Acupuncture and Herbal Medicine

Effect of *Grifola frondosa* polysaccharide on immune function and gut microbiota in mice

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Abstract

Objective: *Grifola frondosa*, a medicinal mushroom, is widely used to enhance immunity and treat cancer. Polysaccharides are its primary active components. We aimed to investigate the effects of the alkaloid *G. frondosa* polysaccharide (GFP) extract on immunity and gut microbiota.

Methods: Alkaloid GFP was extracted using an alkaline extraction method, followed by hollow-fiber microfiltration. The molecular weight of alkaloid GFP was determined by high-performance gel permeation chromatography (HPGPC). Monosaccharide composition was analyzed by pre-column derivatization combined with high-performance liquid chromatography (HPLC). Methylation analysis was performed to characterize glycosidic linkages in alkaloid GFP. The immune function of alkaloid GFP was assessed in a cyclophosphamide (CTX)-induced immunosuppressive mouse model. Splenic lymphocyte proliferation, macrophage phagocytic capacity, and natural killer (NK) cell cytotoxicity were evaluated. The effect of alkaloid GFP on gut microbiota was assessed by 16S rRNA sequencing.

Results: The molecular weight distribution of alkaloid GFP ranged from 17 to 18 kDa. The alkaloid GFP contained a β-(1→6)-glucan backbone branched at O-3 by β-1,3-D-Glcp. Oral administration of alkaloid GFP mitigated the effects of CTX on spleen index, splenic lymphocyte proliferation, and peritoneal macrophage phagocytosis. Additionally, alkaloid GFP improved the gut microbiota composition of immunosuppressed mice, increasing the relative abundances of *Ligilactobacillus* and *Lactobacillus*.

Conclusions: Alkaloid GFP demonstrated immune-enhancing effects and gut microbiota regulatory activity, providing a basis for developing related health food ingredients.

Keywords: *Grifola frondosa*, Gut microbiota, Immune activity, Polysaccharide

Graphical abstract: http://links.lww.com/AHM/A156.

Introduction

Grifola frondosa is a medicinal fungus widely used to boost *qi*, fortify the spleen, moisten the lungs, and protect the liver[1]. It is commonly used in Asia to treat cancer, typically as an adjunct to chemotherapy[2–3]. The main active components of *G. frondosa* are polysaccharides, which have been reported to possess significant anti-tumor[4], immunomodulatory[5], and antioxidant properties[6]. *G. frondosa* polysaccharides (GFP) primarily consist of (1-3,1-6)-β-D-glucan and galactan[7–8]. Various methods have been employed to extract polysaccharides from *G. frondosa*, including hot water extraction[9–11], cold-water extraction[12], acid extraction[13], cold alkali extraction[14–15], hot alkali extraction[14], ultrasonic-assisted extraction[16], microwave-assisted extraction[17], and enzyme hydrolysis[18]. Alkaline solutions can disrupt the fungal cell wall, break down the crude fiber structure, and release intracellular polysaccharides that are not easily extracted by water. The yield of polysaccharides extracted with alkali is higher than that obtained by water extraction[19–20]. Additionally, both cold and hot alkali extracts contain significant amounts of β-glucans[1]. β-D-glucans have been shown to exhibit notable immunomodulatory and anti-tumor activities[21–22].

Studies have demonstrated that highly purified soluble β-glucan (MD fraction) derived from *G. frondosa* can significantly enhance host immune function, with both oral administration and injection of MD fraction significantly inhibiting tumor growth[23]. It has also been shown that a water-soluble polysaccharide from *G. frondosa* can modulate the immune system[24]. Alkali polysaccharide fractions isolated from *G. frondosa* have been reported to exhibit immunostimulatory activities, such as macrophage activation *in vitro*[15] and anti-tumor effects *in vivo*[25]. However, limited information is available regarding the protective effects of alkali

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polysaccharides from *G. frondosa* against immunosuppression *in vivo*.

The gut microbiota and immune system are closely interconnected. Studies have found that the anti-hepatocellular carcinoma effect of GFP may be linked to the gut microbiota[26]. The effects of GFP on intestinal microbiota regulation in diabetic mice, hyperlipidemic rats, and mice with DSS-induced colitis have been reported[27–29]. However, the effects of GFP on the gut microbiota in immunosuppressive mouse models have not yet been investigated.

In this study, we extracted polysaccharides from *G. frondosa* using alkaline extraction. We established a mouse model of immunosuppression induced by cyclophosphamide (CTX) to assess the immunomodulatory activity of alkaloid GFP. Furthermore, we explored its effects on the gut microbiota in immunosuppressed mice.

