

***Dendrobium sonia* polysaccharide regulates immunity and restores the dysbiosis of the gut microbiota of the cyclophosphamide-induced immunosuppressed mice**

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[ABSTRACT] To recognize the potential medicinal value of the *Dendrobium sonia*, polysaccharide (DSP) was extracted, purified, and investigated for its immunomodulatory activity. *In vitro*, DSP was shown to enhance the viability (MTT assay) and phagocytosis of macrophages. In cyclophosphamide-induced immunosuppressed mice, DSP increased serum levels of TNF- α , IL-6 and IFN- γ (enzyme-linked immunosorbent assay, ELISA), and ameliorated the imbalance of the community of gut microbiota as detected by 16S ribosomal RNA gene sequencing. These results suggest that DSP might be beneficial for patients under immunosuppressed conditions.

[KEY WORDS] *Dendrobium sonia*; Polysaccharides; Immunomodulatory activity; Gut microbiota

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Introduction

Dendrobium sonia, one of the most commonly cultivated species of *Dendrobium* spp. [1], has not been used for medicinal purposes and no scholars have studied its potential medicinal value so far. In contrast, *Dendrobium officinale* [2-3] and *Dendrobium huoshanense* [4-7] are often used as medicinal herbs which belong to the same Genus. Formed by a plurality of identical or different monosaccharides linked by glycosidic bonds, polysaccharides are one of the most naturally occurring macromolecules in organisms. Polysaccharides have various bioactivity effects in areas ranging anti-tumor [8], anti-oxidation [9-10], anti-aging, anti-bacterial, anti-viral, hypoglycemic, hypolipidemic effects and so on [11]. As the main active ingredient, polysaccharides have become a hot topic in the research of the pharmacodynamics of the *Dendrobium* plant [12-14]. Research now observes that polysaccharides from the stem of *D. officinale* not only enhance natural killer

cell-mediated cytotoxicity and promote splenocyte proliferation, but they also increase the phagocytosis and nitric oxide production of macrophages [3]. Furthermore, the two fractions significantly promote the cytokine secretion of both splenocytes and macrophages [3]. Moreover, the polysaccharides from *D. huoshanense* could improve the intestinal physical barrier function by modulating mucosal structures and up-regulating the expression of tight junction proteins, reinforcing the intestinal biochemical barrier function by elevating the expression and secretion of mucin-2, β -defensins and sIgA, and regulating the intestinal immunological barrier function by stimulating the production of cytokines and the functional development of immune cells [7]. While the gut microbiota is considered as the forgotten organ of the human body [15], it plays a vital role in the induction, training, and function of the host immune system [16-18]. Therefore, studies with the gut microbiota as an area with drug targeting application continues to attract the attention of scholars [19].

In the present study, the polysaccharide from *D. sonia* was extracted, purified, and investigated for its immunomodulatory activities *in vivo* and *in vitro*. Moreover, the mechanisms potential immuno-modulatory activities on the immunosuppression were assessed. We hope that the findings obtained from the present study would help develop immunity-enhancing medicines in the future.

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Materials and Methods

Materials

Electronic analytical balance of BP-211D (Sartorius Corporate Administration GmbH, Goettingen, Germany); Water bath cauldron (Jintan Jierui Er Electric Co., Ltd., Changzhou, China); Ring vacuum pump (Gongyi Huayi Instrument Co., Ltd., Zhengzhou, China); UV Spectrophotometer (Thermo Fisher Scientific, Waltham, USA); Water purifier of Direct-Q3&Directs (MERCK MILLIPORE, Burlington, USA); Plate reader M200 (Tecan Group Ltd., Männedorf, Switzerland); Freeze dryer and Vacuum (Concentrator Lab-conco Corporation, Kansas City, USA); C₁₈ column Inertsil ODS-3 [4.6 mm × 250 mm × 5 μm, Shimadzu (China) Co., Ltd., Hangzhou, China]; Agilent 1100 series (Agilent Technologies, Santa Clara, USA).

