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Attenuation of DSS induced colitis by *Dictyophora indusiata* polysaccharide (DIP) via modulation of gut microbiota and inflammatory related signaling pathways

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ABSTRACT

Inflammatory bowel disease (IBD) is a lifelong chronic inflammation of the gastrointestinal tract. Polysaccharides from mushrooms have been extensively used to treat inflammatory diseases including IBD. *Dictyophora indusiata* polysaccharide (DIP) modulates gut microbiota and alleviate inflammatory reactions; however, its effect on ulcerative colitis (UC) is yet to explore. In this study DIP effect on DSS induced colitis was determined. Our findings revealed that DIP treatment alleviated the severity of colitis, especially at the high dose. The histopathological alterations and gut epithelial integrity were ameliorated. The inflammatory reactions and oxidative stress were improved. Moreover, proinflammatory cytokines were reduced, conversely, anti-inflammatory cytokines and tight junction proteins (TJs) were elevated. Furthermore, Illumina MiSequencing exhibited perturbation of bacterial community upon DSS treatment; however, the bacterial shift was reversed after DIP treatment. Inclusively, our findings suggest mushroom polysaccharides may have a potential therapeutic effect against colitis and inflammatory-related diseases.

1. Introduction

Inflammatory bowel disease (IBD), which comprises of Crohn's disease (CD) and ulcerative colitis (UC) is a multifactorial disease distinguished by the immune system relapse and inflammation of the gastrointestinal (GI) tract (Binder, 2004; Bouma & Strober, 2003; Maloy & Powrie, 2011). The exact mechanism of IBD is still vague and under debate. However, the emerging evidence strongly supports the hypothesis that the potential players involve a complex interaction of genetic predisposition, epithelial barrier dysfunction, environmental exposure, defective innate and acquired immunity of the host and dysregulation of intestinal flora (Coskun, 2014; Elinav, Thaiss, & Flavell, 2013; Lee et al., 2009; Mar et al., 2014; Packey & Sartor, 2008). Both CD and UC exhibit genetic, phenotypic, immunologic and therapeutic level variations (Sartor, 2006) where inflammation of the entire gastrointestinal tract, abdominal cramp, and bloody mucoid diarrhea are the typical hallmarks of CD (Baumgart & Sandborn, 2012), while UC represents restricted inflammation of the colon and rectum along with symptoms like diarrhea, rectal bleeding, and fatigue (DeRoche, Xiao, & Liu, 2014; Ghosh & Mitchell, 2007).

As far as the innate host mechanisms for the maintenance of gut integrity are concerned, the first line of defense is mucous membrane, epithelial tissues, and intercellular junction proteins that provide protection against pathogenic microbes and the associated LPS (lipopolysaccharides) and toxins (Farhadi, Banan, Fields, & Keshavarzian, 2003; Hansson, 2012). The disruption of the mucus layer and decreased mucins expression leads to enhanced bacterial invasion (Hayashi et al., 2001; Johansson et al., 2014; Johansson & Hansson, 2011). In addition to the mucins, tight junction proteins (TJs) expression, which are an intercellular structure consisting of transmembrane proteins including zonula-occludens (ZO-1, ZO-2), occludin and claudins (Edelblum & Turner, 2009; Mitic & Anderson, 1998) have also been reported to be

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involved in the maintenance of intestinal epithelial integrity by inhibiting the translocation and invasion of microbes across the epithelial membrane (Han et al., 2015; Laukoetter, Nava, & Nusrat, 2008).

On the other hand, studies have shown that the abnormal shifts or dysbiosis of intestinal microflora potentiate inflammatory responses that consequently lead to the development of IBD (Kostic, Xavier, & Gevers, 2014; Packey & Sartor, 2009; Sokol, Lay, Seksik, & Tannock, 2008). In a normal healthy gut, the proportion of *Firmicutes* over *Bacteroidetes* is ~ 0% to ~20% (Gevers et al., 2012; Rup, 2012). However, microbial diversity substantially reduced and perturbed in IBD patients compared to healthy individuals (Morgan et al., 2012; Ott et al., 2004). Dysbiosis in IBD is distinguished by an increment of *Proteobacteria* and *Bacteroidetes* (reduced in some cases), and reduction of *Firmicutes*, *Lactobacillus,* and *Clostridia* (Man, Kaakoush, & Mitchell, 2011; Ott et al., 2004; Sokol et al., 2008; Sokol et al., 2017; Swidsinski et al., 2002; Walker et al., 2011; Walujkar et al., 2014; Wright et al., 2015). Considering the vital role of the intestinal microbiome in IBD, the evaluation of gut microbiota and microbiome linked functional pathways might be one of those targets that provide insight for better understanding of the disease pathogenesis.

The dysregulation of cytokines secretion and the epithelial barrier impairment leads to the progression of IBD (Garrett, Gordon, & Glimcher, 2010; Johansson, Larsson, & Hansson, 2011; Sanchez-Muñoz, Dominguez-Lopez, & Yamamoto-Furusho, 2008). Additionally, UC patient samples and *in vivo* studies also highlighted the altered level of inflammatory cytokines. (Gomes-Santos et al., 2012; He et al., 2016; Jeengar, Thummuri, Magnusson, Naidu, & Uppugunduri, 2017; Kang, Lim, & Kim, 2015). The inflammatory cytokine expression is mainly regulated via inflammatory signaling pathways such as NF-κB and MAPK, and the activation of these signaling pathways are involved in the development of IBD through phosphorylation of p-65, IκB and ERK (Wei & Feng, 2010; Yamamoto & Gaynor, 2001). The dysregulation of cytokines augment intestinal inflammation that eventually induces oxidative stress by stimulating reactive oxygen/nitrogen species (ROS/NOS) such as MPO and iNOS (Aktan, 2004; Rezaie, Parker, & Abdollahi, 2007; H. Zhu & Li, 2012). Moreover, STAT3 and COX-2 have been implicated with IBD and intestinal inflammation. (Mudter et al., 2005; Musso et al., 2005; Wang & DuBois, 2010).

Currently, the cornerstone for the treatment of IBD mainly includes anti-inflammatory drugs, amino-salicylates, anti-diarrheal agents, corticosteroids, antibiotics, immunomodulators, and biologic agents (De Chambrun & Sandborn, 2012; Dethlefsen, Huse, Sogin, & Relman, 2008; Engel & Neurath, 2010). Nonetheless, many of these existing therapeutic strategies have an adverse impact on host health and the long-term intake of these drugs leads to compromised immunity making the patients more vulnerable to infections and diseases (Marehbian, Arrighi, Hass, Tian, & Sandborn, 2009; Rogler, 2010). Thus, there is a demand for the development of alternative and new therapies that have no side effects. Traditional Chinese medicines (TCM) such as medicinal mushrooms have been extensively used for the treatment of multiple human ailments due to the long clinical practice and strong therapeutic efficacy (Joseph et al., 2017; Martel, Ojcius, Lai, & Young, 2014; Stone, 2008). Moreover, studies have revealed the efficacy of prebiotics such as polysaccharides in the treatment of IBD (Diling et al., 2017; Ren et al., 2018). In concordance with these studies, we have previously reported the therapeutic potential of crude polysaccharide (DIP) isolated from mushroom *Dictyophora indusiata* in the restoration of antibiotic-induced gut dysbiosis and ameliorative effect of DIP on gut integrity and inflammatory responses (Kanwal et al., 2018). *D. indusiata* has been used as a medicinal mushroom since 618 A.D (Ker, Chen, Peng, Hsieh, & Peng, 2011) and its bioactive active components have been used to treat several ailments (Deng et al., 2016; Liao et al., 2015; Zhang et al., 2016). However, to the best of our knowledge, so far the activity of *D. indusiata* on UC is unknown. Thus, the present study hypothesizes that DIP polysaccharide possesses a protective effect against DSS-induced colitis via regulating inflammatory reactions and microbial community composition in colitic mice model.

2. Materials and methods

2.1. Materials and chemical reagents

List of materials and chemical reagents is given in Supplementary Table S1.

2.2. Methods

2.2.1. Experimental subjects and housing

Specific-pathogen-free (SPF) level inbred male BALB/c mice aged 6–8 weeks old and weighing 20 ± 2 g were used in this study that was approved by the Animal Care and Research Ethics Committee of Dalian Medical University (Approval Number: SYXK 2016–2018). All the animals were randomly divided and accommodated in separate cages and maintained in conventional conditions in an environmentally controlled room (20–22 °C temperature and 55 ± 5% relative humidity with 12–12 h light/dark cycle), with standard commercial chow (Jiangsu Medison Biomedical Co., Ltd., Yangzhou, Jiangsu Province, China) and water ad libitum. All the animals used in the study were allowed acclimated for a period of one-week prior experimentation. All experimental procedures were approved by the ethics committee of Dalian Medical University.

2.2.2. Extraction and chemical analysis of crude polysaccharide DIP from the mushroom D. Indusiata

The extraction of crude polysaccharide, chemical analysis and monosaccharide composition is described in our previous study (Kanwal et al., 2018). Briefly the total sugar content was 96.66% with 13.2% polysaccharide yield. Glucose 59.84%, Mannose 23.55% and Galactose 12.95% were the main bioactive sugar moieties.

