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Prebiotic, immuno-stimulating and gut microbiota-modulating effects of *Lycium barbarum* polysaccharide

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ABSTRACT

The present study was done to evaluate the prebiotic effect of *Lycium barbarum* polysaccharide (LBP), its effect on murine fecal microbiota composition and innate immune response. Results showed that LBP supports the growth of selective probiotic bacteria with a maximum of 8.23 (log10 cfu/mL) and 6.62 (log10 cfu/mL) for *Lactobacillus acidophilus* and Bifidobacterium longum respectively. *In vivo* studies revealed that the administrations of LBP to mice resulted in an increase in the abundance of the phyla *Proteobacteria* and *Firmicutes*, while reducing the ratio of the phylum *Bacteroidetes*. At the genus level, the administration of LBP stimulated the emergence of some potential probiotic genera (*Akkermansia*, *Lactobacillus*, and *Prevotellaceae*). The concentrations of TGF-β and IL-6 in serum and sIgA in the colon content were enriched significantly after LBP administrations in mice. The thymus index and spleen index of mice treated with LBP displayed significant difference compared to the control group ($P < 0.05$). These findings suggest that LBP is a good source as a potential prebiotic and can enhance the intestinal microbiota and boost beneficial bacteria levels, modulate innate immune response.

1. Introduction

Lycium barbarum (Solanaceae), also named Goji is a well-known traditional medicinal plant in China, having been used for more than 2500 years. The fruits of *L. barbarum* commonly called goji berries (gou qi zi in Chinese) are typically consumed as food supplements and for medicinal purposes. Benefits include the stimulation of liver and kidney function [1–3]. Goji berries contain abundant bioactive molecules such as polysaccharides, scopoletin (6-methoxy-7-hydroxycoumarin), the glucosylated precursor, a stable vitamin C analog 2-O-β-d-glucopyranosyl-l-ascorbic acid, carotenoids, betaine, cerebroside, β-sitosterol, flavonoids, amino acids, minerals, and vitamins [4,5]. Recently, some studies indicated that *Lycium barbarum* polysaccharide (LBP) possesses a wide range of biological properties, including antioxidant and immuno-modulatory effects (reviewed in [6]). LBP can simultaneously induce systemic and local immune responses in H22 tumor-bearing mice [7]. LBP as an adjuvant could improved immune responses against vaccine and increase humoral immunity [8,9]. However, there have been few investigations on potential prebiotic properties of LBP. Research on the properties of LBP is very important due to its potential and its widespread applications in the food and biomedical industries. The objectives of the current study were to investigate the prebiotic effects of LBP *in vitro* and its effect on fecal microbiota composition and innate immune responses in mice.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus acidophilus (CGMCC1.2686) and Bifidobacterium longum (CGMCC1.2186) were obtained from China General Microbiological Culture Collection Center and maintained at −80 °C in de Man Rogosa Sharpe (MRS, Qingdao Top Biotech Co. Ltd., Qingdao, China) broth with 30% (v/v) glycerol. B. longum was cultivated in MRS broth supplied with cysteine-hydrochloride monohydrate (5 g/L, Shanghai Sangon Biotech Co. Ltd., Shanghai, China) at 37 °C under anaerobic conditions. The *L. acidophilus* isolates were cultivated in MRS broth at 37 °C under anaerobic conditions.

2.2. Materials and reagents

LBP was purchased from Xi'an Natural Field Bio-Technique (Xi'an,

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China). Based on GC analysis, the composition of LBP includes arabinose, rhamnose, xylose, mannose, galactose, and glucose [10,11] at molar ratios of 0.18: 0.81: 0.07: 2.17: 0.23: 6.52 [11]. LBP powder was dissolved in normal physiological saline for further experiment, filtered through a 0.22 μm filter, and stored at −20 °C. For oral administration, the concentration of LBP powder was adjusted to 400 mg/mL with physiological saline. Enzyme-Linked Immunosorbent Assay (ELISA) kit for immune factors such as TGF-β, IFN-γ, IL-2, IL-6 and IgA were obtained from Youxuan BioEngineering Co., Ltd. (Shanghai, China), DNA extraction kits were obtained from Qiagen Inc. (Hilden, Germany). All other reagents and chemicals utilized were of an analytical reagent grade.