The dry stems of *Dendrobium Sonia* (Lot No. DSPR150906) was obtained from Jinling Pharmaceutical Company Ltd. (Nanjing, China); D(+)-Anhydrous Glucose (Glu), Cyclophosphamide (CTX), Lentinan, (Purity ≥ 98%) (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China);

L(+)-arabinose (Ara), D-mannose (Man), D-Xylose (Xyl), galactose (Gal) and rhamnose (Rha) (Purity ≥ 98%) was obtained from National Institutes for Food and Drug Control, (Beijing, China);

Enzyme-linked immunosorbent assay (ELISA) kits of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China);

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), Lipopolysaccharides (LPS) was obtained from Sigma-Aldrich (St. Louis, USA); All other reagents obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China) were of analytical grade and used without any further purification.

Extraction and purification of *D. sonia* polysaccharide (DSP)

Briefly, the samples were crushed into coarse powders and subsequently treated with 6 volumes of petroleum in order to be degreased. After being soaked for 2 hours in petroleum at the room temperature, they were let to dry. Then the powders were once extracted for 3 hours with 20 volumes of ultra-pure water in a water bath at 100 °C for reflux extraction. After filtration, the aqueous phase was precipitated over 48 hours by adding absolute ethanol until the alcohol concentration reached 80% at room temperature. The precipitate was lyophilized into crude polysaccharides. The crude polysaccharides were sequentially purified by chromatography with DEAE Sepharose™ Fast Flow (Sigma-Aldrich, St. Louis, USA) and Sephadex® G-100 (Sigma-Aldrich, St. Louis, USA). Specifically, the crude polysaccharides were dissolved in ultra-pure water and eluted with a step gradient of 0–0.5 mol·L⁻¹ NaCl into different fractions through the DEAE Sepharose™ Fast Flow column. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid

method [20]. The major polysaccharide fractions were pooled, dialyzed and further purified in a Sephadex® G-100 column with ultra-pure water as the eluent into different fractions. Again, the fractions were assayed for carbohydrate content by the phenol–sulfuric acid method. Then the major polysaccharide fractions were pooled and lyophilized into *D. Sonia* polysaccharide (DSP).

Monosaccharide analysis

Monosaccharide composition was measured by reversed phased high-performance liquid chromatography (RP-HPLC) after pre-column derivatization. DSP was hydrolyzed with 2 mol·L⁻¹ trifluoroacetic acid (TFA) at 100 °C for 6 h in a sealed tube and then the TFA was removed by methanol vacuum concentrator. Hydrolysate and all reference sugars [D(+)-anhydrous glucose, L(+)-arabinose, D-mannose, D-xylose, galactose and rhamnose] were dissolved in 0.3 mol·L⁻¹ NaOH and then added to 0.5 mol·L⁻¹ methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP, 0.5 mol·L⁻¹) at 70 °C for 1 h. At last, 0.3 mol·L⁻¹ HCl solution was added into the mixture until neutral pH was attained. The supernatant containing the PMP-labeled carbohydrate was filtered through a 0.22 μm nylon membrane, and injected into HPLC with C₁₈ column. The mobile phase was composed of solvent A [Ammonium acetate buffer (10 mmol·L⁻¹, pH 6.8)] and solvent B (Acetonitrile) with the gradient elution: 0 min A : B = 90 : 10, 10 min A : B = 75 : 25, 20 min A : B = 75 : 25, 25 min A : B = 90 : 10. The flow-rate was 1.0 mL·min⁻¹ at a column temperature of 30 °C. The monosaccharide composition of a sample can be determined according to the time of each chromatographic peak in the chromatogram. The molar ratio of various monosaccharides can be calculated from the peak areas of the respective chromatographic peaks.

Immunomodulatory activity of DSP *in vitro*

RAW 264.7 cells (Shanghai Institute of Cell Biology, Shanghai, China) were cultured in RPMI1640 supplemented with 10% (V/V) fetal bovine serum, 100 U·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin. Cells were incubated in an atmosphere of 5% CO₂ at 37 °C.

