Lactiplantibacillus plantarum DLPT4 protects against cyclophosphamide-induced immunosuppression in mice by regulating immune response and intestinal flora

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Abstract

In this study, the strain *Lactiplantibacillus plantarum* DLPT4 was investigated for the immunostimulatory activity in cyclophosphamide (CTX)-induced immunosuppressed BALB/c mice. *L. plantarum* DLPT4 was administered to BALB/c mice by oral gavage for 30 days, and CTX was injected intraperitoneally from 25th to 27th days. Intraperitoneally CTX injection caused the damage to the thymic cortex and intestines, and the immune dysfunction of the BALB/c mice. While *L. plantarum* DLPT4 oral administration exerted immunoregulating effects evidenced by increasing the serum immunoglobulin A (IgA), IgG and IgM levels, and reducing the expression of pro-inflammatory factors (IL-6, IL-1β and TNF-α) of the BALB/c mice with intraperitoneally CTX injection.

The results of the BALB/c mice feces metagenome-sequencing showed that *L. plantarum* DLPT4 oral administration can regulate the intestinal microbial community by changing the ratio of *Lactiplantibacillus* and *Bifidobacterium*.

Meanwhile, the abundance of carbohydrate enzyme (CAZyme), immune diseases metabolic pathways and p38 and AP-1 MAPK signaling pathways were enriched in the BALB/c mice with *L. plantarum* DLPT4 oral administration.

In conclusion, *L. plantarum* DLPT4 administration ameliorated CTX-induced immunosuppression in BALB/c mice by regulating gut microbiota, and further influencing the abundance of carbohydrate esterase in the intestinal flora and increasing the immune metabolic activity. *L. plantarum* DLPT4 could be used as promising probiotics to regulate immune response.

Highlights

• *Lactiplantibacillus plantarum* DLPT4 oral administration could increase the serum immunoglobulin A (IgA), IgG levels of CTX-induced mice.

• *Lactiplantibacillus plantarum* DLPT4 oral administration restored gut microbiota of CTX-induced immunosuppression in mice

• *Lactiplantibacillus plantarum* DLPT4 oral administration could enrich the abundance of carbohydrate enzyme (CAZyme), immune diseases metabolic pathways and p38 and AP-1 MAPK signaling pathways.

Introduction

Immunity is defined as an organism's ability to resist harmful foreign substances through identification and destruction. The immune response is categorized as innate immunity and acquired immunity (Fang et al., 2015). The immune system is a network of cells to protect human beings, where the innate immunity is the first line of defense-enhanced initial protection against infection (Chávez-Sánchez et al., 2014). Hypo immunity may cause fever and lymphadenopathy, and children with weakened immunity are

more likely to suffer from lung infection, leading to acute lung injury or pediatric acute respiratory distress syndrome (PARDS) (Amna et al., 2016). The intestinal mucosa-associated lymphatic tissue as an immunological organ can block the invasion of harmful antigens to the host (M et al., 2001). Cyclophosphamide (CTX) is an effective immunosuppressant, which induces the production of reactive oxygen species (ROS), and excessive production of ROS may lead to tissue damage and loss of many immune functions (Ke et al., 2013, Chao et al., 2018). Moreover, CTX could induce apoptosis of immune cells, which may affect the mucosal barrier, reduce the abundance of intestinal flora (Xu and Zhang, 2015, In and Soo, 2017) and change the composition of host intestinal microorganisms (Hongjie et al., 2017).

Most immunomodulatory drugs are not suitable for long-term or preventive use. Therefore, there is a growing interest in finding new immunomodulators to improve nonspecific host defense mechanisms, including probiotics and fermented products (P et al., 2001, Wang et al., 2010, Junhua et al., 2015). The diversity and abundance of gut microbiota is critical for host health. While probiotics can benefit the intestinal tract, which related to host health. Probiotics prevent some bacteria pathogens from reaching the colonic lamina propria through competitive inhibition, and stimulate the mucosal immune system at the same time (Mudireddy et al., 2014). Kai et al. (2017) reported that *L. plantarum* administration could relieve the duodenal inflammation of mice with enteritis (Kai et al., 2017). Garcia-Castillo et al. (2019) reported that feeding mice with *L. fermentum* UCO-979C significantly increased the production of intestinal IFN-γ and IgA, stimulated intestinal and peritoneal macrophages and increased the number of Peyer's patches $CD4^+$ T cells (Garcia-Castillo et al., 2019). Particularly, probiotics can alleviate adverse reactions caused by colonization of pathogenic bacteria, such as intestinal mucosal damage, flora imbalance (Leimin et al., 2019). Under specific intestinal conditions, intestinal flora of the host can activate the key carbohydrates enzymes and metabolic pathways to improve the production of the secondary metabolite such as short-chain fatty acids (SCFAs) (Makki et al., 2018), which could be used as carbon and energy source by other bacteria (R et al., 2016). Meanwhile, the increase of SCFAs concentration in intestinal tract is also an important contribution of probiotics to regulate intestinal flora in mice (Selvasankar et al., 2018). The development and maturity of the host systemic immune system determines the composition of intestinal flora, and in turn, intestinal flora guides the development of immune response (Yujiao et al., 2019).

In this study, the effect of *L. plantarum* DLPT4 oral administration on immunity function of the CTX-immunosuppressed BALB/c mice was studied via the assessment of the mice body weight, thymus index, and immunoprotein (IgA, IgG, IgM). The intestinal flora composition and functional differences of the BALB/c mice were analyzed by metagenomics to explore how *L. plantarum* DLPT4 oral administration exert influence on the composition and the metabolic activity of intestinal microbes, and the immunity function of the immunocompromised mice.

Materials And Methods

2.1 Bacteria strains and culture condition

Lactiplantibacillus plantarum DLPT4 was isolated from traditional fermented marine fish in Liaoning Province and stored in de Man, Rogosa, and Sharp (MRS) medium (AOBOX biotechnology, Qingdao, China) containing 25% glycerol at -80°C by Dalian probiotics function research key laboratory, Dalian Polytechnic University. *L. plantarum* DLPT4 was cultured in MRS broth (Land Bridge technology) at 37°C for 18 h twice prior to use.

Acid resistance

Acid resistance was preformed according to the method described by Overbeck et al. (2017) (Overbeck et al., 2017). The pH values of MRS medium were adjusted to 2.0, 3.0 and 4.0, *L. plantarum* DLPT4 was inoculated in the medium at the volume ratio of 2% and cultured at 37°C for 12 h. Then, the viable bacteria in the culture at 0 h and 12 h was determined by pouring plate method on MRS agar. The growth rate of *L. plantarum* DLPT4 was calculated as follows:

$$\mathrm{growthrate}\,(\%) = \mathrm{Log}(\mathrm{N}_2 - \mathrm{N}_1)/\mathrm{Log}\mathrm{N}_1 \times 100\%$$

where N_1 (CFU/mL) represents the initial number of viable bacteria in the MRS medium with different pH value at 0 h and N_2 (CFU/mL) represents viable bacteria in the MRS medium with different pH value at 12 h.