2.3. Assessment of prebiotic activity of LBP

The prebiotic activity of LBP was assessed *in vitro* through the growth of *L. acidophilus* and *B.* longum as described by Moreno-Vilet et al. [12] with some modifications. MRS carbohydrate-free broth (Qingdao Top Biotech Co. Ltd., Qingdao, China) was used as the basal medium. For the cultivation of B. longum, 5 g/L cysteine hydrochloride monohydrate was added to MRS basal medium, as a reducing agent. 2% (w/v) glucose (MRS-G) and different concentrations of LBP powder (2.5%, 5%, 10%, 15% (w/v)) were added to MRS basal medium as a carbohydrate source. MRS-G was used as control. The broths were inoculated with 5% (v/v) ($1–2 \times 10^9$ cfu/mL) of stationary phase *L. acidophilus* or *B.* longum. The cultures were then incubated at 37 °C for 24 h with agitation (120 rpm) under anaerobic conditions. After incubation, serial dilutions (10^{-1} – 10^{-8}) were performed using sterile normal saline and plated on MRS agar at 37 °C for 48 h under anaerobic condition. The results were recorded as CFU/mL of culture.

2.4. Animal experimental design using mice

Kunming mice of clean grade (8-weeks old; body weight 20.0 ± 2.0 g) were purchased from the Comparative Medicine Center of Yangzhou University (Jiangsu, China), certificate no. SCXK (Su) 2012-0004. All mice were housed in the laboratory animal center of China Pharmaceutical University with a 12-h-dark/12-h-light cycle at 25 °C. They were allowed free access to standard diet and sterile water throughout the experiments. All experimental procedures were approved by the Animal Care and Use Committee of China Pharmaceutical University, China.

Mice were randomly divided into two groups (7 in each group) and were allowed to acclimate for 1 week before the experiment. Experimental group (S) received LBP for 14 days at a dose of 0.1 mL/10 g body weight [13], whereas control group (D) were given the same volume of physiological saline *via* intragastric administration. Twenty-four hours after the last drug administration, the mice were weighed and killed by decapitation. The blood was collected to separate serum. Moreover, the spleen and thymus were excised from the sacrificed mice and weighed immediately.

2.5. Immune organ indexes and cytokine detection

The viscera indices were measured, to assess any immune function alteration during experimentation. Thymus and spleen indices were calculated according to the following formula: thymus or spleen index = weight of thymus or spleen (mg)/body weight (1000 mg) as described by Xu et al. [14]. The concentrations of immune factors TGF-β, IFN-γ, IL-2, IL-6 and IgA in the serum and sIgA in colon were detected using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (Youxuan BioEngineering, Shanghai, China) according to the manufacturer's instructions.

2.6. Fecal DNA extraction and high throughput sequencing and bioinformatic analysis of sequencing data

Microbial DNA from the cecal content samples was extracted using QIAamp DNA stool mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the DNA was confirmed using a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE). The V3-V4 region of the 16S rDNA was PCR amplified from the microbial genomic DNA using universal primer (341F: 5′−CCTAYGGGRBGCASCAG-3′, 806R: 5′- GGACTACNNGGGTATCT AAT-3′) [15]. The PCR condition were 94 °C for 4 min, followed by 21 cycles of 94 °C for 30 s, 58 °C for 30 s (annealing) and 72 °C for 30 s (extension), and then a final extension 72 °C for 5 min. Amplicons were extracted from 2 % agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Purified PCR products were quantified by Qubit®3.0 (Life Invitrogen) and every twenty-four amplicons whose barcodes were different were mixed equally. The pooled DNA product was used to construct an Illumina Pair-End library following Illumina's genomic DNA library preparation procedure. The amplicon library was paired and sequenced on an Illumina MiSeq platform (Shanghai BIO-ZERON Co., Ltd) according to the standard protocols. The collected data were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP127636). Pyrosequencing paired end reads were assigned, truncated and merged using Pandaseq software (V 2.7) to generate raw tags. High quality clean tags from the raw tags were obtained and compared with reference database to detect and remove chimeric sequences using Usearch (version 7.1 http://drive5.com/uparse/) to obtain effective tags. QIIME platform (http://qiime.org/scripts/assign_taxonomy.html) was used to analyze the effective tags, and tags with ≥ 97% similarity were assigned to the same operational taxonomic unit (OTU). The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed using the RDP Classifier (version 2.2 http://sourceforge.net/projects/rdp-classifier/) against the Silva (SSU115) 16S rRNA database, with a confidence threshold of 70% [16]. Principal component analysis (PCA) and weighted Fast UniFrac principal coordinate analysis (PCoA) based on OTUs were performed to provide an overview of gut microbial dynamics in response to with or without LBP treatments. Analysis of Community richness (Chao1 and Ace), community diversity (Shannon, Simpson), Sequencing depth (Good's coverage) and Observed species, and beta diversity on both weighted and unweighted unifrac distance metrics of OTUs were calculated with the MOTHUR program (http:// www.mothur.org) [17].

