MOP ameliorates colitis by remodelling intestinal mucosal barrier. Its potential protective mechanism may be through inhibiting the activation of the JAK-STAT pathway, regulating the composition and function of gut microbiota, and the level of lipid and amino acid metabolites. These findings suggest that MOP holds promise as a natural and effective drug for supporting to IBD by restoring the intestinal mucosal barrier (Figure 7).

As a new kind of natural medicine, peptides have many advantages, such as their high bioactivity and selectivity, lower toxicity than chemical drugs, easy absorption, and reduced degree of accumulation (30). Similarly, we previously studied the in vitro hypoglycemic activity and stability of MOP, and found that after MOP was digested by pepsin, it still had an inhibitory effect on α-glucosidase activity, with an inhibition rate of 97.60%. And after MOP was digested by trypsin, it still had inhibitory effect on α-glucosidase activity, and the inhibition rate was 86.76% (26). Through the above studies, we believe that MOP has a certain resistance to pepsin and trypsin, and has good stability. Therefore, we believe that MOP is a potential, highly exploitable, functional food for the adjuvant treatment of colitis.

Several studies have shown that many active peptides have anti-inflammatory properties and harbor potential to treat ulcerative colitis (31, 32). For example, Ala-Gln can prevent colitis through PepT1 and by decreasing the abundance of Bacteroidetes and the ratio of Bacteroidetes to Firmicutes (33). Furthermore, food protein-derived VPP and IPP inhibit colitis through NF-κB and MAPK pathways (34). In the present study, the MOP administration attenuated inflammation by inhibiting the JAK-STAT pathway and significantly increased the relative abundances of Lachnospira, Blautia, and Ruminococcus. Although our findings are consistent with previous studies, there are still many noteworthy differences, and these may be related to the peptide sequence and amino acid composition (35).

Intestinal mucosal barrier dysfunction is a key pathology of colitis (36). In the immune barrier, overproduction of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, and IL-6) or reduced production of anti-inflammatory cytokines (IL-10) induces the persistence and severity of IBD, worsen the intestinal environment (37, 38). In agreement with previous studies (39, 40), MOP significantly reversed DSS-induced changes in cytokine (TNF-α, IFN-γ, IL-1β, IL-6, and IL-10) levels. Notably, changes in cytokine levels are often caused by inflammatory cell infiltration (41, 42). Therefore, we examined the expression of immune cells (neutrophils and macrophages), and as expected, MOP significantly reversed the DSS-induced infiltration of F4/80+ macrophages and Ly6G+ neutrophils, which consistent with previous studies (43). Furthermore, MOP attenuated DSS-induced impairment of physical barrier function, as evidenced by enhanced tight junction expression and increased goblet cell numbers and mucus expression, consistent with previous studies (40, 44, 45). Collectively, our results suggest that MOP has anti-inflammatory effects and ameliorates DSS-induced intestinal barrier dysfunction.

It is worth noting that cytokines such as IFN-γ and IL-6 are released and bind to receptors on cells, thereby activating JAK-STAT pathway, further up-regulates claudin-2 expression, and mislocalizes ZO-1 to form intercellular spaces, thereby increasing intestinal epithelial permeability (46–48). In our study, transcriptomics revealed that JAK-STAT pathway is a highly enriched functional pathway. And it was demonstrated by western blot that MOP could inhibit JAK-STAT pathway activation. Therefore, we believe that MOP may inhibit JAK-STAT pathway activation by inhibiting the expression of inflammatory factors, and finally regulate intestinal barrier dysfunction.

Gut microbes can directly or indirectly (through their metabolites) modulate signal transduction and immune responses to remodel the intestinal epithelial barrier. It has been reported that Proteobacteria are the major contributors to IBD (49, 50), whereas Firmicutes are depleted under IBD, resulting in dystrophic gut microbiota (51). In addition, studies have shown that Proteobacteria can invade intestinal epithelial
FIGURE 6 | Metabolic network of MOP-treated mice with colitis. Enriched metabolites are marked in red. The expression of metabolites in the different experimental groups is conveyed by the heatmap blocks, based on Euclidean analysis, corresponding from left to right to the control, DSS, and DSS+H MOP groups. The differing colors denote the magnitude of metabolites’ relative expression in the samples of a given group, with redder colors indicating the higher expression of metabolites and bluer colors indicating the lower expression of metabolites (n = 5).

