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Effects of polysaccharides from wild morels on immune response and gut microbiota composition in non-treated and cyclophosphamide-treated mice†

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Polysaccharides isolated from mushrooms have been identified as potential prebiotics that could impact gut microbiota. In this study, a water-soluble polysaccharide (MP) extracted from wild morels was evaluated for its effects on the gut microbiota of non-treated and cyclophosphamide (CP)-treated mice. The results showed that MP restored the spleen weight and increased the counts of white blood cells and lymphocytes in the peripheral blood and spleen of the CP-treated mice. Mice treated with MP exhibited increased levels of short-chain fatty acid (SCFA)-producing bacteria, especially *Lachnospiraceae*, compared to normal mice, and increased levels of *Bacteroidetes* and SCFA-producing bacteria, especially *Ruminococcaceae*, compared to the CP-treated mice. Moreover, MP treatment increased the production of valeric acid and decreased the production of acetic acid in the non-treated mice and increased the production of acetic acid, propionic acid, butyric acid, and valeric acid in the CP-treated mice. These results show that MP is potentially good for health.

1. Introduction

Gut microbiota consists of a large number of microorganisms that colonize the intestinal tract of mammals.[1] Approximately 10^{13}–10^{14} microorganisms reside in a normal adult bowel, exceeding the total number of human cells in the body.[2] Over the past few years, the development of technology—especially high-throughput sequencing techniques—has increased our understanding of the composition and function of gut microbiota. Numerous studies have suggested that gut microbiota is closely related to the hosts' health,[3,4] metabolic phenotypes,[5] the absorption or production of nutrients,[6–8] and the evolution of the adaptive immune system.[9] Recent studies have also revealed that changes in the composition of gut microbiota have been linked to diseases, such as obesity,[10,11] type I and II diabetes,[11] irritable bowel syndrome (IBS),[12] colon cancer,[13] inflammatory bowel disease (IBD),[14] atherosclerosis,[15,16] and hypertension.[16]

Mushrooms are a kind of fungus that have been valued as edible and medicinal resources for several thousands of years. Studies have suggested that the polysaccharides in mushrooms are potential prebiotics and could induce the growth or action of microorganisms that contribute to the well-being of their host.[17,18] Xu *et al.* reported that *Lentinula edodes*-derived polysaccharides rejuvenated the immune responses and gut microbiota in mice.[19] *L. edodes*-derived polysaccharide L2 could restore age-attenuated immune responses by increasing cytokine levels in peripheral blood and reverse age-related changes to the composition of gut microbiota, such as the reduction in the ratio of *Firmicutes* to *Bacteroidetes*, the increase in the levels of *Bacteroidia* and *Bacteroidaceae*, and the decrease in the levels of *Bacilli*, *Betaproteobacteria*, *Lactobacillaceae*, and *Alcaligenaceae*.[19] Chang *et al.* showed that the presence of *Ganoderma lucidum* reduced obesity in mice by modulating the composition of the gut microbiota, indicating that *G. lucidum* and its high molecular weight polysaccharides may be used as prebiotic agents to prevent gut dysbiosis and obesity-related metabolic disorders.[20]

Morels are edible and medicinal mushrooms appreciated worldwide for both their savory flavor and their nutritional and medicinal properties.[21] Current research has demonstrated that morels possess anti-oxidative and anti-inflammatory activities, as well as immunostimulatory and anti-tumor properties.[21] Several studies over the years have described the immunostimulatory, immunomodulatory, and anti-inflammatory activities of the polysaccharides from morels. Duncan

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et al. reported that a high-molecular-weight galactomannan polysaccharide isolated from *Morchella esculenta* could enhance macrophage activation by increasing the expression of NF-κB.[22] *In vitro* studies of a water-soluble *Morchella conica* polysaccharide (MCP) extracted and isolated from the fermentation broth of the mushroom have shown that MCP may significantly modulate nitric oxide production in macrophages and promote splenocyte proliferation.[23] Two fractions of a water-soluble polysaccharide obtained from the fermentation broth of *Morchella esculenta* (MEP-I and MEP-II) have been shown to have typical immunostimulatory activity *in vivo*.[24] Although morel polysaccharides have been demonstrated to improve immunity, the effects that they have on the gut microbiota and the relationship between this effect and immune functions are poorly understood.

Cyclophosphamide (CP) is an immunosuppressant widely used in cancer therapies and organ transplantation.[2,25] However, CP can cause intestinal microflora dysfunction and intestinal mucosal injury, which eventually threaten intestinal health. Recently, dietary squid ink polysaccharides and fucoidan of *Acaudina molpadioides* were shown to promote the recovery of CP-induced intestinal injury.[26,27] To this end, we examined the effects of morel polysaccharides on gut microbiota and immune responses in both non-treated and CP-treated mice.

In this study, a water-soluble polysaccharide (MP) extracted from wild morels was evaluated for its effects on the gut microbiota of non-treated and CP-treated mice. The gut microbiota of the mice in different treatment groups was compared using α-diversity, taxonomic differences, and β-diversity analyses. Finally, the effects of MP on short-chain fatty acid (SCFA) production and lymphocyte proliferation were determined. Our study revealed for the first time that polysaccharides from wild morels (MP) could promote lymphocyte proliferation and change the gut microbiota in both non-treated and CP-treated mice.

2. Materials and methods

2.1 Materials and chemicals

Wild morels were collected from the Qinling Mountains in Shaanxi province (China). The morels were washed, dried in hot air at 60 °C–70 °C, and then milled and passed through 100-mesh sieves to obtain morel powder. DEAE Sepharose Fast Flow resin was purchased from GE Healthcare (Chalfont St Giles, UK). Standards for gas chromatography, including acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, *n*-valeric acid, hexanoic acid, and 2-ethylbutyric acid, were of chromatographic grade and purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemical reagents were of analytical grade and purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China).

2.2 Extraction of MP

The extraction of MP was performed according to the methods described by Yang *et al.*[28] Dried morel powder (200 g) was extracted with 3000 mL distilled water at 95 °C for 6 h with regular stirring. The liquid fractions and the residue from the extraction were collected separately. The residue was re-extracted twice more. The extracted liquid fraction was collected and enriched by vacuum-rotary evaporation at 50 °C before precipitation with pure alcohol (1 : 4, v/v) at 4 °C for 12 h. The precipitate was collected by centrifugation at 8000*g* for 10 min and dried in a vacuum to obtain the crude morel polysaccharide. After removal of the protein by the Sevag method,[28] the crude morel polysaccharide was loaded onto a column (2.6 × 30 cm) of DEAE Sepharose Fast Flow and irrigated successively with distilled water and 0.125–0.5 mol L^{-1} NaOH solution stage gradient elution separately. The water-eluted fraction was collected, concentrated, dialyzed (molecular weight cut off: 3500) against water, and lyophilized to obtain pure morel polysaccharides.

