Modulation of gut homeostasis by exopolysaccharides from *Aspergillus cristatus* (MK346334), a strain of fungus isolated from Fuzhuan brick tea, contributes to immunomodulatory activity in cyclophosphamide-treated mice

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In this study, the crude exopolysaccharides (CEPSs) from fungus *Aspergillus cristatus* (MK346334, NCBI) isolated from Fuzhuan brick tea and its main purified fraction (EPSs-2) were investigated. Using the RAW264.7 cell model, EPSs-2 exhibited an excellent immunomodulatory effect in vitro. Then, the regulating effects of EPSs on immune function and gut microbiota were evaluated using a cyclophosphamide (Cy)-induced mouse model. It was found that both CEPSs and EPSs-2 improved the body weight loss, immune organ indexes as well as the levels of TNF-α, IL-1β, IFN-γ and IgA, exhibiting potent immunoregulatory activity. Moreover, CEPSs and EPSs-2 not only attenuated the intestinal tissue damage, but also promoted the production of short-chain fatty acids and modulated the microbial composition by increasing the growth of Muribaculaceae, Prevotellaceae_UCG-001, Bacteroides, Parabacteroides and Tidjanibacter, while decreasing the relative abundances of Helicobacter, Bilophila, Mucispirillum, Lachnospiraceae, Ruminococcaceae and Clostridiales. These results indicated that the EPSs, especially EPSs-2, exhibited immunomodulatory activity associated with the modulation of gut microbiota to maintain gut homeostasis, which provided evidence for the development of novel potential prebiotics and immunomodulators.

1. Introduction

The gut microbiota, a complex and plastic network of diverse organisms, has evolved symbiotically with humans over millions of years, profoundly shaping the immune system and intricately connecting with human physiology.1,2 It is clear that the gut microbiota plays an important role in the development and regulation of immune system to keep a mutually beneficial relationship with the host.3 The intestinal microbiota and their metabolites are reported to be essential for human health and maintaining the intestinal immune homeostasis.4–6 For instance, the segmented filamentous bacteria, *Akkermansia muciniphila* and *Bacteroides fragilis*, as beneficial symbionts, could induce protective immune responses.7,8 However, the dysbiosis of gut microbiota, which constituted a disequilibrium in the bacterial ecosystem by inflammation or immunosuppression, may not only damage the intestinal mucosal barrier, but also result in systemic immune disorders and various diseases such as obesity, diabetes, inflammatory bowel disease, and abnormal immune responses.8 Previous studies have revealed that cyclophosphamide (Cy), a potent immunosuppressive agent, could lead to immunodeficiency and affect the intestinal immunity due to gastrointestinal mucosal damage associated with dysbiosis of gut microbiota.9–11 Therefore, it is necessary to search for active substances that can serve as potential immunomodulating agents to regulate gut microbiota and maintain balance of immune function.

Natural polysaccharides can directly or indirectly activate the immune system via stimulating immune cells such as macrophages to promote the secretion of cytokines and antibodies.12,13 Recently and more strikingly, increasing studies have indicated that polysaccharides exhibited immuno-
modulatory activity, which has a close association with the regulation of gut microbial structure and reestablishment of gut homeostasis.\textsuperscript{14,15} It has been demonstrated that polysaccharides have the ability to increase the growth of beneficial bacteria but reduce pathogenic bacteria. For example, the polysaccharides from \textit{Dendroblum huoshanense} could regulate the intestinal immunological function and modulate gut microbiota by increasing the relative abundances of \textit{Lactobacillus}, \textit{Parabacteroides}, \textit{Prevotella}, and \textit{Porphyromonas} with the reduction of \textit{Helicobacter} and \textit{Clostridium}.\textsuperscript{16} As complex carbohydrates, generally, polysaccharides cannot be digested in the human digestive tract, whereas, they can be decomposed by gut microbiota to produce metabolites such as short-chain fatty acids (SCFAs).\textsuperscript{5,6} Moreover, it has been reported that the SCFAs derived from gut microbiota can modulate immune responses via signaling through G protein-coupled receptors on intestinal epithelial cells and immune cells, and enhance the mucosal immunity to maintain homeostasis of the intestinal and immune system.\textsuperscript{5,12} Thus, gut microbiota and metabolites including SCFAs are associated with the contribution of polysaccharides as potential prebiotics in immune regulation and host health.

