



Research Article

Exploring the immunomodulatory activity of *Atractylodes* rhizome polysaccharides from different origins based on structure-gut microbiota-short chain fatty acids

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Abstract: The *Atractylodes* rhizome, derived from the dried rhizomes of *Atractylodes lancea* (Thunb.) DC. or *Atractylodes chinensis* (DC.) Koidz., is a multi-based original medicinal material used in China to treat gastrointestinal diseases. Polysaccharides, a group of active ingredients found in *Atractylodes* rhizome, exhibit excellent immune regulation, intestinal protection, and liver protection activities. However, the structural differences, immunomodulatory and intestinal protective effects, and mechanisms of polysaccharides derived from *Atractylodes lancea* or *Atractylodes chinensis* rhizome remain unclear. This study aims to compare the basic structural differences between *Atractylodes lancea* rhizome polysaccharide (ALP) and *Atractylodes chinensis* rhizome polysaccharide (ACP), and elucidate the differences in pharmacological effects and potential mechanisms of the two polysaccharides in immunomodulation and intestinal protection. The results showed that ALP and ACP were composed of fructose, mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, and arabinose, with minor amount of uronic acid, and exhibited a triple helix structure. Among them, the higher uronic acid (ALP vs ACP: $4.65 \pm 0.68\%$ vs $7.38 \pm 1.76\%$) content of ACP indicated stronger acidity compared to ALP. Both ALP and ACP improved phagocytic activity and cytokine secretion levels in RAW 264.7 cells *in vitro*, with ACP showing a more significant effect. After administering the two kinds of polysaccharides *in vivo*, mice exhibited improved immunosuppression, significantly restored humoral immune indexes, reduced pathological damage in the jejunum, and increased secretion levels of TNF- α , IL-1 β , IL-6, and iNOS. In addition, the number of goblet cells in the jejunum and the expression level of Mucin 2 (MUC2) increased, and the levels of Zonula Occludens-1 (ZO-1), Occludin, Claudin-1, F4/80 antigen, immunoglobulin A (IgA), and secretory immunoglobulin A (sIgA) were significantly higher than those in the model group. Additionally, the activity of ACP is superior to ALP. 16S rDNA sequencing results showed that ACP could not only increase the diversity index of intestinal flora, such as Chao1, observed_features, Pielou_e, Shannon, and Simpson indexes, but also regulate the composition of intestinal flora at the phylum, family and genus levels, reduce the abundance of Firmicutes and *Lachnospiraceae_NK4A136_group*, and increase the abundance of Bacteroidota, Muribaculaceae, *Ruminococcus* and *Prevotellaceae_UCG-001*. Moreover, ACP can also promote the production of short-chain fatty acids (SCFAs) from fermentation products of intestinal flora. These findings indicated that ACP could be used as an effective immunomodulator to improve immunosuppression and intestinal mucosal injury caused by cyclophosphamide (Cy).

Keywords: *Atractylodes lancea* rhizome polysaccharide; *Atractylodes chinensis* rhizome polysaccharide; immunosuppressed; intestinal mucosal barrier; intestinal microbiota; SCFAs

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1 Introduction

The immune system is an important defense system against external threats and functions to recognize and clear foreign antigens, coordinate other bodily systems, and jointly maintain the

stability and physiological balance of the internal environment of the body^[1]. Immune deficiency makes the body more susceptible to bacterial infections, including respiratory infections, urinary tract injuries, sepsis, and meningitis^[2]. Cyclophosphamide (Cy) can impair immune function and cause intestinal barrier

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damage^[2]. The intestine, being the primary site for digestion and absorption, is abundant with lymphatic tissue and ranks as the largest immune organ in the body. The intestinal barrier consists of mechanical, chemical, biological, and immune barriers, which provide innate immune functions. Once the intestinal mucosal barrier is damaged, intestinal permeability increases, allowing pathogenic bacteria to enter the intestinal cavity and raising the risk of infection. Conventional immunostimulants, such as levamisole hydrochloride and isoproterenol, have many side effects (e.g., gastrointestinal effects like nausea, vomiting, and diarrhea caused by levamisole)^[1]. In contrast, natural products have low costs, minimal toxic side effects, and great therapeutic potential, making them highly advantageous^[3].

Many recent studies have found that plant polysaccharides from natural sources exhibit multiple biological activities and excellent biosafety, with minimal toxic side effects^[4]. Studies have shown that plant polysaccharides are effective in treating Cy-induced immunosuppression and intestinal mucosal barrier damage. Polysaccharides^[5] not only regulate the structure of the intestinal microbiota^[6,7] but also can be decomposed by the intestinal microbiota to produce various metabolites, such as short-chain fatty acids (SCFAs)^[8-10]. Research found that ginger polysaccharides (UGP1 and UGP2) enhanced intestinal immune function by regulating the gut microbiota related to immune function and increasing the SCFA levels^[11]. Additionally, polysaccharide from *Cordyceps sinensis* was reported to significantly recover small intestinal pathological damage, increase goblet cell numbers and mucus levels, regulate the balance of Th1/Th2 cells, regulate the secretion of Treg and Th17 cytokines, increase the abundance of beneficial bacteria and total SCFAs in mice with intestinal injuries, and alleviate intestinal injury and the dysbiosis of intestinal flora in immunosuppressed mice^[12]. Longan polysaccharides regulate the gut microbiota, enhance the relative abundance of beneficial bacteria, and protect the intestinal epithelial barrier through metabolizing microbial products^[13]. Therefore, searching for new plant polysaccharides with good immune regulation and intestinal-protective effects is of great importance.

Atractylodes rhizome is a traditional medicinal material derived from the dried rhizomes of *Atractylodes lancea* (Thunb.) DC. and *Atractylodes chinensis* (DC.) Koidz. Both species belong to the Asteraceae family. The herb was first recorded in *Shen Nong's Herbal Classic*^[14]. *Atractylodes lancea* is primarily produced in Jiangsu, Hubei, and Anhui, while *Atractylodes chinensis* is mainly produced in Inner Mongolia, Jilin, Liaoning. The main active components of *Atractylodes* rhizome include both volatile and non-volatile components^[15]. At present, research on the active components of *Atractylodes* rhizome has mainly focused on the volatiles, such as atractylolide A, with relatively less attention given to the non-volatile components. *Atractylodes* rhizome polysaccharides^[16], the main non-volatile components, have many pharmacological activities, including immune regulation^[8], antioxidant^[17], and antitumor effects^[18]. The differences in the medicinal efficacy of different original plants are related to the differences in their chemical components. Studies found that the water decoction of *Citrus aurantium* exhibited better antioxidant activity than *Citrus sinensis* attributed to its higher levels of naringin and neohesperidin^[19]. *Atractylodes lancea* had better efficacy in drying dampness and strengthening the spleen compared to *Atractylodes chinensis*, which may be related to *Atractylodes lancea* was found to have a higher volatile oil content in the same number of decoction pieces^[20]. For polysaccharides,

with higher uronic acid content showed better immunoregulatory activity, this is mainly due to the higher content of uronic acid, which can more effectively activate the signaling pathways of macrophages and membrane receptors (such as TLR4). Previous studies by our research group found immunomodulatory activity in *Atractylodes lancea* rhizome polysaccharide (ALP). However, there is limited comparative research on the structural differences and immunomodulatory activity differences of *Atractylodes* rhizome polysaccharides from different origins. It remains unclear whether the chemical structure and immunomodulatory activity differences of *Atractylodes* rhizome polysaccharides are related to their origin.

This study aimed to explore the structural characteristics of ALP and *Atractylodes chinensis* rhizome polysaccharide (ACP) as well as their pharmacological effects and potential mechanisms for improving Cy-induced immune suppression and intestinal injury. We first compared the structural differences of *Atractylodes* rhizome polysaccharides from different origins. Subsequently, the immunomodulatory effects of ALP and ACP on macrophage phagocytic activity and cytokine expression were studied using RAW 264.7 cells. We further constructed a Cy-induced immunosuppressed and intestinal injury mouse model to investigate differences in the pharmacological effects between ALP and ACP on immune function and intestinal injury in the body. Given that ACP demonstrated a superior effect in activating macrophages and improving immune suppression as well as intestinal mucosal damage compared to ALP, we ultimately investigated the regulatory effect of ACP on the gut microbiota structure and its impact on SCFA metabolism, further revealing the underlying mechanisms by which ACP improves immune suppression and intestinal damage.

2 Materials and methods

2.1 Materials and reagents

Atractylodes lancea rhizome and *Atractylodes chinensis* rhizome were sourced from Hubei Province and Inner Mongolia, China, respectively. Tumor necrosis factor α (TNF- α), interleukin1 β (IL-1 β), and secretory immunoglobulin A (sIgA) kits were sourced from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Zonula Occludens-1 (ZO-1), Mucin 2 (MUC2), Occludin, Claudin-1, immunoglobulin A (IgA), and F4/80 antibodies were acquired from Proteintech (Wuhan, China).

2.2 Preparation of ALP and ACP

ALP and ACP were extracted using a previously described method^[8]. Dried *Atractylodes* rhizome powder was degreased, hot water extraction (material to liquid ratio is 1:30, twice, 3 h each occasion), ethanol precipitation overnight. The resulting sediment was removed protein by the Sevag method (7–8 times) and then freeze-dried to obtain ALP and ACP.

2.3 Structural characterization of ALP and ACP

2.3.1 Chemical composition analysis

Total carbohydrate, uronic acid, protein in ALP and ACP were measured using the phenol-sulfuric acid, *m*-hydroxydiphenyl, Bradford method according to the previously reported method^[8].

2.3.2 Determination of molecular weight of ALP and ACP

The molecular weight of ALP and ACP was determined using

high-performance gel permeation chromatography evaporative light scattering detector (HPGPC-ELSD) (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic column was a TSKgel G4000PWxl column (7.8 mm ID × 30 cm, 10 μm). The mobile phase was ultrapure water, with a flow rate of 0.4 mL/min and a column temperature of 30 °C. The injection volume was 10 μL. The drift tube temperature was 115 °C, and the carrier gas flow rate was 3.4 L/min.

2.3.3 Ultraviolet and FT-IR spectroscopy analysis

1 mg/mL solution of ALP and ACP was scanned using an ultraviolet (UV) Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in the wavelength range of 200–800 nm to detect absorption peaks at 260 nm and 280 nm.

ALP and ACP were ground and mixed with KBr, and their infrared spectrum was scanned in the range of 4,000–400 cm⁻¹ using a DTGS KBr Nicolet 380 detector (Thermo Fisher Scientific).

2.3.4 Congo red experiment

The Congo red assay has been increasingly used to identify the triple-helix conformation, for example, it can be used to detect the triple helix structure of acidic polysaccharides^[21]. Briefly, solutions of ALP and ACP at a concentration of 2.5 mg/mL were prepared, 80 μmol/L Congo red solution (2 mL) was added. Then, NaOH solutions (2 mL) with different concentrations were added so that the final concentration of NaOH in the system was 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mol/L. After 25 min of reaction, the maximum absorption wavelength (λ_{max}) was scanned in the range of 200–600 nm using an UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). A curve using NaOH concentrations as the abscissa and the maximum absorption wavelength (λ_{max}) as the ordinate was generated.

2.3.5 Monosaccharide composition analysis

General method: ALP and ACP sample solutions were hydrolyzed with 4 mol/L trifluoroacetic acid (120 °C, 4 h) and blown dry with nitrogen, then washed three times with 2 mL of methanol. Nine monosaccharides and hydrolyzed ALP and ACP were derivatized using PMP and analyzed by HPLC (Thermo Fisher Scientific, USA). The chromatographic conditions were: AQ-C18 column (4.6 × 250 mm, 5 μm; Yuexu Technology Co. Ltd., Shanghai, China); mobile phase: A (acetonitrile) and B (0.1 mol/L KH₂PO₄ aqueous solution, pH = 6.9) with gradient elution (0–60 min, 15%–25% A); column temperature set at 35 °C; sample volume of 10 μL and detection wavelength at 250 nm^[22].

Fructose method: ALP and ACP samples were pretreated and analyzed by HPAEC (ICS 5000+, Thermo Fisher Scientific, USA). The flow rate was 0.5 mL/min and the injection volume was 5 μL. Solvent system A was double-distilled water, solvent system B was 0.1 mol/L NaOH, and solvent system C was 0.1 mol/L NaOH and 0.2 mol/L NaAc. A gradient elution program was used with the following volume ratios of solutions A, B, and C: 95:5:0 at 0 min; 85:5:10 at 26 min; 85:5:10 at 42 min; 60:0:40 at 42.1 min; 60:40:0 at 52 min; 95:5:0 at 52.1 min; and 95:5:0 at 60 min^[23].