2.3 Determination of the contents of carbohydrates and proteins

The total carbohydrate content of MP was determined using the phenol–sulfuric acid method with glucose as a standard.[2] The protein content was measured by the Coomassie Brilliant Blue method using bovine serum albumin as the standard.[2]

2.4 UV and FT-IR spectroscopic analyses

For UV spectroscopic measurements, 0.5 mg mL^{-1} of MP sample (dissolved in distilled water) was scanned on a Lambda 35 spectrophotometer (PerkinElmer Ltd, MA, USA) with a wavelength range of 200–400 nm. The spectra of MP from Fourier-transform infrared spectroscopy (FT-IR) were recorded on a Nicolet iS50 FT-IR spectrometer (Thermo Fisher Scientific, MA, USA) in the range of 4000–400 cm^{-1}. The dried MP sample was mixed with potassium bromide powder and pressed into a pellet for FT-IR spectroscopic measurements.

2.5 Molecular weight determination

The molecular weight of MP was identified by gel permeation chromatography (GPC). A gel chromatograph instrument (WYATT, CA, USA) was employed, which was equipped with two columns (Shodex OH Pack SB-806 and 803 HQ, JM Science Inc., Buffalo, NY) connected in series. A multi-angle laser light scattering detector (MALLS; DWAN EOS equipped with a GaAs laser at 690 nm, WYATT, CA, USA) and a refractive index (RI) detector (Optilab rEX, WYATT, CA, US) were employed to detect the molecular weight and the molecular weight distribution of MP. The columns were eluted with ddH_2O containing 0.02% $NaNO_3$, and the flow rate was 1.0 mL min^{-1}. The injection volume and concentration were 0.5 mL and 2 mg mL^{-1}, respectively. The temperature of the column oven was 40 °C.

2.6 Monosaccharide composition analysis of MP

The polysaccharide, MP, was hydrolysed with 4 mol L^{-1} trifluoroacetic acid (TFA) for 2 h at 120 °C, then derivatized using trichloro-aldehyde-1-pheny-3-methyl-5-pyrazolone (PMP, 0.5 mol L^{-1}), and determined by the HPLC method with the

245 nm detection wavelength. The chromatograph was fitted with a Shiseido C18 column (Shiseido, Tokyo, Japan), eluted with a solution of KH_2PO_4 (0.1 mol L^{-1}, pH 6.8) and acetonitrile (82 : 18, v/v) at a flow rate of 1.0 mL min^{-1}. The results were compared with the following monosaccharide standards: D-mannose, D-glucose, D-galactose, D-xylose, L-arabinose, L-rhamnose, D-ribose, L-fucose, D-glucuronic acid, and D-galacturonic acid (Sigma).

2.7 Animals and experimental design

Male Kunming mice (KM) (20 ± 2.0 g) were purchased from the Animal Experimental Center at Xi'an Jiaotong University (certificate SCXK (Shaan) 2014-001). All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996) and experiments were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University, China. The mice were raised in cages in a room with controlled temperature (25.0 °C ± 0.5 °C), relative humidity (50% ± 5%), and a 12/12 h period of light and darkness.

After one week of adaptation, the mice were randomly divided into four groups of $n = 8$ as follows: the normal control group (CTC), model control group (MC), MP treatment group (PC), and CP + MP treatment group (MPC). The mice in groups MC and MPC were intraperitoneally injected with 60 mg per kg body weight (BW) of CP from days 1 to 7, while the mice in groups CTC and PC were injected with sterile saline of the same volume using the same method. From days 3 to 8, the mice in groups PC and MPC were administered 75 mg per kg BW of MP by gavage, while the mice in groups CTC and MC received 1.0 mL of sterile saline by gavage. On the 9th day, after intraperitoneal injection of chloral hydrate (0.1 mL), the anesthetized mice were weighed and the peripheral blood was collected from the heart. The mice were then sacrificed by cervical dislocation, and the spleens were removed and weighed. The spleen index was calculated as follows:

$$\text{spleen index} = \text{spleen weight (mg)}/\text{body weight (g)}.$$

Cecum contents and feces were collected in sterile centrifuge tubes and stored at −80 °C until further analysis

2.8 Gut microbe 16S rRNA sequencing

Total microbial genomic DNA from the cecum contents was extracted using the QIAamp DNA stool MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification of the V3–V4 region of bacterial 16S rRNA was performed using a forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and a reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR was carried out in a total reaction volume of 20 μL: 13.25 μL H_2O, 2.0 μL 10 × PCR ExTaq buffer, 0.5 μL DNA template (100 ng mL^{-1}), 1 μL forward and reverse primers (10 mM), 2.0 μL dNTP, and 0.25 μL ExTaq (5 U mL^{-1}). The PCR procedure was as follows: pre-denaturation at 98 °C for 2 min, denaturation at 98 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s. This procedure was repeated for 30 cycles with a final extension at 72 °C for 5 min. Twenty PCR products were purified using an agarose gel electrophoresis method. The barcoded V3–V4 PCR amplicons were sequenced using an Illumina HiSeq platform (Illumina, SD, USA). The extraction of genomic DNA and amplification and sequencing of the 16S rRNA V3–V4 region were completed by Beijing Biomarker Technologies Co., Ltd (Beijing, China). Five repeated samples were arranged in each group.

2.9 Bioinformatics analysis

The bioinformatics analysis in this study was completed using the Biomarker biocloud platform (http://www.biocloud.org). High-quality sequences were clustered using USEARCH,[29,30] and tags with similarity ≥97% were regarded as an operational taxonomic unit (OTU). Taxonomy was assigned to all OTUs by searching against the Silva databases (Release132, http://www.arb-silva.de)[31] using the RDP classifier within QIIME.[32] OTUs were used for α-diversity (ACE, Chao1, and Shannon) analysis with Mothur,[33] and the principal component analysis (PCA) of sequence read abundance was generated using R software.

2.10 Detection of WBC and lymphocyte counts

Five hundred microliters of peripheral blood from each mouse were placed in a sterile, EDTA-anticoagulated tube and counted in a Sysmex KX-21 N Blood Cell Analyser (Sysmex Corporation, Japan). Three repeated samples were tested in each group.