Fuzhuan brick tea (FBT), as one kind of post-fermented tea product, is characterized by its unique fermentation process called “the golden flower blossoming”.\textsuperscript{17} \textit{Aspergillus cristatus}, termed the “Golden Flower Fungus” due to its yellow cleistothecium, is identified as the dominant fungus associated with the fermentation of FBT, and its amount is considered as an important indicator of FBT quality.\textsuperscript{18-20} In our previous studies, it was found that the polysaccharides from FBT (FBTPS) could be utilized by gut microbiota to produce SCFAs and modulate the microbial composition and metabolism.\textsuperscript{21,22} Moreover, FBTPS could prevent obesity and attenuate metabolic syndrome associated with the modulation of gut microbiota.\textsuperscript{23,24} Recently, \textit{A. cristatus}, as a potential probiotic, has been reported to alleviate obesity by regulating gut microbiota,\textsuperscript{25} and its mycelium polysaccharides exhibited immunomodulatory activity.\textsuperscript{25,26} However, there is limited information available on the exopolysaccharides from \textit{A. cristatus} (EPSs),\textsuperscript{27} possible important bioactive substances present in FBT. In our previous study, a strain isolated from FBT was identified as \textit{A. cristatus} (MK346334, NCBI).\textsuperscript{27} Furthermore, it was found that EPSs from the strain of \textit{A. cristatus} could not be digested in the gastrointestinal tract, while it could be degraded by gut microbiota to facilitate the production of SCFAs. In addition, EPSs could regulate the structure of gut microbiota by decreasing the ratio of Firmicutes/Bacteroidetes and increasing the relative abundances of \textit{Prevotella} and \textit{Bacteroides}.\textsuperscript{28} Many studies have confirmed that fungi polysaccharides could exhibit immunomodulatory activities,\textsuperscript{13,29,30} possibly due to their modulation of gut microbiota.\textsuperscript{16,31,32} Nevertheless, the influence of EPSs from \textit{A. cristatus} on the immune system has not been reported. Current knowledge about the interactions among EPSs, immune system and gut microbiota are still unclear. In this study, therefore, crude EPSs (CEPSs) from fungus \textit{A. cristatus} (MK346334) isolated from FBT\textsuperscript{27} were firstly purified, and the immunomodulatory activities of the purified fractions of CEPSs \textit{in vitro} and \textit{in vivo} were then evaluated using the RAW264.7 cell model and a Cy-induced immunosuppressive mice model. In addition, the regulating effect of EPSs on the dysbiosis of gut microbiota induced by immunosuppression was studied to highlight the role in modulating immune responses and in maintaining gut homeostasis.

2. Materials and methods

2.1. Extraction and purification of EPSs from \textit{A. cristatus}

The strain of \textit{A. cristatus} (MK346334) in this work was isolated from FBT as described in our previous report.\textsuperscript{27} As described previously, CEPSs were prepared from the fermentation broth of \textit{A. cristatus}.\textsuperscript{28} Briefly, the fungus \textit{A. cristatus} was inoculated in the potato dextrose broth medium with 5% (w/v) sucrose and cultured with a shaking of 150 rpm at 28 °C for 7 days. The mycelia were removed by filtration, and the filtrate was collected and concentrated at 50 °C to a third of the original volume. Then, 4-fold volume ethanol (95%, v/v) was added into the concentrate and kept overnight at 4 °C. After centrifugation, the precipitates were dialyzed for 3 days and lyophilized to afford CEPSs. Then, CEPSs were deproteinized using the Sevag method and applied to a pre-equilibrated DEAE-fast flow column (2.6 × 45 cm), and the column was eluted with 0.1, 0.3 and 0.5 M sodium chloride (NaCl) solutions at a flow rate of 1.0 mL min\textsuperscript{-1}. The eluate was collected automatically (10 mL per tube), and elution curve was plotted based on the analytical results of phenol–sulfuric acid method (Fig. 1A). The fractions eluted by 0.1 M NaCl were gathered, concentrated, dialyzed and lyophilized, affording the purified fraction 2 (EPSs-2) for further use.

2.2. Characterization of physicochemical properties of EPSs

The contents of total carbohydrates, uronic acid, total phenols and proteins in EPSs were determined by the phenol–sulfuric acid method, \textit{meta}-hydroxydiphenyl method, Coomassie Brilliant Blue method and Folin–ciocalteu method, respectively. The monosaccharide composition of EPSs was analyzed based on the reported method.\textsuperscript{33} The dried EPSs were mixed with the potassium bromide powder and the spectrum was recorded using a Fourier transform-infrared (FT-IR) spectrophotometer (Thermo Fisher Scientific, USA) in the range of 4000–400 cm\textsuperscript{-1}.

2.3. Determination of immune activity of EPSs-2 using the RAW264.7 cell model

2.3.1. Cell culture. The murine RAW264.7 macrophages were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum at 37 °C (5% CO\textsubscript{2}).

2.3.2. Assay of cell viability. The cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, 200 μL per well of RAW264.7 cell suspension (3 × 10\textsuperscript{5} cells per mL) was plated in the 96-well
microplates and incubated (37 °C, 5% CO2) for 12 h, and the supernatant was discarded. Subsequently, the cells were treated with EPSs-2 at serial concentrations (0, 12.5, 25, 50, 100, 200 and 400 μg mL−1) or LPS for 24 h. After that, the culture supernatant was collected to determine the levels of nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 by using enzyme-linked immunosorbent assay (ELISA) kits (Neobioscience, China) according to the instructions of the manufacturer.