The viability of macrophage was measured using MTT assay. RAW 264.7 cells were plated in a 96-well microplate (1 × 10⁵ cells/well, in a volume of 100 μL) for 24 h and then cultured with different concentrations of DSP (100, 200, 300 μg·mL⁻¹) or LPS (10 μg·mL⁻¹) for 24 h. The cells cultured in the absence of DSP and LPS were used as the normal control. After incubation, 20 μL of MTT (5 mg·mL⁻¹) was added to each well, the plates were incubated for 4 h and then the media removed. Aliquots of 150 μL of DMSO were added to each well for 1 h. The absorbance at 570 nm was detected by using the plate reader.

Cell viability was calculated as follows: Index of viability = (A – B)/(C – B). A is the average optical density (OD) of the DSP or LPS treated cells; B is the average OD of the control wells (culture medium without cells); and C is the average optical density of the Blank control (culture medium

with cells).

The phagocytic ability of macrophage was measured by neutral red uptake. The medium was removed after the cells were cultured with DSP at different concentrations (100, 200 and 300 $\mu\text{g}\cdot\text{mL}^{-1}$) or LPS (10 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h, and 100 μL of 0.1% neutral red in phosphate-buffered saline (PBS) was added to each well and incubated for an additional 2 h. The cells were washed with PBS thrice to remove the neutral red that was not phagocytized by the macrophages. Then 100 μL of 1% acetic acid solution (*V/V*) in 50% ethanol (*V/V*) was added to each well to extract the dye phagocytized by macrophages. At last, the *OD* at 540 nm was measured by the plate reader after cells were incubated in room temperature overnight.

Immunomodulatory activity of DSP in vivo

Immunosuppressed mice and drug administration

Male Kunming mice (SPF grade, 23 ± 2 g) were obtained from the Experimental Animal Center, Nanjing University of Chinese Medicine. The mice were housed under normal laboratory conditions (i.e., room temperature 25 ± 2 °C with a normal light/night cycle and had free access to a standard rodent chow and water). The mice (approval No. SYXK 2017-0069, 20171208-20221207) were divided into 6 groups ($n = 7$) for various treatments. After acclimatization for one week, one group was selected randomly as blank control group and i.p. with normal saline while the other groups of mice were injected with cyclophosphamide (CTX, 50 $\text{mg}\cdot\text{kg}^{-1}$) for 5 days for immunosuppression. After the modeling period, all the groups of mice except the blank control group were selected into CTX group, Lentinan group, DSP-Low group, DSP-Medium group and DSP-High group. The mice were treated as follows: blank control group and the CTX group were i.g. administered with 0.3 mL of pure water; lentinan group was treated as the concentration of 200 $\text{mg}\cdot\text{kg}^{-1}$ with 0.3 mL solutions; DSP-Low group, DSP-Medium group and DSP-High group were i.g. administered with 100, 200, and 300 $\text{mg}\cdot\text{kg}^{-1}$ DSP (0.3 mL) respectively. All the mice were i.g. administered once per day for 14 consecutive days with free access water and food.

Cytokine assay

Serum samples were prepared by centrifuging the whole blood at 4500 $\text{r}\cdot\text{min}^{-1}$ at 4 °C for 10 min and stored in -20 °C for further experimentation. The levels of TNF- α , IL-6 and IFN- γ in the serum were measured by ELISA kits according to the manufacturer's instruction.

Gut microbiota

On the last day, all mice were i.g. administered and placed into separate clean metabolic cages for biological sample collection. Fresh feces samples were collected and stored at -80 °C until further analysis.

