Comparative studies on the structure, biological activity and molecular mechanisms of polysaccharides from *Boletus aereus* (BA-T) and *Pleurotus cornucopiae* (PC-1)

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Abstract

In this study, two new polysaccharides were extracted from the fruiting bodies of *Boletus aereus* and *Pleurotus cornucopiae*, respectively. The results of structure identification showed that BA-T was composed of xyllose residues, glucose residues and galactose residues, and the ratio is 3:6:6. The BA-T takes 1,6-glucose and 1,6-galactose as skeletons, extends a branched chain from galactose 2-O to connect 1,4-xyllose, and connects β-4-glucose terminal monosaccharide to xyllose. PC-1 was composed of xyllose, glucose and galactose in the ratio of 2:8:2 and takes 1,6-glucose as skeletons, extends a branched chain from galactose 4-O to connect (1→4,6)-α-D-Glc and (1→2,6)-α-D-galactose, and →4)-β-D-Glc and →1)-α-D-Xylp as terminal group. The results of immunoactivity showed that BA-T and PC-1 have the proliferation activity of B cells, T cells and RAW264.7 cells in vitro, and the effect of BA-T on the proliferation of T cells was the greatest. The results of RNA-sequencing showed that the protein synthesis and metabolism of T cells are more vigorous under the stimulation of BA-T, and oxidative phosphorylation is the main energy source in the process of T cell proliferation.

Keywords: *Boletus aereus*; *Pleurotus cornucopiae*; structure identification; immune activity; molecular mechanism.

Practical Application: Fungal polysaccharides can be used as candidate drugs for immunomodulation and antitumor.

1 Introduction

In recent years, natural polysaccharides have received increasing attention in the biomedical field, because they not only have significant anti-tumor and immunomodulatory activities, but also have low toxic side effects (Valverde et al., 2015). Polysaccharide biology has become a frontier science after nucleic acid and protein. Fungal polysaccharide is a kind of natural macromolecular substance, which usually contains primary, secondary, tertiary and quaternary structures (Meng et al., 2016). The structure of fungal polysaccharide is very complex due to the types of monosaccharide residues, the connection sites and sequence of glycosidic bonds, the branching degree of side chain and the different conformations (Chaturvedi et al., 2018). Since the first confirmation of lentinan's anti-tumor activity in 1968, fungal polysaccharides have been widely concerned in the fields of health food and biomedicine due to their anti-virus, anti-oxidation, anti-tumor, immune regulation and other biological activities, as well as their safety and non toxicity (Chihara et al., 1987). *Craterellus cornucopioides* polysaccharide can induce RAW264.7 cell activation and significantly improve its proliferation activity in a certain concentration range (Guo et al., 2019). *Tricholoma matsutake* polysaccharide can not only promote the proliferation of lymphocytes and macrophages in vitro, enhance the phagocytic capacity of macrophages, but also promote macrophages to secrete cytokines (TNF)-α (Byeon et al., 2009).

*Boletus aereus* and *Pleurotus cornucopiae* are two kinds of rare edible and medicinal fungus with high medicinal value. In recent years, studies have shown that polysaccharide from *Boletus aereus* and *Pleurotus cornucopiae* have some interesting biological activities, such as bacteriostasis, antioxidation, et al. (Hagiwara et al., 2005; Wang et al., 2013; Wu et al., 2014; Minato et al., 2017; Lee et al., 2017; Zhang et al., 2014). However, there are some differences in the structure of polysaccharides from different regions, and there is no report on the study of the polysaccharides of *Boletus aereus* and *Pleurotus cornucopiae* from Litang county of Sichuan Province. (China). In the present study, two novel water-soluble polysaccharide BA-T and PC-1 were extracted, isolated and purified from the fruiting bodies of *Boletus aereus* and *Pleurotus cornucopiae*, respectively. The chemical structure and immune activity of the two polysaccharides were first studied and compared to help determine the molecular mechanism underlying the biological activity of polysaccharide BA-T and PC-1.

2 Materials and methods

2.1 Experimental materials and chemicals

*Boletus aereus* and *Pleurotus cornucopiae* were collected from Qingjiang county of Sichuan Province. (China). The ethanol...
was purchased from Swancor Shanghai Fine Chemical Co., Ltd. (Shanghai, China). Trifluoroacetic acid (TFA), standard monosaccharide and dextran of different molecular weight were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). DEAE-cellulose (DE-52) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Cell counting kit (CCK-8 cell counting kit) was purchased from Dojindo Molecular Technologies, Inc. (Shanghai, China). PBS buffer, lipopolysaccharide (LPS), 0.5% Trypsin-EDTA, neutral red and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (Missouri, USA). RPMI1640 medium (phenol red free) and fetal bovine serum (FBS) were purchased from Gibco Inc. (New York, USA). All analytical reagents were of analytical grade.

2.2 Extraction and purification of polysaccharides

Dry fruiting bodies powder (400 g) was boiled in water for 3 times (6 h for each) (Hu et al., 2021; Liu & Li, 2021). The supernatant was concentrated after centrifugation. Three times volume of 95% ethanol were added in the supernatant to precipitate crude polysaccharides. The crude polysaccharides were purified by DEAE cellulose (DE-52) column and dialysis (7000 Da, Biosharp), drying in vacuum freeze-drying machine (ALPHA2-4LD plus, Christ). The final polysaccharide from Boletus aereus and Pleurotus cornucopiae were named BA-T and PC-1, respectively.

2.3 Determination of the molecular weight

The molecular weight of polysaccharide was determined via high-performance gel permeation chromatography (HPGPC). A total of 10 mg polysaccharide was weighted, dissolved in 1 mL distilled water and filtered (0.22μM pore). The measured data was analyzed via Empower Pro GPC software (version B.01.02; Agilent Technologies Inc., USA) and known dextran standards were used as references.