2.7. Statistical analysis

Statistical analysis was performed with SPSS software (SPSS, Version 22.0, SPSS Inc., Chicago, Ill., U.S.A.). Values were expressed as mean ± SD. Two-tailed Student's *t*-test and one-way ANOVA were used to determine the difference among Goji treated groups and the control group. $P < 0.05$ was considered to be statistically significant. The QIIME pipeline version 1.9.1 was used for the sequencing data analysis. Within community diversity (alpha diversity) was calculated using observed OTUs, Chao1 and Shannon indexes with all sampling repetitions at each sampling depth. Analysis of similarity (ANOSIM) and the ADONIS test were used to determine statistical differences between samples (beta diversity) following the QIIME compare_categories.py script and using weighted and unweighted phylogenetic UniFrac distance matrices. Principal Coordinate Analysis (PCoA) plots were generated using the QIIME beta diversity plots workflow. The biplot function was used to visualize samples and taxa in the PCoA space.

Table 1
Effect of *L. barbarum* polysaccharide on the growth of *Lactobacillus acidophilus* and Bifidobacterium longum in MRS.

Organism	Control	Concentration of LBP (%)			
		2.5	5	10	15
L. acidophilus	7.62 ± 0.16[a]	7.78 ± 0.46[a]	8.23 ± 0.30[b]	8.17 ± 0.21[b]	7.89 ± 0.19[a]
B. longum	5.87 ± 0.14[a]	6.34 ± 0.11[b]	6.41 ± 0.23[b]	6.53 ± 0.32[b]	6.62 ± 0.26[b]

Values are expressed as the mean of the log10 number ± SD (CFU) per mL of MRS. a, b within the same row, mean values with different letters are significantly different at $P < 0.05$, n = 3.

3. Results

3.1. In vitro Prebiotic effect of LBP

The results of the effect of LBP on the growth of *L. acidophilus* and *B. longum* were expressed as the total viable counts (log_{10} cfu/mL) as shown in Table 1. Maximum growth of *L. acidophilus* occurred at 5 % LBP concentration and declined at higher concentrations. In contrast, *B. longum* growth increased with increasing concentrations of LBP present.

3.2. The immune response analysis

The spleen and thymus indices were determined and presented in Table 2. The spleen index and thymus index of mice treated with LBP displayed significant difference as compared to the control group ($P < 0.05$) which increased 22.19% and 44.05% respectively. In addition, the secretion of immune cytokines in serum and gut contents were detected as shown in Fig. 1A and B. The concentrations of TGF-β and IL-6 in serum and sIgA in the colon content were enriched significantly in the LBP treatment group ($P < 0.05$). However, the IFN-γ and IL-2 concentrations and IgA in serum exhibited no significant change after the LBP administration.

3.3. Effects of LBP intake on the composition of cecal gut microbiota

High-throughput sequence analysis of bacterial 16S rRNA V3-V4 region was conducted on cecal content samples. After filtering the low-quality reads, trimming the longer homopolymer runs, adapters, barcodes and primers, removing all cyanobacteria/chloroplast sequences and rarefying the datasets, the analysis revealed a highly diverse microbiota with a total of 612514 sequences, representing 530 OTUs. All novel sequence data have been deposited in NCBI Sequence Read Archive under the accession number SRP127636. The relationships among samples were evaluated based on differences in phylogenetic diversity. Using the weighted-UniFrac metric, principal component analysis (PCA) and principal co-ordinates analysis (PCoA) plots were calculated from weighted UniFrac distances for the evaluation of the community composition, the results demonstrates a clear separation between each groups (Fig. 2A and B), and indicates that there is clear dissimilarity between the gut microbiota fed with or without LBP intake. At the phylum level, the bacterial communities in LBP treated group were dominated by three phyla: *Firmicutes*. *Bacteroidetes* and *Proteobacteria*, but the higher relative abundance of *Firmicutes* and *Proteobacteria* were observed after oral administration of *LBP* (Fig. 2C). From Fig. 2D, we found that at the genus level, the percentage of beneficial bacteria such as *Akkermansia, Lactobacillus,* and *Prevotellaceae* increased significantly after LBP administration. Fig. 2E shows the comparative presentation of the bacterial taxonomic analysis of intestinal microbiota composition between the experimental and control groups for 14 days.