Genomic DNA was extracted by a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions from the fecal samples. The V3-V4 region of the bacteria 16S ribosomal RNA gene were

amplified by PCR (95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 72 °C for 10 min) using primers 338F (5'-ACTCCTACGGGAGGCA GCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), equal quantities of 3 PCR reactions per sample were pooled and purified with a AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega, U.S.). Finally, a mixture of the amplicons from the different samples was sequenced by using an Illumina MiSeq platform (Majorbio BioPharm Technology Co., Ltd., Shanghai, China) according to standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra>). Before analysis, sequences were demultiplexed and quality-filtered by using QIIME (version 1.9.1) [21] with the following criteria: (1) The 300 bp reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp. (2) Exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (3) Only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1) [22] and chimeric sequences were identified and removed using UCHIME [23]. The taxonomy of each 16S ribosomal RNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the SILVA 16S ribosomal RNA database using a confidence threshold of 70%.

Statistical analysis

Data are presented as means \pm standard deviation (SD) unless otherwise indicated. Statistical analyses were performed with GraphPad Prism® 7 (GraphPad Software, San Diego, USA) using the one way-ANOVA for comparison of multiple groups. The *P* values of 0.05 or less are considered significant.

Results

Monosaccharide composition of DSP

Results show that DSP is composed of glucose, arabinose, mannose, D-xylose, galactose and rhamnose. Table 1 shows the ratio of monosaccharide composition of DSP.

Table 1 Monosaccharide compositions of DSP

	Man	Rha	Glu	Gak	Xyl	Ara
Mol%	16.49	20.62	24.74	15.46	16.49	6.19

Immunomodulatory activity of DSP in vitro

Effect of DSP on macrophage viability

The effect of DSP on the viability of RAW 264.7 cells was investigated in this study. Fig. 1A shows that the growth of RAW 264.7 is promoted by either LPS or DSP at concentrations of 100 to 300 $\mu\text{g}\cdot\text{mL}^{-1}$. The viability index of DSP at

concentrations of 100 to 300 $\mu\text{g}\cdot\text{mL}^{-1}$ were significantly higher than the index of blank control group, indicating that DSP could significantly promote the viability of macrophages in the concentration ranging from 100 to 300 $\mu\text{g}\cdot\text{mL}^{-1}$. The

viability of RAW 264.7 cell was promoted by DSP in the concentrations from 100 and 300 $\mu\text{g}\cdot\text{mL}^{-1}$ while the viability enhanced effect did not change significantly with increasing concentration.

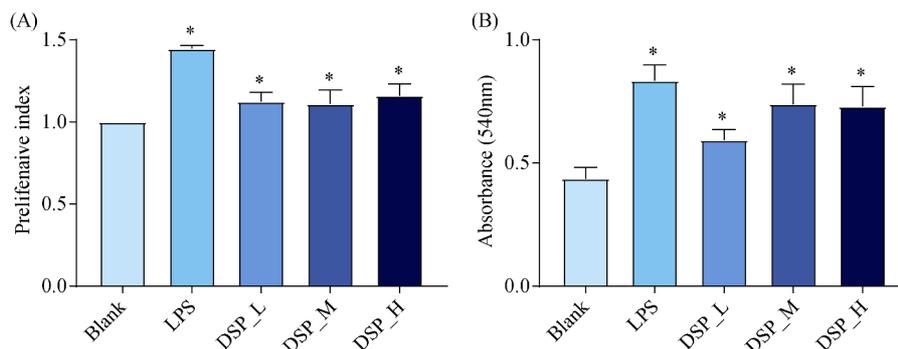


Fig. 1 Effect of DSP on viability and phagocytosis of macrophages. The data are expressed as mean \pm SD, $n = 8$. * $P < 0.05$ vs the Blank group

Effect of DSP on macrophage phagocytosis

Phagocyte phagocytosis is the first and pivotal step in the immune response. In this study, the immune response activation was indicated by the phagocytosis of neutral red. Fig. 1B shows that the phagocytic activity of RAW 264.7 cells is stimulated significantly by LPS and DSP at concentrations from 100 to 300 $\mu\text{g}\cdot\text{mL}^{-1}$ compared with the blank control group. Meanwhile, the enhancement of pinocytic activity was observed in macrophages treated with DSP in the concentration ranging from 100 to 300 $\mu\text{g}\cdot\text{mL}^{-1}$. The polysaccharide effect was similar between DSP_M and DSP_H while significantly higher than DSP_L.