Materials and methods

Reagents and chemicals

The fruit bodies of *G. frondosa* were provided by Infinitus Co., Ltd. (Guangdong Province, China). CTX, concanavalin A (ConA), lipopolysaccharide (LPS), Bradford assay with bovine serum albumin (BSA), and thiazolium blue (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and RPMI-1640 medium were obtained from Gibco BRL (Grand Island, NY, USA). Penicillin and streptomycin were sourced from Tianjin Haoyang Biological Products Co., Ltd. (Tianjin, China). Fluorescent microspheres were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade or higher.

Extraction of polysaccharides

Dried *G. frondosa* fruiting bodies were mixed with 0.5 M NaOH solution at a mass-volume ratio of 1:20. The mixture was heated to 80°C and extracted for 3 hours, followed by filtration. The residue underwent two additional extraction steps under the same conditions. The combined filtrates were neutralized to pH 7.0 using HCl. The neutralized solution was filtered through a 0.2 μm microfiltration membrane, and the permeate was collected. Hollow-fiber membranes with a molecular weight cutoff of 50 kDa were used for ultrafiltration to collect permeate solutions, which were subsequently subjected to a second ultrafiltration step using a 10 kDa cutoff membrane. The retentate was freeze-dried to obtain alkaloid GFP. Total carbohydrate content was measured using the phenol-sulfuric acid method with glucose as the standard[30]. Uronic acid content was determined using the Filisetti-Cosi and Carpita colorimetric method[31]. Protein content was measured using the BSA as the standard[32].

Determination of monosaccharide composition

Alkaloid GFP was hydrolyzed with a 2 M hydrochloric acid methanol solution at 80°C for 16 hours, followed by hydrolysis with 2 M TFA at 120°C for 1 hour. The resulting hydrolysates were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) according to a previously established method[33] and analyzed using a DIKMA Inertsil ODS-3 column (4.6 mm × 150 mm, 5 μm) connected to a high-performance liquid chromatography (HPLC) system (LC-10ATvp pump and SPD-10AVD UV-VIS detector, Shimadzu, Japan). Elution conditions followed previously described methods[34–35].

Molecular weight determination

Alkaloid GFP was dissolved in ddH_2O and analyzed using an HPLC system (RID-20A differential detector, Shimadzu, Japan) equipped with a TSK-gel G3000PWXL column (7.8 mm × 300 mm, TOSOH, Japan). The column temperature was maintained at 40 °C, the mobile phase was 0.2 M NaCl, and the flow rate was 0.6 mL/min.

Methylation analysis

Alkaloid GFP methylation was performed using the method described by Needs and Selvendran[36]. Briefly, alkaloid GFP was dissolved in dimethyl sulfoxide (DMSO) and methylated using a suspension of NaOH/DMSO and iodomethane. The reaction mixture was extracted with CH_2Cl_2, and the solvent was removed by vacuum evaporation. Complete methylation was confirmed by the absence of the -OH band (3,200–3,700 cm^{-1}) in the FT-IR spectrum. The permethylated alkaloid GFP was depolymerized with formic acid, hydrolyzed with TFA, reduced with $NaBH_4$, and acetylated with acetic anhydride. The resulting product was analyzed by GC-MS (7890B-5977B, Agilent, Santa Clara, CA, USA) using a DB-35 ms capillary column (30 m × 0.32 mm × 0.25 mm). The inlet and detector temperatures were 300°C. The mass scan range was 50.0 to 500.0 *m/z*.

Animals and immunosuppressed model

Healthy female C57BL/6N mice (6 weeks old) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed under pathogen-free conditions with unrestricted access to food and water. Following 1 week of acclimation, mice were randomly divided into four groups ($n = 10$): Control group, CTX group, CTX + 50 mg/kg/day of alkaloid GFP group, and CTX + 200 mg/kg/day of alkaloid GFP group. ddH_2O or alkaloid GFP was administered intragastrically for 28 days. On days 23 and 24, CTX (40 mg/kg/day) was injected intraperitoneally. Blood samples were collected at the end of the experiment for white blood cell count analysis using an animal blood cell analyzer. Mice were euthanized to assess spleen and thymus indices. Macrophage phagocytic capacity, lymphocyte proliferation, and splenic natural killer (NK) cell activity were analyzed in five paired samples per group. Cecal contents from six mice per group were collected for gut microbiota analysis. All experiments complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Animal Care and Use Committee of Northeast Normal University.