2.4 Fourier Transform Infrared spectrometer (FT-IR) analysis

A total of 5 mg polysaccharide was weighted, then ground and mixed with dry KBr. The data was collected via the mixture scanning within 4000 cm⁻¹ to 400 cm⁻¹ in the fourier transform infrared spectrometer (Nicolet 5700, Thermo Scientific) (Li et al., 2021).

2.5 Monosaccharide composition analysis

The monosaccharide composition of polysaccharide was analyzed by high performance liquid chromatography (HPLC) (Agilent 1100, USA), respectively (Aydogancoskun et al., 2020; Karaoğlu & Çabaroğlu, 2020). A total of 20 mg polysaccharide was dissolved in 5 mL trifluoroacetic acid (TFA) solution (2 mol/L) and hydrolyzed for 6 h (100°C). The supernatant was dried and washed with distilled water to remove residual TFA (repeated 3 times). Operating conditions were detector: RID detector; injection volume: 10 μl; column temperature: 35 °C. Known monosaccharide samples were used as standards (Li et al., 2020; Zheng et al., 2020).

2.6 Methylation analysis and GC-MS

Methyl iodide reagent was used to obtain methylated polysaccharide. The dry methylated product was dissolved in 2M TFA, then hydrolyzed for 6 h (100 °C). Silane reagent was used to prepare derivatized product detected by GC-MS (Agilent 7890A, USA). The initial temperature set at 80 °C and maintained for 3 min, with a linear increase to 200 °C at a rate of 10 °C/min, then maintaining at 200 °C for 10 min (Ismail et al., 2020; Sun et al., 2020; Yang et al., 2020).

2.7 Nuclear Magnetic Resonance (NMR) assay

A total of 50 mg polysaccharide was dissolved in 500 μl D₂O. The 1H NMR spectra, 13C NMR spectra, 1H-1H COSY spectra, HMQC spectra and HMBD spectra were analyzed by the Varian Unity INOVA 400/45 (Varian Medical Systems, USA) and internal standard was tetramethylsilane (He et al., 2020; Kim et al., 2021).

2.8 Cell lines and reagents

Macrophages RAW264.7 cell line, B cell line, T cell line and S180 cell line (sarcoma cell) were purchased from the cell bank of the Typical Culture Preservation Committee of the Chinese Academy of Science (Shanghai, China). All cells were cultivated in RPMI 1640 medium with 10% FBS, 1% penicillin (100 IU/mL) and streptomycin (100 mg/L) at 37 °C with 5% CO₂. All cell lines came from mice.

2.9 T cells, B cells and RAW264.7 cells proliferation assay

Pharmacological evaluation of polysaccharide on T cells, B cells and RAW264.7 cells proliferation was examined via CCK-8 method. Cells (1 x 10⁶ cells/mL) were added to a 96-well plate (100 µL/well) and incubated for 24 h (5% CO₂, 37 °C). Different concentrations of polysaccharide (final concentration 5, 10, 20 μg/mL) were added to the 96-well plate (100 µL/well), incubated for 24 h. LPS (final concentration 5 μg/mL) and the cell culture medium were used as positive control and negative control, respectively. Proliferation assay was analyzed via instructions of CCK-8. The value of optical density (OD) was detected at 450 nm. The way of calculating cell viability was: cell proliferation rate (%) = [(As-Ac)/(Ac-Ab)] × 100%, where Ac was the absorbance of control group, Ab was the absorbance of blank group. As was the absorbance of experimental groups.

2.10 Transcriptome sequencing

The collected cells of control group, LPS group and BA-T group were used to carry out transcriptome assay. The cells, quickly freezed by liquid nitrogen, were sent to Novogene Bioinformatics Technology Co. Ltd, Beijing, China. The remaining tumor tissues were stored at -80 °C. After the samples were
quantified, the library was constructed and checked, and subsequently sequenced using an Illumina Hiseq platform. Genes with an adjusted P < 0.05 identified by DESeq2 were classified as differentially expressed. Subsequently, the clusterProfiler R package was used for Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. Differentially expressed genes were analyzed using the edgeR program (version 3.11).

2.11 Statistical analysis

Data were showed as the mean ± standard deviation (SD). All statistical comparisons were analyzed using a one-way analysis of variance (ANOVA) test followed by Student-Newman-Keuls test with SPSS 17.0 software. Compared with the blank control group, the significant difference was indicated by *, P < 0.05, and the extremely significant difference was indicated by **, P < 0.01.

3 Results and discussion

3.1 Elution curve of polysaccharide

Distilled water, 0.05 mol/L, 0.1 mol/L and 0.2 mol/L NaCl were used as eluents to elute polysaccharide from Boletus aereus and Pleurotus cornucopiae, respectively. The elution results of polysaccharide were shown in Figure 1A and 1B. With the increase of NaCl concentration, there are three and one main elution peaks, respectively. In this study, all neutral polysaccharide eluates in distilled water were collected as the research objects. According to the calculation, the yield of BA-T and PC-1 in the total fruiting body were about 0.4% and 0.3%, respectively.

3.2 Molecular weight of polysaccharides

The molecular weight of polysaccharide was determined by HPGPC. The weight-average molecular weight (Mw) was 14552 Da, the peak molecular weight (Mp) was 14395 Da, the number-average molecular weight (Mn) was 6957 Da and the polydispersity was 2.09 of polysaccharides BA-T (Figure 1C). The weight-average molecular weight (Mw) was 26606 Da, the peak molecular weight (Mp) was 22369 Da, the number-average molecular weight (Mn) was 14992 Da and the polydispersity was 1.77 of polysaccharides PC-1 (Figure 1D).