Immunomodulatory activity of DSP in vivo

Effects of DSP on the levels of serum cytokines in immunosuppressed mice

Activated macrophages play a key role in the innate and adaptive immune responses by secreting secondary compounds such as cytokines [24]. As shown in Fig. 2, the serum concentrations of IL-6, TNF- α and IFN- γ in the CTX-treated mice decrease significantly. The concentrations of IL-6, TNF- α and IFN- γ in the lentinan group increased significantly compared to the CTX group. Meanwhile the concentrations of IL-6 in the DSP_L, DSP_M and DSP_H groups were signifi-

cantly higher than that of the CTX group. A dose-dependent increase of cytokines concentration was observed in the serum of the mice treated with DSP in the concentrations ranging from 100 to 300 $\text{mg}\cdot\text{kg}^{-1}$.

Effects of DSP on gut microbiota community in immunosuppressed mice

The gut microbiota alteration induced by DSP was evaluated by 16S ribosomal RNA gene sequencing of the samples isolated from mouse feces. The Shannon curves (Fig. 3) suggest that the data covers the most diversity and is sufficient to meet the requirements on data analysis. According to the taxonomy-based analysis, gut microbiota of mice in the blank group quantitatively consisted of bacterial phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* with relative abundances of 63.73%, 30.55% and 2.88%. However, the microbial composition in the CTX group is conspicuously changed by CTX with fewer *Bacteroidetes* (55.94%) and *Firmicutes* (24.96%) but more *Proteobacteria* (12.36%) (Fig. 4). The compositional analysis at bacterial genus level exhibits the effect of CTX on altering the gut microbiota (Fig. 5). An overview of the variation of gut microbiota is provided by operational taxonomic units (OTUs) abundance analysis by principal coordinate analysis (PCoA) (Fig. 6). The CTX cluster

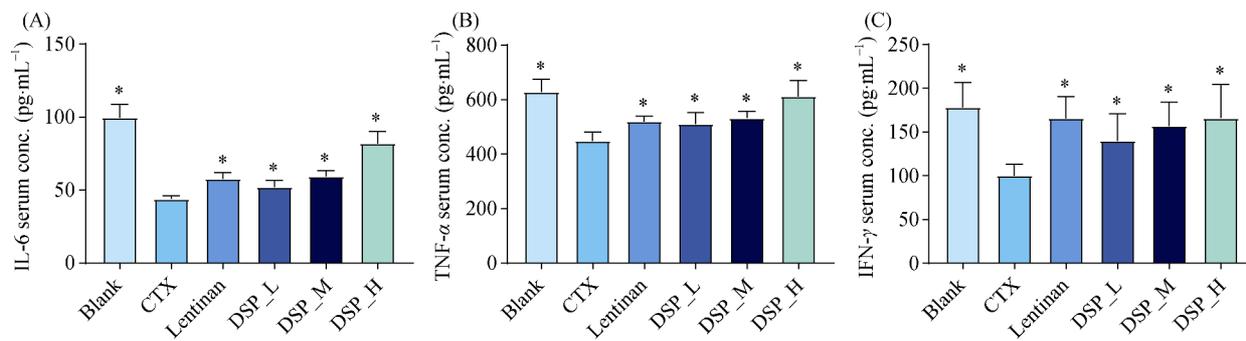


Fig. 2 Effects of DSP on the serum levels of cytokines in immunosuppressed mice. The data are expressed as mean \pm SD, $n = 7$. * $P < 0.05$ vs the CTX group