2.2.3. Induction of colitis and treatment protocol

After acclimation for one-week, experimental subjects were categorized into four groups comprised of the Control group, DSS group, DSS + DIP (low dose 10 mg/kg) mice received 1.5 mg/mL) and DSS + DIP (high dose 33 mg/kg) mice received 5 mg/mL. The treatment procedure was performed for 2 weeks in accordance with the experimental design illustrated in Fig. 1. Acute colitis was induced by 3.5% DSS given in distilled water for seven days. Mice in the DSS + DIP groups received DIP (10 or 33 mg/kg) for two weeks before and during the DSS administration one time daily by oral gavage. Throughout the experiment, the control group was given distilled water, whereas the DSS group received distilled water in the first week followed by DSS treatment in the second week. At day 15th, mice were sacrificed by cervical dislocation. Colon was taken out quickly and washed with cold

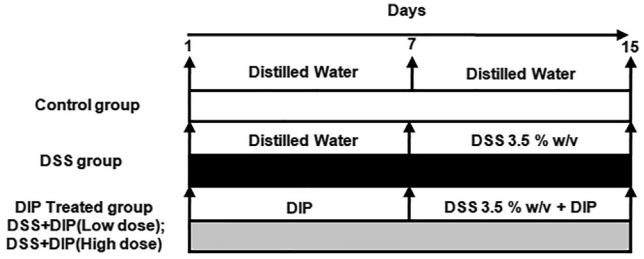


Fig. 1. Schematic representation of the experimental plan. Experimental subjects were categorized into 4 groups. Control group (Ctrl, $n = 6$) was gavaged with distilled water from days 1 to 15. DSS group (DSS, n = 6) received distilled water from day 1 to day 7 followed by DSS (3.5%) treatment till day 15. DIP groups (n = 6), DSS + DIP(L) and DSS + DIP(H) were treated with low dose of DIP and high dose of DIP respectively. From day 7 to onwards DIP groups were treated with DSS along with DIP treatment till day 15.

Table 1
Disease activity index (DAI) evaluation criteria.

Score	Weight Loss (%)	Stool Consistency	Occult/Gross Bleeding
0	None	Normal	Negative
1	1–5		
2	5–10	Loose stool	Positive
3	10–20		
4	> 20	Diarrhea	Gross bleeding

phosphate-buffered saline (PBS). The distal part was fixed in 10% buffered formalin for histological analysis, and other parts were then stored at −80 °C for immunological assays.

2.2.4. Assessment of disease activity index (DAI)

The clinical symptoms and severity of colitis were evaluated daily, as described previously (Jeon et al., 2016). The body weight of each mice was recorded every day and stool consistency was examined. Disease activity index (DAI) reflected the intensity of the disease as presented in Table 1.

2.2.5. Histological analysis

For histological evaluation, colon tissue sections (3 μm thick) were collected and fixed in 4% paraformaldehyde for 24 h at room temperature. The tissue sections were deparaffinized in xylene and rehydrated through decreasing grades of ethanol, and then employed to staining with hematoxylin and eosin (H.E). Images were obtained using a microscope (Leica Microsystems, Wetzlar, Germany) finally; the histological analysis was performed by an independent researcher in a blinded manner. The colonic injury was scored as described previously (Tong et al., 2016). The evaluation criteria was as follows: 0: no evident inflammatory reaction; 1: Low-level inflammatory reaction; 2: Moderate inflammatory infiltration; 3: Severe inflammatory reaction; 4: Large amounts of inflammatory cell infiltration and diminished goblet cell numbers.

2.2.6. Analysis of goblet cells and mucus layer thickness

Periodic acid–Schiff (PAS) staining was employed to determine goblet cells and mucous epithelium thickness in the intestine (M Wlodarska et al., 2011). The tissue sections were deparaffinized in xylene and rehydrated through decreasing grades of ethanol, followed by periodic acid treatment for 5 min at room temperature (RT). Slides were washed in distilled water and Schiff reagent was used for staining for 7 min at RT in a dark chamber. Slides were washed with running water for 10 min followed by counterstain with hematoxylin. Again slides were washed under tap water for 10–15 min and dehydrated with Ethanol. Coverslips were used after slides dried completely. Images were obtained using a microscope (Leica Microsystems, Wetzlar, Germany) finally; the histological analysis was performed by an independent researcher in a blinded manner.

2.2.7. MPO activity assay

The neutrophil influx into the colonic tissue was determined via MPO level which reflects the number of macrophages and monocytes (Masoodi, Tijjani, Wani, Hassan, Khan, & Hussain, 2011; Muthas et al., 2017). The colon tissue was homogenized in 0.1 M phosphate buffer (pH 7.4). The resulting homogenate was centrifuged at 13,000 rpm at 4 °C for 20 min and the supernatant was collected. The MPO and T-SOD levels were assessed by ELISA in accordance with the manufacturer's instructions. The protein quantification was carried out by BCA assay kit.

2.2.8. NO measurement in serum

The blood samples were centrifuged at 3000 rpm for 15 min at 4 °C and the serum was separated. NO levels were determined using the NO assay kit.

2.2.9. Measurement of cytokines by ELISA

The concentrations of various cytokines (TNF-a, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-4, and IL-10) in the colon were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Shang Hai Lengton Bioscience Co., Ltd. Shanghai, China) according to the manufacturer's instructions. Briefly, colonic samples were homogenized in PBS buffer (pH 7.4) and centrifuged at 14,000*g* for 20 min at 4 °C. Supernatant from the colonic homogenates was separated and kept at −80 °C for biochemical analysis.

2.2.10. Immunoblotting assay

Protein was extracted from the colonic tissue samples using RIPA lysis buffer containing a protease inhibitor (Transgene Biotech, Beijing, China) and then centrifuged at 12,000*g* for 5 min at 4 °C. The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Immunoblotting was performed as described in our previous study (Kanwal et al., 2018). Antibodies used in the study are shown in Supplementary Table S2.

2.2.11. DNA extraction and 16S rRNA gene amplification (Illumina MiSeq Sequencing)

DNA extraction and 16S rRNA gene amplification were carried out as described in our previous study (Kanwal et al., 2018). Briefly, the 16S rRNA gene V4 region was amplified from genomic DNA using the universal bacterial primers: (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′, and 806R, 5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were normalized, pooled and sequenced on the Illumina Hiseq4000 sequencer (2 × 150 bp paired-end). Libraries were prepared using Library Quant Kit Illumina GA revised primer-SYBR Fast Universal (KAPA, Wilmington, MA, USA) and sequenced for 600 cycles on an Illumina MiSeq using the MiSeq Reagent Kit (Illumina, San Diego, CA, USA). Low-quality reads were removed and chimeras identified using Chimera.uchime. Taxonomic assignment of individual datasets was performed using the SILVA128. Operational taxonomic units (OTUs) were generated using Vsearch v1.11.1 with a dissimilarity cutoff of 0.03. A Venn diagram was drawn for analysis of group-specific and shared OTUs. Diversity within the community such as species richness and evenness was determined using alpha diversity (α-diversity) indices including an observed species, Chao, abundance-based coverage estimator (ACE), Shannon, and Simpson with Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010; Kemp & Aller, 2004). To evaluate variation between bacterial communities, beta-diversity (β-diversity) indices were analyzed including principal coordinate analysis (PCoA), principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) ordination plots using QIIME with the matrix of Bray-Curtis distance (Caporaso et al., 2010; Kemp & Aller, 2004). BugBase software was used to predict microbial phenotypes (Ward et al., 2017). QLEfSe based linear discriminant analysis (LDA) and cladogram were generated to assess differentially abundant microbial taxa.

2.2.12. Statistical analysis

All the statistical analysis was performed with GraphPad Prism 5.01 software (La Jolla, CA, USA). Statistical significance was determined by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and $p < 0.05$ was considered to be statistically significant. Kruskal–Wallis and Wilcoxon tests were performed to LEfSe analysis. Mann-Whitney test was employed for phenotype and OTU statistical analysis.

3. Results

3.1. DIP attenuated clinical symptoms in the DSS induced colitis mice model

The body weight was recorded on a daily basis. DSS treated mice

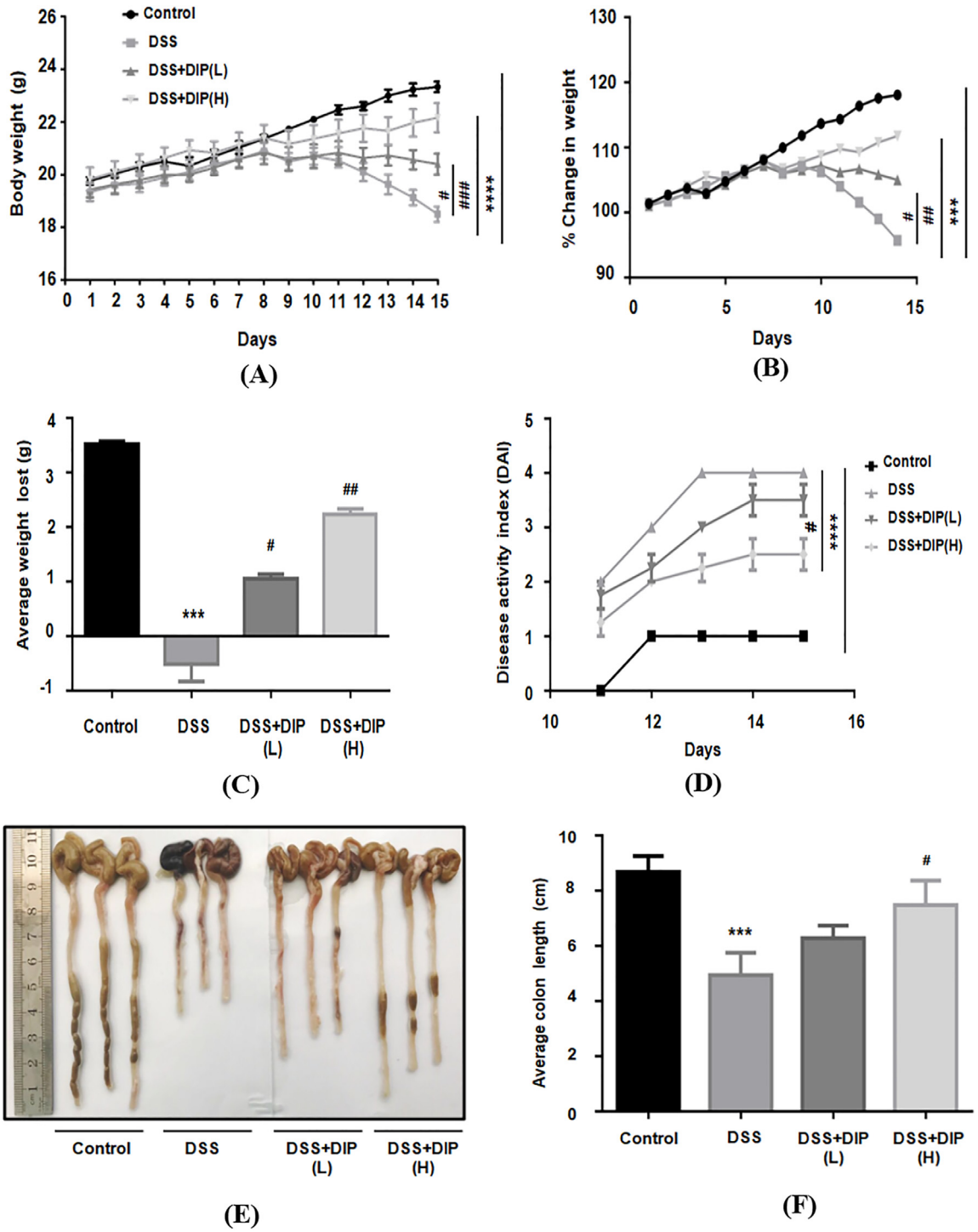


Fig. 2. Effect of DIP treatment on clinical symptoms of DSS induced colitis. (A) Body weight measurement (g). **(B)** Body weight change (%). **(C)** Average weight lost (g). **(D)** Disease activity index **(E)** Representative images of the colon from control, DSS and DIP treated groups. **(F)** Colon length of mice (cm). All data presented as mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs control group. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ and ####$p < 0.0001$ vs DSS group.