Bile salt tolerance

Bile salt tolerance was preformed according to the method described by Hsin et al. (2007) (Hsin et al., 2007). MRS culture medium with bile salt content of 0.1%, 0.2%, 0.3% and 0.4% respectively, were inoculated with *L. plantarum* DLPT4 according to volume ratio of 2% and cultured at 37°C for 12 h. Then, the number of colonies was measured for 0 h and 12 h respectively by pouring plate method on MRS agar. The growth rate of *L. plantarum* DLPT4 was expressed as:

$$\mathrm{growthrate}\,(\%) = \mathrm{Log}(\mathrm{N}_2 - \mathrm{N}_1)/\mathrm{Log}\mathrm{N}_1 \times 100\%$$

where N_1 represents the initial number of viable bacteria in the MRS medium with different bile salt contents at 0 h (CFU/mL) and N_2 represents viable bacteria in the MRS medium with different bile salt contents at 12 h (CFU/mL).

Simulated gastrointestinal fluid tolerance

Simulated gastric juice and intestinal juice was prepared according to the method described by Bao et al. (2010) (Bao et al., 2010). *L. plantarum* DLPT4 was inoculated in simulated gastric juice with pH 2.5 according to volume ratio of 2%. The viable count of *L. plantarum* DLPT4 in the simulated gastric juice (pepsin solution concentration of 3.0 g/L) was measured after 3 hours by pouring plate culture. After culturing in gastric juice for 3 hours, 0.5 mL of the culture was inoculated into 4.5 ml of simulated

intestinal juice (pH 8.0, trypsin (1.0g/L) and bile salts (1.8%) solution) at 37 ℃. The viable count of *L. plantarum* DLPT4 in the simulated intestinal juice was measured after 8 hours by pouring plate method. Survival rate was calculated according to the following equation:

$$\mathrm{survivalrate}\,(\%) = \mathrm{LogN}_1/\mathrm{LogN}_2 \times 100\%$$

where N_1 represents the number of viable bacteria treated by simulated gastric juice or intestinal juice (CFU/mL), and N_2 represents initial viable count of strains (CFU/mL).

Cell Culture

RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (CBCAS), Shanghai, China, and cultured in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 20% fetal bovine serum (Biological Industries, Israel), penicillin (100 U/ml) and streptomycin (100 U/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37°C in the incubator with 90% humidity and 5% CO_2. The culture medium was replaced every 24–48 h to maintain the cells.

Cell Cytotoxicity Assay

RAW264.7 cells were seeded at a density of 5×10^5 cells/mL in a 96-well microplate (Corning Inc.) in 100 μL of DMEM for 24 h at 37°C. After washing twice with PBS (pH 7.4), the cells were treated with 100 μL of sample (10^7,10^8,10^9, or 10^{10} CFU/mL of *L. plantarum* DLPT4 suspended in DMEM) or DMEM alone (control group) for 24h at 37°C. To assess cell viability, cells were washed with PBS twice and then incubated with methylene blue (98% Hanks' Balanced Salt Solution, HBSS, 0.67% glutaraldehyde, and 0.6% methylene blue) at 50 μL/well for 1 h at 37°C. After incubation, the cells were washed with deionized water 6 times until the water clarification, and 100 μL/well of elution solution (49% PBS, 50% ethanol, and 1% acetic acid) was added. Then, the microplate was shaken on a microplate oscillator (IKA, Wilmington, DE) for 20 min and absorbance was measured at 570 nm. Different samples were compared with control. The sample was considered cytotoxic when the absorbance was decreased by > 10% compared to the control (Xing et al., 2017).

Anti-inflammatory Effect of L. plantarum DLPT4 in vitro

Construction of cell inflammation model was prepared according to the method described by Ma et al. (2018) (Ma et al., 2018). The experiment was divided into 6 groups and LPS was used to induce cell inflammation in vitro with LPS concentration of 1 μg/mL: Control group, RAW264.7 cells treated by only DMEM with 200 μL/well, LPS group, RAW264.7 cells treated by only DMEM containing LPS with 200 μL/well, Competition group (CG), RAW264.7 cells treated by LPS with 100 μL/well and *L. plantarum* DLPT4 with 100 μL/well together, Prevention group (PG), RAW264.7 cells treated by *L. plantarum* DLPT4 with 100 μL/well first, prior to LPS with 100 μL/well treatment, Therapy group (TG), RAW264.7 cells treated by LPS with 100 μL/well prior to *L. plantarum* DLPT4 with 100 μL/well treatment.

Cells were seeded in 96-well plates (4×10^5 cells per well) in 100 μL of DMEM and allowed to grow for 24h at 37°C. After that, the cells were incubated as described above. The cell culture supernatant was collected and the detection of NO, TNF-α, IL-8, and IL-10 content was carried out by ELISA kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.2 Animal Experiment

Forty-four male Specific Pathogen-Free (SPF) BALB/c mice with the body weight of 22.0 ± 2.0 g were obtained from Liaoning Changsheng Biotechnology Co, Ltd. (Benxi, Liaoning, China). Before starting the animal experiment, the animals were reared adaptively for one week. All the mice were housed at a constant temperature of 24 ± 1°C with a 12 h light-darkness cycle, free drinking water and feed. The animal experiment was reviewed and approved by the Institutional Animal Care and Use committee of Dalian Polytechnic University (SYXK2017-0005, Dalian, Liaoning, China) and conformed to the National Institute of Health guidelines on the ethical use of animals in China.

2.3 Experimental Design

All BALB/c mice were randomly divided into four groups (n = 11 each group). Groups were named as follows and the experiment period was 30 days. (1) Normal group (NG), were given only saline water by gavage administration once a day; (2) Model group (MG), were given only saline water by gavage administration once a day; (3) Low *L. plantarum* DLPT4 concentration group (DLPT4-L), 1.0×10^7 CFU/mL of *L. plantarum* DLPT4 (10 mL/kg body weight (BW)); and (4) High *L. plantarum* DLPT4 concentration group (DLPT4-H), 1.0×10^9 CFU/mL of *L. plantarum* DLPT4 (10 mL/kg BW). Mice in MG, DLPT4-L and DLPT4-H were intraperitoneally injection with 100 mg/kg BW of CTX on Day 25, 26 and 27, respectively. During experiment, body weight was recorded twice a week. On the 30th day of the experiment, feces were collected. Mice were sacrificed by cervical dislocation and a handsome amount of blood was collected from the eyeball of mice. The colons were separated from the proximal rectum, close to its passage under the pelvisternum. The thymus was also obtained, and their weight was measured.

2.4 Organ index of the mice

The mice were weighed before being sacrificed. And the thymus was weighed immediately. The thymus index was calculated according to follows:

$$\mathrm{Organindex} = \mathrm{thymus\,(mg)\,/bodyweight\,(g)}$$

2.5 Analysis of immunoglobulin in serum of the mice

The levels of the immunoglobulin (IgA, IgM, IgG) in the serum of the BALB/c mice were determined by ELISA kits (ELISAs Kit H108 for IgA, H109 for IgM, H106 for IgG, Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), according to the manufacturer's instructions.