2.3.6 NMR spectroscopy analysis

The ALP and ACP samples were dissolved in 0.5 mL of deuterated water to a final concentration of 40 mg/mL. 1D-NMR (¹H-NMR, ¹³C-NMR) were recorded at 25 °C with a Bruker AVANCE NEO 500 MHz spectrometer (Bruker, Rheinstetten,

Germany) operating at 500 MHz^[24].

2.4 Effects of ALP and ACP on the immunostimulatory activity of RAW 264.7 cells

2.4.1 Cell culture

In this study, mouse macrophage RAW264.7 was used as the experimental object, and DMEM medium was used for *in vitro* culture. The cell culture conditions were: 37 °C constant temperature, 5% CO₂ saturation humidity standard culture environment. All operations ensured that the cells were in the logarithmic growth phase to meet the needs of subsequent experiments.

2.4.2 Determination of cell viability

RAW 264.7 cells were cultured in the logarithmic phase were inoculated into 96-well plates (1 × 10⁴ cells/well) at 100 μL/well, with six wells per group, and 100 μL of ALP and ACP at different concentrations (0, 25, 50, 100, 200, 400, 800, 1,000, 1,600, and 2,000 μg/mL) were added. Then, 10 μL Cell Counting Kit-8 cell counting reagent (CCK8) reagent (Biosharp, Beijing, China) was added, then incubated for 30 min. The absorbance was recorded at 450 nm using a microplate reader (BioTek, Beijing, China).

2.4.3 Neutral red phagocytosis experiment

Inoculate RAW 264.7 cells, fresh medium containing low, medium, and high doses of ALP and ACP (200, 400, and 800 μg/mL) was added. Cells treated with lipopolysaccharide (LPS, 1 μg/mL) served as the positive control group. After 24 h of culture, 100 μL of neutral red solution (1 mg/mL) was added to each well and incubated for 4 h. The supernatant was removed, and the cells were washed three times with phosphate-buffered saline (PBS). Subsequently, 100 μL cell lysate (ethanol:glacial acetic acid = 1:1, v/v) was added for 1 h, and the absorbance was measured at 540 nm using a microplate reader (BioTek, USA).

2.4.4 Detecting the expression level of cytokines

Inoculate RAW 264.7 cells, the experimental group was treated with different doses (200, 400, and 800 μg/mL) of ALP and ACP, and the LPS (1 μg/mL) positive control group was set up for 24 h. Then, extract total RNA, reverse transcription, amplification. The primer sequences are listed in Table S1.

2.5 Effects of ALP and ACP on the immune function of immunosuppressed mice

2.5.1 Animal experiment

Male C57BL/6 mice (20–22 g, 7–8 weeks, *n* = 30) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (license number: Syxk [e] 2017-0067). All mice were raised in a specific pathogen free (SPF) environment. The environmental parameters were set as follows: Temperature 24–26 °C, relative humidity 50%–60%, 12 h day and night cycle, and adequate food and drinking water were provided.

C57BL/6 mice were randomly divided into five groups: Ctrl, Cy, ALP, ACP and LH group. The Ctrl and Cy groups were given 0.9% NaCl solution by gavage, while the ALP, ACP and LH groups were given 1,200 mg/kg of ALP and ACP and 40 mg/kg of LH by gavage respectively for 15 days, the dosages of ALP and ACP groups were based on preliminary experimental results, our research group's published findings, and relevant references^[8].

During the 8th to 10th days of the experiment, mice in other groups except for the Ctrl were intraperitoneally injected with Cy (80 mg/kg) every day, record the weight of each group of mice. After the experiment, euthanasia was carried out. The blood, spleen, thymus, jejunum, colon contents and cecum contents of mice were collected for subsequent detection and analysis. The animal experiment plan was approved by the animal ethics committee of Hubei University of Chinese medicine (approval code: NO.00273385, 14 June 2022).

2.5.2 Blood cell counts

Using BC-5000vet (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China) to analyze the changes in blood cells of mice in each group.

2.5.3 Pathological analysis of jejunum tissue

Following euthanasia, jejunum, spleen tissues were fixed in 4% paraformaldehyde solution, washed, dehydrated, embedded in paraffin, sliced, and stained with hematoxylin and eosin (HE). Tissue morphology was observed under an optical microscope.

2.6 Detecting the expression level of cytokines in jejunum

Extract total RNA, and amplified from jejunum according to the method described in "Detecting the expression level of cytokines". Primer sequences are provided in Table S1.

2.7 Enzyme-linked immunosorbent assay

The expression levels of IgA in the serum, as well as TNF- α , IL-1 β , and sIgA in the jejunum tissue, were analyzed according to the kit instructions (Elabscience, Wuhan, China).

2.8 AB-PAS staining

AB-PAS staining of jejunum was performed according to the reported method, the wax block of jejunum tissue was sliced and stained with Alcian blue-periodic acid Schiff (AB-PAS) dye solution C. The tissue was then acidified with AB-PAS dye solution B, and finally stained with AB-PAS dye solution A, dehydrated and sealed, and examined microscopically^[25].

2.9 Immunohistochemical analysis

After dewaxing paraffin sections of mouse small intestinal tissue, antigen repair was carried out, and serum was dripped into the histochemical circle and sealed. The MUC2 primary antibody and secondary antibody was applied and incubation, followed by DAB color development, hematoxylin double-staining of the nucleus, gradient ethanol dehydration, and sealing.

2.10 Immunofluorescence analysis

Paraffin sections of jejunum tissues from mice in each group were dewaxed, followed by antigen repair and serum sealing. Primary antibodies were added to each section to cover the tissues. Subsequently, fluorescent secondary antibodies of the corresponding species were added, after washing and staining, then, microscopic examination.

2.11 Western blot (WB) analysis

Jejunum tissue proteins (ZO-1, Occludin, and IgA) were separated. The proteins were transferred to a PVDF membrane using the wet transfer method. After transfer, the membrane was sealed with 5% skim milk at room temperature for 2 h, followed

by incubation with primary antibodies and secondary antibodies, and finally developed with enhanced chemiluminescence substrate. Protein bands were quantitatively assessed through the measurement of their intensities utilizing the ImageJ software. (Bethesda, MD, USA).

2.12 16S rDNA sequencing analysis

Genomic DNA was extracted, and amplified (16S rDNA V3-V4 region). PCR products were bead-purified, and libraries were quantified (Qubit/qPCR) before NovaSeq 6000 PE250 sequencing.

2.13 Measurement of SCFAs levels

50 mg freeze-dried colon sample was accurately weighed, 0.2 mL of phosphoric acid (0.5% v/v) was added, and the sample was completely ground in a low-temperature high-speed tissue grinder. Tert-butyl methyl ether (500 μ L) was added, which was then vortexed for 3 min, placed in an ultrasonic ice bath for 5 min, and centrifuged at 4 $^{\circ}$ C, 12,000 r/min for 10 min. Finally, 200 μ L supernatant was removed and stored at -20° C for testing. Reference stock solutions of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, hexanoic acid, and 2-methylvaleric acid were prepared in tert-butyl methyl ether. The gas chromatography-mass spectrometry (GC-MS) method used a DB-FFAP column (internal diameter 30 m $\times$ 0.25 mm, 0.25 μ m; Agilent Technologies, CA, USA), following a previously reported method for detection^[26].

2.14 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.5 software by one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

3 Results

3.1 Structural characterization of ALP and ACP

3.1.1 Preparation, chemical composition analysis, and molecular weight analysis of ALP and ACP

In this study, ALP and ACP were extracted from *Atractylodes lancea* and *Atractylodes chinensis* rhizomes using water extraction and alcohol precipitation, with yields of 12.50% and 17.58%, respectively (Table 1). The total carbohydrate content of ALP and ACP was $97.52 \pm 1.25\%$ and $94.87 \pm 0.72\%$, respectively, and the protein content was $1.60 \pm 0.10\%$ and $0.89 \pm 0.09\%$, respectively, indicating that most of the proteins in ALP and ACP had been removed. The uronic acid content of ALP and ACP was $4.65 \pm 0.68\%$ and $7.38 \pm 1.76\%$, respectively, suggesting that both ALP and ACP were weakly acidic polysaccharides. The higher uronic acid content of ACP indicated stronger acidity compared to ALP (Table 1). Further analysis reveals that the molecular weights of ALP and ACP were 7,396.781 Da and 6,865.306 Da, respectively (Table 1).

3.1.2 UV and Fourier-transform infrared spectroscopy analysis

Fig. 1A presents the UV-Vis spectra of ALP and ACP. ALP and ACP exhibit no absorption peak at 260 nm, suggesting their absence of nucleic acids. Furthermore, ALP and ACP are virtually devoid of proteins, as evidenced by the lack of a prominent absorption peak at 280 nm, consistent with the chemical composition results. The FT-IR spectra for ALP and ACP are

Table 1 Chemical compositions of ALP and ACP

	ALP	ACP
Yield (%)	12.50	17.58
Chemical characteristics		
Total carbohydrate (%)	97.52 ± 1.25	94.87 ± 0.72
Protein (%)	1.60 ± 0.10	0.89 ± 0.09
Uronic acid content (%)	4.65 ± 0.68	7.38 ± 1.76
Average molecular weight (Da)	7,396.781	6,865.306
Monosaccharide composition (molar ratio, %)		
Mannose	3.37 ± 0.02	2.58 ± 0.03
Ribose	3.23 ± 0.01	2.91 ± 0.03
Rhamnose	0.86 ± 0.00	1.53 ± 0.02
Glucuronic acid	1.22 ± 0.00	1.18 ± 0.01
Galacturonic acid	3.17 ± 0.01	7.32 ± 0.07
Glucose	47.14 ± 0.17	48.40 ± 0.12
Galactose	19.46 ± 0.09	15.16 ± 0.05
Arabinose	21.55 ± 0.07	20.92 ± 0.08

Note: ALP, *Atractylodes lancea* rhizome polysaccharide; ACP, *Atractylodes chinensis* rhizome polysaccharide;