depicted variation in the body weight post-DSS administration (Fig. 2A, B and C). DSS group showed a significant reduction of the body weight ($p < 0.0001$) in comparison with the control group (Fig. 2A). Nonetheless, DIP treatment exhibited a protective effect and attenuated body weight loss in a dose-dependent manner (Fig. 2A). In comparison with the DSS alone group (DSS), DIP at low dose (DSS + DIP(L)) and high dose (DSS + DIP(H)) enhanced body weight significantly ($p < 0.05$) ($p < 0.001$). The Disease Activity Index (DAI) parameters were evaluated based on body weight loss, stool consistency and rectal bleeding (Fig. 2D). In the DSS group, the body weight of mice reduced with severe diarrhea and gross bleeding was evidenced compared to the control group. DIP treatment significantly ($p < 0.05$) decreased the severity of DAI symptoms at a high dose in the DSS + DIP(H) group in comparison with the DSS group. At lower dose improvement was observed apparently in DSS + DIP(L) though, it was not significant. The colon length of each mice was measured in all the treatment groups.

DSS group revealed significant shrinkage ($p < 0.001$) of colon length compared to the control group (Fig. 2E, F). Nonetheless, DIP + DSS(H) showed a significant increment of colon length ($p < 0.05$) in comparison with the DSS alone treatment group (Fig. 2E, F). All in all, our results suggested the efficacy of DIP in ameliorating DSS induced colitis.

3.2. DIP improved colonic histological Changes, enhanced mucins and tight junction proteins (TJs) expression in DSS induced colitis mice

To determine histological changes and mucins expression, H&E and PAS staining was performed. Control group depicted normal histological architecture with well shaped compact columnar epithelium, separated and well-defined layer between mucosa and submucosa with deep narrow spaced intact intestinal crypt and abundant goblet cells. On the contrary, the DSS group exhibited severe histological damage with irregular surface epithelium, depleted goblet cells, distorted and shallow crypt structure, and increased inflammatory cell infiltration. The space between mucosa and submucosa was expanded to a larger extent in comparison with the control group. However, in both DIP treated groups, the structure of colon tissue and inflammatory symptoms were ameliorated. The columnar epithelial structure was improved, goblet cells replenished and inflammatory cell infiltrates reduced significantly ($p < 0.001$) in DSS + DIP(L) and DSS + DIP(H) groups (Fig. 3A, B). Moreover, the void between the mucosal and submucosal layer decreased.

Furthermore, Periodic-acid Schiff (PAS) was employed to stain neutral mucins or mucus (purple stained) containing goblet cells in the colon tissue. As indicated in Fig. 3C, the control group revealed a higher abundance of goblet cells (black arrows) and higher expression of mucins in the colon. On the contrary, in the DSS group mucus layer was diminished and goblet cells were apparently demolished compared to the control group. Interestingly, DIP treatment enhanced and replenished the abundance of goblet cells and mucins expression in DSS + DIP(L) and DSS + DIP(H) in a dose-dependent manner.

To examine the impact of DIP on the intestinal barrier function after DSS treatment, tight junction proteins (TJs) expression was determined. The result demonstrated the expression level of Claudin-1, Occludin, ZO-1, and ZO-2 was significantly reduced in DSS treated mice relative to the control group. However, DIP treatment elevated the protein expression of TJs in the DSS treated mice in a dose-dependent manner (Fig. 3D, E). The relative expression of Claudin-1, Occludin, and ZO-1 was significantly ($p < 0.05$) enhanced in DSS + DIP(H) compared to DSS alone treatment. Apparently, a slight improvement was observed at a low dose. Collectively these results suggested that DIP may improve intestinal integrity by inducing expression of mucins and tight junction proteins.

3.3. DIP regulated oxidative stress and inflammatory cytokines secretion in DSS treated mice

To investigate the therapeutic efficacy of DIP on inflammation and oxidative stress, we measured MPO and T-SOD activity in the colon tissue and serum NO levels. MPO enzyme is normally present in a lower quantity in monocytes and its activity is determined to assess the neutrophil infiltration (Pandurangan, Ismail, Saadatdoust, & Esa, 2015; Zhao, Hong, Dong, Meng, & Mu, 2013). As indicated in Fig. 4, MPO and NO levels raised significantly ($p < 0.001$) ($p < 0.01$) in DSS treated group compared to the control group, while T-SOD activity was significantly ($p < 0.01$) reduced in DSS alone treatment in comparison with the control group. Treatment with DIP reduced the MPO levels in DSS + DIP(L) ($p < 0.01$), and DSS + DIP(H) ($p < 0.001$) compared to the DSS alone treatment. NO activity was also reduced in DSS + DIP(H) ($p < 0.01$) compared to DSS alone treatment. Conversely, T-SOD levels enhanced in DSS + DIP(H) ($p < 0.01$) compared to the DSS alone group (Fig. 4A, B and C). No significant difference was observed in NO and T-SOD levels at a low dose of DIP.

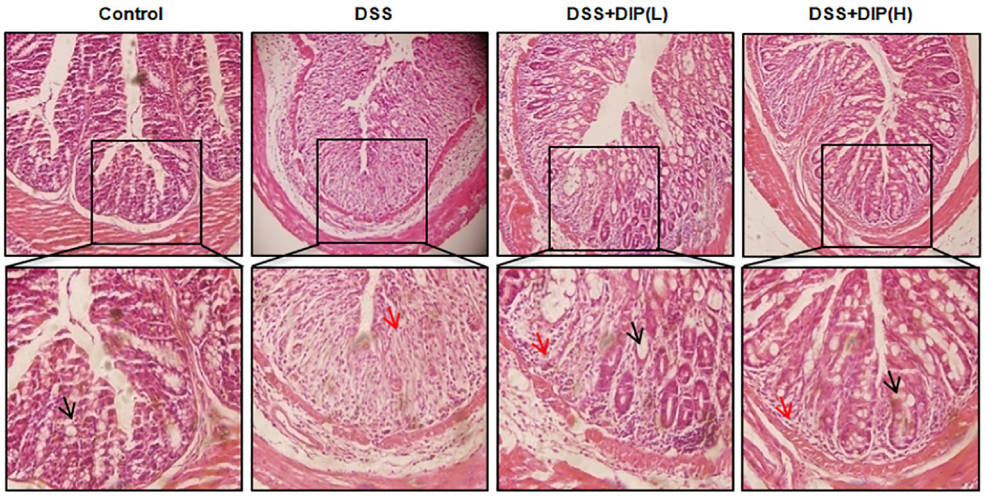
Colitis has been implicated with the cascades of chronic inflammation. It has been associated with the up-regulation of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-17, IFN-γ and downregulation of anti-inflammatory cytokines such as IL-4, and IL-10 (He et al., 2016; Kang et al., 2015; Sanchez-Muñoz et al., 2008; Tao et al., 2017; Y. Zhu et al., 2016). Thus we profiled these inflammatory markers to explore the potency of DIP in the management of DSS induced inflammation via ELISA assay. Our results suggested, the level of pro-inflammatory cytokines were enhanced evidently while anti-inflammatory cytokines were decreased in DSS alone treatment in comparison with the control group (Fig. 4D and E). Treatment with DIP, especially at high dose DSS + DIP(H) significantly reduced the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) ($p < 0.01$), IL-17 ($p < 0.05$) and enhanced anti-inflammatory cytokines (IL-4, IL-10) ($p < 0.01$) compared to DSS alone group, respectively (Fig. 4D and E).

3.4. DIP modulated inflammatory related signaling pathways

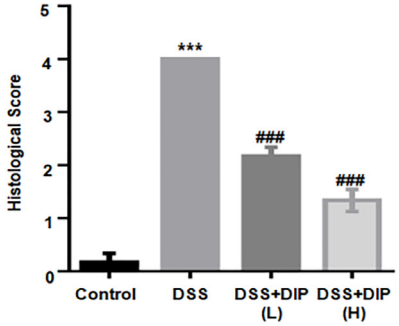
Pathogenesis of IBD has been associated with several signaling pathways including the activation of transcription factors NF-κB, mitogen-activated protein kinases MAPK and inflammatory enzymes (COX-2, iNOS) (B. Liu et al., 2015; Sahu, Kumar, & Sistla, 2016; Wei & Feng, 2010). The effect of DIP on these inflammatory associated signaling pathways was determined in our study (Fig. 5). There was a significant increment in the phosphorylation of NF-κB p65, IκB-α and ERK levels in the DSS alone group in comparison with the control group indicating the NF-Kb and MAPK activation (Fig. 5A, B). The phosphorylation of p65 ($p < 0.001$), IκB-α ($p < 0.05$) and ERK ($p < 0.01$) was significantly decreased upon treatment with DIP at high dose. Previous studies have reported that the up-regulation of inflammatory markers such as COX-2, iNOS, TNF-α, and IL-6 induce inflammatory responses and cause intestinal damage in colitis (Jin, Lin, Lin, Sun, & Zheng, 2016; B. Liu et al., 2015). Moreover, the role of Signal transducer and activator of transcription (STAT3) in the etiology of IBD via up-regulation of several cytokines has been documented in multiple studies (Mudter et al., 2005; Musso et al., 2005; A. Suzuki et al., 2001). In harmony with the previous findings, our data also suggested the increased COX-2, iNOS, TNF-α, IL-6 and p-STAT3 expression in the DSS alone treatment compared to the control group (Fig. 5C, D). Nevertheless, DIP administration at high dose significantly alleviated the expression of iNOS ($p < 0.01$), TNF-α ($p < 0.01$), IL-6 ($p < 0.001$), and p-STAT3 ($p < 0.01$) in comparison with the DSS alone group. The above findings lead us to infer that DIP may attenuate DSS-induced colitis in mice through modulating inflammatory associated signaling pathways.