Splenic lymphocyte proliferation

Spleens were homogenized through a 50-mesh sterile copper mesh, and cell suspensions were filtered through a 200-mesh sterile copper mesh. Filtrates were centrifuged at 800 × g for 5 minutes, and red blood cells were lysed. Cells were resuspended in RPMI-1640 medium, washed twice, and adjusted to a density of 5×10^6 cells/mL. ConA (5 μg/mL) or LPS (10 μg/mL) was added as mitogens for T and B lymphocytes, respectively. Cells were incubated at 37°C with 5% CO_2 for 72 hours. MTT solution (20 μL; 5 mg/mL) was added to each well and incubated for 4 hours, followed by 50 μL of sodium dodecyl sulfate (SDS) to dissolve formazan crystals. Absorbance was measured at 570 nm using a microplate reader.

Phagocytic capacity of macrophages assay

After the mice were sacrificed, peritoneal cells were harvested by peritoneal lavage with 10 mL of cold PBS, centrifuged at 800 × g for 5 minutes, and resuspended in DMEM medium. The cell density was adjusted to 4×10^5 cells/mL using the medium, and cells were cultured in six-well plates, followed by an incubation period of 90 minutes. Fluorescent microspheres were prepared according to the manufacturer's instructions and adjusted to a concentration of 5×10^7/mL with 1% BSA. Subsequently, fluorescent microspheres were added to each well and incubated at 37°C for 90 minutes. The unbound fluorescent microspheres were washed with sterile PBS. Phagocytosis of peritoneal macrophages was assessed using an Agilent NovoCyte Quanteon system (Agilent, Santa Clara, CA, USA). The phagocytic indices of the peritoneal macrophages were calculated using the following formula:

$$\text{Phagocytic index} = \text{number of phagocytic fluorescent microspheres/total number of macrophages.}$$

NK cytotoxicity assay

Splenocytes (1×10^6 cells/well) and YAC-1 cells (4×10^4 cells/well) were seeded in 96-well plates. The color reaction was performed in a 96-well plate following the instructions of the LDH test kit (Promega, Madison, WI, USA). The mixtures were then incubated at 37°C for 4 hours. Subsequently, 50 μL of supernatant was transferred to a new 96-well plate, and 50 μL of color-developing solution was added. Absorbance was measured at 492 nm using a microplate reader. The killing rate was calculated using the formula:

$$\text{Killing rate } (\%) = (\text{experimental release} - \text{natural release}) / (\text{maximum release} - \text{natural release}) \times 100\%.$$

DNA extraction and 16S rRNA sequencing

The cecal contents microbiome (control, CTX, CTX + 200 mg/kg/day of alkaloid GFP) was obtained using the Fast DNA SPIN Kit (MP Bio, CA, USA). Polymerase chain reaction (PCR) amplification of the V4 hypervariable region of the bacterial 16S rRNA was performed using universal primers: V4F (5′-GTGCCAGCMGCCGCGGTAA-3′) and V4R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR products were purified using Vazyme VAHTS™ DNA Clean Beads (Vazyme, Jiangsu, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit. The products were subsequently purified and sequenced on an Illumina MiSeq platform (Illumina). The QIIME data analysis package was used to analyze 16S rRNA data. Sequences were clustered into operational taxonomic units (OTUs) against the database using VSEARCH (1.9.6).

Statistical analysis

Data are shown as the mean ± standard deviation (SD) from at least three independent experiments. Data were analyzed using the Student *t* test for intergroup comparisons. Statistical significance was defined as $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$.

Results

Structural analysis of alkaloid GFP

The molecular weight distribution of alkaloid GFP was determined to range from 17 to 18 kDa using high-performance gel permeation chromatography (HPGPC). The HPGPC results indicated that alkaloid GFP displayed a single symmetric peak, suggesting that alkaloid GFP is a homogeneous polysaccharide (Figure 1A). Monosaccharide composition analysis revealed that alkaloid GFP is composed of glucose (87.4%), glucuronic acid (7.9%), galactose (3%), and mannose (1.7%), with a total sugar content of 89.1%. The uronic acid and protein contents were measured as 4.2% and 1.1%, respectively. Methylation analysis using GC-MS was conducted to determine the linkage types in alkaloid GFP. As shown in Table 1, the glucose linkages in alkaloid GFP predominantly comprised 1,6- and 1,3,6-linkages, indicating that the backbone consists of 1,6-Glcp units branched at O-3. Additionally, 1-Glcp and 1,3-Glcp were identified, likely forming the side chains (Table 1). Collectively, alkaloid GFP is characterized as a β-(1→6)-glucan backbone branched at O-3 by β-1,3-D-Glcp.