2.3.3. Determinations of nitric oxide and cytokines. The RAW264.7 cell suspension (10^6 cells per mL) was loaded onto 24-well microplates. After incubation for 24 h, the supernatant was discarded, and RAW264.7 cells were incubated with EPSs-2 at different concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μg mL−1) or LPS for 24 h. After that, the culture supernatant was collected to determine the levels of nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 by using enzyme-linked immunosorbent assay (ELISA) kits (Neobioscience, China) according to the instructions of the manufacturer.

2.4. Animals and experimental design
Six-week-old specific pathogen free BALB/c male mice, purchased from Qingshui Mountain Animal Breeding Farm (Nanjing, China), were kept at the Animal Center of Nanjing Agricultural University (Nanjing, China). After a week of acclimatization, all mice were randomly classified into five groups (n = 8 per group) as follows: normal control (NC) group, model control (MC) group, CEPSs group, EPSs-2 group and levamisole hydrochloride (LH) group. The mice in the NC group were subjected to intraperitoneal injection of normal saline, while the rest of mice were injected intraperitoneally with Cy at 80 mg per kg body weight (BW) per d for 3 days. For the next 10 days, the mice of the NC and MC groups were administered with sterile water by gavage, while the mice in the CEPSs and EPSs-2 groups were orally treated with CEPSs and EPSs-2 (200 mg per kg BW per d), respectively. Meanwhile, the mice in the LH group were administered with LH (40 mg per kg BW per d) by gavage. The BW of mice was recorded every two days and the percent changes of BW (1− BW in some day/initial BW) were evaluated. All animal procedures were performed in accordance with the National Guidelines for Experimental Animal Welfare (MOST of People’s Republic of China, 2006) and the experiments were approved by the Animal Ethics Committee of Nanjing Agricultural University.

2.5. Determination of immune organ index
The thymus and spleen, immune organs, were surgically separated and weighed. The immune organ index was expressed as the ratio of the weight of immune organ (mg)/BW (g).

2.6. Histomorphological analysis
The jejunums and colon were fixed in 10% formalin solution and then embedded in paraﬃn. Serial paraﬃn sections (thickness, 5 μm) were stained by hematoxylin–eosin (H&E). Pathological conditions were observed under a light microscope, and images were collected using a digital camera. The villus length (the top of villus to crypt transition) and crypt depth (the invagination between two villi) of jejunums were measured. The 5 longest villi and 5 deepest crypts in the field of view were selected and measured in each image. The ratio of villus length to crypt depth (V/C) was also calculated.

2.7. Serum biochemical analysis
The whole blood was collected and centrifuged at 4 °C (3000 rpm, 15 min) to afford the serum. Then, the levels of cytokines including TNF-α, IL-1β, interferon (IFN)-γ and immunoglobulin A (IgA) were determined using mouse ELISA kits.
(Neobioscience, China) referring to the manufacturer's instructions.

2.8. Determinations of SCFAs in cecal contents and feces

As described previously, SCFAs including acetic, propionic, n/i-butyric and n/i-valeric acids were detected using an Agilent 6890 N gas chromatography system (Agilent, USA) equipped with a HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm, Agilent) and a flame ionization detector. Briefly, 100 mg of cecal contents or feces were diluted with deionized water at the ratio of 1:5 (w/v) and vortexed for at least 20 min to release SCFAs sufficiently. After centrifugation (12,000 rpm, 3 min, 4 °C), the supernatant was mixed with an equal volume of internal standard (25 mM 2-ethylbutyric acid dissolved in 0.2 M HCl) and then filtered for SCFA analysis.

2.9. 16S rDNA sequencing and gut microbiota analysis

The feces of mice were collected in sterile microtubes and stored temporarily in liquid nitrogen. Then, the fecal samples were stored at −80 °C until DNA extraction. The total bacterial genomic DNA was extracted from the feces of mice using a QIAamp® Fast DNA stool Mini Kit (Qiagen, Germany) based on the manufacturer’s instructions, and the V3–V4 regions of 16S rDNA were subjected to amplification. The sequencing and bioinformatics study were conducted on an Illumina MiSeq platform purchased from LC-Bio Technology Co., Ltd (Hangzhou, Zhejiang Province, China). The analysis of sequencing was based on the latest version of the QIIME 2. Amplicon data were denoised by DADA2 (Divisive Amplicon Denoising Algorithm), clustered into Feature with a 100% sequence identity. SILVA and NT-16S database were used to assign taxonomy.

2.10. Statistical analysis

The experimental data are presented as mean ± standard deviation (SD). Statistical analysis was performed by using IBM SPSS Statistics 25. The significance between the groups was determined by using the one-way ANOVA procedure, followed by Duncan test, and p < 0.05 was considered as statistically significant. α-Diversity was calculated by Mothur and β-diversity was calculated by using R software packages (Vegan package, V3.5.0). Differences in the relative abundance of features between the two groups were calculated using Tukey’s honest significant difference (HSD) test. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analysis was used to interpret metagenomes and predict Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundances. Statistical analysis of KEGG pathway data was performed by using STAMP v2.1.3 using Welsh’s t-test (p < 0.05).