2.11 Flow cytometric analysis

Flow cytometry was used to detect the effects of MP on spleen lymphocyte subsets in the non-treated and CP-treated mice. The spleens from the mice in each group (CTC, MC, MPC, and PC) were homogenized, filtered using cell strainers (pore size = 40 μm), and removed of red blood cells using Red Blood Cell Lysis Buffer, to yield spleen cells. Spleen cells were stained for 1 h in the dark at 4 °C–8 °C with a variety of anti-mouse CD4, CD8, and CD19 monoclonal antibodies (BD Biosciences). After incubation with the monoclonal antibodies, flow cytometry analysis was conducted using a Guava EasyCyteTM HT (Millipore) and FlowJo software. Three repeated samples were tested in each group.

2.12 Determination of the levels of SCFAs

The determination of SCFA levels in fecal samples from the mice in each group was performed by gas chromatography (GC) according to the method detailed by Zhao *et al.*[34] Fecal samples (0.1 g) were added to 0.5 mL of deionized water and shaken for about 3 min to yield a 17% (w/w) fecal suspension. The pH of the suspension was adjusted to 2–3 by adding HCl, and the suspension was then centrifuged at 12 000*g* for 20 min to yield a clear supernatant. The internal standard, 2-ethylbutyric acid solution, was spiked into the supernatant to a final concentration of 1 mM, and the supernatant was injected into the GC for analysis. Acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, *n*-valeric acid, and hexanoic acid served as standards.

Chromatographic analysis was carried out using a Thermo Trace 1300 GC system equipped with a flame ionization detector (FID). A fused-silica capillary column with a free fatty acid phase (DB-FFAP, J&W Scientific, Agilent Technologies Inc., USA) of 30 m × 0.25 mm i.d. was used. Helium was supplied as the carrier gas at a flow rate of 1.0 mL min^{-1}. The initial oven temperature of 50 °C was maintained for 1.0 min, raised to 120 °C at a rate of 15 °C min^{-1}, increased to 170 °C at a rate of 5 °C min^{-1}, increased to 240 °C at a rate of 15 °C min^{-1}, and then finally held at 240 °C for 3 min. The temperature of the injection port was 250 °C, and the split ratio was 25 : 1. The temperature of the FID detector was 260 °C, and the flow rates of hydrogen, air, and nitrogen as makeup gases were 30, 300, and 20 mL min^{-1}, respectively. The injected sample volume for GC analysis was 1 μL, and the run time for each analysis was 23.5 min. Data handling was carried out using Xcalibur software. Four repeated samples were tested in each group.

2.13 Lymphocyte viability analysis

The effects of MP on lymphocyte proliferation were determined using a Cell Counting Kit-8 (CCK-8). The spleen lymphocytes of the mice were separated using Mouse 1× Lymphocyte Separation Medium (Dakewe Biotech Co., Ltd, ShenZhen, China) according to the manufacturer's protocol. Spleen lymphocytes were seeded into 96-well plates at a density of 2 × 10^5 cells per well, and six repeated wells were arranged in each group. Different concentrations of MP (0, 100, 200 and 400 μg mL^{-1}) were added to cells for 24 h and 48 h. Absorption values were measured 2 h after adding the CCK-8 reagent (MCE, USA). The optical density (OD) was measured at 450 nm.

2.14 Statistical analysis

All data are presented as the means ± standard deviation (SD). Differences between certain groups were analyzed by the *t* test using GraphPad Prism 5.0 software. Differences were considered to be statistically significant if $p < 0.05$.

3 Results

3.1 Preliminary characterization of MP

Crude polysaccharide was obtained from the fruiting body of wild morels and, after removal of protein, loaded onto a DEAE Sepharose Fast Flow column. The water-eluted fraction was collected, concentrated, dialyzed, and lyophilized to yield the pure morel polysaccharide (MP), and its yield rate was 1.48%. The total carbohydrate and protein content of MP was 88.01% and 3.54%, respectively. The UV spectra of MP showed no obvious peak in the 260–280 nm range (Fig. 1), demonstrating that the sample was free of nucleic acids. As shown in Fig. 2, the FT-IR spectra of MP exhibited strong bands around 3405 cm^{-1}, indicative of a hydroxyl group.[2] A band around 2927 cm^{-1} was due to the C–H stretching vibration, and a band at 1639 cm^{-1} was due to bound water.[2,35] A band in the region of 1400–1000 cm^{-1} was due to the C–O–C stretching vibration. These results indicated that MP was a polysaccharide.[2,36]

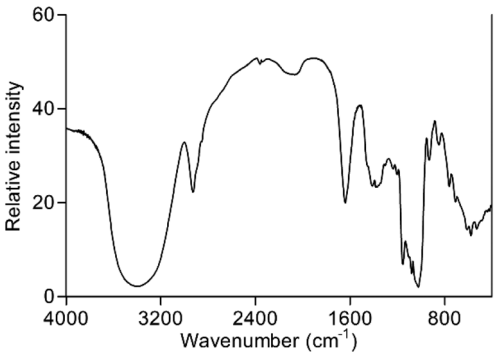

Fig. 1 Ultraviolet (UV) spectra of MP ranging from 200 to 400 nm.

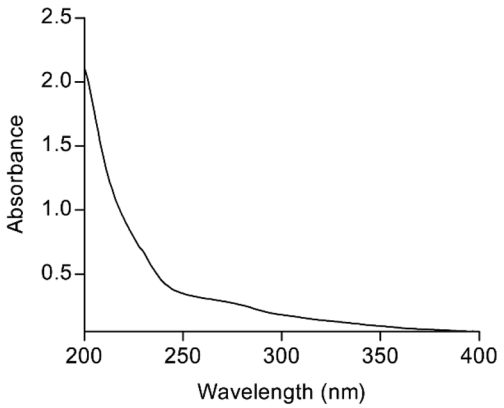

Fig. 2 Fourier-transform infrared (FT-IR) spectra of MP ranging from 400 to 4000 cm^{-1}.

GPC/MALLS was used to elucidate the molecular weights of MP, and the average molecular weight of MP was estimated to be 3.974 × 10^3 kDa. The monosaccharide compositions of MP were analysed by HPLC, and the results showed that MP was a heteropolysaccharide and mainly consisted of D-mannose, D-glucose, D-galactose, and L-rhamnose, with the mass ratio of 43.15 : 19.56 : 20.25 : 1. The monosaccharide composition of the MP is similar to that of MEP-1 prepared by Yang *et al.*, but the average molecular weight of MP is greater than that of MEP-1.[28]

3.2 Effects of MP on spleen index

As shown in Table 1, the body weight, spleen weight, and spleen index in the model control group decreased remarkably compared to the normal control group ($p < 0.05$). Meanwhile, the CP-treated mice administered MP showed a dramatic increase in the spleen weight compared to that in the model control group ($p < 0.05$), with no significant changes in the body weight and spleen index. In addition, there were no obvious changes in the body weight, spleen weight, and spleen index between the mice in the normal control group and group MP.