Regulatory effect of alkaloid GFP on immune activity of immunosuppressed mice

To investigate the effects of alkaloid GFP on immune function, a mouse model of CTX-induced immunosuppression was established. Based on previous studies,

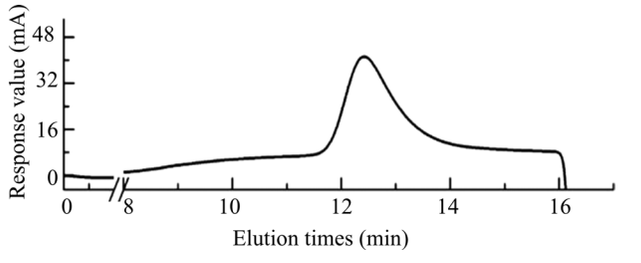


Figure 1. Molecular weight distribution of AGFP extract. AGFP: Alkaloid *Grifola frondosa* polysaccharide.

Table 1

Glycosidic linkage analysis of AGFP as determined by methylation analysis using GC-MS

Methylated sugars	Linkages	Molar percent (%)	Mass fragments (m/s)
2,3,4-Me_3-Glcp	1,6-	41.3	101, 117, 129, 161, 173, 189, 233
2,4-Me_2-Glcp	1,3,6-	21.1	117, 129, 159, 189, 233, 261, 305
2,4,6-Me_3-Glcp	1,3-	18.1	101, 117, 129, 161, 189, 233, 277
2,3,4,6-Me_4-Glcp	1-	19.5	101, 117, 129, 145, 161, 205

AGFP: Alkaloid *Grifola frondosa* polysaccharide; GC-MS: Gas chromatography–mass spectrometry.