3. Results

3.1. Preliminary characterization of EPSs

In the present study, the prepared CEPs contained carbohydrates, uronic acid, proteins and polyphenols for EPSs-2 (the main purified fraction) were 83.89 ± 3.68, 9.84 ± 0.89, 6.68 ± 0.95 and 0.78 ± 0.06, respectively. In addition, CEPSs and EPSs-2 were composed of mannose (Man), ribose (Rib), glucuronic acid (GlaA), glucose (Glc) and galactose (Gal) at a molar ratio of 4.7:0.7:0.6:0.5:3.5 and 4.3:0.5:0.4:1.2:3.6, respectively (Fig. 1B). The results suggested that EPSs were a type of hetero polysaccharide and mainly composed of Man and Gal, and EPSs showed similar monosaccharide composition with the mycelium polysaccharides from Eurotium cristatum.

FT-IR spectrum of EPSs-2 is shown in Fig. 1C. EPSs-2 showed absorption peaks of a typical polysaccharide structure at 3370.96, 2936.09 and 1652.69 cm⁻¹, representing the O–H stretching vibrations, C–H stretching vibrations and asymmetric stretching of the C=O groups, respectively. Moreover, the bands at 1404.88, 1327.75 and 1242.89 cm⁻¹ in the region of 1430–1200 cm⁻¹ could be assigned to the O–H deformation vibrations and C–O stretching vibrations, respectively. The strong absorption at 1057.76 was due to the C–O–C stretching vibrations of glycosidic bonds and pyran structure. In addition, the band at 974.84 cm⁻¹ was attributed to the β-type absorption peak of the furan ring associated with the symmetrical tensile vibrations of the furan ring, while the characteristic absorption peak around 813.81 cm⁻¹ indicated the presence of α-anomeric configuration for α-mannopyranosyl units in ESPs-2.

3.2. Immunomodulatory activity of EPSs-2 in vitro

As shown in Fig. 2A, it was found that EPSs-2 had no apparent cytotoxic effect to RAW264.7 cells. Besides, ESPs-2 could significantly promote (p < 0.05) the proliferation of cells at certain concentrations (100 and 200 μg mL⁻¹). Thus, EPSs-2 at the concentration of 12.5–200 μg mL⁻¹ was selected for the following experiments. The stimulatory effects of EPSs-2 on the production of NO, TNF-α and IL-6 in RAW264.7 cells are shown in Fig. 2B–D. Compared with the blank control, EPSs-2 significantly improved (p < 0.05) the levels of NO, TNF-α and IL-6 in a dose-dependent manner (12.5–200 μg mL⁻¹). These results indicated that ESPs-2 exhibited excellent an immunomodulatory effect on murine macrophage cells of RAW264.7 by promoting NO production and cytokine secretion, which is consistent with the result for mycelium polysaccharides from E. cristatum.

3.3. EPSs attenuated Cy-induced immunosuppression in mice

Based on the immune-stimulating activity of EPSs-2 in RAW264.7 cells, a Cy-induced immunosuppressive mice model was employed to evaluate the immune function of EPSs in vivo.

3.3.1. Effects of EPSs on the BW of mice. As shown in Fig. 3A, the BW of mice in the NC group kept increasing at a certain rate. However, the percentage of weight gain decreased significantly after the Cy treatment (for 3 days). Compared with the MC group, the treatment with CEPs, ESPs-2 or LH could alleviate the symptom of weight loss in the next 10 days.
In particular, EPSs-2, the purified fraction, recovered the BW better than CEPSs under the same dose ($p < 0.05$).

### 3.3.2. Effects of EPSs on immune organ indexes.

As shown in Fig. 3B and C, the thymus and spleen indexes for the MC group decreased significantly compared with those for the NC group, indicating that the immunity was imbalanced due to the treatment of Cy. However, the indexes of the thymus and spleen were significantly increased ($p < 0.05$) after the treatment with ESPs-2 or LH, indicating that EPSs could improve the spleen and thymus indexes in Cy-induced mice. In

![Fig. 2](image-url) Immune-enhancing activity *in vitro* of EPSs-2 (A, cytotoxic effect; B, NO; C, TNF-α; D, IL-6). The different small letters show the significant difference between the groups ($p < 0.05$).

![Fig. 3](image-url) EPSs-2 and CEPSs ameliorated Cy-induced immunosuppression (A, body weight; B, thymus index; C, spleen index, D, TNF-α; E, IL-1β; F, IFN-γ; G, IgA). All values bearing different letters are significantly different between the groups ($p < 0.05$).
addition, compared with CEPSs, the intervention of EPSs-2 could more effectively improve the spleen index \( (p < 0.05) \).

### 3.3.3. Effects of EPSs on the production of cytokines and IgA

The levels of cytokines and immunoglobulins are important indicators of the immune function in the body. As shown in Fig. 3D–G, the contents of TNF-\( \alpha \), IL-1\( \beta \), IFN-\( \gamma \) and IgA in the serum in the MC group were significantly reduced compared with those in the NC group, revealing that the treatment of Cy could significantly reduce the immune function of mice. However, the secretions of these factors increased significantly \( (p < 0.05) \) after the intervention of CEPSs, EPSs-2 or LH. Particularly, the levels of TNF-\( \alpha \), IFN-\( \gamma \) and IgA for the EPSs-2 group showed no significant difference with those for the NC group, indicating that EPSs-2 could dramatically enhance the immune activity through promoting the production of cytokines and IgA in immunosuppressive mice.