FIGURE 7 | Schematic diagram of the mechanism of action of MOP in alleviating colitis. MOP ameliorated colitis by remodeling the intestinal mucosal barrier by inhibiting the activation of the JAK/STAT pathway, modulating the composition and function of the gut microbiota, as well as the levels of lipid and amino acid metabolites.
cells and aggravate intestinal inflammation by releasing endotoxin and lipopolysaccharide (LPS), affecting intestinal permeability (52). Interestingly, our findings show Proteobacteria (including Escherichia-Shigella and Enterobacter genera) as the dominant microbiota in mice with IBD. However, Lachnospiraceae and Ruminococcaceae (Firmicutes) were significantly enriched in the DSS+H MOP group, likely inhibiting the DSS-induced increase in Proteobacteria and improving overall gut dysbiosis. This is consistent with previous studies, in which decreased relative abundances of Lachnospiraceae and Ruminococcaceae were observed in colitis (43, 53, 54). As major producers of butyrate, both taxa enhance the integrity of the intestinal epithelial barrier and suppress inflammation (54–56). Furthermore, also acting as butyrate producers (57–59), Blautia, Enterittinimonas, and Butyrivibrio also occurred at greater relative abundances in the DSS+H MOP group than the DSS group of mice.

Furthermore, as a pathogenic bacteria, Enterococcus is closely related to inflammation-related diseases in vivo (60). It has been shown that Enterococcus produces metalloproteinases, which aggravate intestinal inflammation in mice by damaging epithelial cells and destroying the integrity of the intestinal barrier (61). We detected this bacterial genus increased in the colon of the IBD group, but the MOP intervention reversed this trend. Further, the correlation results showed that Enterococcus was significantly positively correlated with the levels of pro-inflammatory factors in serum and pro-inflammatory genes (IFN-γ) in colon, but negatively correlated with those of anti-inflammatory factor (IL-10) in serum and colonic barrier (ZO-1, Occludin, Claudin-1), mucin (Muc-2), and antimicrobial peptide (Reg3b, Reg3g) in colon. Also, as a butyrate-producing microorganism (62), Lachnospiraceae_NK44A136_group was found significantly increased in abundance after the MOP intervention, being negatively correlated with pro-inflammatory factors and significantly positively correlated with anti-inflammatory factors, intestinal barrier, mucin, and antimicrobial peptide genes. Ruminococcaceae is reportedly more closely related to the Treg/Th17 balance and to resisting DSS-induced ulcerative colitis (63). In our study, as expected, MOP reversed DSS-induced depletion of Ruminococcaceae and was inversely associated with pro-inflammatory factors in the correlations, with anti-inflammatory factors, intestinal barrier, mucin, and antimicrobial peptides genes all positively correlated to it. Taken together, these lines of evidence suggest that the MOP administration ameliorates DSS-induced gut dysbiosis, thereby increasing the abundance of anti-inflammatory-related beneficial bacteria like Lachnospiraceae and Ruminococcaceae, which may help to promote recovery from gut barrier.

As a link coordinating the interaction between gut microbes and the host, the physiologically active compounds produced by the metabolism of the host and gut microbes play an important role. In particular, choline derivatives in lipid metabolism and indole derivatives in amino acid metabolism have many physiological functions, including regulating and maintaining intestinal mucosal immune homeostasis and enhancing intestinal barrier function (18). In our study, we found that MOP significantly regulated the levels of lipid and amino acid metabolites. In fact, lipids play an important role in maintaining the integrity of the intestinal epithelium (64). A previous study revealed an important role for the de novo lipogenic enzyme fatty acid synthase in maintaining gut barrier function by regulating palmitoylation of Mucl (65). Thus, disturbances in lipid metabolism disrupt intestinal barrier integrity. In lipid metabolism (Figure 6), L-serine generates phosphatidylserine with the help of enzymes, which then enters glycerophospholipid metabolism and undergoes a series of metabolic transformations under the action of different enzymes to generate choline-related metabolites. Choline is a metabolite produced from phosphatidylcholine by some gut microbiomes with phospholipase D enzymes, which plays an important role in maintaining the structural integrity of cell membranes (17, 66, 67). Studies have shown that choline supplementation helps maintain intestinal mucosal immune homeostasis and strengthens the intestinal barrier (68). Here, MOP reversed the DSS-induced disturbance of lipid metabolite levels and had a tendency to promote choline increases. Interestingly, we found that lipid metabolites including choline were significantly associated with gut microbes. Therefore, we speculate that this may be the metabolic regulation of choline by gut microbes.