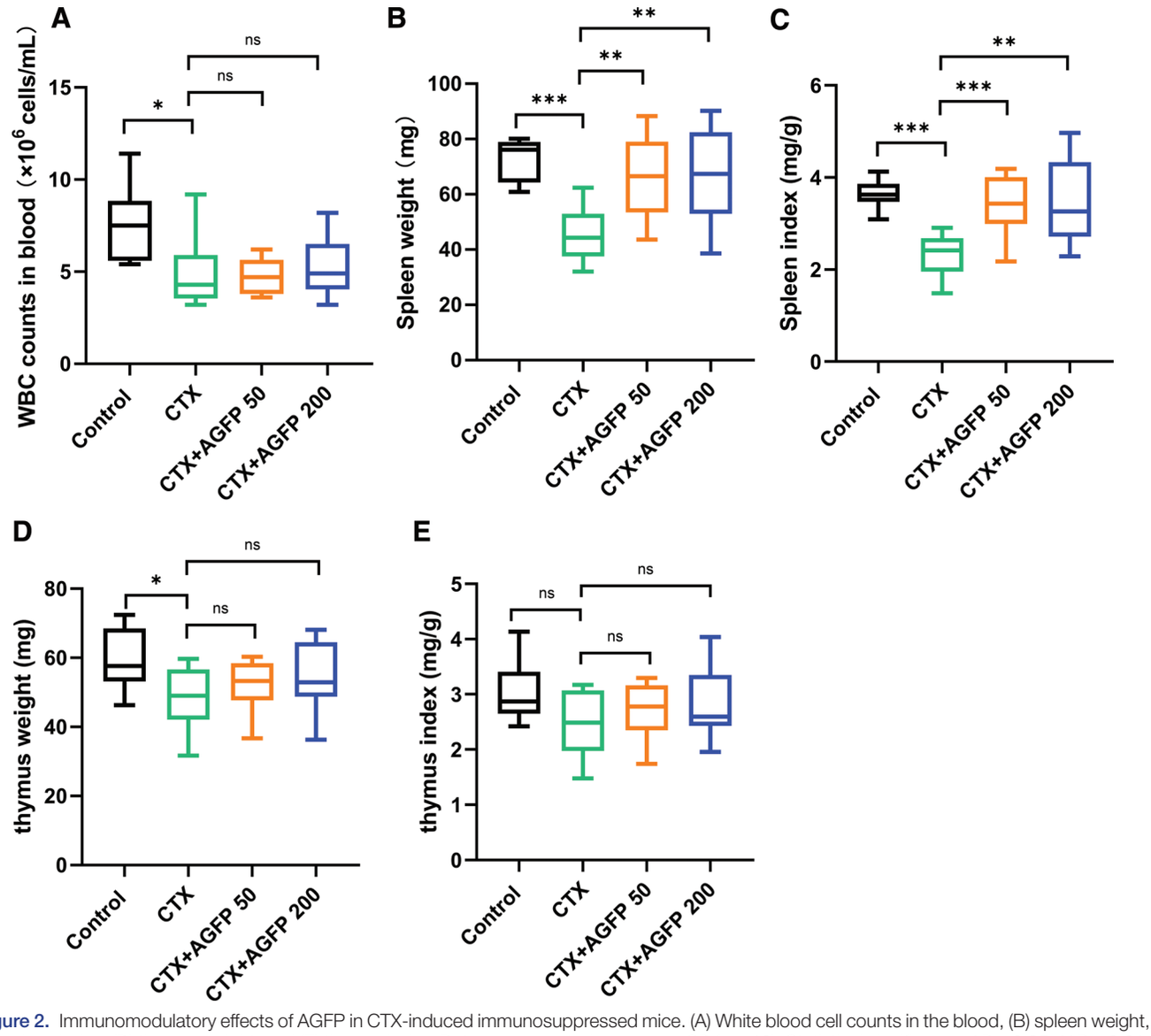


Figure 2. Immunomodulatory effects of AGFP in CTX-induced immunosuppressed mice. (A) White blood cell counts in the blood, (B) spleen weight, (C) spleen index, (D) thymus weight, and (E) thymus index. Data are presented as mean ± SD (n = 10). Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001. AGFP: Alkaloid *Grifola frondosa* polysaccharide; CTX: Cyclophosphamide; ns: no significance; SD: Standard deviation; WBC: White blood cell.

polysaccharide doses ranging from 50 to 800 mg/kg/day have been commonly used to stimulate the immune system in mice[37–38]. Accordingly, the immunomodulatory activity of alkaloid GFP was evaluated at 50 and 200 mg/kg/day. CTX treatment significantly reduced white blood cell counts in the blood (Figure 2A), spleen and thymus weights, and their respective indices (Figure 2B–E). Compared to the CTX group, mice treated with alkaloid GFP exhibited significant recovery in spleen weight and spleen index (Figure 2B, C). However, alkaloid GFP did not affect white blood cell count, thymus weight, or thymus index (Figure 2A, D, E).

Furthermore, the phagocytic activity of peritoneal macrophages, proliferation of splenic lymphocytes, and NK cell cytotoxicity were examined following alkaloid GFP treatment. These functions were significantly suppressed by CTX treatment. As shown in Figure 3A and B, alkaloid GFP at 200 mg/kg/day significantly enhanced phagocytic activity. However, neither 50 nor 200 mg/kg/day of alkaloid GFP improved NK cell cytotoxicity