3.5. Effect of DIP on bacterial diversity in DSS induced colitis mice

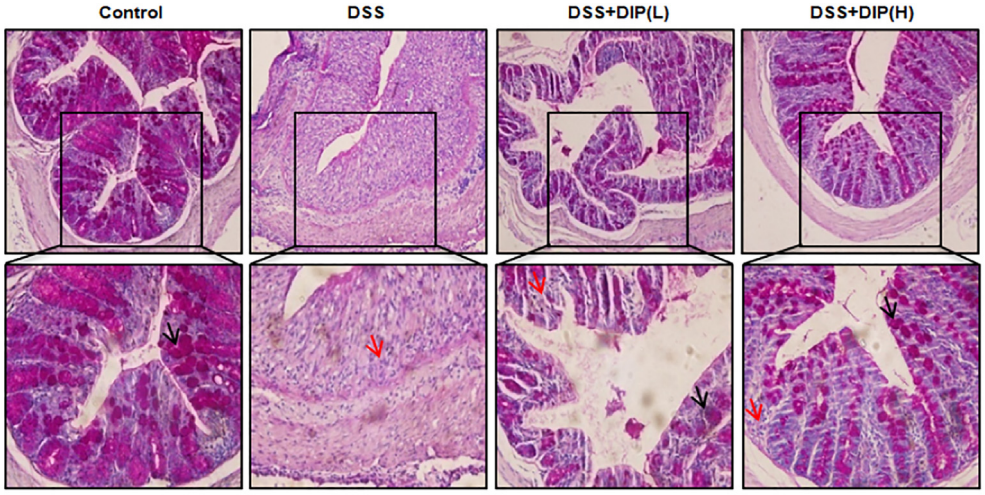
In our study, Illumina MiSeq (16S rRNA gene) has been employed to characterize the overall pattern of gut microbiota community in DSS and DIP treated mice. In order to demonstrate bacterial diversity, richness, abundance, and structural differences in each group, alpha and beta diversity indices were evaluated. The alpha diversity parameters were determined using the rank abundance curve, shannon, and observed species (Fig. 6A). In the DSS group, the relative abundance of alpha diversity indices were reduced compared to the control group. Box plot further confirmed the significant differences between various treatment groups (Supplementary Fig. S1). Nonetheless, DIP treatment evidently enhanced alpha diversity indices in comparison with the DSS group (Fig. 6A) (Supplementary Fig. S1). The alpha diversity parameters are summarized in Supplementary Table S3. Altogether, our findings revealed alpha diversity indices improved and enhanced after DIP treatment especially at the high dose. To elucidate structural variability among different treatment groups, beta diversity parameters



(A)



(B)



(C)

Fig. 3. DIP treatment improved histopathological alterations and enhanced TJs expression in colitis mice model. (A) Hematoxylin and Eosin images showing the effect of DIP in DSS induced colitis. Black arrows indicate the number of goblet cells. Red arrows indicate inflammatory cell infiltrates. Magnification (upper 200×)(lower 400×). **(B)** Histological scores. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ vs control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DSS group. **(C)** PAS staining represents images of colon sections. The abundance of goblet cells and mucin production was determined in all groups. Black arrows indicate the quantity and mucin-producing goblet cells. Red arrows indicate inflammatory cell infiltrates. Magnification (upper 200x) (lower 400x). **(D)** Relative TJs expression of Claudin-1, Occludin, ZO-1, and ZO-2 in the colon tissue using β-actin as a loading control. **(E)** Bar graph showing band relative intensity of respective protein against β-actin as internal control was quantified by NIH image J software. Data was acquired from three independent experiments and is expressed as mean (n = 6) ± SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ vs control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DSS group.

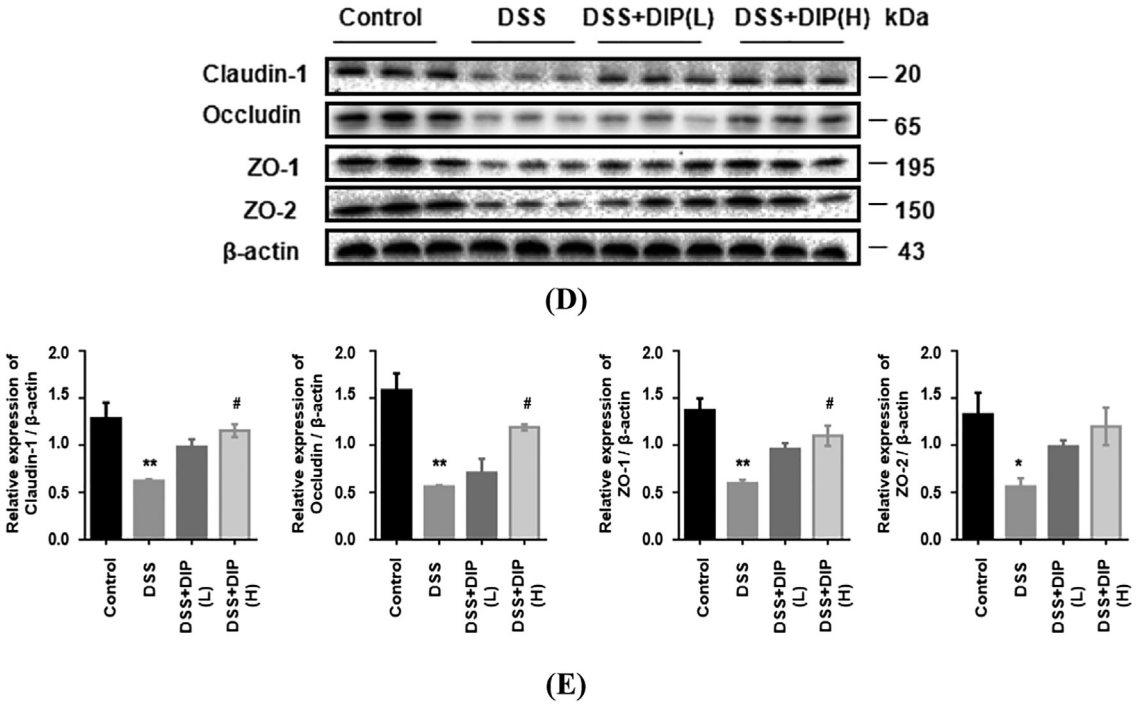


Fig. 3. (*continued*)

were assessed including Principal Coordinate Analysis (PCoA), Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) plot (Fig. 6B). Our findings exhibited, DSS subjects were clustered separately from the control group. However, DIP treatment particularly at a higher dose (DSS + DIP(H)) and the control group clustered more closely relative to the DSS group. These data suggested, control and DIP treated groups are more similar as compared to the DSS alone group.

3.6. Bacterial composition at different taxonomic level in DSS treated mice

Gut flora is one of the essential features in the progression of intestinal inflammation in IBD (Sartor, 2008). In a healthy gut, the major phyla that constitute the gut flora are *Firmicutes, Bacteroidetes, Actinobacteria* and *Verrucomicrobia* (Jandhyala et al., 2015). Previous studies have shown that dysbiosis occurs in IBD and alteration in the normal composition and abundance of bacterial community occurs such as a decrease in the relative abundance of *Bacteroidetes, Firmicutes* and increase in *Proteobacteria* (Frank et al., 2010; Morgan et al., 2012). In order to examine whether DIP treatment regulates gut microbiota structure and composition, we evaluated bacterial distribution at various taxonomic levels in DSS induced colitis mice. In our study 1458676, clean tags were acquired from 1,699,253 raw reads (Supplementary Table S4). A total of 13,132 Operational Taxonomic Unit (OTUs) ranging from 435 to 1306 were obtained (Supplementary Table S4). The bacterial count was decreased at a higher and lower taxonomic level in the DSS group compared to the control and DIP treated groups (Fig. 7A) (Supplementary Table S5). In harmony with the previous findings, the abundance of *Firmicutes* decreased while *Proteobacteria* levels enhanced in the DSS alone treatment compared to the control group at the phylum level (Fig. 7B). However, DIP treatment reversed the dysbiosis pattern effectively at the high dose in the DSS + DIP(H) group compared to the DSS group. At lower dose in DSS + DIP(L) variation persisted as compared to DSS + DIP(H) and control group. Furthermore, at genus, family, order and class level, the bacterial composition varied in the DSS alone treatment compared to the control group (Fig. 7C, D, E, and F). *Bacteroides*, *Lactobacillus*, *Odoribacter*, and *Oscillospira* were dominant flora at the genus level. *Bacteroides* enhanced greatly in the DSS alone in comparison with the control group (DSS 61.94% vs. Control 13.98%) (Fig. 7C) (Supplementary Fig. S2(A), 3)). Nonetheless, DIP treatment modulated the perturbed flora at various degrees. After DIP treatment, *Bacteroides* levels improved compared to DSS group (DSS 61.94% vs. DSS + DIP(L) 21.11%)) (DSS 61.94% vs. DSS + DIP(H) 10.86%). On the other hand, *Lactobacillus* dropped drastically is the DSS alone group in comparison with the control and DIP treated groups (Fig. 7C) (Supplementary Fig. S2(A), 3)). At Family level, *Bacteroidaceae* and *Enterobacteriaceae* prevailed in the DSS alone group which were less abundant in the control group (Fig. 7D) (Supplementary Fig. S2B). At order and class level, *Enterobacteriales* and *Gammaproteobacteria* enhanced markedly as compared to the control group (Fig. 7E, F), conversely, DIP at high dose ameliorates the relative abundance of perturbed flora. Inclusively, our data revealed DSS induced colitis cause alterations in the relative abundance of bacterial flora at various taxonomic levels. However, DIP treatment at low dose partly and at high dose, greatly improved gut microbiota composition and mostly flora returned to the normal state at a high dose of DIP.