Table 2
Effect of *L. barbarum* polysaccharide treatments on viscera index.

Groups	spleen index (mg/1000 mg)	thymus index (mg/1000 mg)
Control	4.19 ± 0.86	2.86 ± 0.75
LBP group	5.12 ± 0.94*	4.12 ± 0.63*

* $P < 0.05$, n = 7.

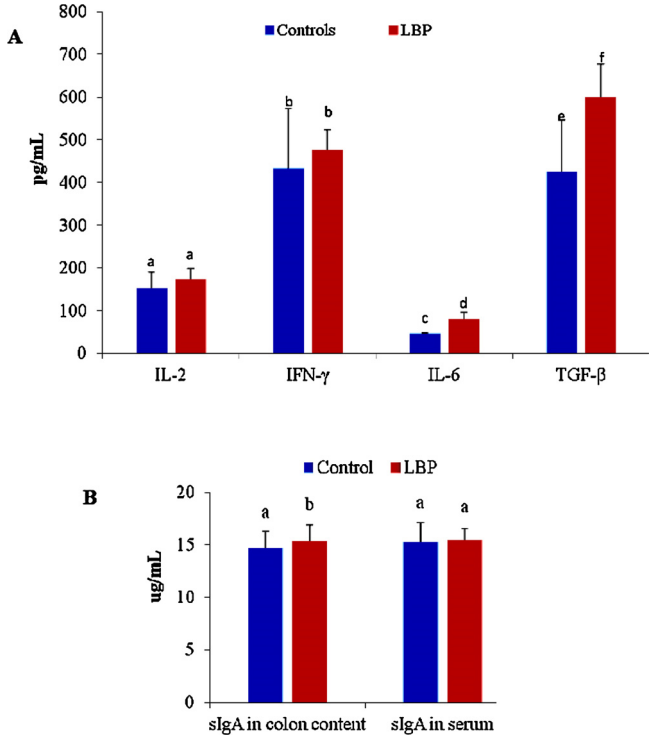
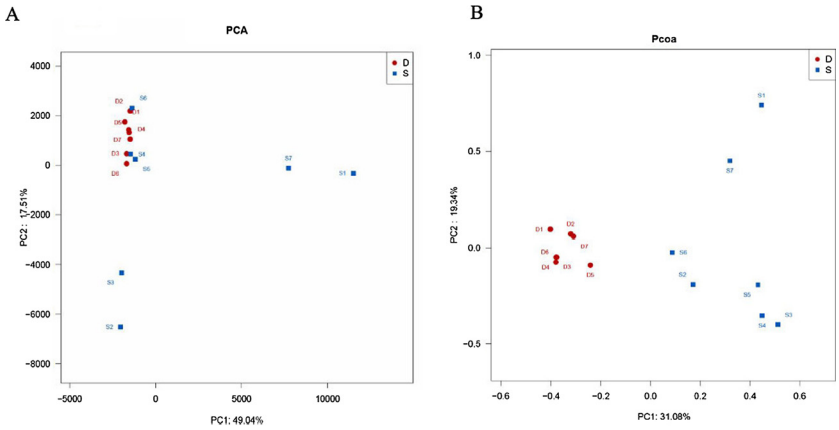
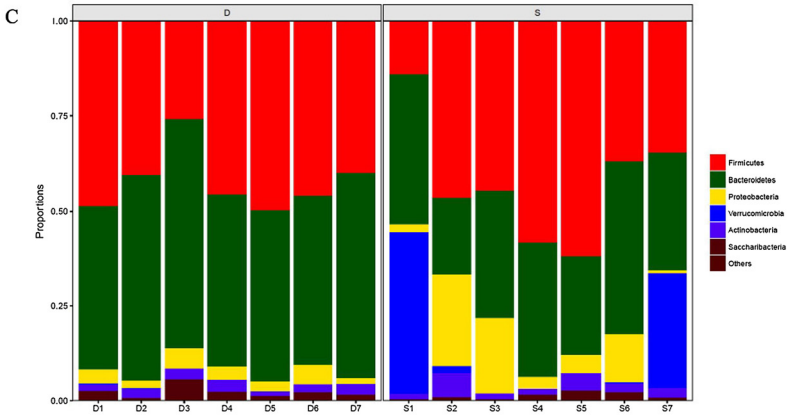