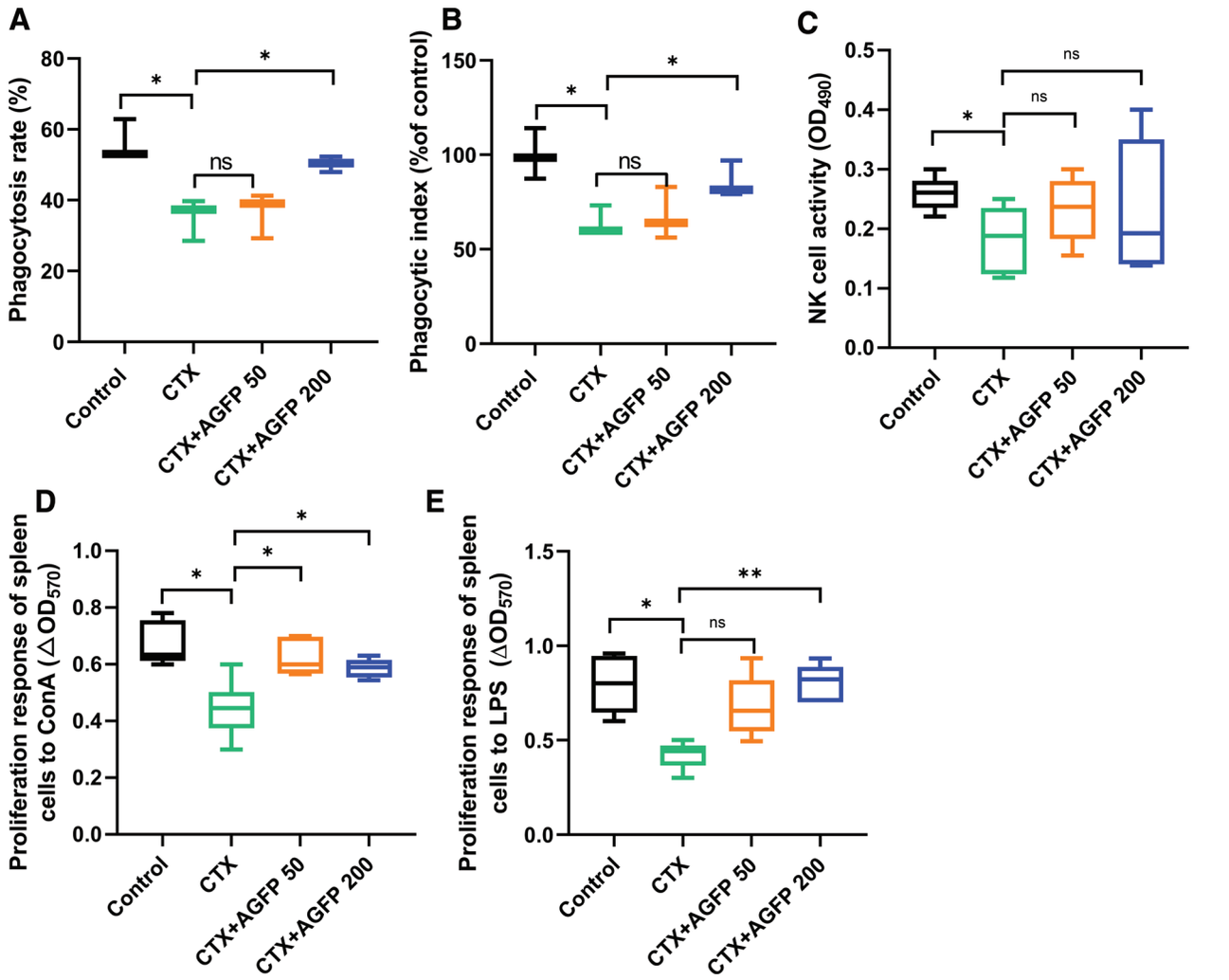


Figure 3. Immunomodulatory effects of AGFP in CTX-induced immunosuppressed mice. (A) Phagocytosis rate of peritoneal macrophages, (B) macrophage phagocytic index, (C) NK cell cytotoxicity, and splenic lymphocyte proliferation rate in response to (D) ConA and (E) LPS. Data are presented as mean ± SD (n = 5). Statistical significance: *P < 0.05, **P < 0.01. AGFP: Alkaloid *Grifola frondosa* polysaccharide; ConA: Concanavalin A; CTX: Cyclophosphamide; LPS: Lipopolysaccharide; NK: Natural killer; ns: no significance; SD: Standard deviation.

(Figure 3C). Alkaloid GFP at 200 mg/kg/day significantly enhanced splenic lymphocyte proliferation (Figure 3D, E). Collectively, alkaloid GFP at 200 mg/kg/day demonstrated enhanced immunomodulatory activity.

Regulation of gut microbiota by alkaloid GFP in mice

Given that polysaccharides are challenging to absorb, their immunoregulatory activity is likely mediated by modulating the gut microbiota. Therefore, the effects of alkaloid GFP on the gut microbiota were investigated, focusing on the 200 mg/kg/day dose. Analysis of α-diversity, which reflects microbial community richness and diversity, revealed that CTX treatment reduced microbiota diversity in the cecal contents (Figure 4A). Compared to the CTX group, alkaloid GFP-treated mice showed a significant increase in α-diversity (Figure 4A). Principal coordinates analysis (PCoA) plots further highlighted significant differences in microbial composition between the CTX and control groups (Figure 4B). Alkaloid GFP treatment mitigated CTX-induced intestinal microbiota dysbiosis (Figure 4B). At the family level, CTX treatment reduced the abundance of *Lactobacillaceae* while increasing *Erysipelotrichaceae* (Figure 4C). Alkaloid GFP treatment significantly increased *Lactobacillaceae* and decreased *Erysipelotrichaceae* (Figure 4C, E). At the genus level, CTX reduced the abundance of *Ligilactobacillus* and *Lactobacillus* while increasing *Dubosiella* (Figure 4D, E). Oral administration of alkaloid GFP reversed these changes, increasing the abundance of *Ligilactobacillus* and Lactobacillus and decreasing *Dubosiella* levels (Figure 4D). These results suggest that *Ligilactobacillus* and *Lactobacillus* may contribute to the immunomodulatory effects of alkaloid GFP.