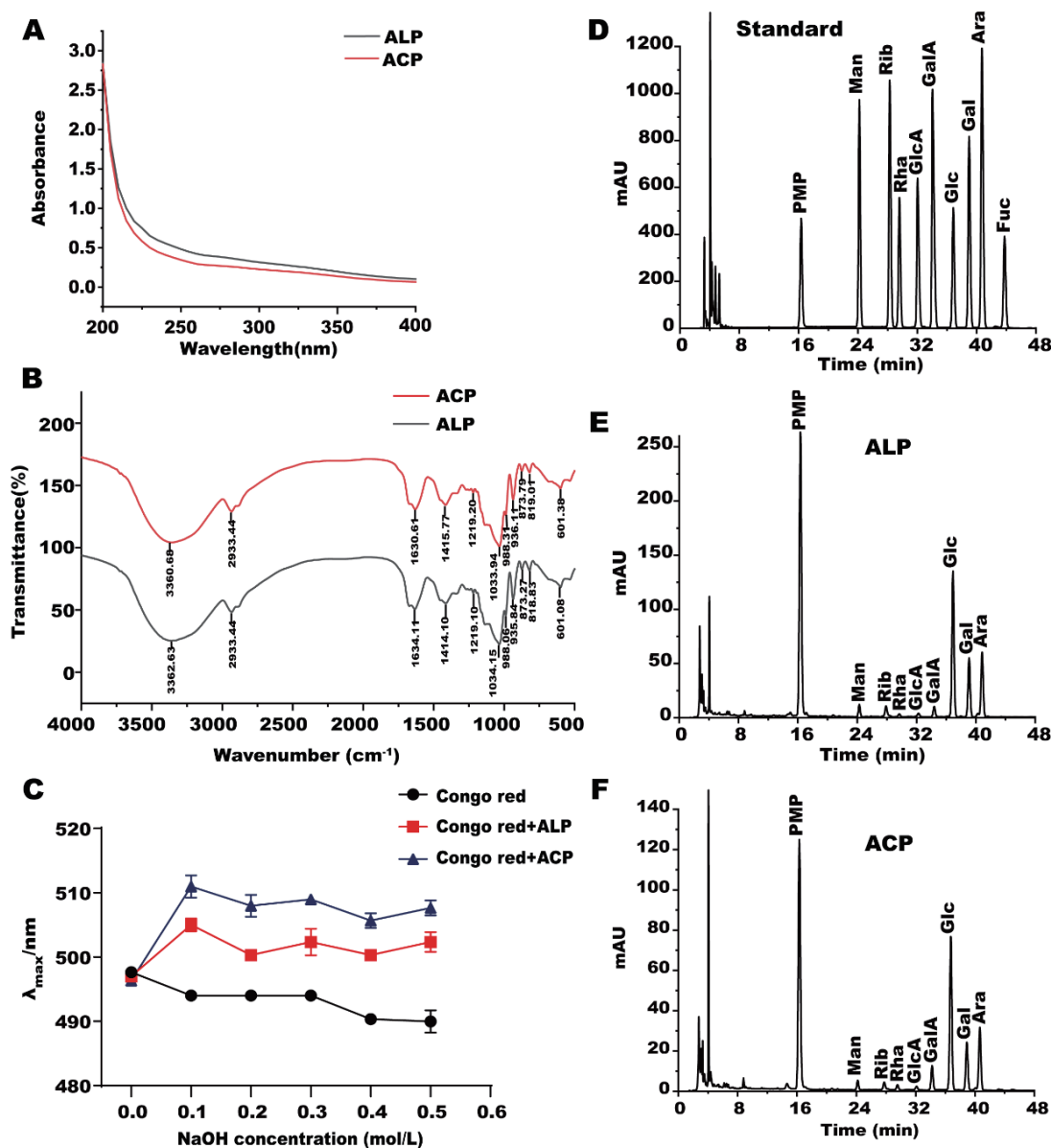


Figure 1 Structure of ALP and ACP. (A-B) UV and FT-IR spectrum. (C) Congo red experiment. (D-F) HPLC chromatogram of monosaccharide mixed standard, ALP and ACP.

presented in Fig. 1B. The absorption peaks at 3,360.68 and 3,362.63 cm^{-1} were due to O-H stretching vibration, and the absorption peak at 2,933.44 cm^{-1} was attributed to saturated C-H stretching vibration; these peaks were the main characteristic absorption peaks of polysaccharides. The absorption peaks at 1,630.61, 1,634.11, 1,415.77, and 1,414.10 cm^{-1} indicated the stretching vibration of carbonyl $\text{C}=\text{O}(-\text{COOH})$ ^[8] and that ALP and ACP contained uronic acid and were acidic polysaccharides, consistent with the chemical composition results. The absorption peaks at 1,033.94, 1,034.15, 601.38, and 601.08 cm^{-1} indicated that the ALP and ACP structures contained pyranose rings. The presence of 873.79, 873.27, 819.01, and 818.83 cm^{-1} indicates that these pyranose rings predominantly exist in the β -configuration. The results indicated that ALP and ACP displayed characteristic polysaccharide absorption peaks, along with a minor quantity of uronic acid.

3.1.3 Congo red experiment

Congo red is frequently employed to identify the existence of a triple helix structure in polysaccharides. The maximum absorption wavelength of ALP, ACP, and Congo red in NaOH solution is shown in Fig. 1C. As the concentration of NaOH increases, the maximum absorption wavelength of both ALP and ACP gradually shifts towards the long wave direction, reaching its peak at a NaOH concentration of 0.1 mol/L, after which it gradually decreases. The aforementioned results demonstrate that ALP and ACP possess a triple helix structure, aligning with the reported findings in the literature^[21].

3.1.4 Monosaccharide composition analysis

As illustrated in Figs. 1D-F, the chromatograms demonstrated that ALP and ACP primarily consist of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, and arabinose. The molar ratios for ALP and ACP were $3.37 \pm 0.02:3.23 \pm 0.01:0.86 \pm 0.00:1.22 \pm 0.00:3.17 \pm 0.01:47.14 \pm 0.17:19.46 \pm 0.09:21.55 \pm 0.07$ and $2.58 \pm 0.03:2.91 \pm 0.03:1.53 \pm 0.02:1.18 \pm 0.01:7.32 \pm 0.07:48.40 \pm 0.12:15.16 \pm 0.05:20.92 \pm 0.08$. The results indicated that both ALP and ACP were weakly acidic polysaccharides, with ACP having a slightly higher total uronic acid content compared to ALP, which may be related to the different origins of *Atractylodes*.

Furthermore, we utilized the fructose method to analyze the monosaccharide composition of ALP and ACP. The findings revealed that the fructose content in ALP and ACP was 92.87% and 92.42%, respectively. (Table S2 and Fig. S1).

3.1.5 NMR spectroscopy analysis

An NMR analysis was utilized to acquire detailed information regarding ALP and ACP. The ^1H and ^{13}C NMR spectra of both ALP and ACP are presented in Figure S2. Based on the ^1H and ^{13}C NMR spectroscopy and references of ALP and ACP, the majority of proton signals are observed in the δ H 3.0–5.5 ppm and δ C 60.0–110.0 ppm regions, which correspond to the characteristic NMR patterns of polysaccharides. Meanwhile, the signals of ALP at δ C 174.42 ppm and ACP at δ C 174.41 ppm indicated the presence of uronic acid in both polysaccharides, corroborating the findings from FT-IR and monosaccharide component analysis. Furthermore, we observed distinct differences in the spectral information of ALP and ACP as revealed by the 1D NMR spectrum. In the ^1H NMR spectrum, ACP has signals of δ H 3.55, 3.58 and 3.96 ppm, while ALP does not. In the ^{13}C NMR spectra, ALP has signals of δ 63.85, 67.52 and 78.97 ppm, while ACP does not. In addition, ALP has five signal peaks in the δ 90–110 ppm

anomeric carbon signal region, which are δ 92.46, 98.04, 101.30, 103.20, 103.65 ppm respectively. ACP has four signal peaks in the δ 90–110 ppm anomeric carbon signal region, which are δ 92.45, 98.04, 103.20, 103.65 ppm respectively. The above results show that ALP and ACP are heteropolysaccharides, and there may be differences in glycosidic linkages or branching patterns between the two. However, the structural characterization of ALP and ACP still has some limitations due to the limited structural information obtained from 1D NMR spectra. We are currently isolating and purifying crude ALP and ACP products, and plan to further analyze their glycosidic linkage characteristics and structural differences through 2D NMR and methylation analysis.

3.2 Effects of ALP and ACP on the immunostimulatory activity of RAW 264.7 cells

3.2.1 ALP and ACP increased RAW 264.7 cell phagocytosis

Macrophages play a crucial role in the immune system and are extensively distributed throughout the body. They can phagocytize and remove pathogens, as well as regulate immune and inflammatory reactions. RAW 264.7 macrophages are a classic model often used to study the immune regulation function of polysaccharides *in vitro*^[4]. The CCK8 results showed that RAW 264.7 cell viability did not differ significantly at ALP and ACP treatment concentrations up to 2,000 $\mu\text{g/mL}$ ($P > 0.05$) (Figs. 2A-B), indicating that ALP and ACP were not obviously toxic.

Concentrations of 200, 400, and 800 $\mu\text{g/mL}$ were chosen as the low, medium, and high doses of ALP and ACP, respectively, to investigate their impact on macrophage phagocytosis (Fig. 2C). The results indicated that, in comparison to the normal control group, the phagocytosis rate of RAW 264.7 cells were increased in the presence of ALP, ACP, and LPS. Notably, significant differences were observed between the ALP group (800 $\mu\text{g/mL}$) and the ACP group (400 and 800 $\mu\text{g/mL}$) ($P < 0.05$). Additionally, the phagocytosis rate of RAW 264.7 cells in the high-dose ACP group was higher than that in the high-dose ALP group. These results suggested that both ALP and ACP increased the phagocytosis of macrophages, with ACP exhibiting a more pronounced effect than ALP.

3.2.2 ALP and ACP promote the expression of cytokines in RAW 264.7 cells

Macrophages not only destroy pathogens through phagocytosis but also regulate immune responses by secreting various cytokines. The expression levels of IL-1 β , IL-6, TNF- α , iNOS, and COX-2 in RAW 264.7 cells were analyzed using RT-qPCR. The results indicated that both ALP and ACP increased the expression levels in a dose-dependent manner (Figs. 2D-H). At equivalent doses, ACP increased cytokine expression compared to ALP across nearly the entire concentration range, exhibiting a similar trend to that observed for ALP and ACP on RAW 264.7 cell phagocytosis. These results showed that both ALP and ACP could regulate the immune responses by increasing cytokine expression, with ACP exhibiting a more pronounced effect.

3.3 ALP and ACP alleviated immune dysfunction in Cy-induced immunosuppressed mice

3.3.1 ALP and ACP alleviate the pathological symptoms in Cy-treated mice

The experimental scheme is shown in Fig. 3A. From day 9, the body weight of mice in the model group decreased significantly

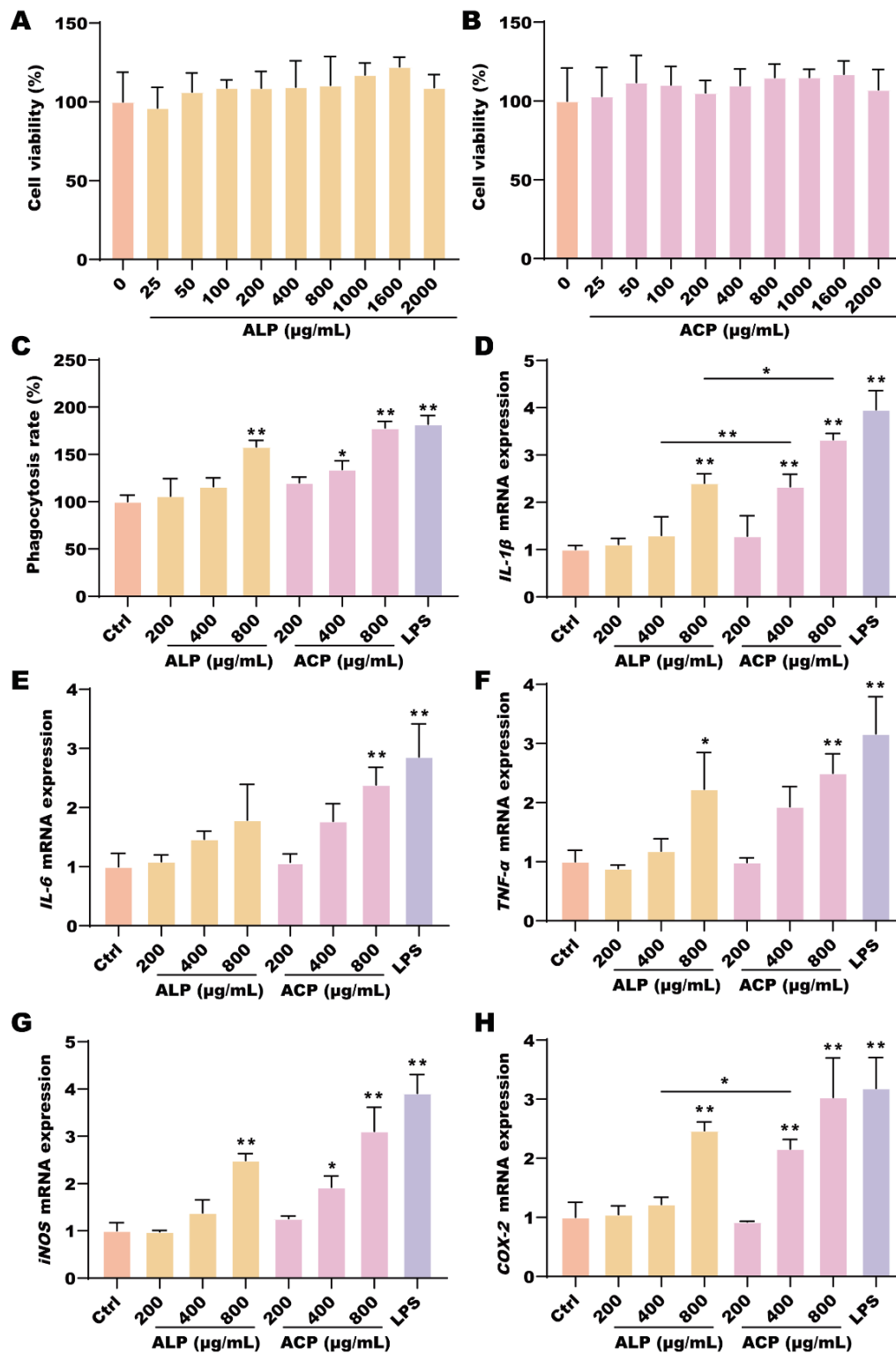


Figure 2 The effects of ALP and ACP on the immunostimulatory activity of RAW 264.7 cells. (A-B) The effects of ALP and ACP on the viability of RAW 264.7 cells. (C) ALP and ACP increased RAW 264.7 cell phagocytosis. (D-H) ALP and ACP increased the secretion levels of *IL-1β*, *IL-6*, *TNF-α*, *iNOS*, and *COX-2* by RAW 264.7 cells. Compared with the Ctrl group, * $P < 0.05$, ** $P < 0.01$.

compared to the normal control group ($P < 0.05$) (Fig. 3B). Additionally, the spleen and thymus indexes decreased significantly ($P < 0.01$) (Figs. 3C-D), indicating successful establishment of the immunosuppressed model. Mice treated with ALP and ACP showed significant increases in spleen and thymus indexes compared to the model group ($P < 0.05$) (Figs. 3C-D), with ACP exhibiting a more pronounced effect.