3.7. Distribution of OTUs and microbial phenotypes evaluation at the organism level with Bug base analysis

In order to demonstrate shared and group-specific OTUs across each treatment, a Venn diagram was generated (Fig. 8A). 474 OTUs were shared by all the treatment groups. Each leaflet depicts the number of OTUs in the respective group. Furthermore, a comparison of differentially abundant taxa between DSS and DIP treated samples is presented with linear discriminant analysis (LDA) (Fig. 8B). To investigate the hierarchical relationship of all the flora from the phylum to the Genus (from the inner circle to the outer circle) in the community, the cladogram tree was constructed (Fig. 8C). The node size corresponds to the relative abundance of the taxon, and the yellow node represents significant differences between groups of classification units.

Bug base is used to assess high-level phenotypes across each treatment group. In our study, BugBase identified phenotype differences associated with aerobic, anaerobic, facultatively anaerobic, biofilms formation, gram-negative bacteria, gram-positive bacteria, potentially

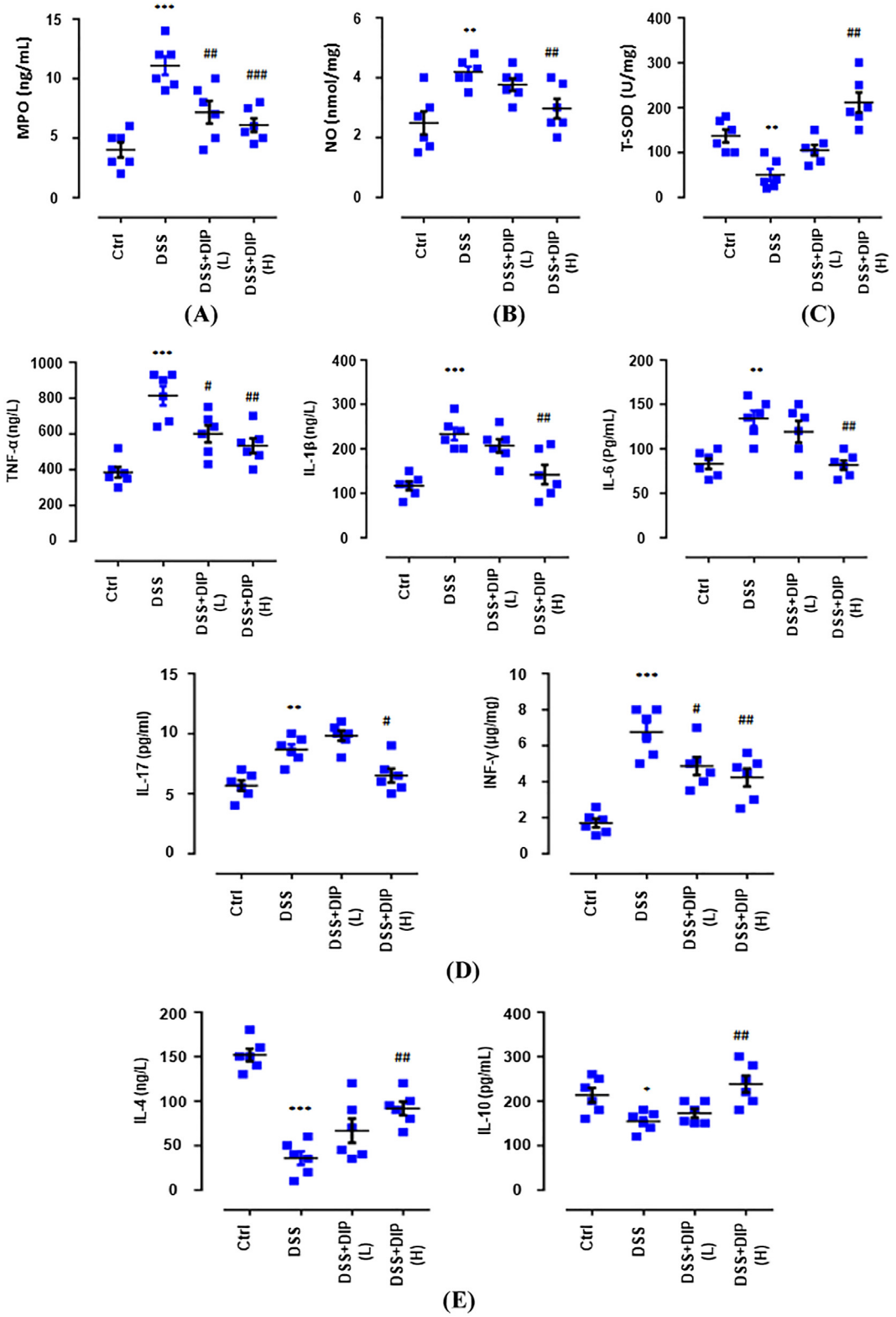


Fig. 4. Effect of DIP on neutrophil infiltration, oxidative stress and inflammatory cytokines in the colonic mucosa of DSS induced colitis mice. (A) MPO activity. **(B)** NO level. **(C)** T-SOD activity. **(D)** Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-17, IFN-γ, **(E)** Anti-inflammatory cytokines IL-4, and IL-10 levels in the colon tissue were analyzed by ELISA kits. Data are expressed as mean ($n = 6$ per group) ± SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ vs control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DSS group.

pathogenic and stress tolerant (Fig. 8D, E). Our data suggested that microbiome phenotypes were more or less similar between control and DIP treated groups. However, DSS treatment exhibited variation in the relative abundance of several microbiome phenotypes. The functional genes encoded for facultative anaerobes, potentially pathogenic, stress tolerant and biofilm-forming bacteria were enriched in the DSS group. *Proteobacteria* (indicated with yellow color in the bar graph) was the most abundant flora in the formation of these high-level microbiome

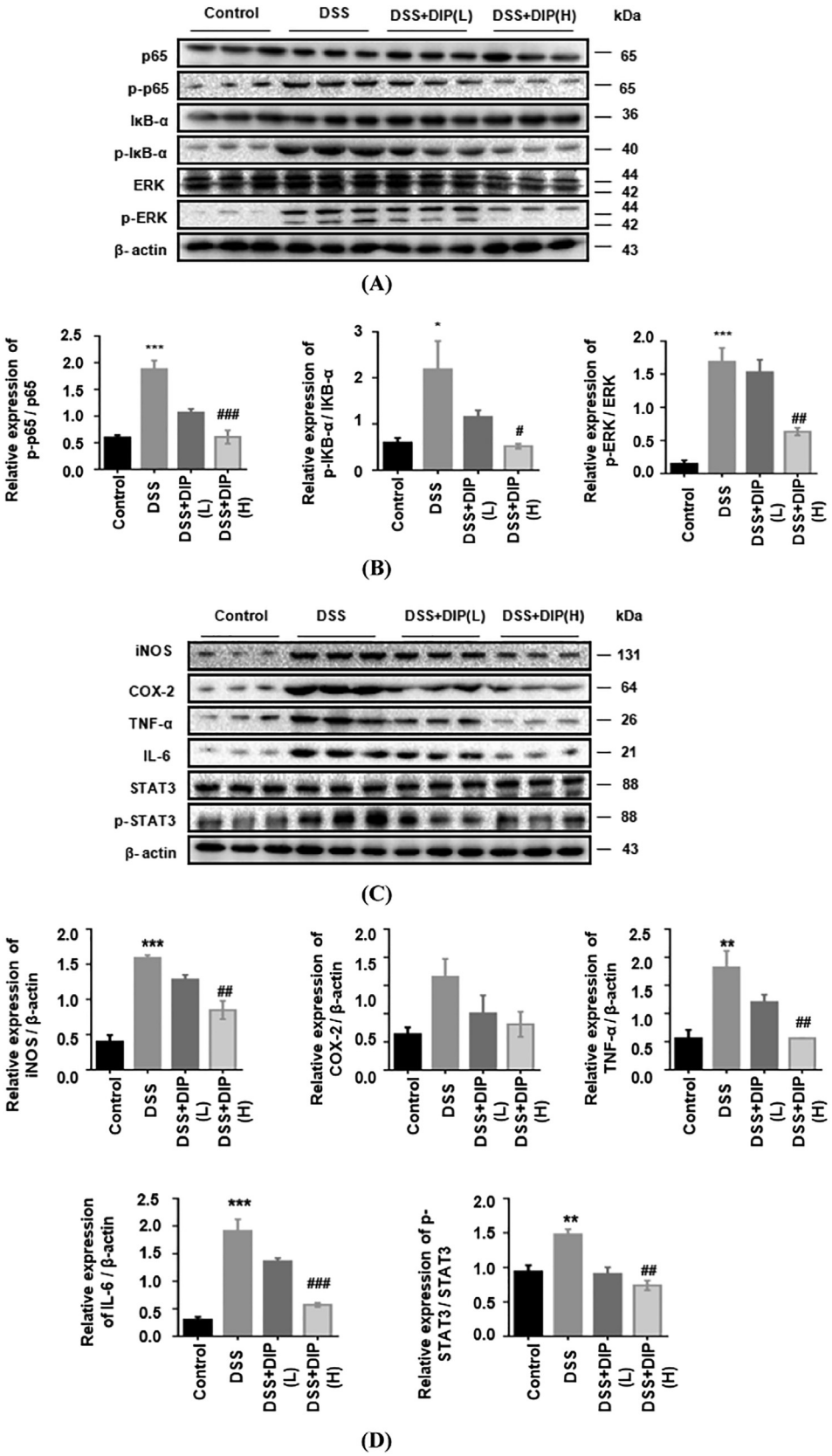


Fig. 5. Effect of DIP in the modulation of colitis-associated signaling pathways. **(A& C)** Western blot images demonstrating the relative expression of protein p65, p-p65, IκB-α, p-IκB-α, ERK, p-ERK, iNOS, COX-2, TNF-α, IL-6, STAT3, and p-STAT3 using β-actin as an internal control**. (B & D)** Bar graph showing relative band intensity of respective protein quantified via NIH image J software. The data was acquired from three independent experiments and is expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ vs control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DSS alone group.