2.6 Histopathology

The isolated thymus, ileum and colon of the mice were immediately fixed with 4% paraformaldehyde for 2 days. Tissue samples of the mice were dehydrated and embedded in paraffin wax, made into 5–8 μm slices. After dewaxing, tissue samples were stained by hematoxylin and eosin (H E) staining and observed under an optical microscope.

2.7 Quantitative expression analysis of cytokines in colon tissue by qPCR

Two-step real-time qPCR was performed to determine the expression of IL-6, IL-1β and TNF-α in colonic tissue of the BALB/c mice according to the methods (Shi et al., 2015, Garcia-Castillo et al., 2019). Total RNA was isolated from colonic tissue sample of each group using TRizol reagent (Invitrogen Ltd., Paisley, United Kingdom). RNA purity and concentration were assessed using NanoDrop TM 1,000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All cDNAs were synthesized using the PrimeScript RT Reagent kit with the treatment of gDNA eraser (Takara-Bio, Japan) according to the manufacturer's recommendations. The PCR cycling conditions were 2 min at 50 ℃, followed by 5 min at 95 ℃, and then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by a dissociation stage of 15 s at 95°C, 1 min at 60°C, 15 s at 95°C and 15 s at 60°C. The reaction mixtures contained 2.5 μL of cDNA and 7.5 μL of master mix, which included the sense and antisense primers. The measurement of β-actin mRNA levels served as internal standards for calibration. The primer sequences are shown in Table 1.

Table 1
Gene primer sequence

Gene name	Primer sequences
β-actin-F	5'-TCA GCA AGC AGG AGT ACG ATG-3'
β-actin-R	5'-AAC GCA GCT CAG TAA CAG TCC-3'
IL-6-F	5'-TGG AAA TGA GAA AAG AGT TGT GC-3'
IL-6-R	5'-CCA GTT TGG TAG CAT CCA TCA-3'
IL-1β-F	5'-TTC ATC TTT GAA GAA GAG CCC AT-3'
IL-1β-R	5'-TCG GAG CCT GTA GTG CAG TT-3'
TNF-α-F	5'-GAT CGG TCC CCA AAG CGA TG-3'
TNF-α-R	5'-GGC TAC AGG CTT GTC ACT CG-3'

2.8 Short chain fatty acids (SCFAs) analysis in the feces of the BALB/c mice

The levels of SCFAs contents in the feces of the BALB/c mice were determined by gas chromatography–mass spectrometry (GC-MS 8890B-5977B Agilent Technologies Inc. CA, UAS). Briefly, fresh feces were dissolved in 900 μL of methanol and 100 μL of 2-ethylbutyric acid and centrifuged at 4°C, 13000 g for 15

min. Then, 50 mg anhydrous sulfuric acid was added to obtain the supernatant for GC-MS assay. HP FFAP column (30 m × 0.25 mm × 0.25 μm, Agilent J & W Scientific, Folsom, CA, USA) was used for separating SCFAs. The electron bombardment ion source (EI) was selected for mass spectrometry analysis. The ion source temperature was 230°C, the quadrupole temperature was 150°C and the transmission line temperature was 230°C and the electron energy was 70 eV. The scanning mode was full SCAN mode (SCAN), and the scanning range was m/z: 30–300.

2.9 DNA extraction and shotgun metagenomics sequencing in the feces of the BALB/c mice

DNA extractions from the feces of the BALB/c mice were performed on 100 mg of feces per sample using A.E.Z.N.A. ™ Soil DNA Kit (Omega Biotek, Inc., Norcross, GA) by following the manufacturer's recommended protocols. The resulting concentrations of genomic DNA were measured by nanodrop, and DNA integrity was examined by agarose gel electrophoresis.

The original sequence of each sample was determined by multi-sample parallel mixed sequencing. The original sequencing data of each sample was subjected to sequencing related quality evaluation, including base quality distribution statistics and A/T/G/C base content distribution statistics. The sequencing linker sequence (adapter sequences at 3' and 5' ends), low-quality base (the average mass value is less than 20), N (N represents uncertain base information) base and sequence with too short length (Length less than 50 bp) in the original sequencing data was deleted (Ruiqiang et al., 2008). Quality control was carried out on the original sequencing data to obtain high-quality clean data to ensure the accuracy of subsequent analysis results. MetaGene software was used for ORF prediction, and CD-HIT was used to build a non-redundant gene catalog (Hideki et al., 2006, Limin et al., 2012). Gene pairs with greater than 95% identity (no gap allowed) and aligned reads covering over 90% of the shorter reads were grouped together. ORFs with a length less than 100 bp were subsequently filtered out (Liang et al., 2017). The raw read data were demultiplexed and converted to fastq format. To assess the taxonomic assignment, genes were aligned to the integrated NR database. All genes in our catalogue were aligned to the KEGG database (http://www.genome.jp/kegg/) and CAZy database (http://www.cazy.org/).

2.10 Statistical analysis

SPSS Statistics 20.0 software (version 20.0, SPSS Inc, Chicago, IL) and the R package analyzed the data. All data were expressed as mean ± standard deviation (SD). The vertical sample t test and one-way ANOVA, $p < 0.05$, showed significant difference. Origin 8.5 software was used for graphs.

Results

3.1 Survival ability of *L. plantarum* DLPT4 in simulated gastrointestinal conditions

As shown in Fig. 1A, L. *plantarum* DLPT4 did not grow in the MRS medium (pH 2.0) with the growth rate of -0.80% after 12 hours incubation, indicating that the acidic condition can inhibit the growth of *L. plantarum* DLPT4 and even lead to the death of *L. plantarum* DLPT4.The mass concentration of bile salt in human small intestine fluid fluctuates from 0.03 to 0.3 g/100 mL (Máire et al., 2005). The growth capabilities of *L. plantarum* DLPT4 in MRS medium with bile salt concentration of 0.1%, 0.2%, 0.3% and 0.4%, respectively, was studied. As shown in Fig. 1B, L. *plantarum* DLPT4 could grow in the MRS medium with bile salt concentration of 0.1%, 0.2%. While *L. plantarum* DLPT4 did not grow in the MRS medium with bile salt concentration of 0.3%, 0.4%, indicating that bile salt at the concentration inhibited the growth of *L. plantarum* DLPT4. As shown in the Fig. 1C, the survival rate of *L. plantarum* DLPT4 in artificial simulated gastric fluid (pH 2.5) for 3 h at 37°C reached to 85.97%. Subsequently the viable *L. plantarum* DLPT4 in artificial simulated gastric fluid was treated by artificial intestinal fluid for 8h at 37°C and the survival rate of the *L. plantarum* DLPT4 was 61.02%. The results indicated that *L. plantarum* DLPT4 had a good survival ability in the simulated gastrointestinal conditions.