3.3 FT-IR analysis of polysaccharides

The Fourier transform infrared spectrum of BA-T is shown in Figure 1E. There were typical polysaccharide absorption peaks at 3436 cm⁻¹, 2923 cm⁻¹ and 1400-1200 cm⁻¹, and there were no other impurity peaks, indicating that the purified BA-T was a kind of polysaccharide. The broad absorption peak at 3436 cm⁻¹ is O-H stretching vibration peak, the absorption peak at 2923 cm⁻¹ is -CH₁ stretching vibration peak, the signal at 1635 cm⁻¹ is C=O stretching vibration peak, the signal at 1400 cm⁻¹ is C-H in-plane bending vibration peak of -CHO, and the signal at 1081 cm⁻¹ is C-O stretching vibration peak. The absorption peak in the range of 1200-1000 cm⁻¹ is the absorption peak of pyranosyl lactone and hydroxyl, indicating that BA-T has pyranosyl. The absorption peak at 878 cm⁻¹ indicates that BA-T contains β-pyranose, and the absorption peak at 562 cm⁻¹ indicates that BA-T contains α-configuration. In addition, there was no absorption peak near 1730 cm⁻¹, indicating that BA-T did not contain uronic acid.

The results of PC-1 infrared spectrum were shown in Figure 1F. The broad absorption peak at 3432 cm⁻¹ is O-H stretching vibration peak, the absorption peak at 2924 cm⁻¹ is -CH₁ stretching vibration peak, the signal at 1637 cm⁻¹ is C=O stretching vibration peak, the signal at 1402 cm⁻¹ is C-H in-plane bending vibration peak of -CHO, and the signal at 1086 cm⁻¹ is sugar C-O stretching vibration peak. The absorption peak in the range of 1200-1000 cm⁻¹ is the absorption peak of pyranosyl lactone and hydroxyl, indicating that PC-1 has pyranosyl. There is an absorption peak at 877 cm⁻¹, which indicates that PC-1 contains β-pyranose, and there is a cyclic symmetric vibration peak of α-hexopyranose at 797 cm⁻¹. There is an absorption peak at 557 cm⁻¹, which indicates that PC-1 contains α-configuration. In addition, there is no absorption peak near 1730 cm⁻¹, indicating that PC-1 does not contain uronic acid, neither.

3.4 Monosaccharide composition analysis of polysaccharides

The monosaccharide composition and ratio of BA-T were analyzed by HPLC. The retention time of the six standards is as follows: Rha: 4.291 min, Xylose: 4.928 min, Ara: 5.619 min, Mannose: 6.434 min, Glucose (Glc): 6.694 min, Galactose (Gal): 7.236 min. According to the peak time of monosaccharide standard, xylose (Xyl) was estimated at 5.048 min, glucose (Glc) at 6.713 min and galactose (Gal) at 7.249 min. The peak area ratio of xylose: glucose: galactose is about 1:2:2.

The retention time of the six standards in PC-1 experiment is as follows: Rha: 4.409 min, Xylose: 5.026 min, Arabinose: 5.785 min, Mannose: 6.625 min, Glucose (Glc): 6.932 min, Galactose (Gal): 7.492 min. According to the peak retention time of monosaccharide standard, xylose (Xyl) was estimated at 5.313 min, glucose (Glc) at 6.953 min and galactose (Gal) at 7.504 min. The peak area ratio of xylose: glucose: galactose is about 1:4:1.

3.5 ¹H-NMR analysis of polysaccharides

NMR could provide related hydrogen and carbon signals of different monosaccharides in polysaccharides. The results show that BA-T and PC-1 both have five anomic protons signals, which are at δ 5.00, δ 4.95, δ 4.90, δ 4.88 and δ 4.42, respectively, and the integral area ratio is 1:0.8:0.21:0.76:0.2, and at δ 5.19, δ 4.95, δ 4.81, δ 4.62 and δ 4.33, respectively, and the integral area ratio is 0.8:1.83:1.29:1.03:0.53 (Figure 1G, H). There was no signal after chemical shift δ 5.40, indicating that BA-T and PC-1 were composed of pyranose, which was consistent with the results of FT-IR analysis. The signal between δ 3.0 and δ 4.2 is attributed to the hydrogen signal of C2-C6 in the monosaccharide residue.

3.6 ¹³C-NMR analysis of polysaccharides

The results of ¹³C NMR spectrum of BA-T and PC-1 show that BA-T and PC-1 both have five anomic carbon, which are at δ 102.44, δ 102.34, δ 101.50, δ 101.35 and δ 97.91, respectively, and at δ 102.65, δ 101.62, δ 98.22, δ 98.39 and δ 98.06, respectively (Figure 1I, J). There was no signal at δ 106-109 ppm and δ 82-
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84 ppm, which indicated that BA-T and PC-1 were composed of pyranose and had no furan ring, which was consistent with the results of $^1$H NMR analysis. The signal between δ 60-78 ppm is attributed to the carbon signal of C2-C6 in sugar residues.