In addition, in amino acid metabolism, L-serine generates L-tryptophan under the enzymatic action of tryptophan synthase alpha chain and this product enters the tryptophan metabolic pathway. It is well known that L-tryptophan is an aromatic amino acid and a biosynthetic precursor of microbial and host metabolites (69, 70). In this study, L-tryptophan generates a series of indole derivatives, including 3-Indoleacetic Acid (IAA), 5-methoxyindoleacetate and indole-3-acetamide. Interestingly, many microorganisms have been shown to synthesize IAA via the indole-3-acetamide and indolepyruvate pathways (71, 72). As a marker of gut microbiota metabolic activity (73), IAA plays a role in gut barrier integrity, immune cell activity and maintaining gut homeostasis (74, 75). Consistent with these findings, our data suggest that MOP is effective in increasing serum concentrations of indole derivatives in colitis and is significantly associated with gut microbes. This may be regulated by the metabolism of tryptophan by gut microbes. Interestingly, we found an interesting phenomenon that serotonin (5-hydroxytryptamine [5-HT]) and its derivative N-acetyl serotonin, produced by some gut microbes, were significantly enriched in the DSS+H MOP group, these signaling molecules are thought to regulate intestinal motility (76). It is worth noting that intestinal peristalsis dysfunction is a common symptom in patients with inflammatory bowel disease (77). Therefore, whether MOP can affect intestinal peristalsis through the intestinal microbial metabolite 5-HT to improve ulcerative colitis warrants future investigation.

In conclusion, we hypothesized that gut microbiota imbalance in UC mice causes abnormal lipid and amino acid metabolism, promoting gut barrier disruption. MOP may improve intestinal barrier integrity and improve colitis by regulating the disturbance of gut microbes and their metabolites (including lipid and amino acid metabolism).
MATERIALS AND METHODS

Preparation of the Moringa oleifera Peptide (MOP) in Seed

The peptide (KETTTIVR) was synthesized (by GUOPING Pharmaceutical, Anhui, China) using a solid phase procedure that relied on Fmoc-protected amino acid synthesis methods (78). The peptide’s purity was > 95%, and the MOP formally identified by GUOPING Pharmaceutical (Supplementary File 1: Supplementary Figure 1).

Animal Experiments

The animal protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee of Yunnan Agricultural University (Yunnan, China) for ethical issues and scientific care [license no. SYXX (Dian) K2020-0006]. Six-week-old male C57BL/6J mice (each 15–18 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd., China [license no. SCXK (Hunan) 2019-0004]. Prior to the experiments, the mice were housed in a climate-controlled rearing box at 55% ± 5% relative humidity with a 12-h day/night cycle. After 1 week of acclimatization, the mice were randomly divided into five groups of 8 mice each: control, DSS, DSS+200 mg/kg MOP (DSS+L MOP), DSS+400 mg/kg MOP (DSS+M MOP), and DSS +800 mg/kg MOP (DSS +H MOP) (Figure 1A). The UC model was induced by administering to mice 3% (w/v) DSS (molecular weight 50,000 kDa; MP Biomedicals, UK) in their drinking water for 10 days. The MOP was dissolved in water. At the same time, the mice in the control group and the DSS group were gavaged with an equal volume of water. Body weight and disease activity index (DAI), including stool consistency and rectal bleeding, were monitored daily.

Histology and Immunohistochemistry

Fresh colon tissues were fixed in 4% paraformaldehyde solution (Sigma-Aldrich), embedded in paraffin, sectioned (3-μm thickness), and stained with hematoxylin and eosin (H&E) and Alcian Blue. Tissue sections were incubated with Ly6G (Abcam, ab25377) and F4/80 (Abcam, ab6640). Images of each were collected using a microscope (Olympus, Tokyo, Japan) and then analyzed with Image pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA). Western blotting analysis was performed to observe the protein bands. -Tubulin was used as an internal control. Image J software was used to analyze the gray value of obtained protein bands.