3.2 Effects of *L. plantarum* DLPT4 on the inflammatory symptoms of RAW264.7 induced by LPS

As shown in Fig. 2A, L. *plantarum* DLPT4 at different bacterial concentrations showed no effect on cell viability of the RAW264.7 cells, indicating that *L. plantarum* DLPT4 had no cytotoxic effect on the RAW264.7 cells. The *L. plantarum* DLPT4 concentration of 10^9 CFU/mL was selected for further study.

NO acts as a cytotoxic molecule in the immune function (O et al., 2009, Lin et al., 2017). As shown in the Fig. 2B, LPS can stimulate RAW264.7 cells to produce a large amount of NO, while the *L. plantarum* DLPT4 treatment significantly inhibited the secretion of NO in these RAW264.7 cells ($p < 0.05$). When RAW264.7 cells were treated by LPS together with *L. plantarum* DLPT4, or treated by *L. plantarum* DLPT4 prior to LPS treatment, NO secretion of the treated RAW264.7 cells were markedly suppressed. LPS also stimulated RAW264.7 cells to produce cytokines. As shown in Fig. 2C, the cytokines (TNF-α and IL-8) secretion of RAW264.7 cells were significantly increased after the LPS treatment ($p < 0.05$). While treatment with *L. plantarum* DLPT4 significantly reduced the overexpression of the cytokines ($p < 0.05$), The results showed that *L. plantarum* DLPT4 ameliorate the inflammatory symptoms of RAW264.7 cells induced by LPS. Suppressing LPS-induced NO and inflammatory cytokine production in RAW264.7 cells could alleviate inflammation (Mingyan et al., 2021).

3.3 Effect of L. plantarum DLPT4 oral administration on body weight and organ indices of CTX-treated BALB /c mice

As shown in the Fig. 3A, the body weight of the BALB/c mice in all groups showed stable upward trend in the first 25 days with no significant difference ($p > 0.05$). After 3 times of continuous treatment with CTX, it was observed that the body weight of the mice in the MG decreased dramatically, significantly lower than those of the mice in other three groups ($p < 0.05$) on 28th day.

Thymus are important immune organs of animals. Thymus have a positive correlation with immune ability in BALB/c mice (Wang et al., 2014). As shown in Fig. 3B, the thymus index of the mice in the NG were significantly higher ($p < 0.05$) than those of the mice in the other groups injected with CTX, indicating that CTX damaged the immune system of the mice. And there was no significant difference in thymus index among the mice in the *L. plantarum* DLPT4 administration group and the model group ($p > 0.05$). Wang et al. (2014) reported that a large number of lymphocytes underwent apoptosis within the thymus than the spleen in male BALB /c mice exposed to perfluorooctanoic acid (Wang et al., 2014). In this study, we found there was no difference in the spleen index of the mice in all groups (data no shown).

3.4 Effect of *L. plantarum* DLPT4 oral administration on tissue histopathology of CTX-treated BALB /c mice

HE staining sections of the mice thymus are shown in the Fig. 4A. The yellow arrow in the figures indicates the dividing line between cortex and medulla in the thymus. The cortex and medulla of the mice thymus in the NG were clearly demarcated, and thymocytes in cortex were uniformly dense. The boundary between cortex and medulla of the mice thymus in the MG was not clear, some of which were occupied by adipocytes, the tunica becomes thinner and the medulla shrinks. The boundary between cortex and medulla of the mice thymus in the *L. plantarum* DLPT4 oral administration group was clearer than that in the MG, and more thymocytes can be seen, and the tunica was uniform, and medulla atrophy was less than that in the MG.

HE staining sections of the mice ileum are shown in the Fig. 4B indicated the arrangement of goblet cells and the villi of ileum. The ileum tissue morphology was complete, villi showed a slender, closely structure; goblet cells were arranged neatly, and submucosa and muscularis were evenly distributed in the ileum tissue of the mice in NG group. While the goblet cells were disorganized, submucosa and muscular were recorded abnormal in the ileum tissue of the mice in MG group. The goblet cells were arranged neatly, and the villi were arranged neatly and the histomorphology was relatively completed of the mice in the high *L. plantarum* DLPT4 concentration group.

HE staining sections of the mice colon are shown in the Fig. 4C that the arrangement of goblet cells and intestine glands. The crypt structure of the mice in the NG group was regular, and goblet cells were arranged in order, and there are complete mucosal muscles, and large intestine glands were arranged in order. Mucosal muscle thickening, crypt structural changes, irregular arrangement of goblet cells and disappearance of mucus in goblet cells can be seen in colon pathological tissues of the mice in the MG and DLPT4-L groups. While the integrity of colon epithelial cells of the mice in the DLPT4-H groups was higher than those of the mice in MG and DLPT4-L group, evidenced by the improved crypt morphology, increased goblet cells, and recovered mucus in goblet cells.

3.5 Effect of L. plantarum DLPT4 administration on immune protein regulation and intestinal inflammation of CTX-treated BALB /c mice

The effect of *L. plantarum* DLPT4 oral administration on humoral immune response of the immunosuppressed BALB /c mice was further evaluated. As shown in the Fig. 5(A-C), the IgA, IgG and IgM levels in the serum of the immunosuppressed BALB /c mice in the MG were significantly different from those in the NG ($p < 0.05$). Compared with those in the MG, the IgA and IgG levels in the serum of the immunosuppressed BALB /c mice with *L. plantarum* DLPT4 oral administration significantly increased ($p < 0.05$). The contents of IgM were significantly increased by injecting the CTX and high-dose *L. plantarum* DLPT4 oral administration could significantly relieve the symptoms ($p < 0.05$).

IL-6, IL-1β and TNF-α are regarded as the most important inflammatory mediators involved in fever during the induction of acute phase reaction protein (Depeng et al., 2013). As shown in the Fig. 6, the expression quantity of IL-6, IL-1β and TNF-α of the BALB /c mice in the MG significantly increased ($p < 0.05$) compared with those in the NG. While, *L. plantarum* DLPT4 oral administration at high dose significantly reduced the levels of IL-6, IL-1β and TNF-α in DLPT4-groups when compared to those in MG ($p < 0.05$).

3.4 Effect of L. plantarum DLPT4 administration on fecal short chain fatty acids (SCFAs) contents of CTX-treated BALB /c mice

Non-digestible carbohydrates reached the cecum and proximal colon and were metabolized by microbe in colon to produce short-chain fatty acids, which are beneficial to the health of the host (Canani et al., 2011). As shown in Table 2, the SCFAs contents (propionic acid-2-methyl, pentanoic acid, butanoic acid-3-methyl, hexanoic acid and pentanoic aci-4-methyl) in the feces of the CTX-induced immunosuppressed BALB /c mice were distinctly lower than those of the mice in NG ($p < 0.05$). However, the content of SCFAs in the feces of the BALB /c mice with *L. plantarum* DLPT4 oral administration did not change significantly ($p > 0.05$).

Table 2
Effect of *L. plantarum* DLPT4 oral administration on the content of short-chain fatty acids in feces of different groups of BALB/c mice.