### 3.3.4. Effects of EPSs on histological changes of intestinal tissue

As shown in Fig. 4A, the morphology of jejunum tissues in the NC group was normal with intact, neat, slender villus, integrated epithelium and a tightly arranged structure. However, the villi in the MC group were shorter with a looser structure, exhibiting a certain level of intestinal mucosa damage. Nevertheless, the intestinal tissue injury was alleviated obviously by CEPSs and EPSs-2 treatments and restored with complete, tall, tidily, tightly arranged villus. Villus and crypt are important indicators of intestinal morphology. Specifically, the length of villi significantly decreased in the MC group was increased remarkably \( (p < 0.01) \) in the CEPSs and EPSs-2 groups (Fig. 4B). As expected, the significant decrease of the ratio of V/C in the MC group was significantly reversed \( (p < 0.05) \) in the EPSs-2 group (Fig. 4C). As shown in Fig. 4D, the colon histological morphology in the NC group exhibited intact colonic mucosa, crypts, and a fold with a tidily and tightly arranged structure. While the colon in the MC group showed relatively severe mucosa damage with hyperplasia of crypts, epithelial cell damage and basal lymphoid aggregates. Nevertheless, this colon tissue damage was ameliorated by CEPSs and EPSs-2 treatments, almost near to the status of the NC group. In a word, EPSs could improve the Cy-induced injury in intestinal tissues.

### 3.4. Effects of EPSs on SCFAs in cecal contents and feces

The concentrations of SCFAs including acetic, propionic, \( n/\text{i-} \) butyric and \( n/\text{i-} \) valeric acids and total SCFAs in the cecal contents and feces were measured. As shown in Table 1, all SCFAs in the cecal contents and feces in the MC group decreased significantly \( (p < 0.05) \) compared with those in the NC group. As expected, the SCFA contents were improved by the intervention of CEPSs or EPSs-2, and EPSs-2 treatment remarkably enhanced all kinds of SCFAs and total SCFAs in the cecal contents \( (p < 0.05) \). In particular, the levels of acetic acid, \( n/\text{butyric acid and total SCFAs in the cecal contents for the EPSs-2 group were significantly higher} \ (p < 0.05) \) than those for the NC group. In the feces, the levels of acetic, propionic,
i-butyrlic, n-valeric acids and total SCFAs for the EPSs-2 group were increased obviously ($p < 0.05$) compared with those for the MC group. However, there were no significant differences in the contents of n-butyrlic and i-valeric acids. In brief, EPSs-2 treatment could promote the production of SCFAs more efficiently compared with CEPSs.

3.5. EPSs regulated gut microbiota in Cy-induced mice

The α-diversity indexes evaluate the richness and diversity of the gut microbiota among groups. As shown in Fig. 5A, Chao1 index, reflecting the species richness, was significantly reduced ($p < 0.05$) in the MC group compared with the NC group. When compared with those in the MC group, the values of Chao1, Shannon and Simpson indexes were significantly increased ($p < 0.05$) in the EPSs-2 group, indicating that the treatment of EPSs could increase the microbial community diversity. Moreover, EPSs-2 could significantly improve ($p < 0.05$) the richness and diversity of microbial community compared with the MC group.

Hierarchical cluster and principal component analysis (PCA) were used to assess the differences among groups in microbial composition. As shown in Fig. 5B, it was found that the MC group was clustered far from the NC group, suggesting the microbial composition was obviously altered by Cy treatment. The CEPSs, EPSs-2 and LH groups were adjacent to the NC group, and the EPSs-2 group was completely separated from the MC group, indicating that the treatment of EPSs-2 could regulate the microbial composition altered by Cy treatment and returned towards that of the NC group. Moreover, PCA results (Fig. 5C) showed the same trend obtained by hierarchical cluster analysis. The microbial structures of the NC and MC groups were obviously different, while the microbial compositions of the CEPSs and EPSs-2 groups shifted in varying degree towards that of the NC group. These results indicated that EPSs had substantial effect on regulating gut microbiota composition in Cy-treated mice.

To figure out the specific microbial variation, the bacterial taxa at different taxonomic levels were analyzed. At the phylum level, gut microbiota was mainly composed of Bacteroidetes and Firmicutes (Fig. 5D), followed by Epsilonbacteriota, Proteobacteria and Deferribacteres. As shown in Fig. 5E, EPSs-2 treatment could significantly increase ($p < 0.05$) the relative abundance of Bacteroidetes that were suppressed in the MC group. Meanwhile, compared with the MC group, the relative abundances of Firmicutes, Epsilonbacteriota and Proteobacteria decreased remarkably ($p < 0.05$) after the treatment with EPSs-2. At the family level, the families with significant differences among groups are shown in Fig. 6A–I. It was found that EPS treatment could notably increase ($p < 0.05$) the relative abundances of Muribaculaceae and Prevotellaceae that were significantly decreased in the MC group. In contrast, the remarkably increased relative abundances of Lachnospiraceae, Helicobacteraceae, Ruminococcaceae, Clostridiales, Desulfovibrionaceae and Deferribacteraceae in the MC group were significantly reversed ($p < 0.05$) by EPSs-2 treatment.