### 3.7 $^1$H-$^1$H COSY analysis of polysaccharides

Each proton signal of monosaccharides in polysaccharides could be obtained by $^1$H-$^1$H COSY spectrum. In the $^1$H-$^1$H COSY spectrum of BA-T (Figure 2A), the cross signal A (δ
5.00/3.79), B (δ 5.00/3.96), C (δ 4.95/3.73), D (δ 4.90/3.74), E (δ 4.88/3.73) and F (δ 4.42/3.20) represented the correlation between H-1 and H-2 of the (1→6)-α-D-galactose residues (A), (1→2,6)-α-D-glucose residues (B), (1→6)-α-D-glucose residues (C), (1→2,6)-α-D-glucose residues (D), (1→4)-α-D-xylose residues (E) and (1→2,6)-α-D-galactose residues (F), respectively. According

Figure 2. (A) ¹H-¹H COSY spectrum of BA-T; (B) ¹H-¹H COSY spectrum of PC-1; (C) HMQC spectrum of BA-T; (D) HMQC spectrum of PC-1; (E) HMBC spectrum of BA-T; (F) HMBC spectrum of PC-1; (G) Predicted chemical structure of BA-T; (H) Predicted chemical structure of PC-1.
to the correlation between adjacent hydrogen nuclei, the signals of H2, H3, H4, H5 and H6 in part A are at δ 3.79, δ 3.90, δ 3.76, δ 3.50 and δ 3.20, respectively. By analogy, the hydrogen signal attribution of B, C, D, E and F is shown in Table 1.

In the 1H-1H COSY spectrum of PC-1 (Figure 2B), the cross signal A (δ 5.19/3.54), B (δ 4.95/3.83), C (δ 4.81/3.73), D (δ 4.62/4.01) and E (δ 4.33/3.20) represented the correlation between H-1 and H-2 of the →4)-β-D-Glcp (A), →1)-α-D-Glcp (B), (1→2,6)-α-D-Galp (C), →1)-α-D-Xylp (D) and (1→4,6)-α-D-Glcp (E), respectively. According to the correlation between adjacent hydrogen nuclei, the signals of H2, H3, H4, H5 and H6 in part A are at δ 3.54, δ 4.01, δ 3.73, δ 3.38 and δ 4.12, respectively. By analogy, the hydrogen signal contribution of B, C, D, E and F is shown in Table 2.

### 3.8 HMOC analysis of polysaccharides

The direct correlation of carbon and hydrogen signals could be obtained by HMOC spectra. In the HMOC spectrum of BA-T (Figure 2C), the signal A (δ 5.00/102.34), B (δ 5.00/101.50), C (δ 4.95/97.91), D (δ 4.90/101.35), E (δ 4.88/97.91) and F(δ 4.42/102.44) represented the correlation between H-1 and C-1 of the (1→6)-α-D-galactose residues (A), →4)-β-D-glucose residues (B), (1→4)-α-D-glucose residues (C), (1→2,6)-α-D-galactose residues (D), (1→4)-α-D-xylene residues (E) and (1→2,6)-α-D-galactose residues (F), respectively. According to the correlation between directly connected 1H and 13C, the signals of C2, C3, C4, C5 and C6 in →4)-β-D-Glcp (A) are δ 60.76, δ 70.06, δ 69.55, δ 66.81 and δ 68.30, respectively. By analogy, the hydrogen signal assignments of parts B, C, D, E and F are shown in Table 1.

In the HMOC spectrum of PC-1 (Figure 2D), the signal A (δ 5.19/99.28), B (δ 4.95/98.59), C (δ 4.81/98.06), D (δ 4.62/101.62) and E (δ 4.33/102.65) represented the correlation between H-1 and C-1 of the →1)-α-D-Xylp (D), (1→4)-α-D-xylose residues (E) and (1→2,6)-α-D-galactose residues (F), respectively. According to the correlation between directly connected 1H and 13C, the signals of C2, C3, C4, C5 and C6 in →4)-β-D-Glcp (A) are δ 60.97, δ 72.84, δ 66.71, δ 78.68 and δ 65.00 respectively. By analogy, the hydrogen signal attribution of parts B, C, D and E is shown in Table 2.

### 3.9 HMBC analysis of polysaccharides

HMBC spectrum reflects the coupling relationship between 1H and 13C, which can provide not only hydrogen signal and carbon signal, but also the connection sequence of monosaccharide residues. In the HMBC spectrum of BA-T (Figure 2E), the signal (δ 5.00/70.06), signal (δ 5.00/73.40) and signal (δ 4.88/69.25) conformed to the correlation between H-1 and C-3 of the (1→6)-α-D-galactose residues (A), →4)-β-D-glucose residues (B) and (1→4)-α-D-xylene residues (E). The chemical signal (δ 5.00/70.06) corresponded to the correlation between H-5 and C-3 of (1→6)-α-D-glucose residues (C). The signal (δ 3.90/69.55) and (δ 3.52/73.40) corresponded to the correlation between H-3 and C-5 of (1→2,6)-α-D-glucose residues (D) and (1→2,6)-α-D-galactose residues (F), respectively.

In the HMBC spectrum of PC-1 (Figure 2F), the signal (δ 3.87/72.84) and signal (δ 3.91/60.97) conformed to the correlation between H-5/C-3 of (1→6)-β-D-Glcp (A) and (1→6)-α-D-Glcp (B), respectively. Signal (δ 3.91/72.84) conformed to the correlation between H-3/C-5 of (1→2,6)-α-D-Glcp (C). Signal (δ 4.62/70.34) and signal (δ 4.62/76.88) conformed to the correlation between H-1/C-3 and H-1/C-5 of →1)-α-D-Xylp (D), respectively. Signal (δ 3.91/66.71) conformed to the correlation between H-6/C-4 of (1→4,6)-α-D-Glcp (E). The above results are consistent with those of 1H-1H cosy and HMQC.