Table 1 Effects of the polysaccharides extracted from morels on body weight (g), spleen weight (mg), and spleen index (mg g^{-1}) in the non-treated and CP-treated mice

Group	Body weight (g)	Spleen weight (mg)	Spleen index (mg g^{-1})
CTC	34.15 ± 1.78	137.20 ± 16.54	4.03 ± 0.54
MC	29.39 ± 1.55**	49.40 ± 20.53***	1.70 ± 0.74***
MPC	29.85 ± 3.48	95.40 ± 38.41^{+}	3.27 ± 1.54
PC	34.90 ± 2.24	118.40 ± 20.26	3.38 ± 0.43

T tests were used to evaluate the significant differences in body weight, spleen weight, and spleen index. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. **$p < 0.01$, ***$p < 0.001$ *vs.* CTC; $^{+}p < 0.05$ *vs.* MC.

3.3 Effect of MP on peripheral blood white blood cells and lymphocytes

As shown in Fig. 3, the model control group had decreased counts of peripheral blood white blood cells (WBCs) and lymphocytes compared to the normal control group ($p < 0.05$). Administration of MP was shown to increase the counts of WBCs and lymphocytes in the CP-treated mice ($p < 0.05$), suggesting that MP could promote lymphocyte proliferation in the CP-treated mice.

3.4 Effects of MP on spleen lymphocyte subsets

The effects of MP on the levels of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}CD19^{+}$ lymphocytes were determined by flow cytometry. As shown in Fig. 4, the model control group showed decreased levels of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}CD19^{+}$ lymphocytes compared to the normal control group ($p < 0.05$), implying that CP treatment could suppress immune activities. Group PC showed an increase in $CD4^{-}CD8^{-}CD19^{+}$ lymphocyte levels compared to the normal control group ($p < 0.05$), and there was no significant change in the $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ lymphocyte levels between Groups CTC and PC, indicating that MP had a limited immunoregulatory effect in the non-treated mice. However, the levels of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}CD19^{+}$ lymphocytes were greatly enhanced in the CP-treated mice after the administration of MP. Therefore, MP could restore CP-induced immunosuppression and improve immunity through increasing the levels of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}CD19^{+}$ lymphocytes.

3.5 Effects of MP on the overall structure of intestinal bacteria

On average, 128 422 clean bacterial 16S rRNA gene reads were obtained from each fecal sample by pyro-sequencing. OTUs in each group were defined based on a sequence identity greater than 97% (Table 2). The number of OTUs was the lowest in the model control group (Table 2). Compared to the model control group, group MPC showed a significant increase in OTUs ($p < 0.05$), and the administration of MP was shown to increase the OTUs in normal mice ($p < 0.05$). Species richness at the taxonomic level was reflected by Chao1 and ACE indices (Table 2). As shown in Table 2, the model control group had decreased ACE and Chao1 indices compared to the normal control group ($p < 0.05$), while the administration of MP increased both ACE and Chao1 indices in the CP-treated mice ($p < 0.05$). Species richness and evenness, reflected by the Shannon index, also significantly changed between the groups CTC and MC and between the groups MC and MPC, which was consistent with the ACE and Chao1 indices. Moreover, the mice receiving MP showed a dramatic increase in the Shannon index compared to the mice in the normal control group ($p < 0.05$).

The phylogenetic differences and similarities within the gut microflora were assessed by principal component analysis (PCA) (Fig. 5). The results indicated a high variation among individuals. The second principal coordinate (PC2), which accounted for 13.11% of variance in the data, could completely separate the group MPC from the model control group. PC2 could also separate the normal control group from the model control group with 80% accuracy, except for one sample in the normal control group.

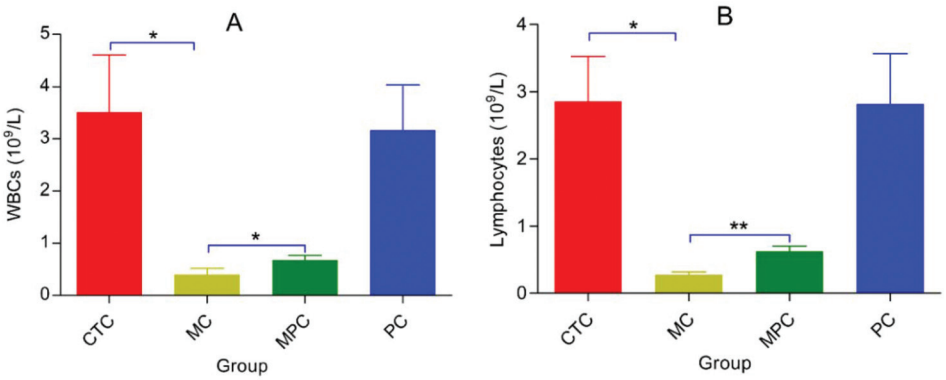

Fig. 3 Effects of the polysaccharides from morels on WBCs (A) and lymphocytes (B) in the non-treated and CP-treated mice. *T* tests were used to evaluate the significant differences in the counts of WBCs and lymphocytes. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. *$p < 0.05$, **$p < 0.01$.