Based on linear discriminant analysis (LDA), the relative abundances of 84 features altered between groups are manifested in a heatmap (Fig. 7). It was found that 28 features (22 down-regulated and 6 up-regulated) were significantly altered due to Cy treatment ($p < 0.05$). Nevertheless, compared with the MC group, the treatments of CEPSs and EPSs-2 could notably alter ($p < 0.05$) 17 (14 increased and 3 decreased) and 45 (33 increased and 12 decreased) features, respectively. Specifically, the features belonging to the genera Muribaculaceae_II, Prevotellaceae_UCG-001 and Muribaculum that obviously decreased in the MC group were significantly enriched ($p < 0.05$) by the administration of EPSs-2. In contrast, EPSs-2 intervention effectively inhibited ($p < 0.05$) the growth of genera Helicobacter, Bilophila, Mucispirillum, Lachnospiraceae_NK4A136_group, Clostridiales_unclassified and Ruminiclostridium that dramatically increased in the MC group.

Finally, LDA effect size (LEfSe) analysis was performed to identify the key phenotypes of gut microbiota altered by Cy and EPSs-2 treatments. As shown in Fig. 8, the relative abundances of Muribaculaceae, Prevotellaceae (Prevotellaceae_UCG-001), Bacteroidaceae (Bacteroides) and Tannnerellaceae (Parabacteroides and Tidjanibacter) depleted in the MC group were enriched in the EPSs-2 group. As shown in the

### Table 1. Concentrations of SCFAs in the cecal contents and feces of mice

<table>
<thead>
<tr>
<th>SCAFs (μmol g⁻¹)</th>
<th>Group</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>i-Butyric acid</th>
<th>n-Butyric acid</th>
<th>i-Valeric acid</th>
<th>n-Valeric acid</th>
<th>Total SCFAs</th>
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<tbody>
<tr>
<td><strong>Cecal contents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NC</td>
<td>28.55 ± 5.02b</td>
<td>7.92 ± 1.49c</td>
<td>0.49 ± 0.03d</td>
<td>11.86 ± 1.07c</td>
<td>0.63 ± 0.06d</td>
<td>0.99 ± 0.07d</td>
<td>50.43 ± 4.46c</td>
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<tr>
<td>MC</td>
<td>23.39 ± 2.75a</td>
<td>3.73 ± 0.86a</td>
<td>0.15 ± 0.02a</td>
<td>6.52 ± 1.78a</td>
<td>0.14 ± 0.01a</td>
<td>0.18 ± 0.04a</td>
<td>34.10 ± 3.40a</td>
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<tr>
<td>CEPSs</td>
<td>26.66 ± 3.03ab</td>
<td>4.91 ± 0.69ab</td>
<td>0.28 ± 0.04ab</td>
<td>9.89 ± 0.79b</td>
<td>0.33 ± 0.06b</td>
<td>0.58 ± 0.05b</td>
<td>42.66 ± 3.88b</td>
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<tr>
<td>EPSs-2</td>
<td>35.12 ± 2.28c</td>
<td>6.05 ± 1.29b</td>
<td>0.32 ± 0.02c</td>
<td>15.90 ± 1.91d</td>
<td>0.46 ± 0.15c</td>
<td>0.90 ± 0.05c</td>
<td>58.75 ± 4.75d</td>
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<td>LH</td>
<td>24.31 ± 2.06a</td>
<td>5.55 ± 0.42b</td>
<td>0.52 ± 0.03d</td>
<td>10.70 ± 0.78bc</td>
<td>0.75 ± 0.06e</td>
<td>0.91 ± 0.11cd</td>
<td>42.74 ± 2.35bc</td>
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<tr>
<td><strong>Feces</strong></td>
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<tr>
<td>NC</td>
<td>24.15 ± 2.09b</td>
<td>4.25 ± 0.74b</td>
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<tr>
<td>MC</td>
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<td>2.48 ± 0.70a</td>
<td>0.21 ± 0.04a</td>
<td>2.72 ± 0.46a</td>
<td>0.41 ± 0.04a</td>
<td>0.28 ± 0.08a</td>
<td>23.80 ± 4.52a</td>
<td></td>
</tr>
<tr>
<td>CEPSs</td>
<td>22.93 ± 3.79b</td>
<td>2.94 ± 0.76a</td>
<td>0.31 ± 0.06b</td>
<td>2.90 ± 0.80a</td>
<td>0.47 ± 0.07ab</td>
<td>0.43 ± 0.09b</td>
<td>29.98 ± 3.78bc</td>
<td></td>
</tr>
<tr>
<td>EPSs-2</td>
<td>24.23 ± 5.05b</td>
<td>4.06 ± 0.83b</td>
<td>0.32 ± 0.07b</td>
<td>3.42 ± 1.29a</td>
<td>0.49 ± 0.09ab</td>
<td>0.49 ± 0.07b</td>
<td>33.01 ± 6.25bc</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>20.10 ± 3.93ab</td>
<td>4.09 ± 0.37b</td>
<td>0.40 ± 0.07c</td>
<td>3.07 ± 0.59a</td>
<td>0.52 ± 0.09b</td>
<td>0.22 ± 0.06a</td>
<td>28.39 ± 3.61ab</td>
<td></td>
</tr>
</tbody>
</table>