SCFAs	NG(ug/mg)	MG(ug/mg)	DLPT4-L(ug/mg)	DLPT4-H(ug/mg)
Ace	2.4592 ± 0.6164^{a}	2.1417 ± 0.3298^{a}	2.3773 ± 0.1161^{a}	2.2607 ± 0.2029^{a}
Pro	0.6640 ± 0.0476^{a}	0.6026 ± 0.0231^{a}	0.6462 ± 0.0728^{a}	0.6257 ± 0.0592^{a}
But	0.0925 ± 0.0064^{a}	0.0535 ± 0.0018^{a}	0.0632 ± 0.0033^{a}	0.0515 ± 0.0053^{a}
Isobut	0.533 ± 0.0773^{a}	0.3965 ± 0.0315^{b}	0.3629 ± 0.0152^{b}	0.3573 ± 0.1104^{b}
Val	0.1017 ± 0.0236^{a}	0.0468 ± 0.0043^{b}	0.0447 ± 0.0060^{b}	0.0476 ± 0.0044^{b}
Isoval	0.4490 ± 0.03392^{a}	0.2270 ± 0.0045^{b}	0.2789 ± 0.0295^{b}	0.2349 ± 0.0129^{b}
Hex	0.1472 ± 0.0208^{a}	0.0764 ± 0.0002^{b}	0.0900 ± 0.0076^{b}	0.0738 ± 0.0011^{b}
Isohex	0.5421 ± 0.0568^{a}	0.2886 ± 0.0012^{b}	0.3001 ± 0.0034^{b}	0.3035 ± 0.0030^{b}

Note: Marking different lowercase letters means that each row is significantly different ($p < 0.05$). NG mean: normal group; MG mean: model group; DLPT4-L mean: Low concentration group (10^7 CFU/mL); DLPT4-H mean: High concentration group (10^9 CFU/mL).

3.5 Overview of metagenome sequencing

To distinguish whether the changes of gut microbial and function are related to *L. plantarum* DLPT4 oral administration, shotgun metagenomic sequencing was performed for the mice feces samples from four groups. The feces DNA was extracted, fragmented and sequenced by Illumina Hiseq to obtain raw reads (PE150, 150 bp paired end reads), which was 48,185,894, 47,273,730, 46,862,357 and 46,881,374 in NG, MG, DLPT4-L and DLPT4-H, respectively. After filtering the low-quality reads, there were 47,812,681, 46,810,825, 45,848,378 and 46,464,971 high-quality clean reads, which were obtained from NG, MG, DLPT4-L and DLPT4-H, respectively. The high-quality reads of contig lengths greater than 300 bp was reserved for splicing assembly (Dinghua et al., 2015). By performing MetaGene software, final gene prediction results were 964.23 million genes with 53.68 billion bps. After assembling, 2.73 million catalog genes with 16.99 billion bps were remained.

3.6 Effect of L. plantarum DLPT4 oral administration on intestinal bacterial diversity of CTX-treated BALB /c mice

Venn diagram shows unique and shared bacteria genus in the feces samples of the BALB /c mice in NG, MG, DLPT4-L and DLPT4-H groups. As shown in Fig. 7A, a total of 2,021 genus overlapped in NG, MG, DLPT4-L and DLPT4-H groups, and NG, MG, DLPT4-L and DLPT4-H contained 18, 20, 28 and 26 unique genus, respectively. Meanwhile, NG and MG have 16 genus in common, and NG and DLPT4-L have17

genus in common, and NG and DLPT4-H have 31 genus in common, indicating that *L. plantarum* DLPT4 oral administration restored the abnormal intestinal flora of CTX-induced BALB/c mice.

The percent of community abundance on Phylum level (Fig. 7B) showed the distribution of bacterial phylum in the feces samples of the BALB /c mice in NG, MG, DLPT4-L and DLPT4-H groups. *Bacteroidetes* was the most abundant phylum in NG, MG and DLPT4-L groups, the average percent of *Bacteroidetes* was 62.46%, 67.15% and 53.69%, respectively. Whereas the most abundant phylum in the mice of DLPT4-H was *Firmicutes* with a proportion of 57.29%, and *Bacteroidetes* accounts for 33.87%. While the *Firmicutes* proportion in the NG, MG and DLPT4-L groups were 30.01%, 26.04% and 36.75%, respectively. The proportion of *Proteobacteria* was 2.34%, 2.50%, 5.09% and 4.1% in the NG, MG, DLPT4-L and DLPT4-H groups, respectively. As shown in Fig. 7C, the top four genera of the microbes in the four groups are *Bacteroides*, *Prevotella, Alistipes* and *Clostridium* respectively. As shown in Fig. 7D the proportions of *Lactiplantibacillus* in NG, MG, DLPT4-L and DLPT4-H group were 0.82%, 0.076%, 0.18%, 2.2%, respectively. The ratio of *Bifidobacterium* in each group was analyzed separately, which was 0.026%, 0.023%, 0.032%, 0.039% in the NG, MG, DLPT4-L and DLPT4-H, respectively. The results showed that the contents of *Lactiplantibacillus* and *Bifidobacterium* significantly improved through oral gavage *L. plantarum* DLPT4.

3.7 Effect of L. plantarum DLPT4 administration on functional differences in gut microbiome of CTX-treated BALB /c mice

Through Kruskal-Wallis rank sum test, the differential genes between groups were compared to KEGG database to obtain the metabolic pathways information. As illustrated in the Fig. 8A, the ko05323 in DLPT4-H was significantly improved compared with MG ($p < 0.05$) at the functional level of immune diseases of Pathway Level 2. The ko05323 is related to IL-17 signaling pathway, TNF signaling pathway, MAPK signaling pathway and other pathways, which was found by comparing the database (Supplementary information 1). As shown in the Fig. 8B, the ko04659 and ko04657 in DLPT4-H group was significantly decreased compared with MG at the functional level of immune system of Pathway Level 2. The ko04660, ko04062, ko04650 and ko04664 were simultaneously elevated in DLPT4-H group ($p < 0.05$) compared with MG. The ko04659 and ko04657 involved in FoxO signaling pathway, HTLV-I infection, PI3K-Akt signaling pathway and other signaling pathways (Supplementary information 1). The ko04660, ko04062, ko04650 and ko04664 were related to T cell receptor signaling pathway, MAPK signaling pathway, Adherens junction, Tight junction and a whole lot more (Supplementary information 1).

Carbohydrate-active enzymes (CAZyme) are a kind of important active protein, which act on various carbohydrates. Among the CAZyme family level found (Fig. 8C), Glycoside Hydrolases (GHs) were the most abundant, followed by Glycosyl Transferases (GTs) and Carbohydrate Esters (CEs) especially GT2, GH2, GT4. As shown in the Fig. 8D, CE 10, GH 20 and GH 92 in DLPT4-H group were significantly decreased compared with MG at the CAZyme family level.