The effects of ALP and ACP on blood cell counts and IgA levels in Cy-treated mice were further evaluated. Compared to the normal control group, the model group exhibited a decrease in the numbers of neutrophils, red blood cells (RBCs), platelets (PLTs), hemoglobin (HGB), and lymphocyte percentages, along with a significant reduction in serum IgA levels ($P < 0.01$). After treatment with ALP and ACP, these indexes recovered

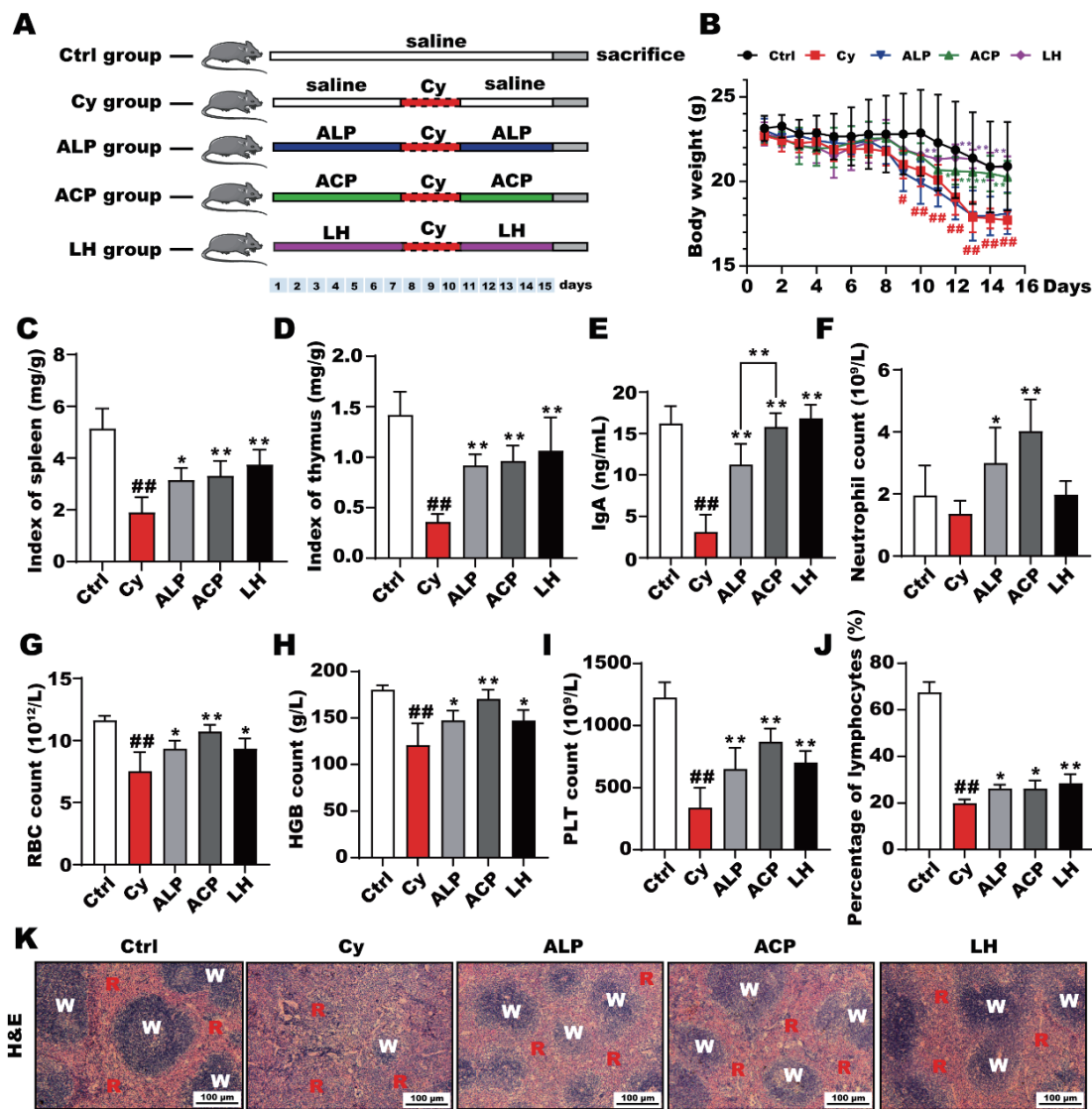


Figure 3 ALP and ACP improve the pathological symptoms in Cy-induced mice. (A) Animal dosing regimen. (B) The change in trends of mice weight. (C-D) Spleen and thymus index. (E) IgA levels. (F) Neutrophil count. (G) RBC count. (H) HGB level. (I) PLT count. (J) Percentage of lymphocytes (%). (K) HE staining of the spleen, W: White pulp. R: Red pulp. Compared with the Ctrl group, * $P < 0.05$, ** $P < 0.01$; Compared with the Cy group, * $P < 0.05$, ** $P < 0.01$.

significantly in immunosuppressed mice ($P < 0.05$), and the effect was more significant in ACP. In addition, ACP was more effective than LH in preventing decreases in neutrophils, RBCs, PLTs, and HGB levels, with values approaching those of the normal group (Figs. 3E-J).

The results of the HE staining reveal that the spleen structure of normal control mice was well-defined, with distinct boundaries between the red pulp (R) and white pulp (W), and a significantly large white pulp area. In contrast, the spleen structure of model mice was vague, with unclear boundaries between red pulp and white pulp and a noticeably reduced white pulp area. After treatment with ALP, ACP, and LH, the spleen structure was obviously improved, and the boundary between red pulp and white pulp gradually became clear (Fig. 3K). The above results showed that both ALP and ACP could improve the immune function in immunosuppressive mice induced by Cy, and ACP showed a more pronounced improvement.

3.3.2 ALP and ACP reduced jejunum tissue damage in Cy-treated mice and improved the expression level of cytokines

The intestine serves not only as the main place for digestion

and absorption but also as a key region for the distribution of immune cells, forming the largest mucosal immune system in the body. HE staining of the jejunum in normal control group showed that the intestinal structure was intact, the villi were closely arranged, the outline was clear, the villi were long, and the depth of the recesses was shallow. In the model group, most of the intestinal villi were swollen and atrophied, the structure was loose, and some were broken and detached. The crypt depth increased significantly ($P < 0.01$), while the villi length and the ratio of villi length to crypt depth decreased significantly ($P < 0.01$). Treatment with ALP, ACP, and LH improved these findings ($P < 0.01$), with the ACP group showing a more significant effect than the ALP group (Figs. 4A-D).

The results obtained from RT-qPCR and ELISA analysis demonstrated that the expression levels of cytokines TNF- α , IL-1 β , IL-6, and iNOS in the jejunum of mice in the model group were significantly reduced compared to those in the normal control group ($P < 0.05$) (Figs. 4I-N). Following ALP and ACP treatments, the expression levels of cytokines increased, with ACP showing a significant effect ($P < 0.01$). The AB-PAS staining results showed that mucus in the jejunum mucosa was significantly reduced after

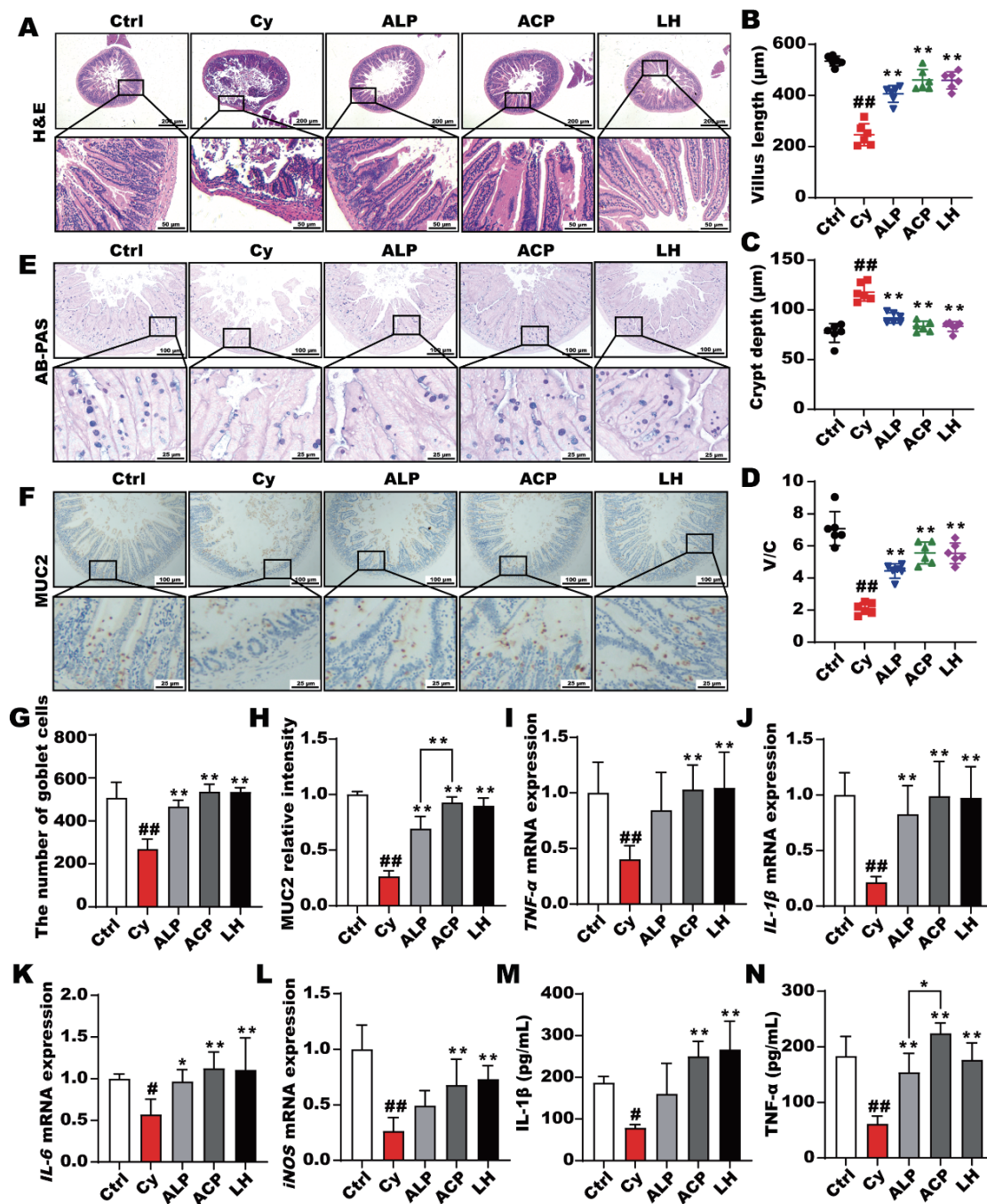


Figure 4 The effects of ALP and ACP on jejunal tissue damage and cytokine expression levels in Cy-treated mice. (A) HE staining of the jejunum. (B) Jejunum villi length. (C) The depth of jejunum crypts. (D) Villus length/crypt depth values. (E-F) AB-PAS and immunohistochemical staining of the jejunum. (G-H) Goblet cells and MUC2 statistical chart. (I-N) The expression level of cytokines in the jejunum. Compared with the Ctrl group, [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with the Cy group, ^{*} $P < 0.05$, ^{**} $P < 0.01$.