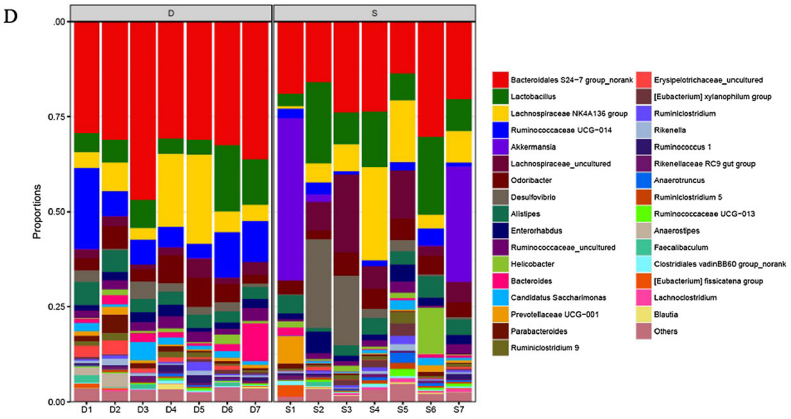
Fig. 1. Concentrations of cytokines in serum (A) and IgA in serum and sIgA in colon content (B) in mice with/without *L. barbarum* polysaccharide intake. Values are expressed as the mean ± standard deviation (n = 7). Different lowercase letters mean significant difference ($P < 0.05$).

4. Discussion

Prebiotics are non-digestible ingredients which can selectively promote the growth and/or activity of specific "good" bacteria and can modulate gut microbiota [18]. Dietary polysaccharides can increase the ratio of probiotic bacteria, regulate the intestinal microenvironment such as declining the gut pH, and stimulate the innate immune like macrophages or lymphocytes in intestinal mucosa to fight against infections [19]. *Lycium barbarum* is an old food-herbal medicine used in China. Carbohydrates are major components in *Lycium barbarum* (about 51%) [20] and contains different monosaccharides such as arabinose, mannose, glucose, rhamnose, xylose, galactose and galacturonic acid [21]. Several animal studies show that LBP diets enhance immune response to infections and tumors, as well as scavenging free radicals [6,22,23]. Different monosaccharide composition and molecular structure of LBPs are reported in the literature and likely reflect the biodiversity of *L. barbarum* and different purification and analysis protocols [24]. In this study, we found that LBP has the ability to







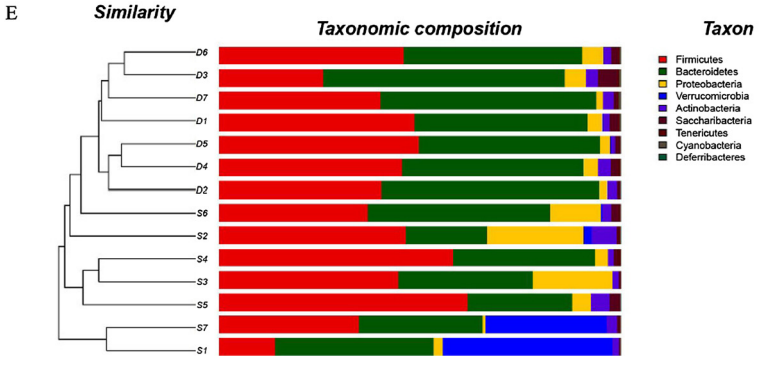


Fig. 2. *L. barbarum* polysaccharide intake promotes modification of gut microbiota composition in mice. Cecal gut microbiota was analyzed using 16S rRNA sequencing in mice. Experimental group (S) was fed with *L. barbarum* polysaccharide and was compared to a group without *L. barbarum* polysaccharide (D). (A), (B) Principal component analysis (PCA) and principal coordinates analysis (PCoA) plots for different bacterial communities using Bray-Curtis metric and weighted Unicfrac distances, respectively. (C) Bars represent the relative abundance (%) of bacterial phyla (D) The relative abundance of bacterial genera (%) that with and without *L. barbarum* polysaccharide supplementation. (E) Taxonomic diversity of the cecal microbiota of mice with or without supplementation with *L. barbarum* polysaccharide.

support the growth of probiotic bacteria such as *L. acidophilus* and B. longum when cultured in MRS medium *in vitro*. The results of our study confirmed the growth promotion activity of LBP and support previous work [8,25,26].