Discussion

In this study, *L. plantarum* DLPT4 could maintain viability in artificial gastrointestinal condition, indicating that the strain could reach host colon with high viable bacterial cells. When probiotics pass through gastrointestinal tract with gastric acid, bile salts and pancreatin respectively, and successfully reach and colonize the host colon, their probiotic function can be exhibited (Aslim, 2004, Lye et al., 2009).

Macrophages were activated to phagocytize and digest pathogens, at the same time, macrophages indirectly activated lymphocytes and other immune cells, making the body produce immune responses to inflammatory reactions (Lian et al., 2017, Zheng et al., 2017) and secrete pro-inflammatory cytokines such as IL-6, TNF-α (Andrina et al., 2019). The lipopolysaccharide (LPS) is a compound of the cellular wall of Gram-negative bacteria, could induce inflammatory response in RAW264.7 cells. (Cao et al., 2019) In the study, *L. plantarum* DLPT4 treatment inhibited the inflammatory response of RAW 264.7 cells exposed to LPS. Cao et al. (2019) (Cao et al., 2019) observed that Punicalagin could reduce the content of NO, IL-8 and TNF-α by LPS-induced cells. It was speculated that that *L. plantarum* DLPT4 inhibited the pro-inflammatory cytokines production LPS-treated RAW 264.7 cells through NF-κB and MAPK signaling pathway. In the RAW 264.7 cell experiment, the treatment by *L. plantarum* DLPT4 prior to LPS exhibited higher ameliorating effect on the inflammatory response induced by LPS. Therefor in the subsequent animal experiment, *L. plantarum* DLPT4 was administered to BABL/c mice for 25d firstly, and then cyclophosphamide was given to the mice for the subsequent 3 days.

The host immune system is composed of immune organs, immune cells and immune reactive substances (J et al., 2013). The dysregulation of immune response may cause inflammation (J et al., 2006) and autoimmune diseases (Garry and John, 2014). CTX as an alkylating agent can inhibit the cellular, and humoral immune response and the mice treated by CTX can be used as an animal model with weakened immune system to validate the immunoregulatory capacity of probiotics. Different probiotics show different immunomodulatory effects (Bahman et al., 2019, M et al., 2019). In this study, we found that *L. plantarum* DLPT4 oral administration could mitigate the weight loss, reduce the production of pro-inflammatory factors, and regulate the abundance of gut flora to relieve inflammation symptoms of CTX-treated BALB /c mice, indicating that *L. plantarum* DLPT4 ameliorate the immunosuppression of the BALB /c mice treated by CTX.

CTX is an effective alkylating agent for treating malignant tumors (Ashkan et al., 2009). However, long-term use of CTX can lead to the decline of host immune function, and the destruction of the intestinal mucosal barrier (Gaoxiang et al., 2019). The intestinal mucosal barrier is essential for the host defense system to eliminate invading pathogenic microorganisms (Krajmalnik-Brown et al., 2012, Ren et al., 2019). Intraperitoneal injection of CTX can reduce the diversity of the intestinal flora in mice, and cause intestinal inflammation of the mice (Xu and Zhang, 2015, In and Soo, 2017). In this study, the decrease of the number of *Lactiplantibacillus* and *Bifidobacterium* of the mice feces in MG may be attributed to the intraperitoneal injection of CTX, which led to intestinal flora imbalance of the BALB/c mice. While the CTX-treated BALB/c mice receiving *L. plantarum* DLPT4 showed the increased levels of

Lactiplantibacillus and *Bifidobacterium* in the feces. Enhancement of *Lactiplantibacillus* and *Bifidobacterium* levels are reported to ameliorate impaired intestinal immunity (Feng et al., 2018, Yueyue et al., 2019). The CTX-evoked damage to the colon of immunosuppressed BALB/c mice could be ameliorated by regulating gut microbiota such as *Lactiplantibacillus*, *Coriobacteriaceae*, *Bacteroides* (Yujiao et al., 2019), *Alloprevotella* (Kong et al., 2020), and up-regulating the secretory immunoglobulin A (SIgA) and mucin2 (Yu et al., 2019, Yujiao et al., 2019, Kong et al., 2020).Oral administration of *L. plantarum* DLPT4 could improve the ratio of *Firmicutes* / *Bacteroides*, which may improve the immune functions and relieve weight loss of the CTX-treated mice. *Firmicutes* and *Bacteroides* are generally believed to help the body to collect energy (Xin et al., 2018).

Gut commensal microbes colonize on the intestinal mucosa to produce organic acids and bacteriocins to inhibit the adhesion of pathogenic bacteria presenting as decreasing proinflammatory signaling (Ulla and Airi, 2013, Young et al., 2015). In this study, *L. plantarum* DLPT4 promote the recovery of intestinal flora of the CTX-treated mice. We speculate that the recovery of the mice intestinal barrier function may be due to the expression of Occludin like intestinal tight junction proteins and the recovery of intestinal flora by *L. plantarum* DLPT4 oral administration.

Intestinal flora plays an important role in the maintenance of intestinal barrier function. CTX induced intestinal mucosal injury, resulting in intestinal immune disorder. According to the HE stained histopathology images, *L. plantarum* DLPT4 induced crypt epithelial proliferation and increased goblet cells, indicating that *L. plantarum* DLPT4 oral administration increase the secretion of tight junction proteins and enhance the intestinal barrier function

The oral administration of *L. plantarum* DLPT4 stimulated the secretion of IgA, and IgG, and reduced the expression level of pro-inflammatory factors (IL-6, IL-1β and TNF-α) in the CTX- immunosuppressed BALB /c mice. Immunoglobulin plays an important role in the body's humoral immunity, for example, IgA, IgG and IgM have antibacterial and antiviral effects (Feng et al., 2014). The expression of cytokines and immunoglobulins plays important roles in the host immune system (Huang et al., 2016, Gaoxiang et al., 2019). Persistent releases of pro-inflammatory cytokines cause deleterious effects and affect the repair process of damaged tissues at the site of immune reaction (Jose and Kurup, 2017).

Non-digestible carbohydrates reach the cecum and proximal colon, and are metabolized by gut microbes to produce short-chain fatty acids, which is beneficial to host health (A et al., 2005, Yu et al., 2018). The decrease of SCFAs content in intestinal tract can increase intestinal permeability and promote inflammation (V et al., 2014). Conversely, the increase of SCFAs can promote intestinal cell metabolism, promote antibody production by B cells, and regulate intestinal mucosal immunity by influencing various immune cells (Young et al., 2015). Probiotics such as *Bifidobacterium* (Tian et al., 2019) and *Lactiplantibacillus* (Yujun et al., 2020) can affect the production of SCFAs in colon of the host. In this study, intraperitoneal injection of CTX reduced the fecal SCFAs contents of the CTX-treated mice. *L. plantarum* DLPT4 oral administration could stimulate the production of part of SCFAs in the CTX-treated mice but no significant difference. The results were similar to Liu (Wenwei et al., 2021). But *L. plantarum*

DLPT4 oral administration increased the *Bifidobacterium* and *Lactiplantibacillus* proportion of the gut flora in the mice.