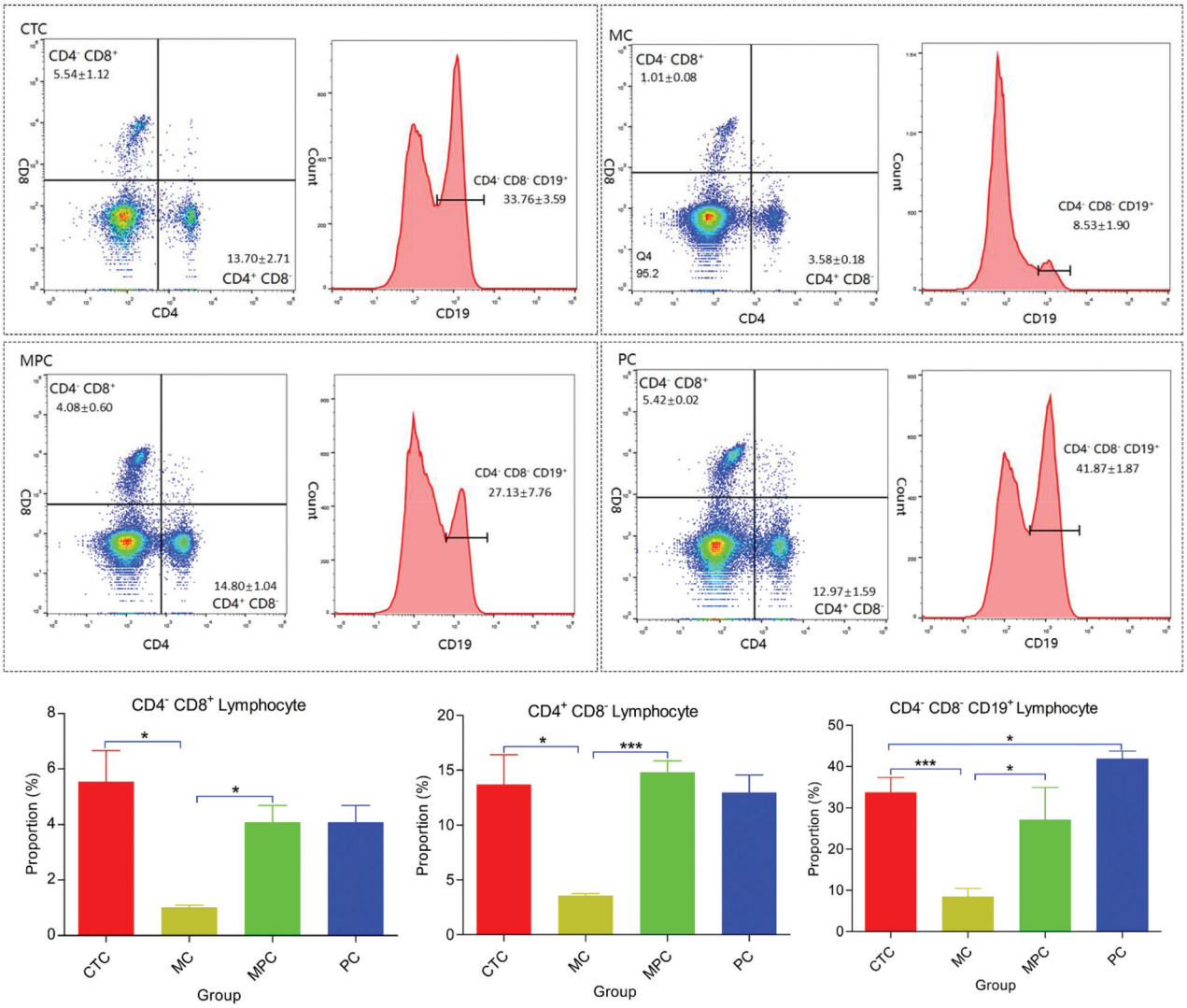

Fig. 4 Comparison of the proportion of different lymphocyte subsets in each group. *T* tests were used to evaluate the significant differences in the proportion of different lymphocyte subsets. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. *$p < 0.05$, ***$p < 0.001$.

Table 2 Comparisons of OTUs and α-diversity index in each group

Group	OTUs	Shannon	ACE	Chao1
CTC	365.20 ± 27.96	3.77 ± 0.17	399.60 ± 18.35	412.40 ± 25.66
MC	337.40 ± 28.59	3.23 ± 0.36*	370.10 ± 21.27*	374.30 ± 25.72*
MPC	392.80 ± 18.27^{++}	4.23 ± 0.21^{+++}	415.90 ± 16.71^{++}	425.30 ± 18.47^{++}
PC	404.40 ± 20.38^{x}	4.45 ± 0.17^{xxx}	425.00 ± 16.48	435.10 ± 13.38

T tests were used to evaluate the significant differences in OTUs and α-diversity index. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; MPC: CP + MP treatment group. *$p < 0.05$ *vs.* CTC; $^{++}p < 0.01$, $^{+++}p < 0.001$ *vs.* MC; $^{x}p < 0.05$, $^{xxx}p < 0.001$ *vs.* CTC.

3.6 Effects of MP on the taxonomic composition of intestinal microbiota

As shown in Fig. 6, the phylum-level distribution patterns for all of the groups were different. To assess specific changes in the gut microbiota, several predominant taxa from each group were selected for analysis (Table 3). At the phylum level, *Firmicutes* and *Bacteroidetes* were the two most predominant phyla, accounting for almost 90% of total bacteria, and *Proteobacteria* constituted the next predominant phylum. The model control group exhibited a striking decrease in the rela-

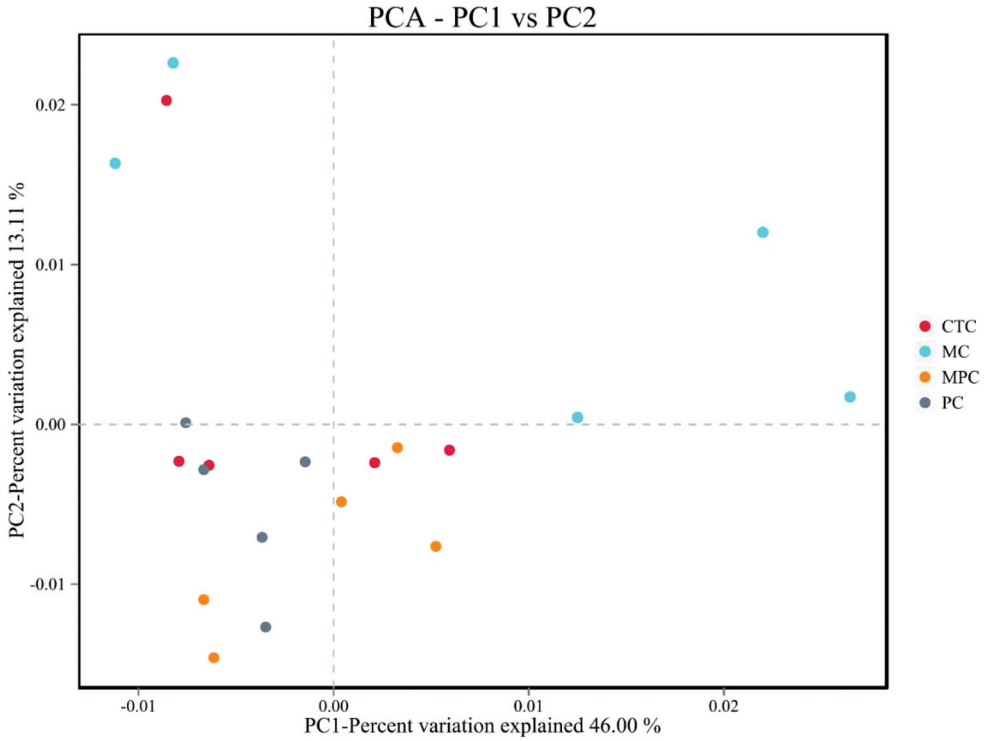

Fig. 5 Principal component analysis (PCA) of the gut microbiota in each group. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group.