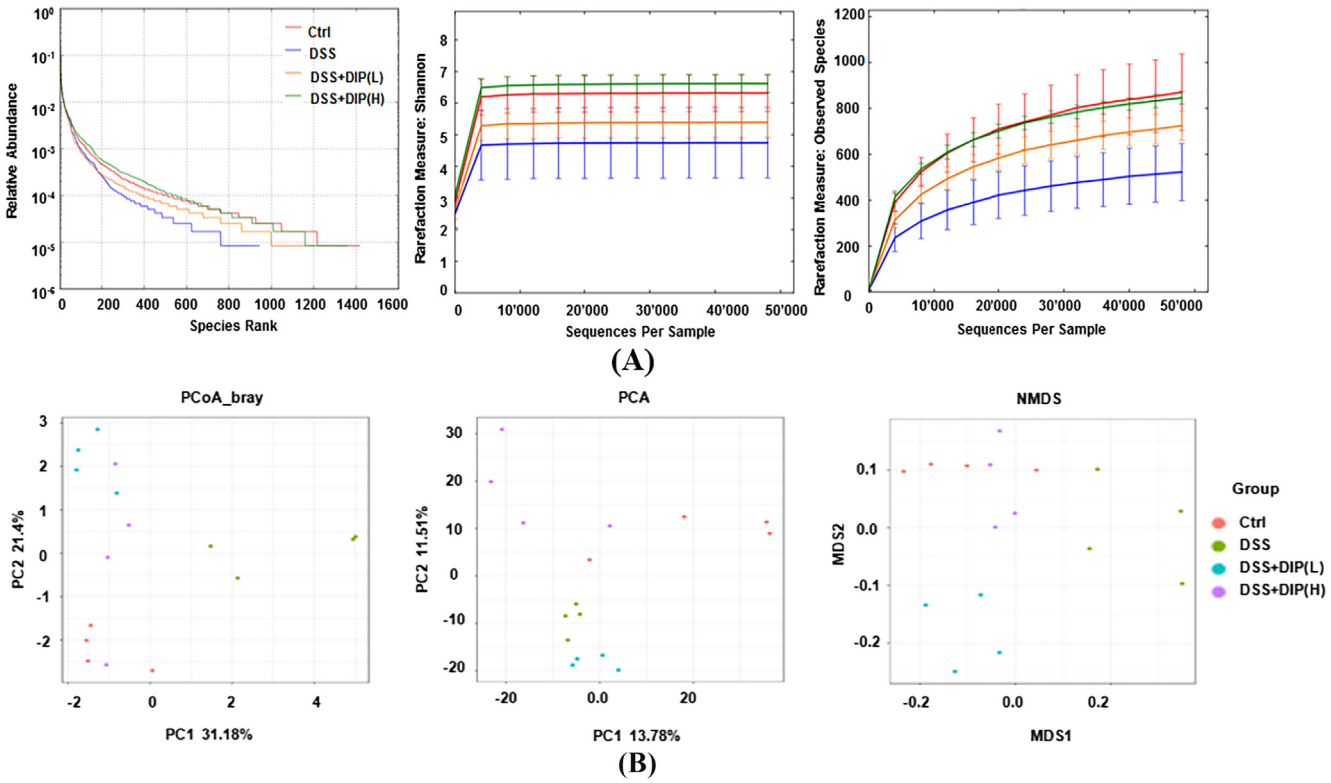


Fig. 6. Effect of DIP on alpha and beta diversity indices in DSS induced colitis mice. (A) Rank abundance curve, Shannon index, and Observed species represents the alpha diversity indices. Rank abundance curve shows bacterial richness and abundance, respectively. Rarefaction curves including Shannon index and Observed species represent species diversity, abundance, and evenness. **(B)** Beta diversity parameters were measured using PCoA, PCA and NMDS plot. Each point designates an individual sample, and the points of distinct colors belong to different treatments. The distance between different points exhibits the similarity or differences of the microbial community structure.

phenotypes in the DSS group (Fig. 8E). The role of *Proteobacteria* in the development of IBD has been addressed already in several studies (Morgan et al., 2012; Sokol et al., 2017). Conversely, treatment with DIP redirected the abundance of high-level phenotypes.

4. Discussion

Inflammatory bowel disease (IBD) is a chronic inflammatory illness, characterized by an anomalous immune response in the gastrointestinal (GI) tract (Miao et al., 2015). Chemically induced colitis mice models have been extensively used to investigate the molecular mechanism of colitis and in search of effective therapies in the management of the disease. Natural products such as polysaccharides from various sources (plant/mushroom) have been used as an alternative therapy in several inflammatory diseases such as gastric ulcers, ulcerative colitis and chronic gastritis (L. Liu et al., 2008; Miao et al., 2015; Muszyńska, Grzywacz-Kisielewska, Kała, & Gdula-Argasińska, 2018; Qin et al., 2016; M. Wang, Gao, Xu, & Gao, 2015). Our previous study has shown the modulatory effect of *Dictyophora Indusiata* mushroom polysaccharide (DIP) on gut microbiota in mice model (Kanwal et al., 2018). Keeping in mind the beneficial properties of *D. Indusiata* polysaccharide, we proposed that DIP may exert an ameliorative effect on DSS induced colitis.

Herein, the disease activity index (DAI) revealed that DSS treatment resulted in the induction of chronic colitis. The DAI enhanced significantly showing a decrease in body weight, severe diarrhea, and gross rectal bleeding in DSS alone treatment compared to the control group. Furthermore, the colon length was decreased markedly upon DSS treatment. However, DIP treatment at a higher dose in particular evidently alleviated the clinical symptoms of colitis (Fig. 2). The body weight, colon length, and DAI improved significantly. These findings revealed the preventive effect of DIP in DSS induced colitis.

In the present study, we observed histological changes and lower TJs expression in the DSS treated group (Fig. 3). DSS induced colitis damaged intestinal and epithelial integrity; however, DIP treatment ameliorated the architecture of colon tissue. The structure of epithelial cells improved and the number of goblet cells elevated. The pathological symptoms including inflammatory cell infiltration, mucosal abrasion, submucosal edema, loss and disruption of crypts and villi were improved after DIP treatment particularly at high dosage (DSS + DIP(H)).

Furthermore, some clinical studies have shown decreased tight junction proteins (TJs) expression in ulcerative colitis (Gonzalez-Mariscal, Betanzos, Nava, & Jaramillo, 2003; Mees et al., 2009). The intercellular tight junction proteins (TJs) account for epithelial permeability, paracellular diffusion, and cell–cell adhesion. TJs including cytoplasmic scaffolding proteins (e.g ZO family) and transmembrane barrier proteins (e.g. Claudins and Occludin) have a key role in the preservation of intestinal integrity (Dokladny, Zuhl, & Moseley, 2015; Sánchez de Medina, Romero-Calvo, Mascaraque, & Martínez-Augustin, 2014). Various inflammatory responses influence the expression of TJ proteins. For instance, a higher level of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α, and IL-1β) diminish the tight junction proteins (TJs) expression in the intestine (Al-Sadi, Boivin, & Ma, 2009). Considering the fact that TJs have a pivotal role in the maintenance of mucosal integrity, we determined tight junction proteins expression in the colonic mucosa of mice. Consistent with the previous literature, our results revealed a decreased tight junction proteins expression in the DSS induced colitis mice. Nevertheless, DIP elevated the expression of TJs in DIP treated groups (Fig. 3D, E). These findings revealed that DIP potentially ameliorates histological structure and intestinal integrity by augmenting mucins expression, and TJs expression.