T cell receptor (TCR) signal transduction plays a key role in the development and function of T cells, while T cells affect the host immunity (Warner et al., 2013). Several studies showed that TCR signal strength is important to Treg and Th17 Cell Differentiation (Hidehiro and E, 2013). Further the Tec family kinase ITL is activated, leading to the activation of MAPK pathway (Berg et al., 2005). *L. plantarum* DLPT4 activated T cell receptor signaling pathway and MAPK signaling pathway in CTX-immunosuppressed BALB/c mice. It was reported that the immunity of CTX-immunosuppressed mice could be enhanced by regulating the MAPK pathway mediated by TLR2/4 and activating the transcription activities of activator protein-1 and NF-κB (Ting et al., 2017, Bai et al., 2019). It was speculated that oral administration of *L. plantarum* DLPT4 might activate phosphorylated p38 and AP-1 MAPK signaling pathways to exert immunomodulatory activity.

Overall data suggested that *L. plantarum* DLPT4 oral administration modulated the intestinal microbiota of the CTX-immunosuppressed BALB/c mice, which affect certain metabolic pathways and immune system of the mice.

Conclusions

The present study showed that oral administration of *L. plantarum* DLPT4 could alleviate the immune dysfunction of CTX-immunosuppressed BALB/c mice. *L. plantarum* DLPT4 could enhance the immune protein secretion and relieve intestinal inflammation of CTX-immunosuppressed BALB/c mice. In addition, *L. plantarum* DLPT4 could increase the *Lactiplantibacillus* and *Bifidobacterium* abundance, regulate intestinal flora imbalance caused by cyclophosphamide, effectively participate in the regulation of various metabolic pathways and immune system pathways, and improve the abundance of carbohydrate enzymes. These results indicated that *L. plantarum* DLPT4 has the potential to enhance the immunity of immunosuppressed mice.

Declarations

Ethics statements

All animal experiments were conducted in strict accordance with the Institutional Animal Care and Use committee of Dalian Polytechnical University (SYXK2017-0005, Dalian, Liaoning, China) and conformed to the National Institute of Health guidelines on the ethical use of animals in China.

CRediT authorship contribution statement

Yinglong Song: validation, formal analysis, writing – original draft, and data curation.

Mengying Sun: validation, formal analysis, writing – original draft, and data curation. Fenglian Ma: validation, formal analysis, writing – original draft. Dongxue Xu: conceptualization, methodology, validation. Guangqing Mu: conceptualization, project administration and supervision. Yang Jiao: validation and formal analysis. Changlu Ma: validation and formal analysis. Ping Yu: validation and formal analysis. Yanfeng Tuo: conceptualization, writing – review and editing, funding acquisition, visualization, and supervision.

Conflicts of interest

The authors declare no competing financial interest.

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Figures

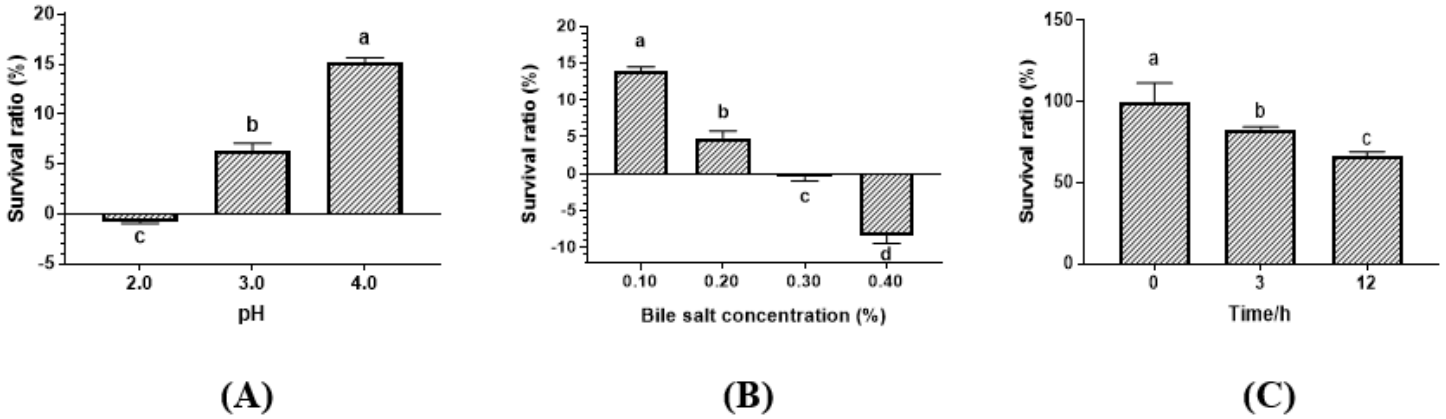


Figure 1

Survival ability of *L. plantarum* DLPT4 in simulated gastrointestinal conditions. (A) acidic conditions, (B) different bile salt concentration, (C) simulated gastrointestinal fluid.