Discussion

Edible fungal polysaccharides exhibit diverse biological activities, including regulation of immunity and gut microbiota, anti-tumor effects, and hypoglycemic and hypolipidemic properties[39–44]. *G. frondosa* is both a medicinal and edible fungus[45] containing various bioactive substances, making it a promising candidate for functional food development. Polysaccharides, one of its main components, have been reported to possess a range of biological activities[46–47]. Previous studies identified several types of polysaccharides in *G. frondosa*,

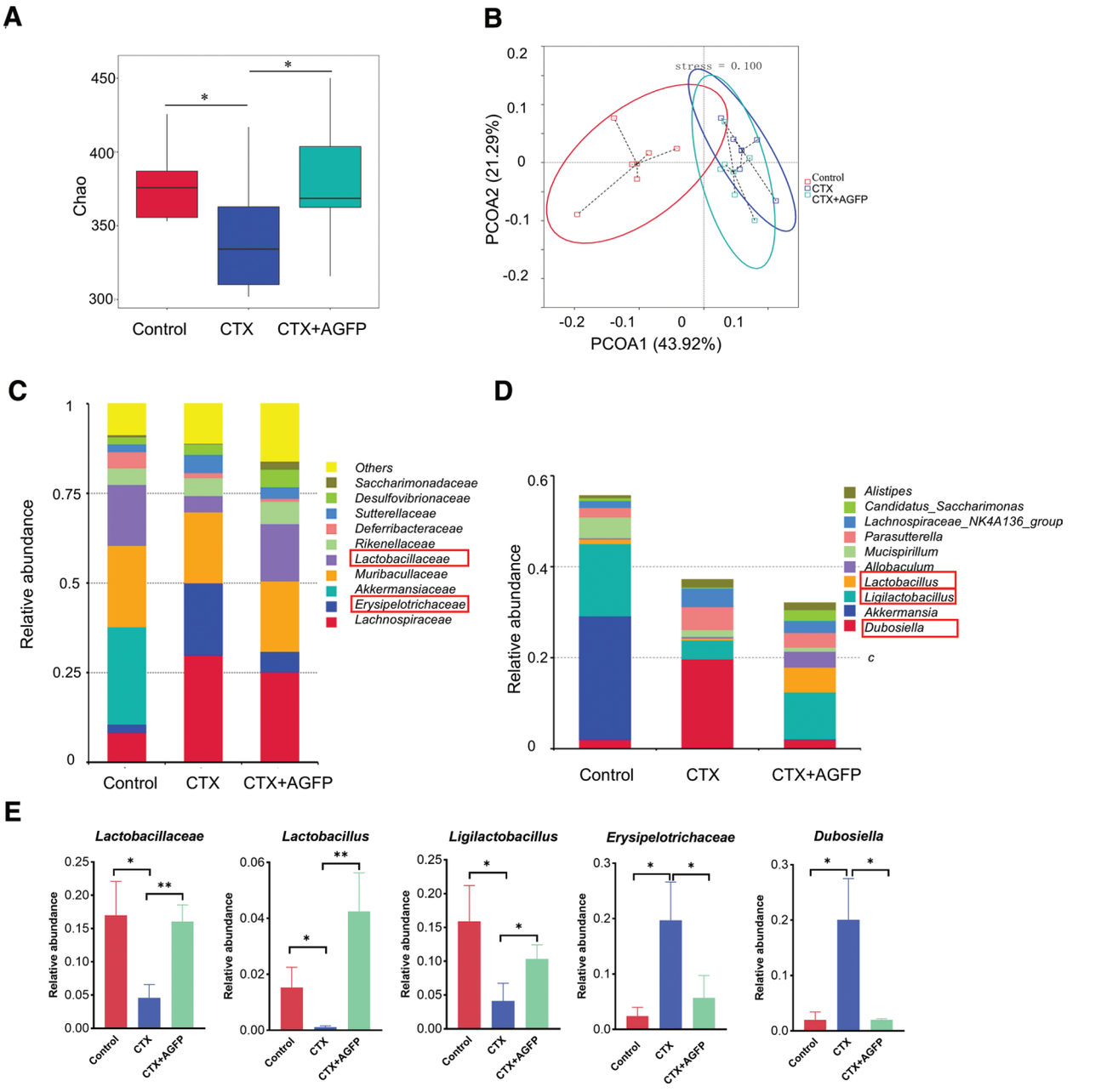


Figure 4. Effects of AGFP on gut microbiota composition in CTX-induced immunosuppressed mice. Cecal contents were analyzed by 16S rRNA sequencing. (A) α-diversity (Chao1 index), (B) PCoA of microbial communities, (C) relative abundance of microbiota at the family level, (D) relative abundance of microbiota at the genus level, and (E) statistical analysis of key bacterial groups. Data are presented as mean ± SD (n = 6). Statistical significance: *P < 0.05, **P < 0.01. AGFP: Alkaloid *Grifola frondosa* polysaccharide; CTX: Cyclophosphamide; PCoA: Principal coordinates analysis; SD: Standard deviation.