Cy modeling, indicating that the number of goblet cells synthesizing mucus was significantly reduced ($P < 0.01$). ALP and ACP treatments significantly mitigated the mucus reduction caused by Cy, increased the number of goblet cells ($P < 0.01$), and alleviated intestinal injury, thus protecting the intestinal mucosa chemical barrier, in which ACP was more effective than ALP (Figs. 4E and G).

The mucus secreted by normal goblet cells is mainly composed of MUC2, which covers the surface of the intestinal mucosa for lubrication. The immunohistochemical results showed that MUC2 expression in the jejunum of the model group significantly decreased ($P < 0.01$), while it significantly increased following ALP

and ACP treatments, with more significant effects seen in ACP ($P < 0.01$) (Figs. 4F and H). These results suggested that ALP and ACP may enhance the intestinal chemical barrier by increasing the number of goblet cells to promote MUC2 secretion.

3.3.3 ALP and ACP upregulated the expression of tight junction proteins in the jejunum of Cy-treated mice and enhanced the intestinal mechanical barrier

The results showed that the expression levels of ZO-1, Occludin, and Claudin-1 in the jejunum of mice in the model group were significantly lower than those in the normal control group ($P < 0.01$). After treatment with ALP and ACP, the

expression of these proteins increased in the ALP group compared to the model group, with significant increases observed for Occludin and Claudin-1 ($P < 0.05$). ACP treatment notably upregulated the expression of these tight junction proteins ($P < 0.01$) (Figs. 5A-E). The WB results showed that both ALP and ACP significantly increased the levels of ZO-1 and Occludin, and ACP had a more significant effect (Figs. 5F-H). The consistency between immunofluorescence and WB results indicated that ALP and ACP protected the intestinal mechanical barrier from Cy-induced damage by increasing the expression of tight junction proteins, with ACP exhibiting a stronger protective effect.

3.3.4 ALP and ACP increased the expression of F4/80, IgA, and sIgA in the jejunum of CY-treated mice and enhanced the intestinal immune barrier

Macrophages are important antigen-presenting cells, and F4/80

is a marker of macrophage maturation and activation. IgA and sIgA are key indicators for assessing the intestinal immunity. The immunofluorescence analysis of the jejunum revealed a significant reduction in the expression levels of F4/80 and IgA in the model group ($P < 0.01$), suggesting that the intestinal immune barrier was destroyed. The expression levels of F4/80 and IgA in the jejunum were significantly increased by both ALP and ACP ($P < 0.05$), with the ACP group exhibiting a more pronounced effect that was significantly different from the ALP group ($P < 0.01$) (Figs. 6A-D). Further WB analysis showed that the protein expression level of IgA increased in both the ALP and ACP groups, with a more significant increase observed in the ACP group, approaching levels close to those in the normal control group ($P < 0.01$) (Figs. 6F-G). Consistent results were observed between immunofluorescence and WB analyses. The ELISA results showed a significant reduction in the secretion level of sIgA in the model group ($P < 0.05$), which was notably improved by

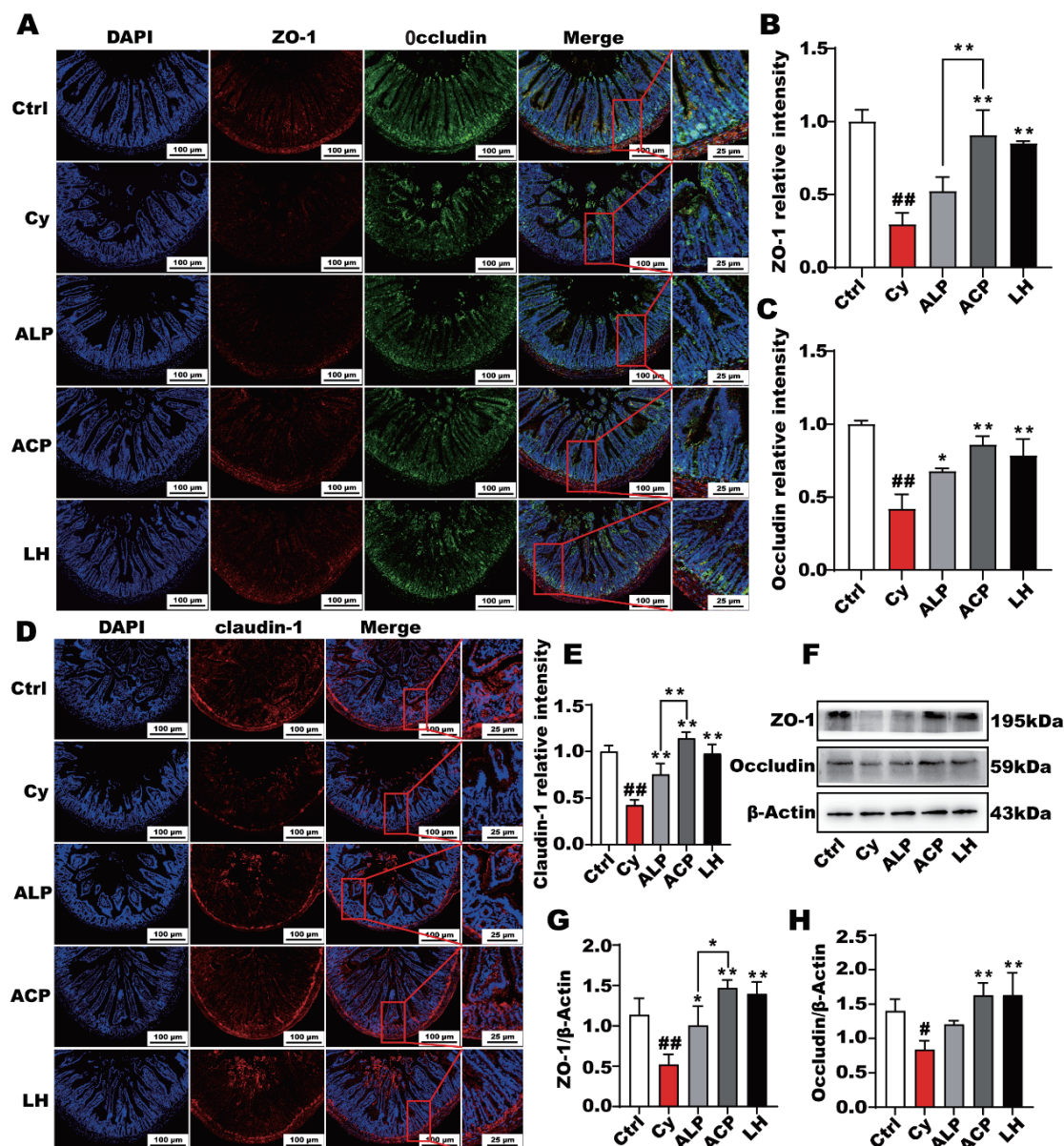


Figure 5 ALP and ACP increased the expression of tight junction proteins in the jejunum of Cy-treated mice and strengthened the intestinal mechanical barrier. (A) ZO-1 and Occludin immunofluorescence staining. (B) ZO-1 statistical chart. (C) Occludin statistical chart. (D) Claudin-1 immunofluorescence staining. (E) Claudin-1 statistical chart. (F) ZO-1 and Occludin WB analysis. (G-H) Statistical chart of Occludin and ZO-1 protein expression. Compared with the Ctrl group, * $P < 0.05$, ** $P < 0.01$; Compared with the Cy group, # $P < 0.05$, ## $P < 0.01$.

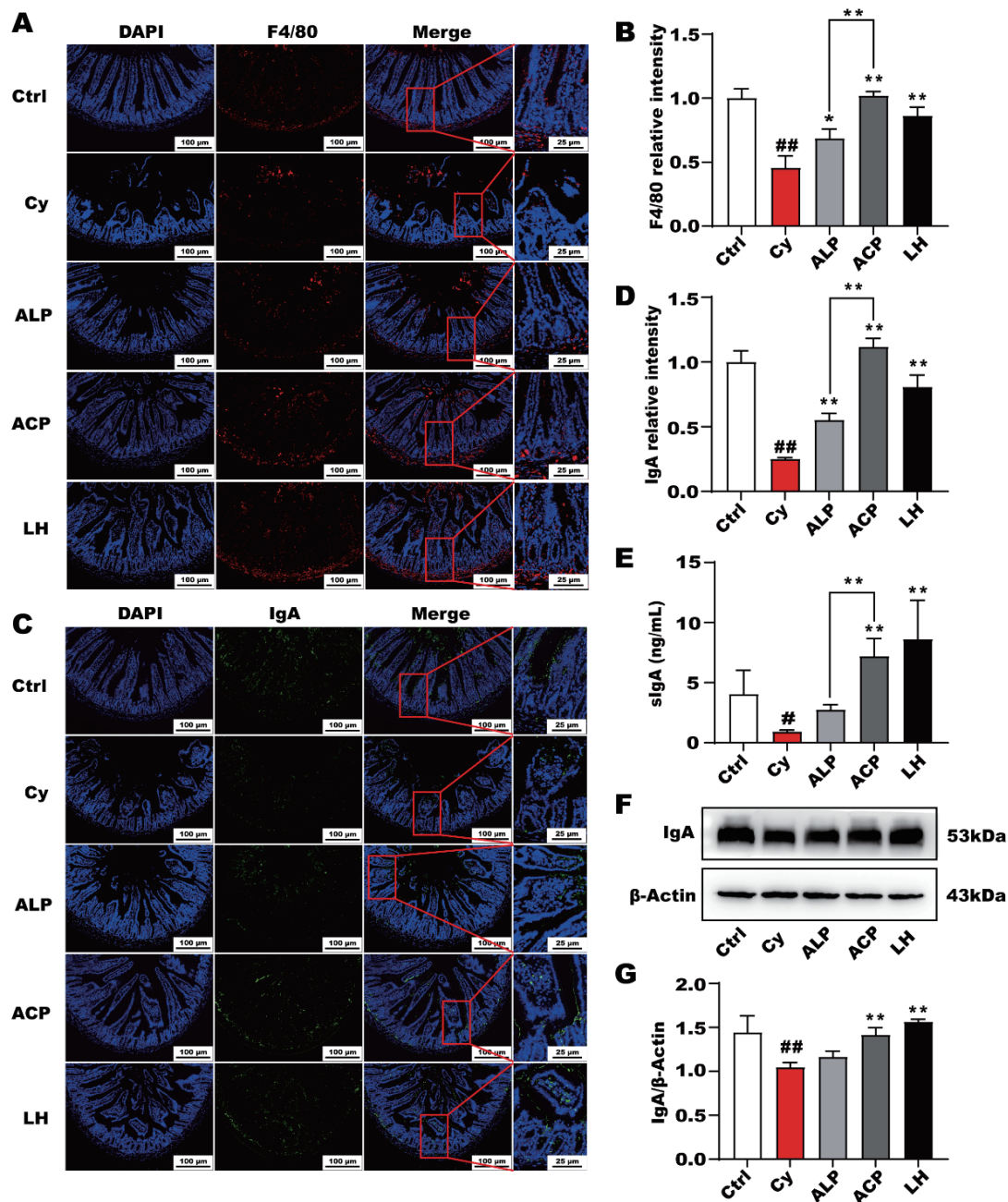


Figure 6 ALP and ACP enhanced the intestinal immune barrier of Cy-treated mice. (A) F4/80 immunofluorescence staining. (B) F4/80 statistical chart. (C) IgA immunofluorescence staining. (D) IgA statistical chart. (E) The level of sIgA in jejunum. (F) IgA WB analysis. (G) Statistical chart of IgA protein expression. Compared with the Ctrl group, ^{*} $P < 0.05$, ^{##} $P < 0.01$; Compared with the Cy group, ^{*} $P < 0.05$, ^{**} $P < 0.01$.

ACP treatment ($P < 0.01$), with significant difference compared to the ALP group ($P < 0.01$) (Fig. 6E). These results indicated that ALP and ACP enhanced the intestinal immune barrier by stimulating macrophage activation and promoting IgA expression and sIgA secretion.