The diet supplementation of LBP lead to increase in the abundance of phylum *Proteobacteria, Firmicutes*, while reducing the ratio of the phylum *Bacteroidetes* in the gut (Figs. 2C and E). At the genus level, the administration of LBP tended to stimulate the emergence of some potential probiotic bacteria such as *Akkermansia, Lactobacillus,* and *Prevotellaceae*. Plant polysaccharide-rich diet can balance the microbial communities [27]. Many traditional Chinese medicines are food and medicine homologs, which have prebiotic properties and can alter the composition of the faecal flora. For example, Chinese yam, the rhizome of various species of genus *Dioscorea opposita Thunb.* (Dioscoreaceae), is an extensively cultivated food in China. Dietary fiber and polysaccharide from Chinese yam can increased the number of *Bifidobacterium longum* JCM1217 *in vitro* [28], and in mouse studies, the yam powder increased the total bacteria concentration and *Bifidobacteria* composition, and suppressed *Clostridium perfringens* composition [29]. Another famous Chinese medicine, Panax ginseng, also has prebiotic activity. It has been reported that ginseng can stimulate *Lactobacillus, Bifidobacterium* and can inhibit pathogens such as *Escherichia coli, Staphylococcus aureus* and *Salmonella in vitro,* also it can stimulate *Lactobacillus* proliferation significantly *in vivo* [30,31]. In human volunteer trials, ingestion of Panax ginseng extracts, caused fecal bacterial composition changes. The dominant genera switched from *Blautia*, *Bifidobacterium*, and *Anaerostipes* to *Bifidobacterium*, *Blautia*, and *Faecalibacterium* [32]. The prebiotic function of many Chinese medicines is mainly due to high levels of dietary fiber, polysaccharides and polymerized polyphenols and certain other substances, which are beneficial to probiotic bacteria [33].

Plant polysaccharides have the function of regulating host defense against infection through gut microbiota [34]. The gut microbiota prevents exogenous pathogen infection through direct (competition for common nutrients and niches) and indirect (enhancement of host defense) mechanisms. Polysaccharides have prebiotic activities which can stimulate beneficial bacteria growth and host immune response [35]. Benefits of probiotic bacteria include production of some acids, bacteriocins, short chain fatty acids (SCFAs), *etc.* that can stimulate host innate immune response [36,37]. In this study, we found that LBP can enhance innate immune immunity. As shown in Table 2, the thymus index and spleen index of mice treated with LBP displayed a significant difference compared to the control group ($P < 0.05$). The concentrations of TGF-β and IL-6 in serum and sIgA in the colon content enriched significantly in the LBP treatment group ($P < 0.05$) (Fig. 1). The results indicated that LBP could effectively enhance the host innate immune response. These findings are consistent with other reports. Tang et al. reviewed the anticancer and immunomodulatory effects of *L. barbarum* fruit [38]. Chen et al. found that polysaccharide from *L. barbarum* can enhance the host immunity by activating macrophages [39]. It can also increase the expression of TNF-a, IL-1b, IL-12, and enhance the production of TNF-α in a dose dependent manner. Mo et al reported that *L. barbarum* extracts have the ability to enhance the growth and innate immunity of grass carp and Nile tilapia [40].

5. Conclusion

In conclusion, the present study provided the experimental support for the prebiotic effects of LBP, and results showed that it can enhance the growth of beneficial bacteria *in vitro*, also can modulate the intestinal microbial communities and enhance the innate immunity in mice. With the increasing concerns about prebiotic use in the health industry, we suggest that the combination of LBP with probiotics to symbiotics may be needed to obtain beneficial effects in practice.

Author contributions statement

WC, JL, and RM designed the research. SZ performed sampling. WZ and SZ performed experimental work. SZ performed data analysis, WC wrote the manuscript with critical revision by RM and input from all authors.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

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