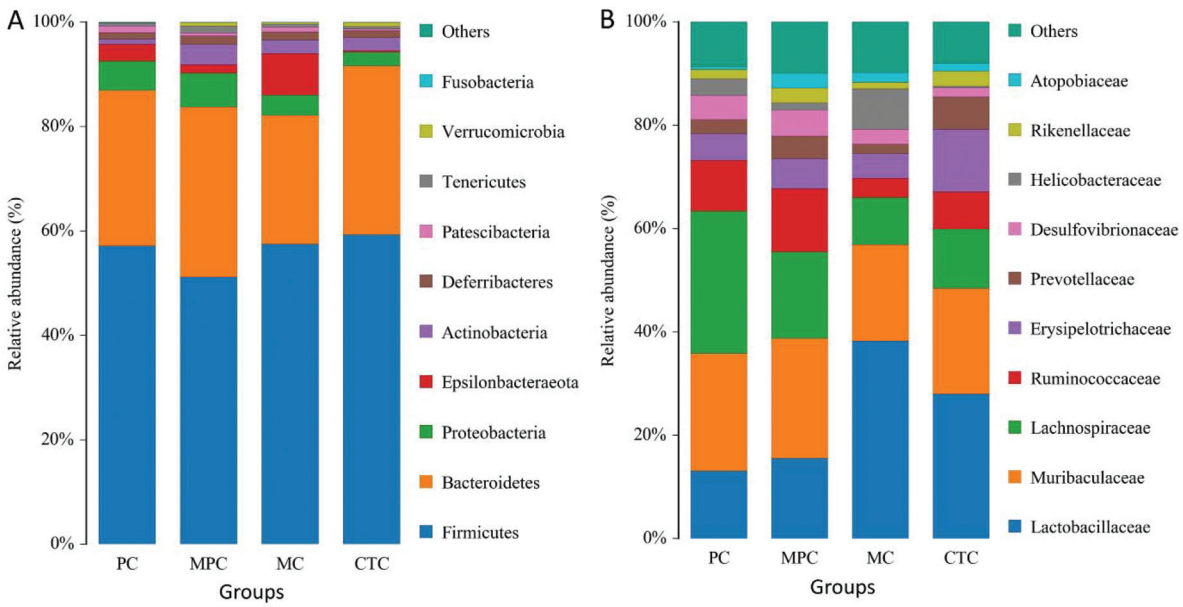

Fig. 6 Comparisons of gut microbiota at the phylum (A) and family levels (B). Each taxonomic phylum and family accounted for the proportion of identified classification. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group.

tive abundances of *Bacteroidetes* ($p < 0.05$) compared to the normal control group, and the administration of MP increased the *Bacteroidetes* level in the CP-treated mice ($p < 0.05$). No significant changes were observed in the *Firmicutes* and *Proteobacteria* levels in each group.

Obvious changes were observed in the gut microbiota at the family level (Fig. 6). Several predominant taxa for all the treatment groups are presented in Table 4. Compared to the normal control group, the mice in group PC exhibited a dramatic increase in the relative abundance of *Lachnospiraceae* (p

Table 3 Relative abundance of predominant taxa at the phylum level

Group	*Firmicutes* (%)	*Bacteroidetes* (%)	*Proteobacteria* (%)
CTC	59.29 ± 4.79	32.31 ± 5.83	2.66 ± 1.71
MC	57.49 ± 12.99	24.65 ± 1.43*	3.88 ± 1.78
MPC	51.17 ± 6.84	32.61 ± 2.39+++	6.55 ± 5.74
PC	57.18 ± 7.33	29.76 ± 8.29	5.53 ± 3.09

T tests were used to evaluate the significant differences in each bacterial population at the phylum level. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. *$p < 0.05$ *vs.* CTC; $^{+++}p < 0.001$ *vs.* MC.

< 0.05). MP administration significantly increased the levels of *Ruminococcaceae* in the CP-treated mice compared to those in the model control group ($p < 0.05$). Some species in the predominant families of *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* are capable of producing SCFA, especially butyrate and propionate.[37] The total levels of *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* (SCFA-producing bacteria; Table 4) were significantly decreased in the model control group compared to those in the normal control group ($p < 0.05$). MP administration significantly increased the levels of SCFA-producing bacteria in the CP-treated mice compared to those in the model control group ($p < 0.05$). Moreover, compared to the normal control group, group PC showed a significant increase in the levels of SCFA-producing bacteria ($p < 0.05$).

To validate the differences in the gut microbiota of some groups, several predominant microorganisms at the genus level were analyzed and compared. Compared to the normal control group, the model control group exhibited distinct decreases in the relative abundances of Ruminococcaceae_UCG-014 ($p < 0.05$) (Table 5). Table 5 shows that the mice in group PC exhibited an increase in the relative abundance of *Lachnospiraceae*_NK4A136 and *Helicobacter*, and a decrease in the relative abundances of *Lactobacillus* ($p < 0.05$). MP administration increased the levels of *Lachnospiraceae*_NK4A136 and decreased the levels of *Lactobacillus* in the CP-treated mice compared to those in the model control group ($p < 0.05$).

To evaluate the effect of MP on gut microbiota at the level of species in the non-treated and CP-treated mice, LEfSe was employed to identify specific species that were statistically different between groups CTC and PC, MC and MPC. A pairwise comparison between the CTC and PC groups indicated that 4 species were significantly different (Fig. 7), one of them belonging to the family *Ruminococcaceae* was higher in the PC group compared with the CTC group ($p < 0.05$). While there were 3 significantly different species between MC and MPC (Fig. 7), one of them belonging to the family *Lachnospiraceae* was higher in the MPC group compared with the MC group ($p < 0.05$).

3.7 Effects of MP on SCFA production

Acetic acid, propionic acid, butyric acid, and valeric acid are the four main SCFAs in the intestine, and all are beneficial for human health. As shown in Table 6, the concentrations of acetic acid, propionic acid, butyric acid, and valeric acid were notably decreased in Group MC compared to those in Group CTC ($p < 0.05$). MP administration improved the production of the four SCFAs in the mice pretreated with CP compared to those in the model control group ($p < 0.05$). MP treatment also increased valeric acid production and decreased the production of acetic acid compared to the normal control group ($p < 0.05$).