3.3.5 ACP increased the diversity of intestinal flora in Cy-treated mice

Cy has been shown to disrupt both the intestinal immune barrier and the balance of intestinal flora. It was demonstrated that ACP exerted superior immune regulation and intestinal protection compared to ALP. Consequently, further evaluate the impact of ACP on intestinal flora. Specacum curves were used to determine the adequacy of the sample number and estimate

species richness. We found that with the increases in sample numbers, the specacum curve first increased and then remained stable (Fig. 7A), indicating that the measured datas were sufficient to reflect the richness and evenness of the microbial community in the sample. The large span of the horizontal axis in the tank-abundance curve indicated a high species abundance, while the flattening of the vertical axis indicated a uniform distribution of species in the sample (Fig. 7B).

The influence of ACP on the α -diversity of the intestinal flora was further investigated. The α -diversity indexes include Chao1, dominance, observed_features, Pielou_e, Shannon, and Simpson indexes. Chao1, observed_features, Shannon, and Simpson indexes can be used to evaluate the richness of intestinal flora, while the dominance and Pielou_e indexes can reflect species

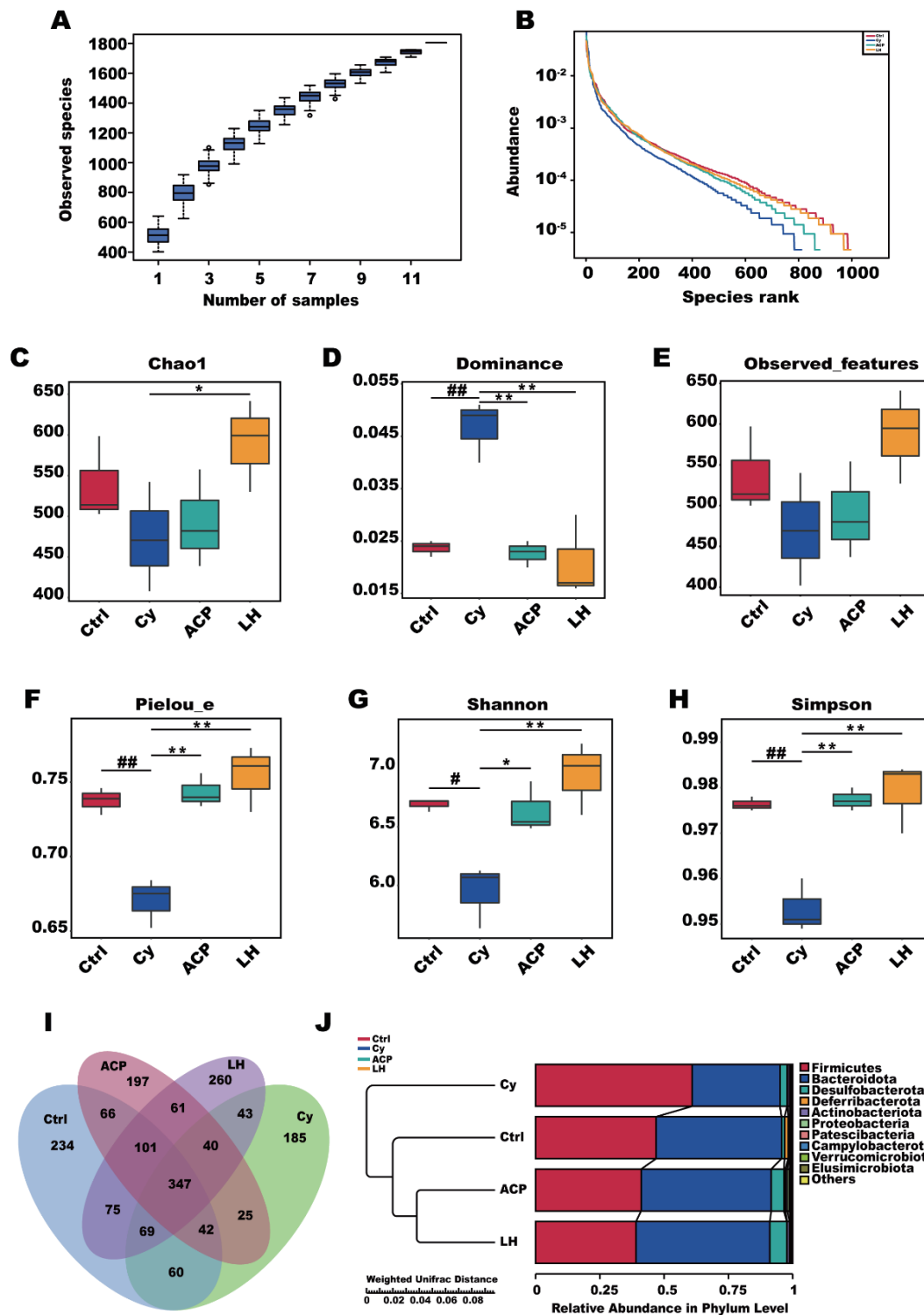


Figure 7 ACP increased the diversity of the intestinal microbiota in Cy-treated mice. (A-C) Specaccum curve, rank-abundance curve, and Chao1 index. (D) Dominance index. (E) Observed_features index. (F) Pielou_e index. (G) Shannon index. (H) Simpson index. (I) Venn diagram. (J) UPGMA cluster tree diagram. Compared with the Ctrl group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the Cy group, $^*P < 0.05$, $^{**}P < 0.01$.

evenness. Compared to the normal group, the Chao1, observed_features, Pielou_e, Shannon, and Simpson indexes in the model group had a downward trend, and the Pielou_e, Shannon, and Simpson indexes decreased significantly ($P < 0.05$). In addition, the dominance index in the model group increased significantly ($P < 0.01$), and species richness and uniformity decreased. However, following ACP treatment, the Chao1, observed_features, Pielou_e, Shannon, and Simpson indexes all showed a

tendency to increase, while the dominance index tended to decrease. Among these, the changes in Pielou_e, Shannon, Simpson, and dominance indexes were statistically significant ($P < 0.05$) (Figs. 7C-H). The Venn diagram illustrating the number of OTUs in each group showed that the common number of OTUs in the Ctrl, Cy, ACP, and LH groups was 347, and the number of unique OTUs in each group was 234, 185, 197, and 260, respectively. This indicated that ACP and LH increased

the number of OTUs in the intestines of mice. In addition, the number of common bacteria between the Ctrl and ACP groups was greater than that between the Ctrl and model groups, with the intersection of Ctrl and ACP being larger than that of Ctrl and Cy (Fig. 7I). β -diversity is a comparative analysis of the composition of bacterial communities in different samples or groups of samples. The UPGMA clustering tree (Fig. 7J) was obtained by clustering the samples. The results showed that ACP and LH were initially clustered together and subsequently formed a second branch with the Ctrl group, indicating high species similarity among the ACP, LH, and Ctrl groups at the phylum level. In contrast, the Cy group was clustered separately at the outermost side, indicating high species diversity of the Cy group and other groups. These results suggested that ACP mitigated Cy-induced intestinal flora disorders.

3.3.6 ACP regulated the composition of intestinal flora in Cy-treated mice at the phylum, family, and genus levels

The composition of the intestinal flora was analyzed at the phylum, family, and genus levels to further explore the influence of ACP on the flora community structure. At the phylum level, Cy treatment increased the relative abundance of Firmicutes compared to the normal group, while the relative abundance of Bacteroidota decreased, and the Firmicutes/Bacteroidota ratio increased. Of note, ACP treatment reversed this trend, showing a flora composition close to that of the normal control group at the phylum level (Figs. 8A-D). At the family level, the abundance of Muribaculaceae in the Cy group decreased, and the abundance of Muribaculaceae increased after ACP treatment (Figs. 8E-F). At the genus level, the abundance of *Lachnospiraceae_NK4A136_group* in the Cy group increased, while the abundance of *Ruminococcus* and *Prevotellaceae_UCG-001* decreased. However, ACP treatment significantly decreased the abundance of *Lachnospiraceae_NK4A136_group* ($P < 0.01$) and significantly increased the abundance of *Ruminococcus* and *Prevotellaceae_UCG-001* (Figs. 8G-J). These results showed that ACP improved the intestinal flora imbalance caused by Cy at the phylum, family, and genus levels.

3.3.7 LEfSe comparison and function prediction of intestinal flora

The intestinal flora of mice was analyzed using LEfSe to determine the specific changes in the distribution of intestinal flora induced by ACP. Figs. 9A-D shows strains with LDA scores greater than 4.0. The results indicated that the main marker bacteria in the Ctrl group were Bacteroidota, Prevotellaceae, and *Alloprevotella*, while those in the Cy group were Firmicutes, Clostridia, and Lachnospiraceae. In comparison to the Cy group, ACP group showed Muribaculaceae as the marker bacteria (Figs. 9A-D). Based on the 16S rDNA sequencing results, the function of the sample was predicted using the Tax4Fun method. The thermogram results revealed significant metabolic differences between the Cy and Ctrl groups, and the metabolic profile of the ACP group was more similar to that of the normal control group (Fig. 9E). These results suggested that metabolic dysfunction may be related to intestinal flora imbalance and intestinal immune dysfunction.

3.3.8 ACP improves abnormal metabolism of SCFAs in Cy-treated mice

SCFAs are essential metabolites produced by intestinal flora that serve a pivotal role in preserving the intestinal mucosal

barrier. The results showed that the acetic acid, propionic acid, isobutyric acid, butyric acid, and valeric acid content in the model group decreased to different degrees compared to the normal control group. After ACP treatment, the content of these SCFAs increased, with significant increases observed in acetic acid, propionic acid, and total SCFA content compared to the model group ($P < 0.01$) (Figs. 9F-K). These results suggested that ACP improved the SCFA metabolic disorder induced by Cy. Furthermore, Spearman's correlation analysis showed that the SCFA content was positively correlated with immune-related indicators, including immune organ indexes, the levels of immune cells and immunoglobulins in the blood (spleen index, thymus index, PLT, HGB, lymphocytes, RBCs, neutrophils, and IgA), the levels of cytokines in the jejunum (TNF- α , IL-1 β , IL-6, and iNOS), and the level of secretory immunoglobulin (sIgA) in the jejunum (Fig. 9L). These results suggested that ACP might enhance the immune function and intestinal protection in mice by increasing the content of SCFAs.

4 Discussion

Cy is a commonly used broad-spectrum chemotherapy drug, which not only kills tumor cells but also destroys normal immune cells. Long-term use may lead to many adverse reactions, including acute cytotoxicity, immunosuppression, and gastrointestinal mucosal barrier damage, which in turn increases intestinal permeability and reduces goblet cell numbers and mucin levels^[9]. In recent years, natural polysaccharides have garnered significant research interest owing to their lower toxicity and side effects, better safety, and physiological compatibility with the human body than chemical drugs^[27,28], as well as their ability to improve Cy-induced immunosuppression by affecting various immune regulatory pathways. *Atractylodes* rhizome is a commonly used product in the healthcare industry^[29]. As a functional plants^[30], it has a long history of use in China and is widely studied for its effects on intestinal and immune-related diseases^[8,25]. *Atractylodes* rhizome was divided into South and North sources namely *Atractylodes lancea* (Thunb.) DC. or *Atractylodes chinensis* (DC.) Koidz. Studies show that the components and efficacy of *Atractylodes* rhizome from different sources have significant differences^[20]. The primary active component of *Atractylodes* rhizome is its polysaccharide. Our research group previously found that raw ALP (SALP) significantly improved Cy-induced immunosuppression, and the activity of bran-fried ALP was stronger than that of SALP, which may be attributed to the influence of bran-frying on the chemical composition, monosaccharide composition, and preliminary structure of polysaccharides^[8]. However, it is not clear whether *Atractylodes* rhizome polysaccharides will show efficacy differences due to different sources of *Atractylodes*. In addition, preliminary studies have shown that *Atractylodes* rhizome polysaccharides have immunoregulatory activity. However, the current research on the immunoregulatory activity of *Atractylodes* rhizome polysaccharides is limited to *in vitro* experimental evaluation. And that the comparative study on the efficacy of *Atractylodes* rhizome polysaccharides from different sources in improving cyclophosphamide induced immunosuppression and intestinal injury has not been reported. In this study, we first compared the structural differences of *Atractylodes* rhizome polysaccharides from different sources (ALP and ACP), and then evaluated the immunoregulatory activities of ALP and ACP by combining *in vitro* and *in vivo* experiments. As far as we know,