### 3.10 GC-MS analysis of polysaccharides

Through gas chromatography and mass spectrometry (GC-MS) analysis of BA-T after methylation and silylation, the total ion current spectrum of BA-T analyzed by GC-MS, and the mass spectra of different retention times are shown in

| Table 1. Chemical shifts of 1H and 13C NMR spectra of BA-T. |
| Glycosyl residues | Chemical shifts (ppm) |
|-------------------|-----------------------|
|                   | H1/C1 | H2/C2 | H3/C3 | H4/C4 | H5/C5 | H6/C6 |
| (1→6)-α-D-Galp(A) | 5.00/102.34 | 3.79/60.76 | 3.90/70.06 | 3.76/69.55 | 3.50/66.81 | 3.20/68.30 |
| (1→4)-β-D-Glcp(B) | 5.00/101.50 | 3.96/77.70 | 3.74/73.40 | 4.06/67.22 | 3.65/61.08 | 3.88/66.81 |
| (1→6)-α-D-Glcp(C) | 4.95/97.91 | 3.73/77.70 | 3.96/68.30 | 3.67/67.22 | 3.76/70.06 | -- |
| (1→2,6)-α-D-Glcp(D) | 4.90/101.35 | 3.74/69.82 | 3.90/67.22 | 3.67/61.08 | 3.39/69.55 | 3.20/70.06 |
| (1→4)-α-D-Xylp(E) | 4.88/97.91 | 3.73/67.22 | 3.79/69.25 | 3.55/61.08 | 4.09/68.38 | 3.88/77.63 |
| (1→2,6)-α-D-Galp(F) | 4.42/102.44 | 3.20/61.08 | 3.52/71.76 | 3.76/69.55 | 3.65/73.40 | 3.50/69.90 |

| Table 2. Chemical shifts of 1H and 13C NMR spectra of PC-1. |
| Glycosyl residues | Chemical shifts (ppm) |
|-------------------|-----------------------|
|                   | H1/C1 | H2/C2 | H3/C3 | H4/C4 | H5/C5 | H6/C6 |
| (1→4)-β-D-Glcp(A) | 5.19/99.28 | 3.54/60.97 | 4.01/72.84 | 3.73/66.71 | 3.38/78.68 | 4.12/65.00 |
| (1→6)-α-D-Glcp(B) | 4.95/98.59 | 3.83/68.69 | 3.54/60.97 | 3.66/68.46 | 3.40/66.71 | 3.50/69.41 |
| (1→2,6)-α-D-Galp(C) | 4.81/98.06 | 3.73/76.88 | 3.91/70.34 | 3.54/66.71 | 3.45/72.84 | 3.38/68.69 |
| (1→4)-α-D-Xylp(D) | 4.62/101.62 | 4.01/68.69 | 3.58/70.34 | 3.66/69.41 | 3.42/76.88 | -- |
| (1→4,6)-α-D-Glcp(E) | 4.33/102.65 | 3.20/76.88 | 3.42/68.46 | 3.54/66.71 | 3.77/60.97 | 3.91/69.41 |
Table 3. The results showed that the β-D-glucose residue was 2,3,6-tri-O-methyl-1-O-trimethylsilyl-Glc, indicating that there was a 4-linked β-D-glucose residue in BA-T. The α-D-glucose residues are 2,3,4-tri-O-methyl-1,6-bis-O-trimethylsilyl-Glc, indicating that there are 1,6-linked α-D-glucose residues in BA-T. The xylose residues are 2-O-methyl-1,3,4-tris-O- (trimethylsilyl) -Xylp and 3-O-methyl-1,2,4-tris-O- (trimethylsilyl) -Xylp and there are two forms of α-D-galactose residues: 2,4-di-o-methyl-1,3,6-tris-o-trimethylsilyl-Galp and 3,4-di-o-methyl-1,2,6-tris-o-trimethylsilyl-Galp. Considering that the steric hindrance of C2 and C3 on the monosaccharide ring is large, it is easy to cause incomplete methylation, so it is speculated that the linkage mode of Xylose residues in BA-T is 1,6-linkage, and the galactose is connected by 1,6-linkage.

The results of GC-MS analysis of PC-1 showed that the β-D-glucose residue was 2,3,6-tri-O-methyl-4-O-trimethylsilyl-Glc, indicating that there was a 4-linked β-D-glucose residue in PC-1. There are 2,3,4-tri-O-methyl-1,6-bis-O-trimethylsilyl-Glc and 2,3-di-O-methyl-1,4,6-bis-O-trimethylsilyl-Glc in the α-D-glucose residues, indicating that there are 1,6-linked and 1,4,6-linked α-D-glucose residues in PC-1. The xylose residue is 2,3,4-tri-O-methyl-1-O- (trimethylsilyl)-Xylp, which indicates that there is a xylose residue linked at C1 in PC-1. The α-D-galactose residue is 3,4-di-O-methyl-1,2,6-tris-O- (trimethylsilyl) -Galp, which indicates that there are 1,2,6-linked galactose residues in PC-1 (Table 4).

The results of structure identification by GC-MS, HPGPC, HPLC, FT-IR and NMR showed that BA-T was composed of xylose residues, glucose residues and galactose residues, and the ratio is 3:6:6. The BA-T takes 1,6-glucose and 1,6-galactose as skeletons, extends a branched chain from galactose 2-O to connect 1,4-xylose, and connects β-4-glucose terminal monosaccharide to xylose. PC-1 was composed of xylose, glucose and galactose in the ratio of 2:8:2. In accordance with FT-IR, monosaccharide composition and methylation results of PC-1, it was inferred that PC-1 takes 1,6-glucose as skeletons, extends a branched chain from galactose 4-O to connect (1→4,6)-α-D-Glcp and (1→2,6)-α-D-galactose, and (1→4)-β-D-Glc and (1→α-D-Xylp as terminal group. The structure of BA-T and PC-1 were shown in Figure 2G, H, respectively.