Mean values in the same column with different letters were significantly different among different groups in cecal contents and feces, determined by Duncan test ($p < 0.05$).
cladogram, the species in Epsilonbacteraeota, Deferribacteres, Proteobacteria and Firmicutes were enriched in the MC group. However, the treatment of EPSs-2 suppressed the growth of species belonging to Helicobacteraceae (*Helicobacter*), Deferribacteraceae (*Mucispirillum*), Desulfovibrionaceae (*Bilophila* and *Desulfovibrio*), Ruminococcaceae (*Ruminiclostridium*), Lachnospiraceae (*Lachnospiraceae_NK4A136_group*) and Clostridiales that were promoted in the MC group.

### 3.6. Effects of EPSs-2 on the functional changes of gut microbiota

KEGG pathway enrichment analysis was performed by PICRUSt to predict the metabolic function and interpret the functional changes of gut microbiota (Fig. 9). As shown in Fig. 9A, 29 pathways (7 enriched and 22 depleted) were significantly altered in Cy-treated mice. However, 6 pathways (signal transduction,
environmental adaptation, cell motility, membrane transport, transcription, xenobiotics biodegradation and metabolism) enriched in the MC group were remarkably decreased in the EPSs-2 group. In addition, 22 pathways (immune system, digestive system, energy metabolism, amino acid metabolism, enzyme families, etc.) depleted in the MC group were all notably enriched by EPSs-2 treatment. Interestingly, another 2 enriched pathways (carbohydrate metabolism and lipid metabolism) were also enriched in the EPSs-2 group. In a word, the metabolic function altered by Cy treatment was reversed and recovered to the normal status by EPSs-2 treatment.

4. Discussion

As a potent immunosuppressive agent, Cy can affect the organism's immune system and induce the imbalance of immune function, which is manifested by a decrease in body mass, immune organ indexes, cytokines and immunoglobulin. The treatment of Cy in mice not only causes damage to the mucosal barrier (increasing the amounts of potentially pathogenic bacteria and intestinal permeability), but also affects the composition of gut microbiota with the disruption of gut immunity. Therefore, it is necessary to develop effective functional components from daily diets to regulate immune system and manipulate gut microbiota to maintain intestinal homeostasis. Recently, the regulating effects of polysaccharides on immune response associated with gut microbiota in Cy-induced mice have received increasing attention. In the present study, therefore, the interactions among EPSs from A. cristatus, immune function and gut microbiota were investigated by the Cy-induced immunosuppressive mice model.

As reported previously, the mice induced by Cy exhibited rough fur and weight loss, and were anorectic and apathetic. Herein, the treatment with CEPSs or EPSs-2 could alleviate the symptom of weight loss. In particular, the EPSs-2 group obviously exhibited a better recovery, indicating that EPSs-2, as the main fraction of CEPSs, could recover the body injury induced by Cy. It is well known that the spleen and thymus are vital immune organs to reflect the immune functions of the
The spleen can produce immune cells (lymphocytes, monocytes, and plasma cells) and synthesize antibodies in response to most blood-borne antigens, while the thymus is considered as the main site for the development, differentiation, maturation and immune response of T and B cells. It was found that Cy-induced immunosuppressive mice exhibited shrinking of the spleen and thymus, indicating the decline in immune function. Notably, the present results demonstrated that EPSs-2 significantly increased thymus and spleen indexes compared with the MC group, alleviating the immunosuppressive activity induced by Cy to a certain extent.

Cytokines, such as TNF-α and IL-1β, as intercellular signaling proteins, play vital roles in the host defense system and induce the expression of other immune-regulatory and inflammatory mediators in the organism. IgA, as the main type of immunoglobulin secreted by plasma cells (differentiated B cells), is an important component of humoral immunity. Moreover, cytokines and immunoglobulins could transfer from the intestinal immune system to spleen and peripheral lymph nodes, which enhances the systemic immune response. Cy could lead to the suppression of the immunocyte-mediated action, and as expected in this study, Cy treatment significantly inhibited the expressions of TNF-α, IL-1β, IFN-γ and IgA in the organism with an imbalance of immunity.