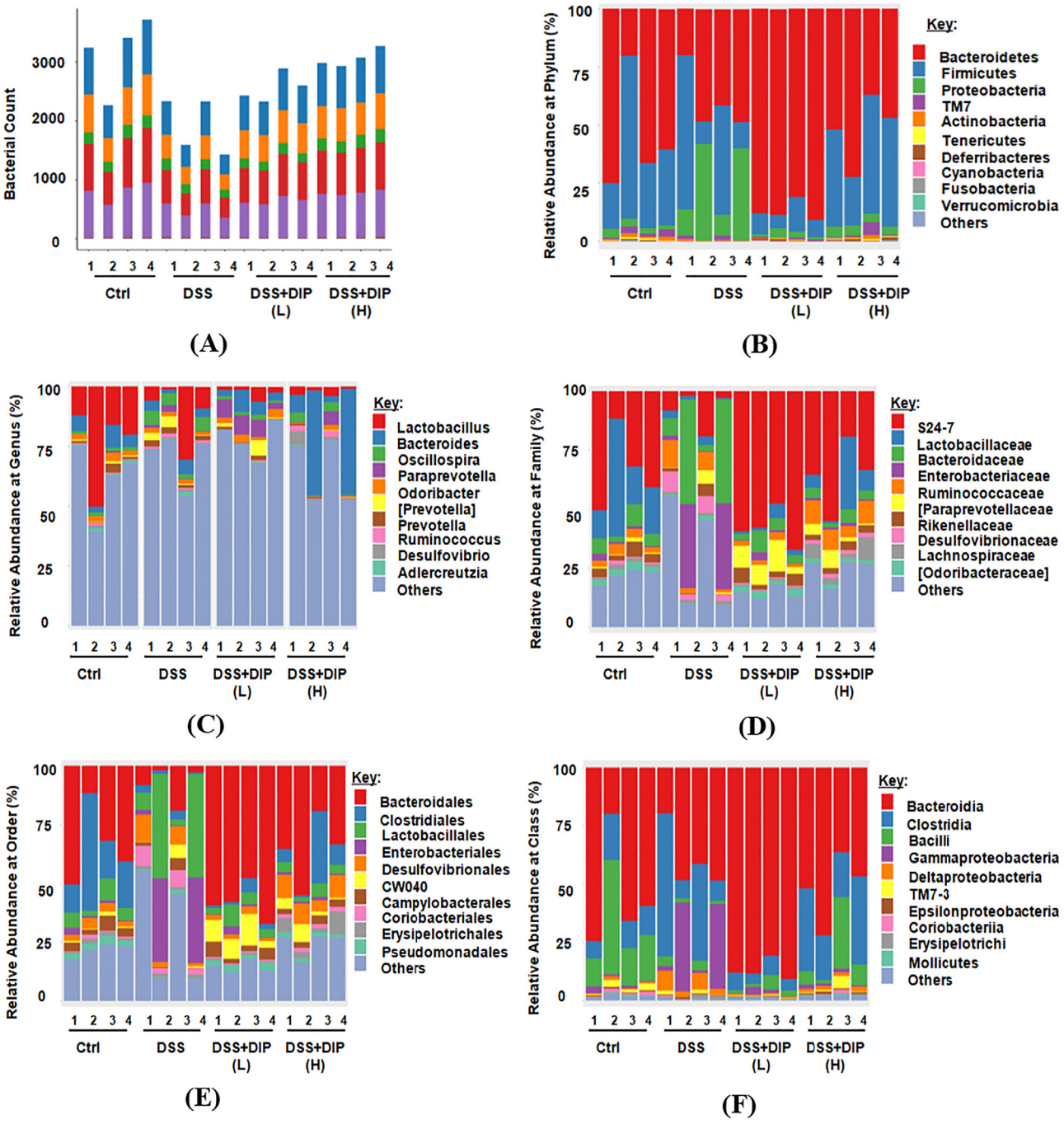


Fig. 7. Microbial composition at various taxonomic levels in the DSS induced colitis mice. **(A)** Bacterial count at all taxonomic levels. **(B)** Relative abundance (%) at the phylum level. **(C)** Relative abundance (%) at the genus level. **(D)** Relative abundance (%) at the family level. **(E)** Relative abundance (%) at the order level. **(F)** Relative abundance (%) at the class level.

The association of myeloperoxidase enzyme (MPO) with ulcerative colitis is documented in earlier studies (He et al., 2016; R. Li et al., 2016). MPO is an enzyme that is used as an inflammatory marker to determine inflammatory cell infiltration and tissue damage (Ren et al., 2018). In accordance with these studies, our findings revealed higher MPO levels in DSS alone treated group in comparison with the control group. Nonetheless, DIP treatment alleviated the MPO levels markedly in a dose-dependent manner (Fig. 4). Moreover, the involvement of oxidative stress and the formation of oxygen free radical species in the progression and development of colitis has been confirmed in various studies (Ren et al., 2018; H. Zhu & Li, 2012). The oxygen free radicals generate upon higher nitric oxide (NO) production (R. Li et al., 2016) eventually causing tissue damage and trigger inflammatory responses. Conversely, antioxidant enzymes such as superoxide dismutase (SOD) alleviated the oxidative stress in clinical and in-vivo studies (Ren et al., 2018; Suzuki, Matsumoto, Okamoto, & Hibi, 2008). Therefore, we investigated the NO, and T-SOD activity. Our findings indicated that DSS treatment increased NO levels and reduced T-SOD activity. On the contrary, DIP treatment alleviated NO activity and elevated T-SOD levels. (Fig. 4B, C). These findings demonstrate the anti-oxidative properties of DIP via scavenging oxygen free radical species.

Dysregulation of pro-inflammatory cytokines is a hallmark of IBD. Accumulating literature has shown the pivotal role of pro-inflammatory cytokines in the pathogenesis of IBD (Fina & Pallone, 2008; Neurath,

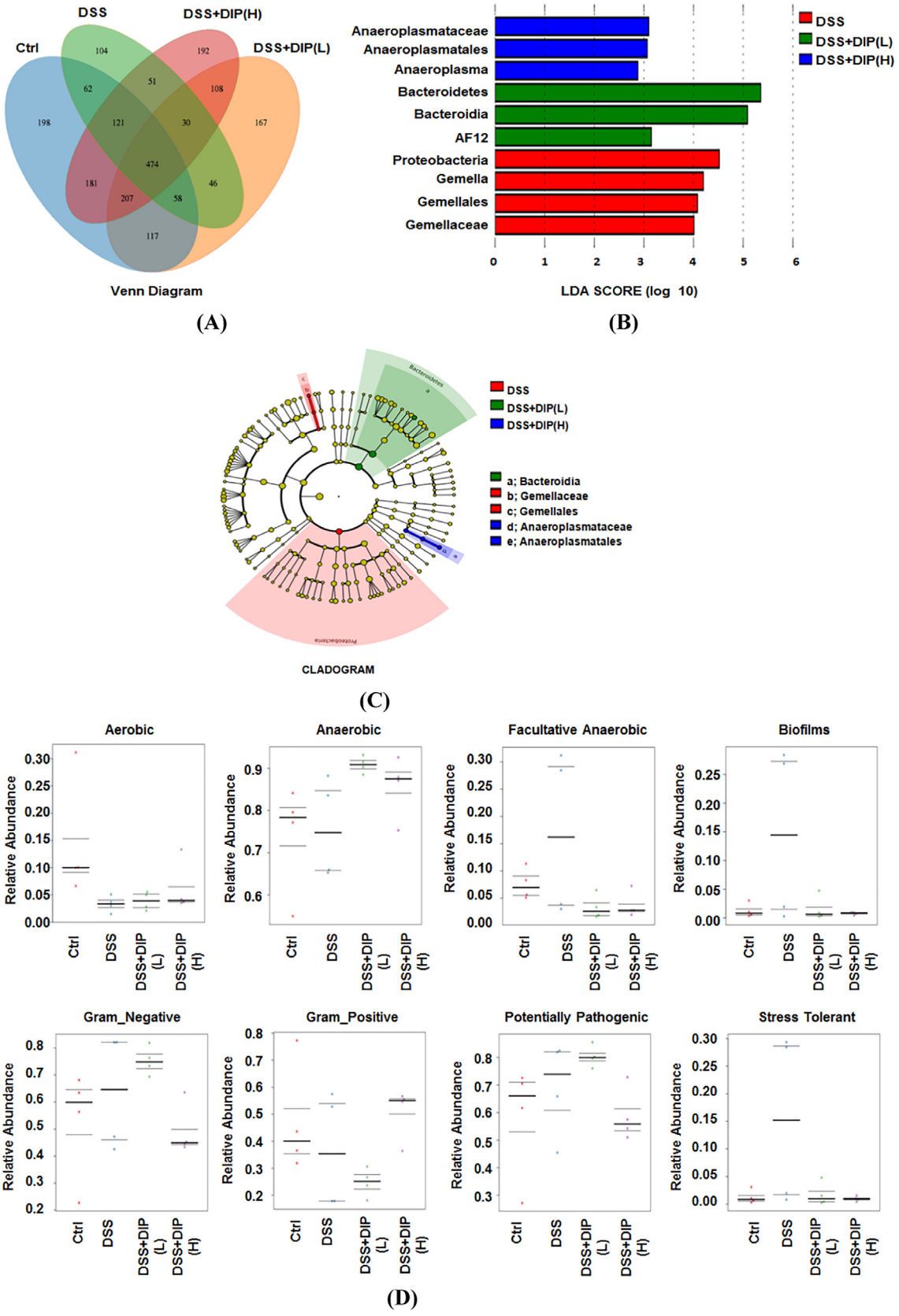
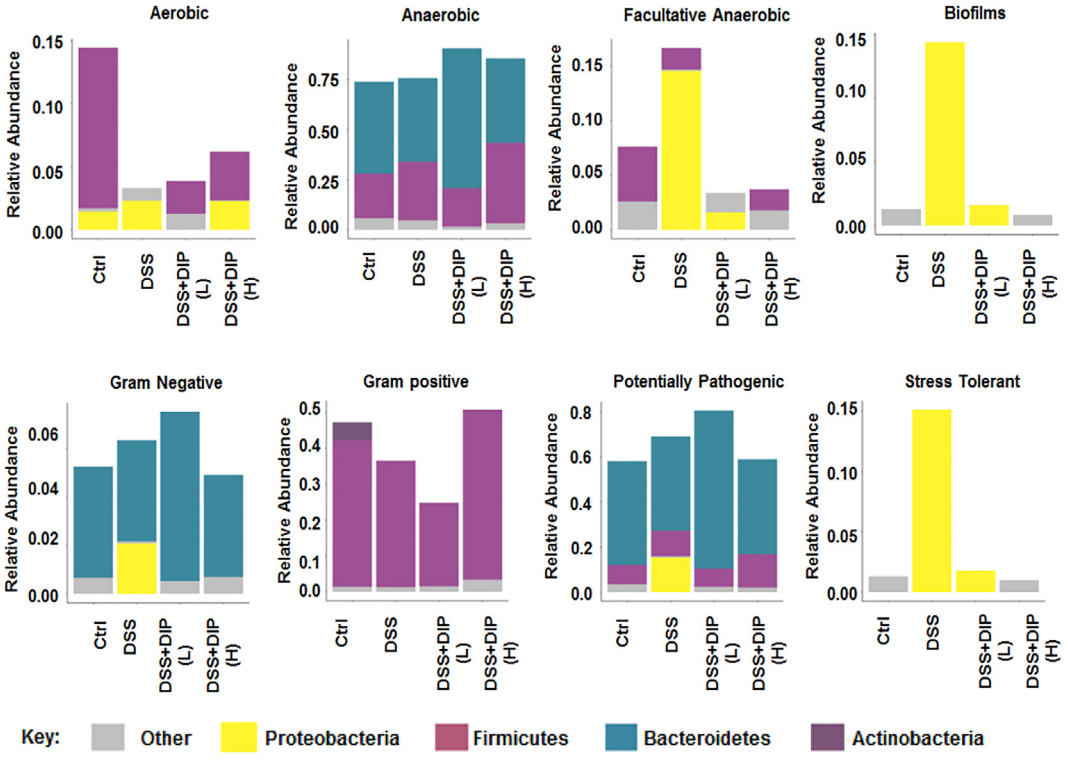


Fig. 8. Operational Taxonomic Unit (OTUs), Linear Discriminant Analysis (LDA), effect size (LEfSe) analysis of bacterial taxa and Bug Base predicted phenotype analysis. (A) The distribution of operational taxonomic units (OTUs) among four different treatment groups is represented via the Venn diagram. **(B)** Linear Discriminant Analysis (LDA) indicates most differential abundant bacterial taxa in specific samples. **(C)** Cladogram illustrating highly abundant taxa across various treatments. **(D)** BugBase predicted phenotype differential analysis among all the groups. Aerobic, anaerobic, facultative anaerobic, biofilms formation, gram-negative, gram-positive, potentially pathogenic and stress tolerant were the identified phenotypes. **(E)** Bar graph represents the proportion of each phyla in the respective phenotypes.