RNA Sequencing

Total RNA was extracted from colon tissue samples according to the manufacturer’s instructions (Invitrogen). Then RNA quality was determined by 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). RNA-seq transcriptome library was prepared following TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA) using RNA samples with high quality. Paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq xten/NovaSeq 6000 sequencer.

Sequencing of 16S rRNA Genes of Gut Microbiota

Total microbial genomic DNA was extracted from mouse colon contents. The quality and concentration of DNA were determined by agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., USA). PCR amplifications were performed, and their ensuing products were extracted and purified. Purified amplicons were pooled in equimolar amounts and paired on the Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA), this done according to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) End Sequencing.

Non-Targeted Metabolomics

Serum samples from mice were extracted and transferred to a sample vial with an inner cannula for its machine analysis carried out using the UPLC-TripleTOF System of AB SCIEX. The resulting LC-MS raw data were processed using the metabolomics software program Progenesis QI (Waters Corporation, Milford, USA), to obtain the final data matrix for use in the formal analysis. Meanwhile, their MS and MSMS mass spectral information was searched against and matched with that in two public metabolic databases, HMDB (http://www.hmdb.ca/).
and Metlin (https://metlin.scripps.edu/), to obtain each metabolite’s information.

**DATA AVAILABILITY STATEMENT**

The sequences generated in this study are stored in the National Center for Biotechnology Information (NCBI) and the project numbers are PRJNA822105 and PRJNA824237. The raw spectral data for metabolome analysis are freely available at https://data.mendeley.com/datasets/rrsn78z2bnpj/1. Additionally, all other data is contained within the article and Supplementary Material.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Yunnan Agricultural University.

**AUTHOR CONTRIBUTIONS**

JS and YT conceived and designed the experiments. Z-SH, JX, and YT performed the experiments. YT, JX, and Z-SH analyzed the data. JS wrote the paper. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.924178/full#supplementary-material
58. Liu X, Mao B, Gu J, Wu J, Cui S, Wang G, et al. Blautia—A New Functional Genus With Potential Probiotic Properties? Gut Microbes (2021) 13(1):1–21. doi: 10.1080/19490976.2021.1875616

59. Eckhart V, Machiels K, Perrier C, Romero C, Maes S, Flahou B, et al. Butyrivibrio Pullicinivorans in Inflammatory Bowel Disease. Gut (2013) 62(12):1745–52. doi: 10.1136/gutjnl-2012-303611

60. Garcia-Solache M, Rice LB. The Enterococcus: A Model of Adaptability to Its Environment. Clin Microbiol Rev (2019) 32(2):e00058–18. doi: 10.1128/CMR.00058-18

61. Martin-Gallausiaux C, Marinelli L, Blottiere HM, Larrau I, et al. Intestinal Microbiota at Confluence in Allo-Hsct Recipients. Front Immunol (2020) 11:57. doi: 10.3389/fimmu.2019.00576

62. Steck N, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, et al. Microbiota and Lipid Metabolism in Weaned Piglets. Modulates Growth Performance and Gut Inflammation by Altering the Gut Microbiota. Front Cell Infect Microbiol (2020) 9:669. doi: 10.3389/fcimb.2020.541178

63. Han L, Jin H, Zhou L, Zhang X, Fan Z, Dai M, et al. Intestinal Microbiota at Engraftment Influence Acute Graft-Versus-Host Disease Via the Treg/Th17 Balance in Allo-Hsct Recipients. Front Immunol (2018) 9:669. doi: 10.3389/fimmu.2018.00669

64. Li Y, Yan H, Zhang Y, Li Q, Yu L, Li Q, et al. Alterations of the Gut Microbiome Composition and Lipid Metabolic Profile in Radiation Enteritis. Front Cell Infect Microbiol (2020) 10:541178. doi: 10.3389/fcimb.2020.541178