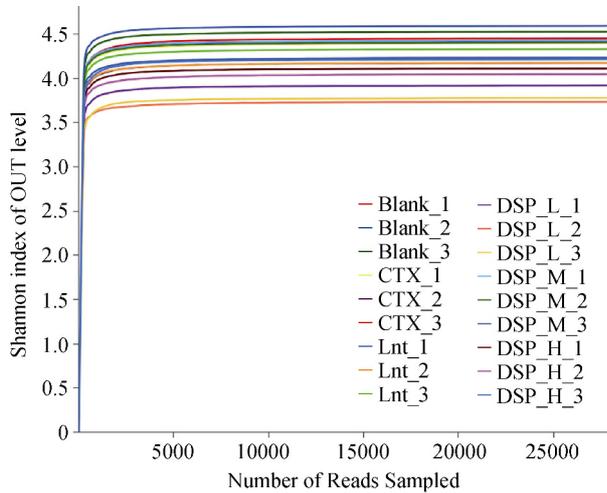


Fig. 3 Shannon curves of 18 samples

is distinctly away from the blank one, indicating robust differences in the membership of gut bacteria between the blank

and CTX groups. Additionally, *Lactobacillus* spp. and *Bacteroides* spp. are two crucial beneficial symbiotic bacteria that should be particularly concerned that they both are substantially decreased in the CTX group (Fig. 7). In following study, the effects of DSP on the gut microbiota of immunosuppressed mice were investigated. Fig. 4 provides evidence that all groups of DSP at doses ranging from 100 to 300 mg·kg⁻¹ have the ability to increase the relative abundance of *Bacteroidetes*, *Firmicutes* and decrease that of *Proteobacteria* in order to reverse the CTX-induced gut microbial dysbiosis at phyla level to approach the homeostasis of blank group. The tendency is further confirmed by the PCoA results of OTUs that the clusters of the DSP_L, DSP_M, DSP_H, lentinan and blank groups intertwined mutually, but detached with that of the CTX group (Fig. 6). The relative abundances of *Lactobacillus* spp. and *Bacteroides* spp. are increased by DSP_L, DSP_M and DSP_H but significantly ($P < 0.05$) different from the CTX group (Fig. 7). Altogether, it could be concluded that CTX led to dysbiosis of the gut microbiota in the mice, and DSP was able to substantially restore the dysbiosis.