Table 4 Relative abundance of predominant taxa at the family level

Group	*Lachnospiraceae* (%)	*Ruminococcaceae* (%)	*Erysipelotrichaceae* (%)	SCFA-producing bacteria (%)
CTC	11.52 ± 9.06	7.19 ± 3.98	12.03 ± 11.06	30.73 ± 4.25
MC	9.15 ± 6.88	3.73 ± 3.64	4.67 ± 3.88	17.56 ± 4.01***
MPC	16.71 ± 6.57	12.21 ± 5.15^{+}	5.78 ± 6.60	34.70 ± 3.77^{+++}
PC	27.54 ± 10.48^{x}	9.78 ± 3.31	5.18 ± 6.61	42.49 ± 4.58^{xx}

T tests were used to evaluate the significant differences in each bacterial population at the family level. Each value is presented as mean ±SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. ***$p < 0.001$ *vs.* CTC; $^{+}p < 0.05$, $^{+++}p < 0.001$ *vs.* MC; $^{x}p < 0.05$, $^{xx}p < 0.01$ *vs.* CTC.

Table 5 Relative abundance of predominant taxa at the genus level

Group	*Lactobacilluceae* (%)	*Lachnospiraceae*_NK4A136 (%)	*Helicobacter* (%)	*Ruminococcaceae*_UCG-014(%)
CTC	27.97 ± 6.71	3.00 ± 3.68	0.30 ± 0.18	2.88 ± 1.54
MC	38.27 ± 13.19	0.48 ± 0.24	7.93 ± 12.66	0.31 ± 0.26*
MPC	18.40 ± 6.58^{+}	4.90 ± 2.29^{+}	1.46 ± 2.21	3.50 ± 2.71
PC	15.12 ± 4.73^{x}	8.33 ± 2.10^{x}	3.29 ± 1.43^{xx}	2.23 ± 1.06

T tests were used to evaluate the significant differences in each bacterial population at the genus level. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. *$p < 0.05$ *vs.* CTC; $^{+}p < 0.05$ *vs.* MC; $^{x}p < 0.05$ *vs.* PC; $^{xx}p < 0.01$ *vs.* PC.

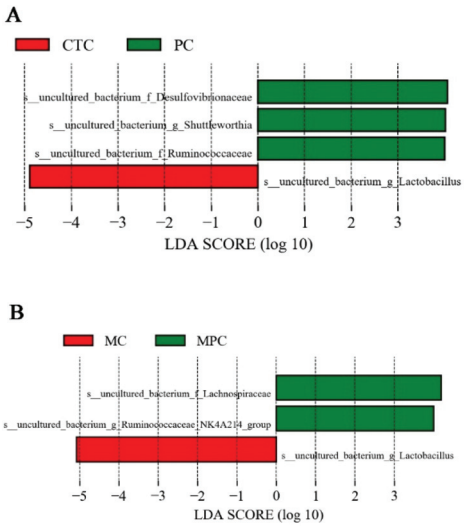

Fig. 7 Comparisons of gut bacteria between group CTC and PC (A), MC and MPC (B) using LEfSe. The histogram shows the LDA scores computed for features at the species level. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group.

3.8 Effects of MP on lymphocyte proliferation

In this study, lymphocytes isolated from the spleen of mice were treated with different concentrations of MP for 24 and 48 h. As shown in Fig. 8, all of the different concentrations of MP tested resulted in an increase in lymphocyte viability, suggesting that MP promotes the proliferation of lymphocytes.

4 Discussion

CP is a frequently-used immunosuppressant, and its mechanism is similar to that of virus immunosuppression. CP can damage the DNA structure, kill immune cells, interfere with the proliferation and differentiation of macrophages, T cells, and B mother cells, and inhibit cell-mediated immunity and humoral immunity.[38–42] Several recent studies have focused on the effect of CP on gut microbiota.[25,43] CP has been shown to increase the count of potentially pathogenic bacteria (*e.g.*, *Escherichia coli* and *Enterobacteraceae*) and disrupt the mechanical mucosal barrier and colonization resistance in the gut.[43] It has also been shown that CP could alter the composition of microbiota in the small intestine, induce the translo-

Table 6 Effects of the polysaccharides extracted from morels on SCFA production (mM per 100 g) in the non-treated and CP-treated mice

Group	Acetic acid (mM per 100 g)	Propionic acid (mM per 100 g)	Butyrate acid (mM per 100 g)	Valeric acid (mM per 100 g)
CTC	14.07 ± 1.32	2.43 ± 0.74	1.26 ± 0.37	0.22 ± 0.08
MC	6.34 ± 1.02***	0.54 ± 0.06*	0.28 ± 0.04*	0.07 ± 0.03**
MPC	$11.10 \pm 2.17^{++}$	$1.34 \pm 0.37^{+}$	$1.31 \pm 0.47^{+}$	$0.30 \pm 0.08^{++}$
PC	11.53 ± 1.58^{x}	1.92 ± 0.26	1.72 ± 0.27	0.41 ± 0.04^{xx}

T tests were used to evaluate the significant differences in SCFA production. Each value is presented as mean ± SD. CTC: normal control group; MC: model control group; PC: MP treatment group; and MPC: CP + MP treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* CTC; $^{+}p < 0.05$, $^{++}p < 0.01$ *vs.* MC; $^{x}p < 0.05$, $^{xx}p < 0.01$ *vs.* CTC.

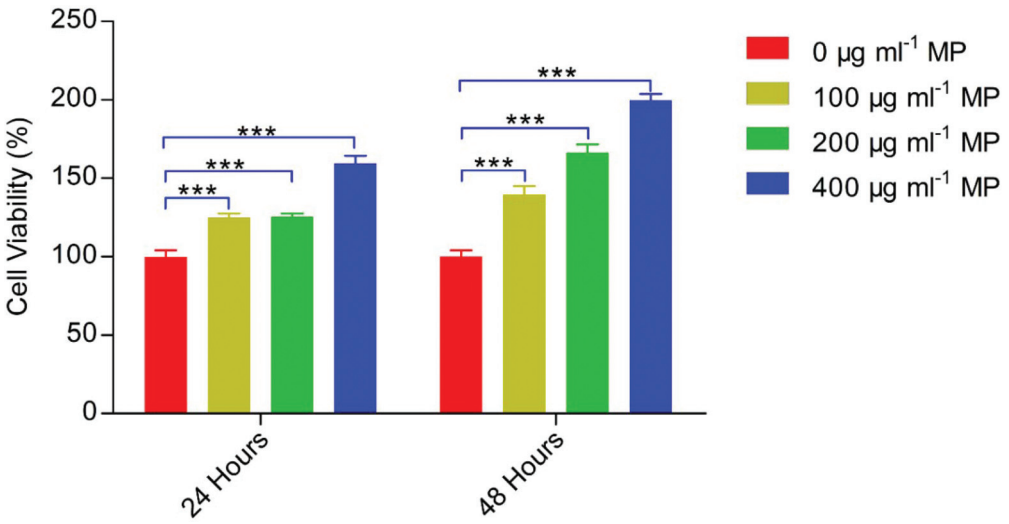

Fig. 8 Analysis of lymphocyte proliferative activity at different concentrations of morel polysaccharide (MP). *T* tests were used to evaluate the significant differences in cell viability. Each value is presented as mean ± SD. $^{***}p < 0.001$.