65. Wei X, Yang Z, Rey FE, Ridaeva VK, Davidson NO, Gordon JJ, et al. Fatty Acid Synthase Modulates Intestinal Barrier Function Through Palmitoylation of Mucin 2. Cell Host Microbe (2012) 11(2):140–52. doi: 10.1016/j.chom.2011.12.006

66. Goh YQ, Cheam G, Wang Y. Understanding Choline Bioavailability and Utilization: First Step Toward Personalizing Choline Nutrition. J Agric Food Chem (2021) 69(37):10774–89. doi: 10.1021/acs.jafc.1c03077

67. Wang Z, Klipfell E, Bennett BJ, Koether R, Levison BS, Dugar B, et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. Nature (2011) 472(7341):57–63. doi: 10.1038/nature09922

68. Qiu Y, Liu S, Hou L, Li K, Wang L, Gao K, et al. Supplemental Choline Modulates Growth Performance and Gut Inflammation by Altering the Gut Microbiota and Lipid Metabolism in Weaned Piglets. J Nutr (2021) 151(1):20–9. doi: 10.1093/jn/nxaa331

69. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe (2018) 23(6):716–24. doi: 10.1016/j.chom.2018.05.003

70. Alkhalfal LM, Ryan KS. Biosynthetic Manipulation of Tryptophan in Bacteria: Pathways and Mechanisms. Chem Biol (2015) 22(3):317–28. doi: 10.1016/j.chembiol.2015.02.005

71. Nutravat R, Srisuk N, Arunratnavorn P, Limtong S. Indole-3-Acetic Acid Biosynthetic Pathways in the Basidioymycetous Yeast Rhodosporidium Paludigenum. Arch Microbiol (2016) 198(5):429–37. doi: 10.1007/s00203-016-1202-z

72. Tsavkelova E, Oeser B, Oren-Young L, Israeli M, Sasson Y, Todorovsky B, et al. Identification and Functional Characterization of Indole-3-Acetamide-Mediated Iaa Biosynthesis in Plant-Associated Fusarium Species. Fungal Genet Biol (2012) 49(1):48–57. doi: 10.1016/j.fgb.2011.10.005

73. Pavlova T, Vidova V, Bienertova-Vasku J, Janku P, Almasi M, Klabanov J, et al. Urinary Intermediate of Tryptophan as Indicators of the Gut Microbial Metabolism. Anal Chim Acta (2017) 987:72–80. doi: 10.1016/j.aca.2017.08.022

74. Lamas B, Michael ML, Leducz V, Pham HP, Michel ML, Da Costa G, et al. CARD9 Impacts Colitis by Altering Gut Microbiota Metabolism of Tryptophan Into Aryl Hydrocarbon Receptor Ligands. Nat Med (2016) 22 (6):598–605. doi: 10.1038/nm.4102

75. Zhang J, Zhu S, Ma N, Johnston LJ, Wu C, Ma X. Metabolites of Microbiota Response to Tryptophan and Intestinal Mucosal Immunity: A Therapeutic Target to Control Intestinal Inflammation. Med Res Rev (2021) 41(2):1061–88. doi: 10.1002/med.21752

76. Mawe GM, Hoffman JM. Serotonin Signalling in the Gut—Functions, Dysfunctions and Therapeutic Targets. Nat Rev Gastroenterol Hepatol (2013) 10(8):473–86. doi: 10.1038/nrgastro.2013.105

77. Barros LL, Farias AQ, Rezaie A. Gastrointestinal Motility and Absorptive Disorders in Patients With Inflammatory Bowel Diseases: Prevalence, Diagnosis and Treatment. World J Gastroenterol (2019) 25(31):4414–26. doi: 10.3748/wjg.v25.i31.4414

78. Schutznerova E, Pribylka A, Krchnak V. N(Alpha)-Amino Acid Containing Privileged Structures: Design, Synthesis and Use in Solid-Phase Peptide Synthesis. Org Biomol Chem (2018) 16(29):5359–62. doi: 10.1039/c8ob01485y

79. Rachmilewitz D, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, et al. Immunostimulatory DNA Ameliorates Experimental and Spontaneous Murine Colitis. Gastroenterology (2002) 122(5):1428–41. doi: 10.1016/s0016-5085(02)70307-4

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