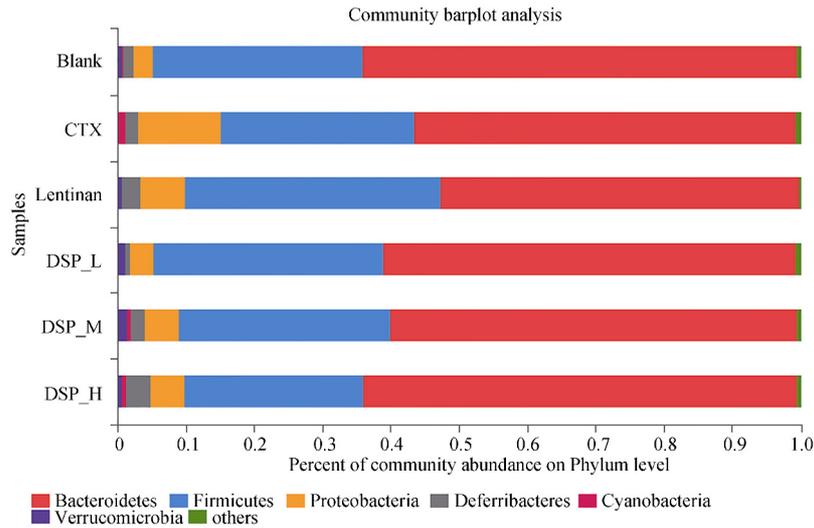


Fig. 4 Percent of community abundance on Phylum level

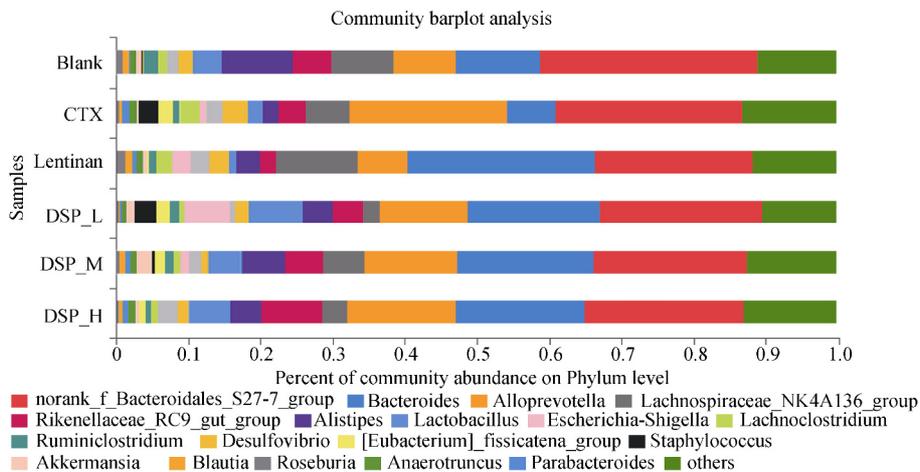


Fig. 5 Percent of community abundance on Genus level

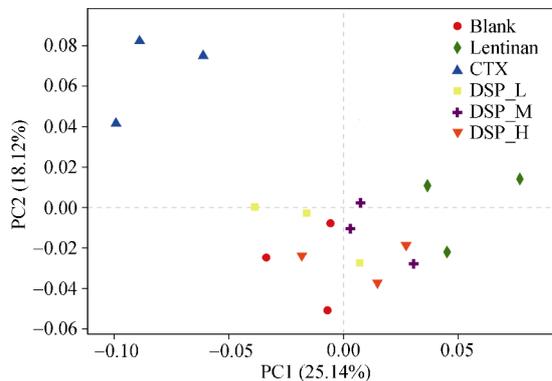


Fig. 6 OTUs abundance analysis by PCoA

Discussion

Plant polysaccharides are a large class of active ingredients which are very common in many kinds of natural products [25]. Many studies have suggested that most of polysaccharides have immunomodulatory function as well as other pharmacological effects [26-28]. Macrophages are monocyte-derived phagocytic cells that play crucial roles in adaptive and innate immunities [29]. The viability and phagocytotic function of macrophages can reflect the immune function more or less [30]. It has been proven that DSP can activate the immune system by increasing the viability of macrophages and enhancing their phagocytic capacity *in vitro* experiments, suggesting that DSP might be a potent activator of macrophage.

Lymphocytes are the foundation of the immune system and play a key role in the immune response. Lymphocytes consist of T, B, NK, K, LAK cells etc., among which T cells and B cells are respectively involved in cell-mediated immunity and humoral immunity *in vivo* by accepting specific antigen stimulation to generate specific immune response. T_H cells and cytotoxic T (TC) cells are two different kinds of T cells. T_H cells interact with B cells, helping them to divide, differentiate and produce antibody, interact with mononuclear phagocytes and helping them to destroy intracellular pathogens. T_H cells exhibit their effects by releasing soluble cyto-

kines and/or by direct cell-cell interactions. TC cells are classified into three T_H types (0, 1, 2, or 3) based on the types of cytokines that the pathogens infected CD4⁺ cells produce. T_H1 cells can become directly or indirectly cytotoxic by secreting the cytokines TNF- α and IFN- γ . T_H2 cells produce IL-6 while the proliferation of T cells, B cells, macrophages can be promoted by both IL-6 and IFN- γ . In our study, as shown in Fig. 2B, IL-6, TNF- α and IFN- γ levels were significantly inhibited in CTX group as compared with Blank group. The serum concentration of IL-6 was significantly higher in the DSP (100 to 300 mg·kg⁻¹) groups as compared with the CTX group. It was observed that there was a similar trend with the production of TNF- α and IFN- γ among the groups. These results give evidence that DSP might enhance the immune function of cyclophosphamide-induced immunosuppressed mice by promoting the release of cytokines.