including 1,4-linked-D-Glcp glucan, β-(1→4)-linked backbones with β-(1→6)-linked branches[48], β-(1→3)-linked glucan with a β-(1→6)-linked terminal glucose residue[25], β-d-(1→3)- and β-d-(1→4)-linked glucopyranosyl residues[15], (1→3)-β-D-Glcp main chains branched with (1→3)-α-D-Manp[49], and α-glucopyranose[12]. In this study, a homogeneous branched β-(1→6)-glucan with branches of β-(1→3)-D-Glcp was extracted using alkaline extraction and membrane filtration, providing a technical reference for GFP extraction.

Acid-soluble *G. frondosa* polysaccharide (GFAP) exhibits anti-tumor activity in mice[49]. Chen et al.[17] optimized the GFP extraction process and prepared polysaccharides with inhibitory effects on HepG-2 cell proliferation. Soluble polysaccharides from *G. frondosa* have been shown to increase thymus and spleen indices, demonstrating cytotoxic activity against HepG-2 cells[50]. Additionally, selenium-enriched *G. frondosa* polysaccharides (Se-GFP) were reported to prevent CTX-induced immune suppression in mice *via* the MAPK signaling pathway[51]. In this study, a CTX-induced immunosuppression model was employed to simulate impaired immunity[52–53]. Within this model, alkaloid GFP provided protection against CTX-induced immunosuppression, demonstrating its potential as an immunomodulator.

The gut microbiota significantly influences energy metabolism, nutrient absorption, and immune function[54–55]. Natural polysaccharides serve as prebiotics, modulating immunity by altering gut microbiota

composition[56]. The gut microbiota impacts the intestinal barrier, reducing the invasion of pathogenic bacteria[57]. Meanwhile, gut microbiota metabolites, including short-chain fatty acids, bile acids, and tryptophan[58], regulate gut and systemic immunity. Bacterial components such as exopolysaccharides (EPS)[59] and S-layer protein A (SlpA)[27–29] have been reported to display immunostimulatory activities. Several studies have confirmed the activity of GFP in modulating gut microbiota[27–29]. However, the effects of GFP on the gut microbiota in immunosuppressive mouse models have not been previously reported. Our results indicate that alkaloid GFP treatment reduced the abundance of Erysipelotrichaceae, which was increased by CTX. Erysipelotrichaceae are known to be enriched in colorectal cancer and inflammatory bowel diseases (IBD)[60]. Additionally, alkaloid GFP significantly increased the abundance of Ligilactobacillus and Lactobacillus. These probiotics have been described as major contributors to gastrointestinal health and immunity[26]. Studies have shown that cold-water extraction of polysaccharides from *G. frondosa* increased the abundance of *Bacillus* while decreasing the abundance of *Lactobacillus* in mice[26]. The cold-water extraction method produced polysaccharides with higher protein content and molecular weight[26]. This finding suggests that different methods of extracting polysaccharides from the same source may have distinct effects on gut microbiota. The limitation of this research is the study focused on the effects of GFP on the gut microbiota in immunosuppressive mouse models but did not verify these bacteria could modulate the mouse immune, which could be addressed in future research.

Conclusions

This study employed alkaline extraction and membrane filtration to isolate a homogeneous β-(1→6)-glucan with branches of β-(1→3)-D-Glcp from the fruit body of *G. frondosa*. Alkaloid GFP demonstrated immune-enhancing and gut microbiota-regulating activities, providing a foundation for developing related health food ingredients.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Linlin Ma and Xiaoliang Lin participated in the research design. Linlin Ma and Hairong Cheng participated in the writing of the paper. Ming Liang, Jieyi Long and Xian Qu participated in the performance of the research. Xiaoliang Lin, Yifa Zhou, and Hairong Cheng contributed new reagents or analytic tools. Yi Yu and Linlin Ma participated in the data analysis.

Ethical approval of studies and informed consent

Animal experiments followed NIH guidelines (Pub. no. 8523, revised 1996) and were approved by the Science and Technology Ethics Committee of Northeast Normal University (202502003).

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Data availability

The datasets generated during and/or analyzed during the current study are not publicly available due to the need for patenting but are available from the corresponding author on reasonable request.

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