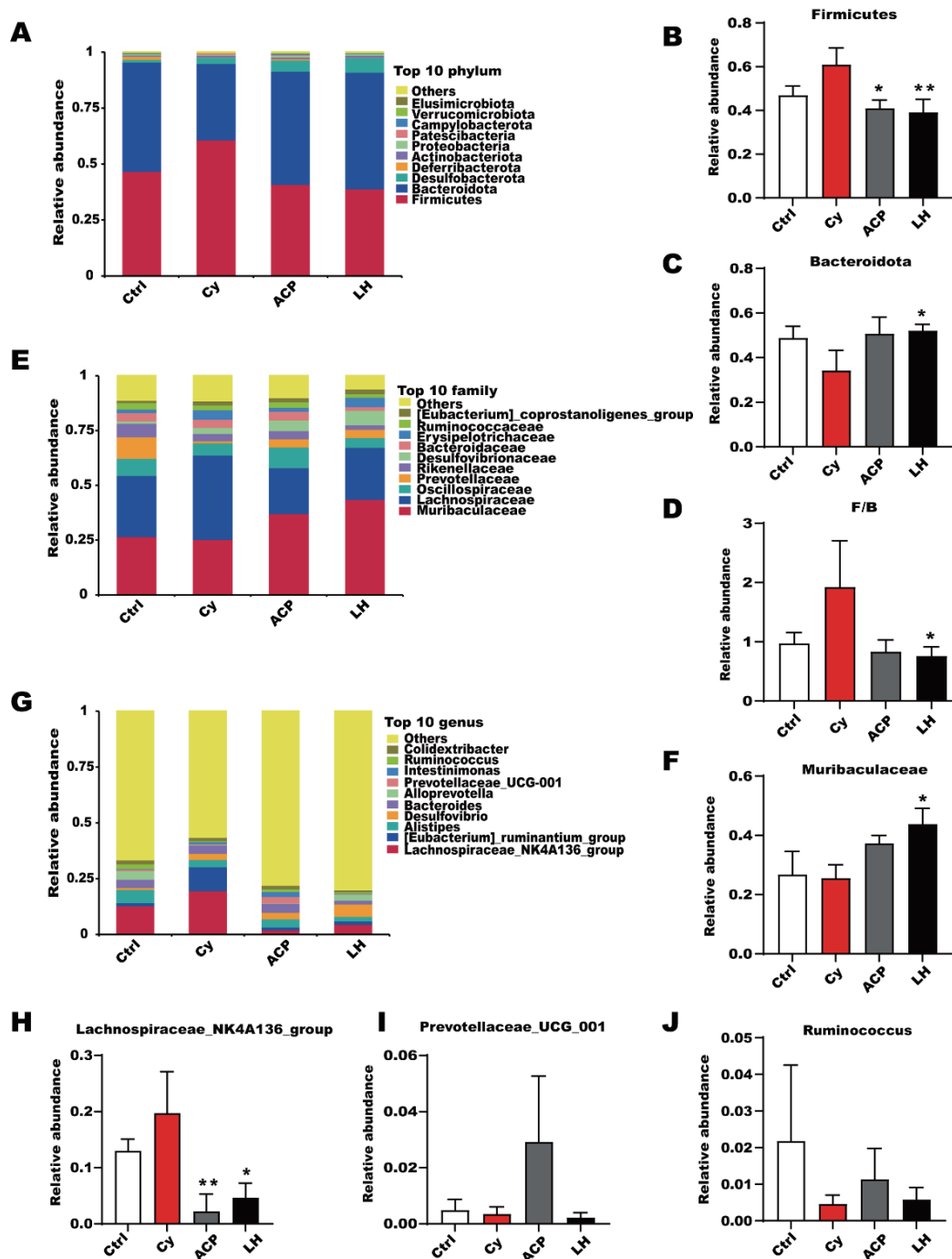


Figure 8 ACP regulated the composition of the intestinal microbiota. (A) Distribution of species at the phylum level. (B-C) Firmicutes and Bacteroidota abundance. (D) The Firmicutes/Bacteroidota ratio. (E) Distribution of species at the family level. (F) Muribaculaceae abundance at the family level. (G) Distribution of species at the genus level. (H-J) *Lachnospiraceae_NK4A136_group*, *Prevotellaceae_UCG_001*, and *Ruminococcus* abundance at the genus level. Compared with the Cy group, * $P < 0.05$, ** $P < 0.01$.

this is the first time to evaluate the difference of immunoregulatory effect of *Atractylodes* rhizome polysaccharides from different sources by combining chemical methods with *in vivo* and *in vitro* experiments. The findings revealed significant differences in the chemical composition, molar ratio of the monosaccharide components, molecular weight, and other preliminary polysaccharide structures from *Atractylodes lancea* rhizome and *Atractylodes chinensis* rhizome, which may contribute to the significant differences in their immunomodulatory activities *in vitro* and *in vivo*. The results of

this study help to comprehensively evaluate the quality differences and pharmacodynamic advantages of *Atractylodes* rhizome polysaccharides from different sources, and provide reference for the application of ALP and ACP.

Macrophages constitute the primary defensive barrier against pathogenic invasions and can engulf and digest bacteria, viruses, fungi, foreign bodies, and dead cells^[31]. Studies have demonstrated that polysaccharides can recognize and bind with specific receptors on the surface of macrophages and promote their activation and the release of cytokines, thereby exerting

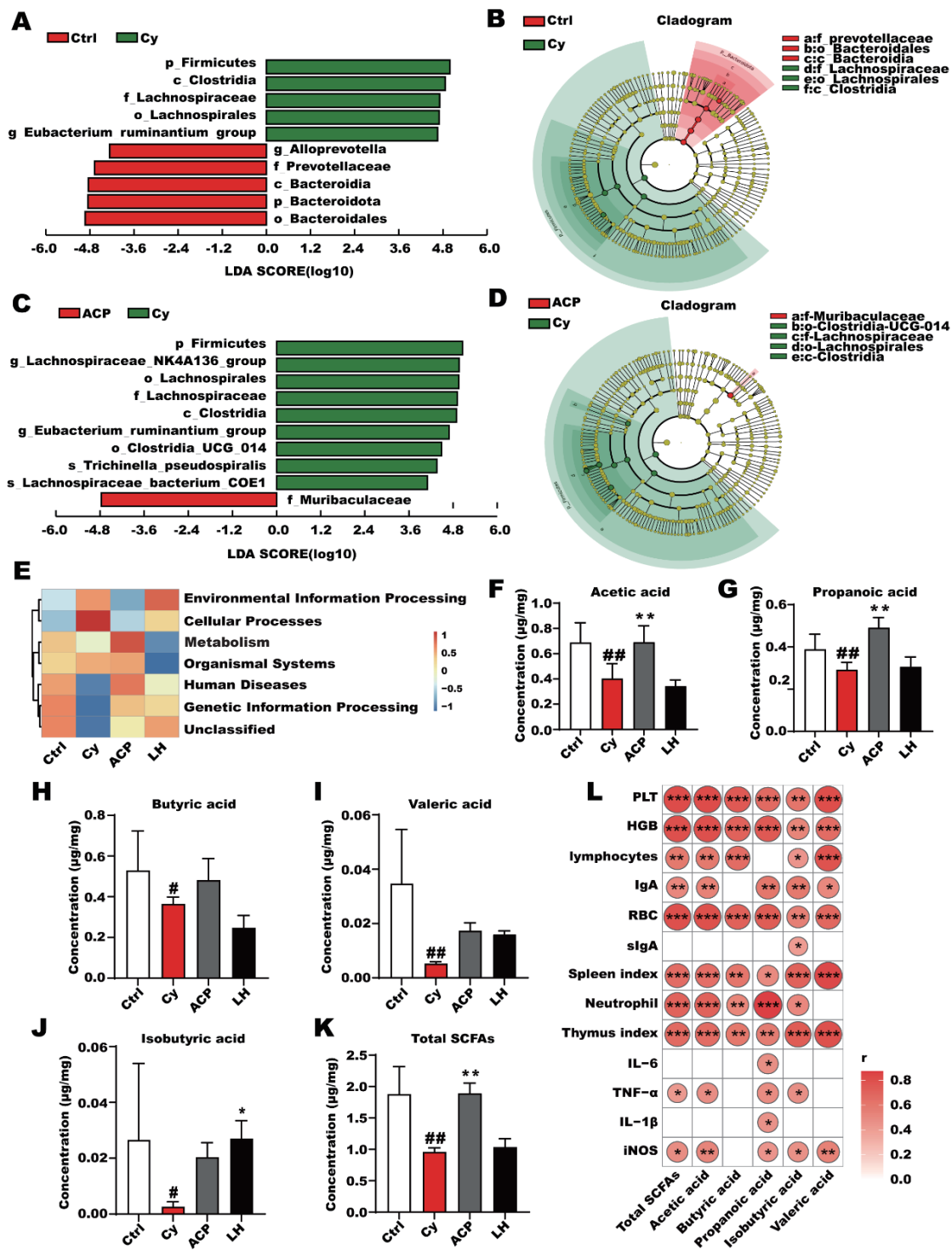


Figure 9 The effect of ACP on SCFAs metabolism. (A-B) Ctrl group and Cy group. (C-D) ACP group and Cy group. (E) Functional annotation relative abundance cluster analysis heat map. (F-K) The content of SCFAs. (L) Spearman's correlation analysis heat map of SCFAs and immune indexes. Compared with the Ctrl group, * $P < 0.05$, ** $P < 0.01$; Compared with the Cy group, * $P < 0.05$, ** $P < 0.01$.

immunomodulatory effects^[32]. In this study, RAW 264.7 cells were used to study the activation of macrophages by ALP and ACP. ALP and ACP were found to increase the phagocytosis rate of neutral red by RAW 264.7 cells, indicating that they can activate these cells to a certain degree. Notably, ACP exhibited a stronger immunostimulatory effect than ALP at the same concentration. During phagocytosis, macrophages produced and released a variety of cytokines, which were important for regulating inflammatory responses, activating other immune cells, and

promoting tissue repair. Therefore, the expression levels of IL-1β, IL-6, TNF-α, iNOS, and COX-2 in RAW 264.7 cells were further evaluated. The results indicated that both ALP and ACP could exert immunomodulatory activity by promoting the release of inflammatory factors from macrophages, consistent with previous findings^[33].

The above *in vitro* results suggested that both ALP and ACP possessed good immunomodulatory activity, with ACP demonstrating a stronger effect. However, it was not clear whether

ACP also had stronger immunomodulatory activity *in vivo*. Therefore, we used Cy to construct an immunocompromised mouse model and further verified the immunomodulatory effects of ALP and ACP along with their potential mechanisms through *in vivo* experiments. The spleen and thymus are important immune organs, and their respective indexes are important indicators of overall immune function^[34]. In this study, we found that both ALP and ACP increased the spleen and thymus indexes and improved the pathological damage to spleen tissue. Notably, ACP exhibited a more pronounced effect, indicating that ACP also had stronger immunomodulatory activity *in vivo*, which was consistent with the *in vitro* experimental results. White blood cells, including lymphocytes, monocytes, and granulocytes, are very important immune cells in the body. The number of white blood cells can directly reflect the body's humoral immune ability. Studies have shown that Cy can cause the alkylation of functional groups in protein, thus inhibiting the hematopoietic function of the medulla, reducing the number of white blood cells in the blood, and inhibiting medullary and extramedullary blood production^[1]. Our results indicated that Cy significantly reduced the number of lymphocytes, neutrophils, RBCs, PLTs, and HGB levels in the blood of mice. Conversely, ALP and ACP were effective in reversing these reductions in blood cell counts, suggesting that ALP and ACP could alleviate Cy-induced bone marrow suppression, with ACP demonstrating high activity.