cation of selected species of Gram-positive bacteria into secondary lymphoid organs, and further affect immune responses.[2,44] In this study, both the non-treated and CP-treated mice were used to explore the effects of MP on gut microbiota and immune responses. We found that CP could decrease the spleen index, levels of WBCs and lymphocytes, and α-diversity of gut microbiota in mice and lessen the proportion of *Bacteroidetes.* These results indicated that CP could alter the structure and composition of gut microbiota and affect immune responses, which was in accordance with previous studies.[6,25,43,44]

The molecular weight of polysaccharides has been considered to have a great relationship with their biological activity; most polysaccharides with medicinal properties are molecules with a molecular weight above 100 kDa.[23] In this study, the average molecular weight of MP was 3.974×10^3 kDa, implying that MP has a certain medicinal value.

Polysaccharides from mushrooms can activate the body's immune system by stimulating immune cells and immune-associated cells.[23,45–47] *Ganoderma* polysaccharide has been shown to improve macrophage function by promoting cell proliferation and phagocytic function, as well as by stimulating the release of NO, TNF-α, and cytokines.[45,47] In addition, *Ganoderma* polysaccharide can activate splenocyte function by stimulating cell proliferation and differentiation and enhancing the expression of cytokines and related RNA.[45] It has also been demonstrated that a water-soluble *Morchella conica* polysaccharide (MCP) could promote splenocyte proliferation *in vitro*.[23] A study by Yu *et al.* demonstrated that *Ganoderma atrum* polysaccharide (PSG-1) enhanced the immunity of CP-induced immunosuppressed mice.[48] They also described the immunomodulatory mechanisms behind the PSG-1-mediated stimulation of spleen lymphocyte function *via* the NO/cGMP, cAMP/PKA, and Ca^{2+}/PKC/calcineurin/NFAT pathways.[49] *Ganoderma* polysaccharide was found to also function as a T cell adjuvant. Pi *et al.* reported that a polysaccharide obtained from *Ganoderma formosanum* was a novel adjuvant that stimulated dendritic cell (DC) maturation, antibody production, cytotoxic T lymphocyte (CTL) activation, and a Th1-polarized immune response.[50] In this study, we showed that MP significantly enhanced lymphocyte levels—especially those of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-CD19^+$ lymphocytes—in the CP-treated mice, as well as promoted the proliferation of spleen lymphocytes *in vitro*. Moreover, MP could also increase the levels of $CD4^-CD8^-CD19^+$ lymphocytes in the non-treated mice, CD19 is an important membrane antigen related to B cell proliferation, differentiation, activation and antibody production. Thus, MP might be able to promote B lymphocyte proliferation in the non-treated mice. These results indicated that MP may function as a kind of adjuvant that can promote the proliferation of lymphocytes. However, the signaling pathway involved in the immunomodulatory effect of MP in lymphocytes has to be clarified, which needs further investigation.

The human gastrointestinal (GI) tract carries a diversity of microbial organisms, and the gut microbiota lives in a symbiotic relationship with the host and plays an important role in the development of the immune system.[51] In this study, we examined the effects of MP on the structure of gut microbiota in both the non-treated and CP-treated mice and found that MP increased the number of OTUs and the α-diversity index. Recent studies have shown that low levels of microbiota species and diversity are associated with abnormal immune function.[52] Our results indicated that MP was beneficial for host health. Comparisons of the predominant gut microbiota at different taxonomic levels (phylum and family) revealed that *Firmicutes* and *Bacteroidetes* were the two most prevalent phyla in cecum microbiota in all groups, and the relative abundance of *Bacteroidetes* was notably increased in the CP-treated mice administered MP compared to the mice in the model control group. At the family level, MP was shown to increase the relative abundance of *Lachnospiraceae* in the non-treated mice, and in the CP-treated mice, MP administration significantly increased the relative abundance of *Ruminococcaceae.* It has been reported that *Ruminococcaceae* and *Lachnospiraceae* are responsible for the degradation of polysaccharides and therefore may degrade MP.[53,54] The *Ruminococcaceae* levels are inversely related to intestinal permeability, hepatic encephalopathy, non-alcoholic fatty liver disease, and alcoholic cirrhosis.[53] *Lachnospiraceae* and *Ruminoccaceae* may directly inhibit toxins produced by *Clostridium difficile* through their 7α-dehydroxylation activity to increase secondary bile acids and decrease primary bile acids.[55]

The total levels of *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* (SCFA-producing bacteria[37]) were increased in both the non-treated and CP-treated mice after the administration of MP. Moreover, MP administration increased the amount of acetate, propionate, butyrate, and valerate in the CP-treated mice. These results indicated that MP may increase the production of SCFAs by increasing the population of SCFA-producing bacteria. Zeng *et al.* revealed that lotus seed-resistant starch increased the contents of SCFAs in mice through augmenting the levels of SCFA-producing bacteria, such as *Lachnospiraceae* and *Ruminococcaceae*.[56] However, the concentration of acetate was decreased in the non-treated mice with MP administration. This may be due to SCFA-producing bacteria consuming acetate to produce butyrate *via* butyryl-CoA: acetate CoA-transferase.[37] It is believed that increased levels of SCFAs produced by SCFA-producing bacteria can acidify the intestinal environment to protect it against several pathogenic bacteria.[57] In addition, SCFAs can directly act on immune cells. For instance, it has been shown that acetate promotes the regulation of T cell (Treg cell) differentiation by inhibiting the histone deacetylase HDAC9, while acetate and butyrate both induce Th1 and Th2 cells.[58,59] Moreover, SCFAs can promote the secretion of secretory IgA (sIgA) from B cells.[60] Considering these positive effects of increased SCFAs on host physiology and immunity, our results indicated that MP enhanced immunity and improved gut microbiota composition and SCFA production in both the non-treated and CP-treated mice. Further investigation of the relationship between MP and other metabolites produced by the gut microbiota

could shed more light on the effect of MP on the host immune system.

5 Conclusion

Our results demonstrated that MP stimulated the proliferation of lymphocytes and had health-promoting potential by improving gut microbiota composition and SCFA production. MP increased the production of SCFAs, possibly by increasing the population of SCFA-producing bacteria, such as *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae*. Furthermore, MP may function as a kind of adjuvant that promotes the proliferation of lymphocytes. However, more detailed mechanisms of MP on promoting health still need to be explored.

Conflicts of interest

There are no conflicts of interest to declare.

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