### 3.11 Effect of BA-T and PC-1 on T cell proliferation

T cells were differentiated from bone marrow stem cells, which could participate in the cellular immunity of the body (Park et al., 2005). The analysis of results showed that polysaccharide group of BA-T and PC-1 (final concentration 2.5, 5, 10 μg/mL, respectively) could significantly promote the proliferation of T cells compared with the blank control group (Figure 3A, B). When the concentration of BA-T was 5 μg/mL and PC-1 was 15 μg/mL, the proliferation efficiency of T cells reached the maximum and increased by 101.98% and 57.62%, respectively, and was higher than that of the positive control group (LPS was 5 μg/mL), which were 29.60% and 26.70%, respectively. Cell morphology observation demonstrated that compared with cells in blank control group, cells stimulated by BA-T and PC-1 formed large clusters and increased in number.

### 3.12 Effect of BA-T and PC-1 on proliferation of B cells

B cells were derived from the pluripotent stem cells of bone marrow, which could proliferate and differentiate into a large number of plasma cells when stimulated by antigens. Plasma cells could synthesize and secrete antibodies in the blood circulation, which plays an important role in the development and regulation of the immune system (Whillock et al., 2021). The results indicated that when the final mass concentration of polysaccharide was 2.5-20 μg/mL, the proliferation effect of B cells was enhanced. When the final concentration of BA-T and PC-1 was 10 μg/mL, the effect of promoting B cell proliferation was the best, and the proliferation rate reached 68.88% and 34.58%, respectively, and was higher than that of the positive control group (LPS was 5 μg/mL), which were 34.97% and 22.47%, respectively (Figure 3C, D). B cells grew well, mostly round, and grew in clusters. When polysaccharide

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### Table 3. Methylation analysis of monosaccharides in BA-T.

| Methylated sugar          | Linkage | m/z                      |
|---------------------------|---------|--------------------------|
| 4-O-trimethylsilyl- Glcp  | 4-      | 59 73 85 88 101 116 133 146 159 174 187 207 219 229 261 |
| 1,6-bis-O-trimethylsilyl-Glc | 1,6-   | 59 73 88 101 117 133 145 159 174 205 229 245 265 287 319 351 |
| 1,2,4-tris-O-(trimethylsilyl)-Xylp | 1,2,4- | 59 73 89 101 133 146 159 173 191 217 233 265 290 305 333 |
| 1,3,4-tris-O-(trimethylsilyl)-Xylp | 1,3,4- | 59 73 85 89 101 116 133 146 159 169 189 204 217 231 247 259 275 291 305 333 348 |
| 1,3,6-tris-O- trimethylsilyl-Galp | 1,3,6- | 59 73 89 146 159 191 207 233 259 303 345 377 |
| 1,2,6-tris-O-trimethylsilyl-Galp | 1,2,6- | 59 73 89 103 117 133 146 159 173 189 205 232 259 277 317 345 377 |

### Table 4. Methylation analysis of monosaccharides in PC-1.

| Methylated sugar          | Linkage | m/z                      |
|---------------------------|---------|--------------------------|
| 1,6-bis-O-trimethylsilyl-Glc | 1,6-   | 59 73 88 101 117 133 145 159 174 185 205 217 233 229 245 265 287 319 |
| 1,4,6-bis-O-trimethylsilyl-Glc | 1,4,6- | 59 73 88 103 117 133 147 159 191 205 232 229 259 287 319 345 377 |
| 4-O-trimethylsilyl- Glcp  | 4-      | 59 73 88 101 116 133 146 159 174 187 207 229 261 293 |
| 1-O-(trimethylsilyl)-Xylp  | 1-      | 58 73 85 88 101 115 121 133 149 159 174 185 217 249 |
| 1,2,6-tris-O-trimethylsilyl-Galp | 1,2,6- | 59 73 89 103 117 133 146 159 173 189 205 232 259 277 317 345 377 |
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3.13 Effect of BA-T on proliferation of RAW264.7 cells

RAW264.7 cells have a variety of immune functions and strong ability of adhesion and phagocytosis of antigens. They not only participate in innate immunity of the body, but also play an important role in immune regulation by participating in acquired immunity (Iwamoto et al., 2005). As shown in Figure 3E, F, when the final mass concentration of polysaccharide was 5-15 μg/ml, the proliferation effect of RAW264.7 cells increases with the increase of different concentrations of polysaccharide. When the final concentration of BA-T and

![Figure 3](https://example.com/fig3.png)

**Figure 3.** (A) Effect on the proliferation of T cells by BA-T; (B) Effect on the proliferation of T cells by PC-1; (C) Effect on the proliferation of B cells by BA-T; (D) Effect on the proliferation of B cells by PC-1; (E) Effect on the proliferation of RAW264.7 cells by BA-T; (F) Effect on the proliferation of RAW264.7 cells by PC-1. Note: Compared with the control group, the difference was significant (P < 0.05) expressed by * and very significant (P < 0.01) expressed by **.
PC-1 was 10 μg/ml, the proliferation rate of RAW264.7 cells was 76.91% and 64.61%, respectively, and was higher than that of the positive control group (LPS was 5 μg/mL), which were 18.54% and 13.72%, respectively. RAW264.7 macrophage was in the state of high refraction circle and part of cells extend pseudopodia, and adheres to the wall under the stimulation of polysaccharide BA-T and PC-1.