The intestinal tract serves multiple roles, including digestion, nutrient uptake, waste elimination, and immune defense^[35]. Cytokines, released by activated immune cells, are crucial for regulating immune balance. We analyzed the levels of cytokines in the jejunum, and found that ACP treatment improved decreases in cytokine levels caused by immunosuppression, significantly increasing the expression of TNF- α , IL-1 β , IL-6, and iNOS. ACP alleviates the effects of immunosuppression caused by Cy by regulating the levels of cytokines in the jejunum. The intestinal mucosal barrier of normal mice is mainly composed of four barriers: The mechanical barrier, the chemical barrier, the biological barrier, and the immune barrier^[8]. The mechanical barrier is formed by intestinal epithelial cells and tight junction proteins^[35]. In this study, we found that intraperitoneal injection of Cy caused jejunum tissue damage, resulting in destroyed and severely broken intestinal villi. Crypt cells secreted a large number of immature cells within a short period, resulting in increases in crypt depth, decreases in the levels of tight junction proteins which increased intestinal mucosal permeability and the risk of disease. MUC2, secreted by goblet cells, is an important part of the chemical barrier. It forms a hydrophobic layer on the surface of the intestinal epithelium to protect intestinal epithelial cells from bacteria invasion^[36]. Therefore, the number of goblet cells and the level of MUC2 secreted by goblet cells are crucial for the effective functioning of the intestinal chemical barrier. In this study, the number of goblet cells in the intestine of Cy-treated mice decreased, and the expression of MUC2 was reduced, indicating the damage to the intestinal chemical barrier. Intestinal-associated lymphoid tissue contains more than 70% of the immune cells in the body. The immune barrier includes mucosa-associated lymphoid tissue and secretory antibodies, such as sIgA, produced by intestinal plasma cells. IgA-secreting cells are transformed from B cells in the intestine, and the IgA produced by them enters the intestinal cavity through the cell membrane to become sIgA, which exerts immune effects^[37]. F4/80 is widely distributed in the gut-associated lymphoid region and is often used to detect and label intestinal macrophages^[38]. This study found that Cy

significantly reduced the levels of IgA, sIgA, and F4/80 in the intestine, indicating that intestinal mucosal immunity was inhibited and that the intestinal chemical and mechanical barriers were damaged to varying extents. However, treatment with ALP and ACP repaired damage to the mechanical, chemical, and immune barriers of mice to varying extents, with ACP demonstrating a notably superior effect compared to ALP.

The intestinal flora is closely related to host health. Most polysaccharides from natural sources cannot be degraded by digestive enzymes synthesized by the body. However, the intestinal flora produces a variety of active enzymes that decompose these polysaccharides^[39], producing a variety of SFCAs, such as acetic acid, propionic acid, butyric acid, valeric acid, and isobutyric acid. SFCAs can not only regulate the composition of the intestinal flora, increase the diversity of the intestinal flora, and promote the colonization of beneficial bacteria, but also provide energy for colonic epithelial cells to play an immunomodulatory role^[40]. We sequenced the contents of mouse cecum using 16SrDNA to further investigate the effect of ACP on intestinal flora diversity. The results showed that the Chao1, observed_features, Pielou_e, Shannon, and Simpson indexes were significantly lower in the Cy group compared to normal mice, but all indexes were restored after ACP treatment. The cluster tree results demonstrated that the species composition in the ACP group was similar to that in the Ctrl group at the phylum level but differed significantly from the Cy group. These findings suggested that ACP treatment enhanced both α -diversity and β -diversity of intestinal flora. Firmicutes and Bacteroidota are relatively abundant flora of mice, in healthy organisms, the ratio of Firmicutes to Bacteroidota (F/B) is regulated and remains within a specific range. Cy-induced immunosuppressed mice showed increases in the relative abundance of Firmicutes and decreases in the relative abundance of Bacteroidota^[41]. Our results demonstrated that ACP decreased the relative abundance of Firmicutes while increasing that of Bacteroidota, a major producer of SCFAs, suggesting a beneficial modulatory effect on gut microbiota composition and function. At the family level, the dominant flora in the ACP group compared to the Cy group was Muribaculaceae, a major producer of SCFAs, indicating that ACP may promote the production of SCFAs by increasing the relative abundance of Muribaculaceae^[42]. Conversely, *Lachnospiraceae_NK4A136_group*, known as a characteristic flora representative of intestinal dysfunction, was observed to be less dominant^[43]. Studies have shown that *Chrysanthemum morifolium* polysaccharide enhanced intestinal homeostasis and improved functional constipation by reducing the relative abundance of *Lachnospiraceae_NK4A136_group* and increasing the relative abundance of beneficial bacteria such as *Lactobacillus* and *Romboutsia*^[44]. In this study, following an intraperitoneal injection of Cy, the intestinal flora became disordered, with a notable increase in the relative abundance of the pathogenic bacteria *Lachnospiraceae_NK4A136_group*. Treatment with ACP reduced the relative abundance of *Lachnospiraceae_NK4A136_group*, thereby reducing intestinal homeostasis, which was consistent with previous findings^[44]. *Prevotellaceae_UCG-001* is a commonly recognized bacterium. A heteropolysaccharide from the fruit of *Crataegus pinnatifida* was found to play an immunomodulatory role by increasing the relative abundance of beneficial bacteria such as *Prevotellaceae_UCG-001*^[45], while *Ruminococceae* and *Lachnospiraceae* were involved in the degradation of polysaccharides^[46]. The increase in the relative abundance of *Ruminococceae* may be beneficial to the degradation of ACP. Our

results demonstrated that ACP significantly reduced the relative abundance of *Lachnospiraceae_NK4A136_group*, increased the relative abundance of *Ruminococcus* and *Prevotellaceae_UCG-001*, and improved the intestinal flora structural imbalance induced by Cy at the phylum, family, and genus levels. Studies have shown that among the metabolites of various bacterial populations, acetic acid, propionic acid, and butyric acid are the most abundant and key metabolites that promote the development of the immune system^[8,43]. In the intestinal mucosa, these acids exert beneficial effects on intestinal epithelial and immune cells by inducing intracellular or extracellular processes. Specifically, butyric acid can directly provide energy for colonic epithelial cells, promote the expression of Occludin, ZO-1, and MUC2, and enhance the integrity of the intestinal mucosal barrier^[47]. In this study, ACP significantly increased the levels of acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and total SCFA while alleviating the disordered SCFA metabolism caused by Cy. Correlation analysis between SCFA levels and immune-related indexes revealed a positive association, indicating that SCFA levels were positively correlated with immune indexes. These results suggest that ACP played an immunomodulatory role by regulating intestinal flora to improve disordered SCFA metabolism.

The biological function of a polysaccharide is highly dependent on its structural configuration^[48]. In this study, ALP and ACP exhibited distinct activities *in vitro* and *in vivo*, which may be related to their structural differences. ALP and ACP were prepared through hot water extraction, a widely used method for extracting polysaccharides from *Atractylodes* rhizome. The yields of ALP and ACP were 12.50% and 17.58%, respectively, which may be due to the different origins of *Atractylodes lancea* and *Atractylodes chinensis* as well as the significant differences in their growing environments. The chemical structure of polysaccharides is the basis for their biological activity. The protective effect of polysaccharides on the intestinal barrier and the enhancement of immunity is closely related to their molecular weight, monosaccharide composition, molar ratio, uronic acid, triple helix structure, functional group type, glycosidic bond characteristics, and apparent morphology^[49]. Next, we will focus on exploring the relationship between structure and activity from the following two aspects:

(1) The effect of monosaccharide composition and uronic acid on immune regulatory activity

The monosaccharide composition of polysaccharides is a key determinant of their immunomodulatory activity. Studies have found that heteropolysaccharides effectively improve intestinal mucosal barrier damage and immunosuppression^[49]. ALP and ACP are heteropolysaccharides consisting of fructose, mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, and arabinose, which may contribute to their ability to enhance intestinal mucosal barrier damage and modulate immunoregulatory responses. Research shows that the activity is positively correlated with galacturonic acid content: Polysaccharides with a high galacturonic acid molar ratio, such as polysaccharide from *Saposhnikovia Radix* (SP4002501) and the leaves of *Hippophae rhamnoides* L. (HRLP-II-b), significantly promote macrophage proliferation and phagocytosis, repair the intestinal barrier, regulate the microbiota, promote SCFAs production, and activate Toll-like receptor pathways. Similarly, rhamnose-rich capsular polysaccharide markedly enhances the secretion of IFN- γ , TNF- α , and IL-6, exhibiting potent immune regulatory activity^[50-52]. As a key acidic group in polysaccharides,

the high content of uronic acid is beneficial for enhancing immune regulatory activity. For example, *Hericum erinaceus* polysaccharides (HEPs) rich in uronic acid, significantly promote the secretion of NO, IL-10, IFN- γ , TNF- α , and IL-6, and act through the NF- κ B, MAPK, and PI3K/Akt pathways. In terms of intestinal protection, the negative charges conferred by uronic acid residues promote polysaccharide adhesion to intestinal mucus, extend the retention time of polysaccharides in the intestine, and thereby improve fermentation efficiency, increase SCFAs production, and enhance immune regulatory function^[53,54]. In this study, we found that ACP contains higher levels of galacturonic acid, rhamnose and uronic acid than ALP, which may explain its superior immune regulatory and intestinal protective activities, as evidenced by enhanced macrophage phagocytosis, cytokine secretion, gut microbiota regulation, and SCFAs production. While the difference in uronic acid content appears to be the primary determinant of activity variation, the potential contributions of other structural characteristics (such as molecular weight and glycosidic bond type) require further investigation.

(2) The effect of triple helix structure on immune regulatory activity

The triple-helical conformation of polysaccharides is closely linked to their immune regulatory activity. The novel triple-helical *Craterellus cornucopioides* polysaccharide (CCP) and *Ganoderma lucidum* polysaccharides (RGLP-1) exhibit stronger immune regulatory effects, markedly promoting the secretion of NO, TNF- α , IL-6 and the proliferation and activation of macrophages^[55,56]. The Congo red assay confirmed the triple-helical structure of ACP, which may further contribute to its immunomodulatory activity.

It should be noted that the current study has some limitations: First of all, this study focused on comparing the immunoregulatory activities of ALP and ACP, but because the two polysaccharides obtained in this study were not isolated and purified by ion exchange column or gel column, they were crude polysaccharides. Although we added the 1D NMR of ALP and ACP in this study, the precise structures of these two polysaccharides remained undetermined owing to the limited structural insights provided by 1D NMR. Therefore, it is essential to further investigate the glycosidic linkages or branching of ALP and ACP using 2D NMR and methylation analysis, and to elucidate how their structural characteristics (e.g., molecular weight, monosaccharide composition, glycosidic bond type) affect immune regulation and intestinal protective activity. Secondly, this study lacks the molecular mechanism of ACP to exert its immunoregulatory activity. Our current research only shows that ACP plays an immunomodulatory role by regulating intestinal flora and SCFAs. However, the specific molecular mechanism is still unclear. Further research through transcriptomics, untargeted metabolomics and proteomics may be an important means to explore the immunomodulatory mechanism of ACP. Third, although this study has found that ACP plays an immunomodulatory role by regulating intestinal flora and SCFAs, the specific structure information of ACP, as well as toxicological studies in mice have not been obtained in this study, so the relationship between the structure and biological function of ACP is still worth further exploration.

5 Conclusion

In conclusion, this study explored the structural characteristics and immunoregulatory effects of ALP and ACP from different origins. The uronic acid content was higher in ACP compared to

ALP, while ALP had a slightly higher molecular weight, which may be related to the different origins, planting terrain, and growing altitude of *Atractylodes* rhizomes. Both ALP and ACP exhibited notable immunomodulatory activities *in vitro* and *in vivo*, with ACP demonstrating superior efficacy. ACP can not only increase the diversity of intestinal flora, regulate the structure and composition of intestinal flora, reduce the abundance of Firmicutes and *Lachnospiraceae_NK4A136_group*, but also increase the abundance of Bacteroidota, Muribaculaceae, *Ruminococcus* and *Prevotellaceae_UCG-001*. It can also promote the production of SCFAs from fermentation products of intestinal flora, so as to improve Cy-induced immunosuppression and intestinal injury. However, the necessity of intestinal flora for ACP's immunomodulatory effects remains unclear. Future research will focus on aseptic verification and fecal bacteria transplantation experiments to verify the role of the intestinal flora and explore the molecular mechanisms of ACP in its immunomodulatory role.

Electronic Supplementary Material (ESM)

The online version contains supplementary material available at <https://doi.org/10.26599/FMH.2027.9420173>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Author contribution statement

Yan Dong: Data curation, project administration, validation, writing manuscript, experimental design. Dong-Peng Wang: Data curation, project administration, validation, writing manuscript, experimental design. Chang Ke: Project administration. Wen-Lv Nie: Data curation and methodology. Li-Jiao Zhong: Data curation and methodology. Ji-Yuan Tu: Data curation and methodology. Ling-Hang Qu: Project administration, funding acquisition. Yan-Ju Liu: Project administration, funding acquisition, writing manuscript. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All experiments were approved by the animal ethics committee of Hubei University of Chinese medicine (approval code: NO.00273385, 14 June 2022).

Use of AI statement

The authors declare that no generative AI or AI-assisted technologies were used in the writing or preparation of this manuscript.

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