(E)

Fig. 8. (*continued*)

2014; Sanchez-Muñoz et al., 2008; Strober & Fuss, 2011). An elevated level of pro-inflammatory cytokines including (TNF-α, IFN-γ, IL-1β, IL-6, and IL-17) is the main feature of colitis (Ito et al., 2008; Jeengar et al., 2017; Kang et al., 2015; Műzes, Molnár, Tulassay, & Sipos, 2012). Plant-derived polysaccharides, particularly from mushrooms, mitigate the inflammatory reactions (Diling et al., 2017; Lv et al., 2017; Nie, Lin, & Luo, 2017; Tao et al., 2017; Tao et al., 2018). We measured the level of pro-inflammatory and anti-inflammatory cytokines, respectively. Our results suggested the level of pro-inflammatory cytokines enhanced in the DSS alone group. However, DIP treatment significantly reduced the pro-inflammatory cytokines secretion. The tendency of IL-4 and IL-10 to ameliorate mucosal damage in IBD has been reported previously (Alex et al., 2008; Kang et al., 2015; Olszak et al., 2014; Tao et al., 2017). Thus we targeted these anti-inflammatory cytokines. Our findings indicated an elevated IL-4 and IL-10 levels upon DIP administration in DSS treated groups (Fig. 4E). These results suggested that DIP regulate inflammatory responses by augmenting anti-inflammatory cascades in colitic mice.

Given the capacity of DIP in improving DSS induced colitis, we further targeted the inflammatory-related signaling pathways that are implicated with the etiology of IBD. Studies have shown that NF-κB and MAPKs signaling pathways have a role in the induction of ulcerative colitis (J. Li et al., 2014; Sahu et al., 2016; Schwanke et al., 2013). The activation of NF-κB mitigates inflammatory responses via up-regulating infiltration of neutrophils and macrophages (J. Li et al., 2014; Schreiber, Nikolaus, & Hampe, 1998). Additionally, it can induce several cytokines expression including TNF-α, IL-6, and IL-12 and also enhance the expression of inflammatory enzymes such as COX-2 and iNOS (Barnes & Karin, 1997; Chen et al., 2004; He et al., 2016; Sahu et al., 2016; Schottelius & Baldwin Jr, 1999). Our results demonstrated the anti-ulcerative colitic action of DIP via dephosphorylation of NF-κB and MAPK, inhibition of iNOS, COX-2, and reducing the level of TNF-α and IL-6 respectively in DIP treated groups (Fig. 5).

The intestinal microbiota plays a rudimentary role in the maintenance of the host immune system via its symbiotic relationship with immune cells. Perturbation in the intestinal microbiome has been implicated with the pathogenesis of IBD (Kramer & Genco, 2017; Manichanh, Borruel, Casellas, & Guarner, 2012; Marta Wlodarska, Kostic, & Xavier, 2015). 16S rRNA Illumina MiSequencing was employed in our study to investigate the composition of the gut microbiome. Previous studies have shown reduced bacterial diversity and richness in UC patients and also in animal models (Andoh et al., 2007; Manichanh et al., 2006; Samanta, Torok, Percy, Abimosleh, & Howarth, 2012). In agreement with these studies, we also found reduced bacterial abundance and richness in the DSS alone treatment compared to the control group (Fig. 6). Nonetheless, bacterial diversity and abundance were augmented after DIP treatment. Moreover, beta diversity parameters were assessed to find the similarities among various treatment groups. As expected, variation in the clustering pattern was evidenced in DSS and DIP groups. Control and DIP groups clustered more closely, while DSS alone treatment revealed more deviation from these respective groups.

The main phyla that shape the most of microbial community are; *Firmicutes, Bacteroidetes,* and *Proteobacteria.* Multiple reports have shown the increment of *Proteobacteria* and reduction of *Firmicutes* in IBD (Liguori et al., 2015; Ott et al., 2004; Sokol et al., 2017). Consistent with the previous findings, our results revealed a higher abundance of *Proteobacteria* and a lower abundance of *Firmicutes* in DSS alone treatment. However, DIP treatment potentially reversed the microbial dysbiosis at the phylum level and revealed a similar trend like the control group, particularly at high dose (DSS + DIP(H)). To our surprise, DIP at low dose depicted a high abundance of *Bacteroidetes* in the DSS + DIP (L) group. Possibly, DIP at a lower dose is not much effective compared to a high dose. We further investigated the microbial flora at lower taxonomic levels. Previously the higher abundance of *Bacteroides* and associated species such as *Bacteroides fragilis* have been correlated with the pathology of IBD and colorectal cancer (Sears, 2009). In concordance with the previous studies, we found an increase abundance of *Bacteroides* (61.9%) in the DSS group (Fig. 7C) (Supplementary Fig. S2(A), 3)). Conversely, *Lactobacillus* levels dropped to a greater extent

in the DSS group, Nonetheless, after DIP treatment the abundance of *Lactobacillus* increased particularly at the high dose. *Lactobacillus* is well known for modulating health and a number of studies have shown its protective effect against several infections and inflammatory disorders (Lara-Villoslada et al., 2007). Several *Lactobacillus* species are lactate producing bacteria that is a precursor for short chain fatty acid (SCFA) producing bacteria (Tsukahara, Koyama, Okada, & Ushida, 2002). Furthermore, at the family level, the abundance of *Bacteroidaceae* and *Enterobacteriaceae* greatly enhanced in the DSS alone group and the higher abundance of these taxa has been correlated with ulcerative colitis (Xu, et al., 2018). However, the bacterial shift was reversed after DIP treatment (Fig. 7D) (Supplementary Fig. S2(B)). The percentages of abundant flora at the genus and family level is illustrated in Supplementary Fig. S2. Additionally, bacterial abundance at the order and the class level is presented in Fig. 7E, F. The bacterial abundance of certain species belonging to *Proteobacteria* including *Gammaproteobacteria* has been reported to increase in IBD (Sokol & Seksik, 2010). In harmony with the previous findings, our results also suggested an increased abundance of *Gammaproteobacteria* in DSS alone treatment compared to the control group (Fig. 7F). Contrarily, DIP treatment reversed the bacterial shift and reduced the level of *Gammaproteobacteria* in DIP treated groups especially at a high dose. Comprehensively, our data at all taxonomic levels suggested the bacterial shift in DSS treatment alone; however, DIP treatment reversed the trend to a greater extent, particularly at the high dose. These findings suggested the immunomodulating properties of DIP.

Shared and group-specific OTUs were demonstrated in Venn diagram (Fig. 8A). LDA and Cladogram were generated to exhibit hierarchy and abundance of gut microbiome (Fig. 8B, C). In order to elucidate the high-level microbiome phenotypes in different treatment groups, Bugbase analysis was employed. Our findings revealed a higher abundance of facultative anaerobes, stress tolerant and biofilm-forming bacteria in the DSS alone treatment in comparison with the control group (Fig. 8D, E). The composition of these phenotypes mainly came from *Proteobacteria* which has been associated with IBD and UC in multiple studies as mentioned above. Yet, DIP alleviated the relative abundance of these phenotypes suggesting its modulatory effect on the gut microbiome community.

5. Conclusions

Altogether, our study corroborates that the crude polysaccharide (DIP) isolated from the mushroom *D. indusiata* contains potentially bioactive polysaccharide components that have shown a promising effect against DSS induced colitis by ameliorating intestinal injury, oxidative stress, and pro-inflammatory cytokine production. Additionally, DIP treatment modulated inflammatory related signaling pathways, enhanced mucins, and tight junction proteins (TJs) expression. Moreover, DIP restored the intestinal microbiome by redirecting the bacterial shift to a normal state and suppressing harmful taxa including, *Proteobacteria, Gammaproteobacteria, Bacteroides, Bacteroidaceae, Enterobacteriaceae* and enhancing beneficial flora such as *Lactobacillus*. Since at this stage, we have used crude polysaccharide in order to demonstrate its therapeutic potential, so it is suggested that after specifically identifying the active polysaccharide component it can be used to investigate the protective and curative effects against other diseases. It is our hope that after concrete preclinical and clinical studies, the clinical implications of these bioactive polysaccharides can be made possible in the near future in order to treat multiple human ailments.

Author contributions

Conceptualization, S.K., Y.X., T.P.J., and S.A.; Methodology, S.K., S.S., Y.M., and T.P.J.; Software, S.K., and S.A.; Formal Analysis, S.K. and M.Z.S.; Investigation, S.K., M.A.N., and T.P.J.; Resources, Y.X.; Data Curation, S.K.; Writing—Original Draft Preparation, S.K.; Writing—Review and Editing, T.P.J., S.A., and Y.X.; Validation, Y.M., and Y.X.; Visualization, Y.X.; Y.W., A.M.; Supervision, Y.X; Project Administration, Y.X.; Funding Acquisition, Y.X.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Ethical approval statement

Approval for this study was obtained from the Dalian Medical University Committee for animal experiments, and animal handling practices agreed with guidelines on Care and Use of Laboratory Animals sanctioned by the National Institutes of Health. All animal experiments comply with the ARRIVE guidelines and has been carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103641.

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