3.14 Quantitative analysis of gene expression

In the screening of the two polysaccharides on the proliferation of immune cells, BA-T has a strong immune proliferation activity on T cells. Transcriptome analysis was done to explore the molecular mechanism of BA-T on T cells proliferation. After RNA-sequencing of T cells of control, BA-T and LPS groups, raw data (raw reads) were obtained, respectively. After removing the spliced reads and low-quality reads, 8.21 G (Control), 8.30 G (BA-T) and 8.26 G (LPS) analysis data (clean Reads) were obtained, respectively. In control group, Q20 (%) was 97.87%, Q30 (%) was 93.93%, GC content was 49.47%; in BA-T group, Q20 (%) was 97.87%, Q30 (%) was 93.94%, GC content was 49.47%; in LPS group, Q20 (%) was 97.87%, Q30 (%) was 93.86%, GC content was 49.69%, and the base error rate was all 0.03%. According to the reference sequence alignment analysis, 53385212 readings (97.59%) were successfully compared in the control group, of which 52061527 (95.18%) were the only readings compared to the reference genome, and 1323685 (2.41%) were compared to multiple locations in the reference genome. In BA-T group, 54012626 readings (97.66%) were compared successfully, of which 57360186 (95.21%) were the only readings compared to the reference genome, and 1359758 (2.45%) were compared to multiple locations of the reference genome. In LPS group, 53754649 readings (97.60%) were compared successfully, of which 54632832 (95.16%) were the only readings compared to the reference genome, and 1345592 (2.44%) were compared to multiple locations of the reference genome. The above results show that the sequencing results were reliable and could be used for the next analysis and research.

In this experiment, FPKM method was used to estimate the gene expression level (Hart et al., 2013). It is generally believed that genes with FPKM over 60 are expressed at a high level, while genes with FPKM over 0-0.1 are expressed at a low level or not at all. The results of gene expression level analysis showed that although the gene expression levels of control group, BA-T group and LPS positive control group were similar, there were still some differences. In the control group, 13243 genes were expressed in the state of high refraction circle and part of cells extend pseudopodia, and adheres to the wall under the stimulation of BA-T and PC-1.

Further analysis showed that there were 7 over expressed genes in the control group (MT-CO1, MT-ND4, MT-CO2, MT-CO3, MT-ATP6, MT-CYB, MT-ND2, MT-ND4L), 7 over expressed genes in the BA-T group (MT-CO1, MT-ND4, MT-CO2, MT-CO3, MT-ATP6, MT-ND2, MT-ND4L), and 7 over expressed genes in the LPS positive control group (LPS)(MT-CO1, MT-ND4, MT-CO2, MT-CO3, MT-ATP6, MT-ND2, MT-ND4L).

3.15 Differential gene analysis

After quantitative analysis of gene expression, statistical analysis of gene expression data was carried out to screen the genes with significant expression differences in different samples under the same conditions. Based on the three biological repeats in this experiment, the significance of gene differential expression was analyzed by using DESeq2 software, and log2 (foldchange) | > 0 and Padj < 0.05 was used as the screening criteria. The results are shown in Figure 4A. B. Compared with the blank group, there were 600 genes up-regulated and 822 genes down regulated in BA-T group (Figure 4A), among which 10 genes up-regulated were Smn1, Gapdh, Psmnb7, Pkm, Snrph, Rps5, Cdc123, Mrps34, Ac139272.1 and ps13. Compared with the blank control group, there were 702 genes in the LPS positive control group, of which 544 genes were up-regulated and 71 genes were down regulated (Figure 4B). Among them, 10 genes were up-regulated, including Rabggtb, Nop53, Sta3s1, Hnrnpa1, Pkm, Rip13A, H3j3b, Runx1, Atf4 and Eef1a1.

Gapdh is the gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GAPDH is the key enzyme in the process of glycolysis (Hara et al., 2005); Pkm is the gene encoding pyruvate kinase (PKM), PKM promotes the production of pyruvate in the process of glycolysis (Shema et al., 2007), and Gapdh and Pkm are up-regulated in BA-T group, which indicates that the intracellular glucose metabolism process is more vigorous under the stimulation of BA-T. Psmnb7 is a gene encoding proteasome β7 subunit (Munkácsy et al., 2010). Psmnb7 is up-regulated in BA-T group, suggesting that BA-T contributes to increase intracellular protein metabolism. Cdc123 is a gene of cell cycle division protein 123 (CDC123) (Imamura et al., 2011). CDC123 is a cell cycle regulator required for eukaryotic initiation factor 2 (eIF2) assembly. Cdc123 is up-regulated in BA-T group, suggesting that BA-T plays an important role in regulating protein synthesis, cell growth and proliferation.

3.16 Go enrichment analysis of differential genes

Go (Gene Ontology) is a comprehensive database describing gene function, which is divided into three parts: biological
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**Figure 4.** (A) Volcanic map analysis of differential gene expression of BA-T group compared with control group; (B) Volcanic map analysis of differential gene expression of LPS group compared with control group; (C) Classification and analysis of gene function of BA-T group compared with Control group; (D) Classification and analysis of gene function of LPS group compared with Control group; (E) Top 20 KEGG pathways enriched for up-regulated differentially expressed genes between BA-T and Control group; (F) Top 20 KEGG pathways enriched for down-regulated differentially expressed genes between BA-T and Control group; (G) Top 20 KEGG pathways enriched for up-regulated differentially expressed genes between LPS and Control group; (H) Top 20 KEGG pathways enriched for down-regulated differentially expressed genes between LPS and Control group.
process, cellular component and molecular function. A total of 9116 differential genes (padj < 0.05) between BA-T and blank group were successfully annotated, among which 7191 genes were up-regulated and 1925 genes were down regulated (Figure 4C). Among them, ribonucleoprotein complex biogenesis is the most abundant gene in biological process, ribosome is the most abundant gene in cell component, and structural construct of ribosome is the most abundant gene in molecular function. A total of 5751 differential genes (padj < 0.05) were successfully annotated between LPS group and blank group, of which 4729 genes were up-regulated and 1022 genes were down regulated (Figure 4D). Among them, translation initiation is the most abundant in biological processes, ribosome is the most abundant in cell components, and structural construct of ribosome is the most abundant in molecular functions. The results showed that there were differences between BA-T group, LPS group and blank group in gene types of biological process, cell composition and molecular function. However, it should be noted that the effect of BA-T on T cells may be similar to that of LPS.