The gut microbiota is a major part in the modulation of immune system [31-32]. The enteric microbiome functions as a potent bioreactor; It controls numerous metabolic functions, of which many still remain unclear, while producing thousands of important and unique substances of the greatest benefits to the body, as indigestible food substances are converted by fermentation to simple sugars, short-chain fatty acids, various nutrients, antioxidants and vitamins [33-35]. Most bacterial species cannot be cultured, so in this study high-throughput sequencing of 16S ribosomal RNA was a feasible method to amplify bacterial nucleic acid extracted from feces, indicate evolutionary divergence that can be used to identify and classify bacteria. In this study, the growth of two most important probiotics: *Lactobacillus spp.* and *Bacteroides spp.* in the CTX-induced immunosuppressed mice was simultaneously stimulated by DSP. Studies have shown that *Lactobacillus spp.* can stimulate the gut macrophage and promote the secretion of cytokines, and consequently activate the immune system [36]. In the meantime, keeping the balance of gut microbiota can shape and maintain normal mucosal immunity in gut [37]. DSP holistically restored the gut microflora perturbed by CTX.

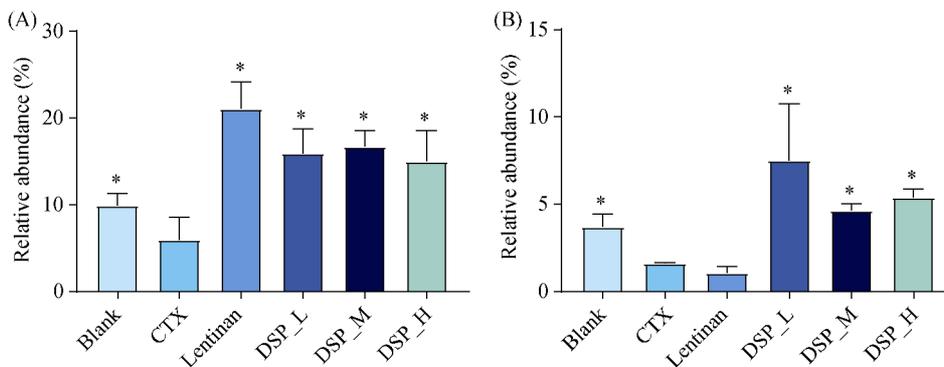


Fig. 7 (A) The relative abundances of *Bacteroides spp.* (B) The relative abundances of *Lactobacillus spp.* The data are expressed as mean \pm SD, $n = 3$. * $P < 0.05$ vs the CTX group

Except for the direct effects as prebiotics, it is worthy of special note that indirect effects of DSP on gut microbiota of CTX-induced immunosuppressed mice potentially occurred as well. In this study, we suggest that the composition of gut microbiota was perturbed by CTX significantly. These findings clearly demonstrate that the growth of *Lactobacillus* spp. and *Bacteroides* spp. was stimulated by DSP, and more meaningfully, the entire gut microbiota perturbed by DSP was restored by DSP at the same time. It is worth mentioning that like the polysaccharides from *D. aphyllum* and *D. huoshanense*, in phylum level, the relative abundance of the *Firmicutes* was elevated and the relative abundance of the *Proteobacteria* was reduced by DSP. Meanwhile, the abundance of the *Bacteroidetes* was increased by polysaccharides from *D. aphyllum* and decreased by polysaccharides from *D. sonia* and *D. huoshanense*^[7,38]. Of course, in consideration of the differences in various research conditions, the mechanism remains to be further studied.

Conclusion

In the present study, we find that DSP was mainly composed of glucose, arabinose, mannose, D-xylose, galactose and rhamnose. DSP not only promotes macrophage viability and enhances macrophage phagocytosis *in vitro*, but it can also increase the concentration of cytokines in serum in cyclophosphamide-induced immunosuppressed mice. At the same time, DSP can substantially restore the dysbiosis of the gut microbiota in cyclophosphamide-induced immunosuppressed mice, develop the community of gut microbiota and increase the proportion of probiotics. All in all, *D. sonia* polysaccharide has potential immunomodulatory activity.

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