It has been reported that immunomodulatory activities of polysaccharides may be initiated by activating macrophages, lymphocytes and other various effector cells via various receptors such as toll-like receptors. The activated receptors further trigger the intracellular signaling cascades, promoting the secretion of cytokines, which enhances adaptive immune responses. In the present study, the treatment of CEPSs or EPSs-2 could significant promote the production of TNF-α, IL-1β and IFN-γ as well as IgA that were reduced in Cy-treated mice. The results are consistent with the report that exopolysaccharides from Cordyceps sinensis could enhance the release of cytokines TNF-α and IFN-γ in Cy-treated mice.
Fig. 8  LEfSe taxonomic cladogram (LDA score >3) between the NC and MC groups (A), and the MC and EPSs-2 groups (B). Enriched taxa (LDA score >3.6) are shown in the histogram: NC group vs. MC group (C), and MC group vs. EPSs-2 group (D).
The intestine is the largest immune organ in the body, and its integrity of intestinal barrier is vital for defense against the invasion of microorganisms. However, previous studies have demonstrated that Cy treatment could damage the intestinal mucosa with impaired intestinal epithelia, destroyed structure of the intestinal villi and the increase of intestinal permeability.11,39 In this study, the MC group showed severe damage to the morphology of jejunum and colon. However, the treatments of CEPSs and EPSs-2 attenuated the intestinal tissue damage, increasing the villus length and the V/C ratio in jejunum as well as returning to normal and intact colon tissues. It has been reported that polysaccharides from Cordyceps sinensis were beneficial for intestinal morphology in the Cy-treated mice of intestinal injury.39 The restoration of intestinal barrier injury by polysaccharides is often related to the increase of tight junctions as well as the expression of mucin, β-defensins and secretory IgA to reinforce intestinal immunity.16

The dysregulation of immune system is often accompanied by the dysbiosis of gut microbiota.40 Accumulating evidence indicates that polysaccharides could regulate the richness and microbial composition to maintain immune function balance and host health.14,15 Our present data revealed that EPSs-2 could enhance the diversity of microbial community to some extent that was reduced by Cy treatment. The results of PCA and hierarchical cluster analysis demonstrated that EPSs-2 could modulate the structure of gut microbiota and exhibit a positive regulatory role in Cy-treated mice. It has been reported that Cy-induced immunosuppression is associated with the dysbiosis of gut microbiota, decreasing the proportion of Bacteroidetes and promoting the proliferation of Firmicutes.9 In this study, their relative abundances were reversed by the treatment of EPSs-2. In Bacteroidetes, Muribaculaceae (called S24-7 previously) is the dominant family in the mouse gut microbiota and versatile with respect to degradation of complex carbohydrates.44 It is positively correlated with immune responses including nuclear factor-κB and natural killer cell signaling.45 Furthermore, Prevotellaceae, often enriched due to the treatment of polysaccharides,34 is associated with the transforming growth factor β3 that regulates the intestinal barrier function.46 In our present study, Muribaculaceae and Prevotellaceae including the corresponding genera Muribaculum and Prevotellaceae_UCG-001 were suppressed by Cy treatment, while EPSs-2 significantly increased the growth of these beneficial bacteria. Besides, EPSs-2 treatment could enhance the relative abundance of Bacteroides, which is consistent with the previous report.28 Bacteroides can ferment polysaccharides to produce volatile fatty acids as an energy source for intestine and secrete lipopolysaccharides as a potent innate immune activator.4,31 Some Bacteroides species can promote host health through stimulating immune system and B. fragilis can regulate gut immune homeostasis by its surface polysaccharide A to induce immune regulatory function.47 Also, Bacteroides can improve the
immune defense including increasing the production of TNF-α and capacity of dendritic cells to induce a T-cell proliferation response. In addition, *Parabacteroides*, as the beneficial microbe, is associated with the maintenance of the intestinal regulatory T cell repertoire to keep the homeostatic balance of intestine and can promote the production of SCFAs to stimulate immune response accompanied with the increase of immune indices. Herein, *Parabacteroides* as well as *Tidjanibacter* belonging to Tannerellaceae were increased due to the treatment of EPSs-2, which is reported for polysaccharides. The present results indicated that intervention of EPSs-2 could balance the structures and metabolic activities of gut microbiota to sustain the immunity. Overall, it could be speculated that EPSs-2 from *A. cristatus*, as prebiotics, affected the immune system associated with the modulation of gut homeostasis, including the restoration of intestinal barrier function, regulation of microbial composition and production of SCFAs in Cy-treated mice.

5. Conclusion

In conclusion, EPSs-2 from *A. cristatus* was demonstrated to have a potent immunoregulatory effect in Cy-induced immunosuppressive mice. The restoration of physical signs was manifested by the increase in BW and immune organ indexes. The levels of cytokines and immunoglobulin (TNF-α, IL-1β, IFN-γ and IgA) were promoted by the treatment with CEPSs or EPSs-2. Moreover, EPS intervention attenuated the intestinal tissue damage of the jejunum and colon in their morphology, improving the intestinal barrier function. Furthermore, EPSs promoted the production of SCFAs in the gut and modulated the microbial composition to maintain gut homeostasis. In particular, EPSs-2, as the main purified fraction of CEPSs, exhibited more efficient biological activity than CEPSs.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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