### 3.17 KEGG enrichment analysis of differential genes

In order to investigate the effect of BA-T and LPS on T cell proliferation, the KEGG (Kyoto Encyclopedia of genes and genomes) pathway was analyzed by cluster profiler software. The differential genes among BA-T group, LPS group and blank group were annotated in KEGG database. A total of 476 (8.36%) differential genes between BA-T group and blank group were successfully annotated into 283 pathways, and 445 (7.95%) differential genes between LPS group and blank group were successfully annotated into 281 pathways. By analyzing the top 20 pathways of differentially expressed genes (padj < 0.05) between BA-T group and blank group, it was found that there were 11 up-regulated genes in BA-T, mainly including ribosome, proteasome and oxidative phosphorylation (Figure 4E). The down regulated genes in BA-T significantly enriched include Fanconi anemia pathway and homologous recombination (Figure 4F). By analyzing the top 20 pathways of differentially expressed genes (padj < 0.05) between LPS group and blank group, it was found that there were 4 up-regulated genes in LPS, including ribosome, central carbon metabolism in cancer, proteasome, and glutathione metabolism pathway (Figure 4G), while there were no significantly enriched down regulated genes in LPS (Figure 4H).

Further analysis of the signal pathway between BA-T group and blank group showed that 62 differentially expressed genes (13.03%) were up-regulated in ribosomal pathway, 20 (4.20%) were up-regulated and 8 (1.68%) were down regulated in oxidative phosphorylation pathway. The expression of Gapdh (glyceraldehyde 3-phosphate dehydrogenase), Bpgm (phosphoglycerate mutase), Pgk (phosphoglycerate kinase), Pgam (phosphoglycerate kinase), Eno (enolase), Pkm (pyruvate kinase), Pdh (pyruvate dehydrogenase) and Lidh (lactate dehydrogenase) in glycolysis pathway were up-regulated, while the expression of Aldh (aldehyde dehydrogenase) was down regulated. The expression of Pdh (pyruvate dehydrogenase), Aco (Acetonitum hydrase), Lidh (isocitrate dehydrogenase) and Ogdh (oxoglutarate dehydrogenase) in the tricarboxylic acid cycle were up-regulated. It is suggested that the protein synthesis and metabolism of T cells are more vigorous under the stimulation of BA-T, and oxidative phosphorylation is the main energy source in the process of T cell proliferation.

### 4 Conclusion

In this study, hot water extraction, ethanol precipitation, DEAE-52 cellulose and column chromatography were used to extract and purify the crude polysaccharides of *Boletus aereus* and *Pleurotus cornucopiae*. Two new polysaccharides were extracted from the fruiting bodies of *Boletus aereus* and *Pleurotus cornucopiae*, respectively. In this study, two new polysaccharides were extracted from the fruiting bodies of *Boletus aereus* and *Pleurotus cornucopiae*, respectively, by hot water extraction, ethanol precipitation and DEAE column chromatography. The results showed that the molecular weight of *Boletus aereus* polysaccharide (BA-T) was 14552 Da, and that of *Pleurotus cornucopiae* (PC-1) was 26606 Da. The results of structure identification by GC-MS, HPGPC, HPLC, FT-IR and NMR showed that BA-T was composed of xylose residues, glucose residues and galactose residues, and the ratio is 3:6:6. The BA-T takes 1,6-glucose and 1,6-galactose as skeletons, extends a branched chain from galactose 2-O to connect 1,4-xyllose, and connects β-4-glucose terminal monosaccharide to xylose. PC-1 was also composed of of xylose, glucose and galactose in the ratio of 2:8:2. In accordance with FT-IR, monosaccharide composition and methylation results of PC-1, it was inferred that PC-1 takes 1,6-glucose as skeletons, extends a branched chain from galactose 4-O to connect (1→4,,6)-α-D-Glcp and (1→2,6)-α-D-Galp and (1→3)-β-D-Glcp and (1→1)-α-D-Xylp as terminal group. The results of immunoactivity showed that BA-T and PC-1 have the proliferation activity of B cells, T cells and RAW264.7 cells in vitro, and the effect of BA-T on the proliferation of T cells was the greatest. In addition, BA-T could significantly promote the secretion of IgA, IgE, IgG and IgM in B cells and enhance the phagocytic ability of RAW264.7 cells. The results of RNA-sequencing showed that the protein synthesis and metabolism of T cells are more vigorous under the stimulation of BA-T, and oxidative phosphorylation is the main energy source in the process of T cell proliferation. In conclusion, BA-T and PC-1 were both natural polysaccharides with novel structure, which had immune and proliferation activity for specific immune cells. To explore the structure of *Boletus aereus* polysaccharide (BA-T) and *Pleurotus cornucopiae* polysaccharide (PC-1) and the structure-activity relationship between them can provide a better theoretical basis for the research and development of medicinal value of BA-T and PC-1.

### Conflict of interest

The author declare that they have no conflict of interest.

### Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors’ contributions

Yiling Hou conceived and designed the experiments of the present study. Xiang Ding, Xian Tang and Yiling Hou performed the experiments and acquired data. Xiang Ding and Yiling Hou drafted the manuscript and revised it critically. All authors read and approved the final manuscript.

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