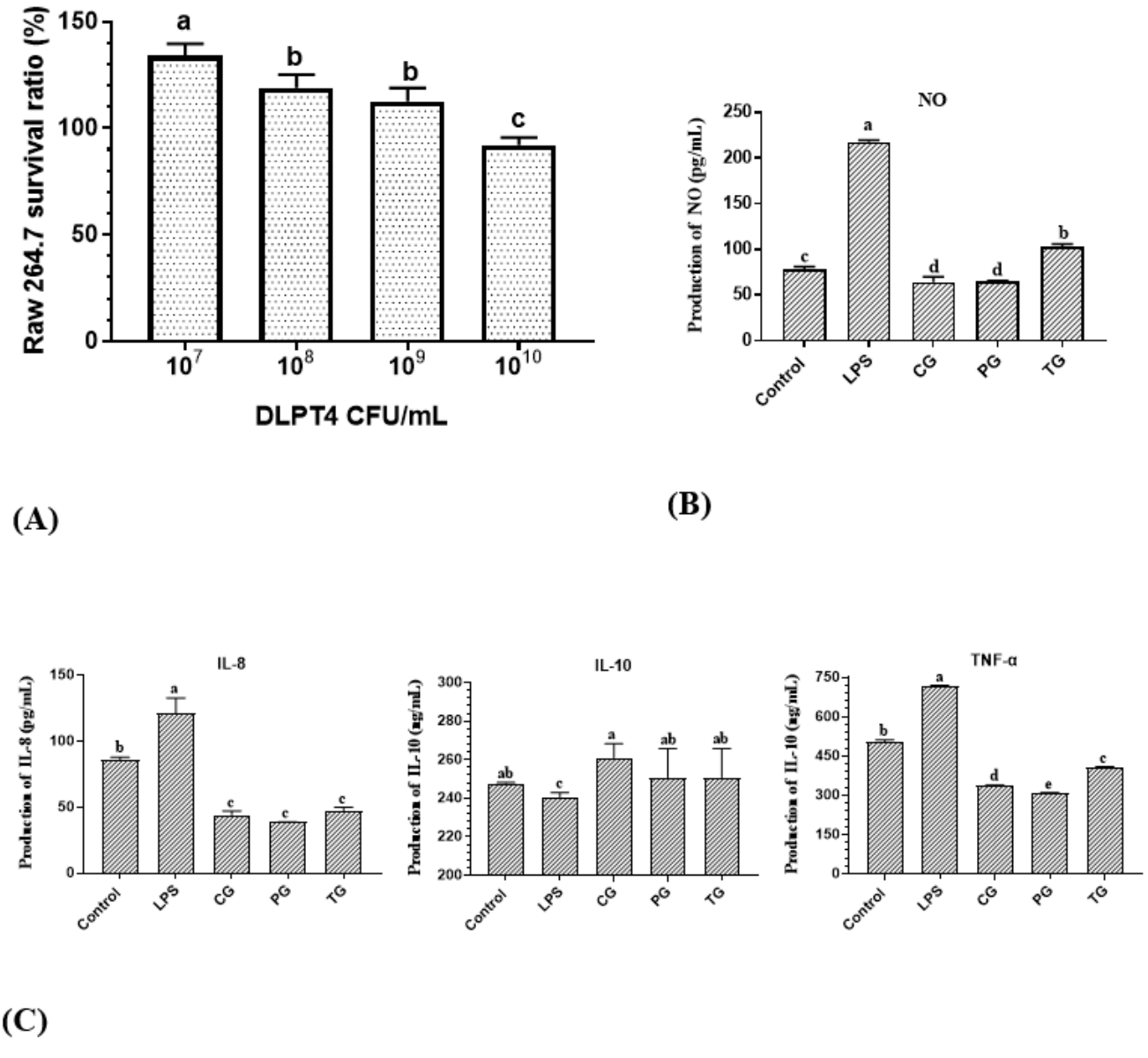


Figure 2

Effects of *L. plantarum* DLPT4 on the inflammatory symptoms of RAW264.7 induced by LPS. (A) RAW264.7 cell viability, (B) NO production, (C) the expression of inflammatory factors (IL-8, IL-10, TNF-α). Marking with different lowercase letters means significant difference (p<0.05). CG, Competition group; PG, Prevention group; TG, Therapy group.

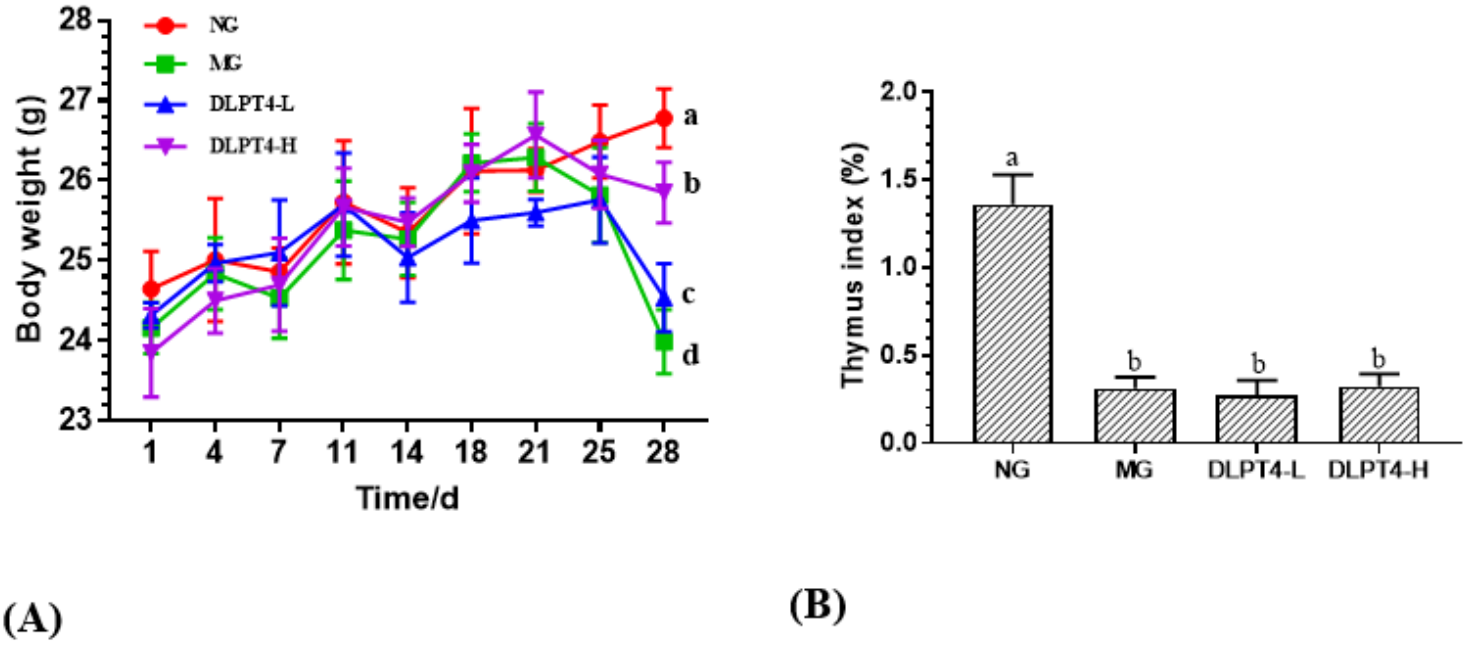
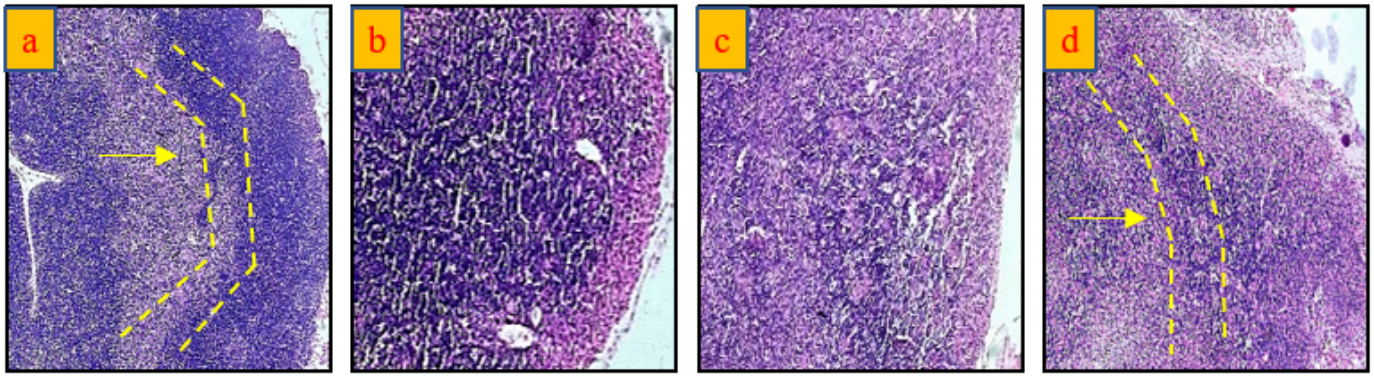
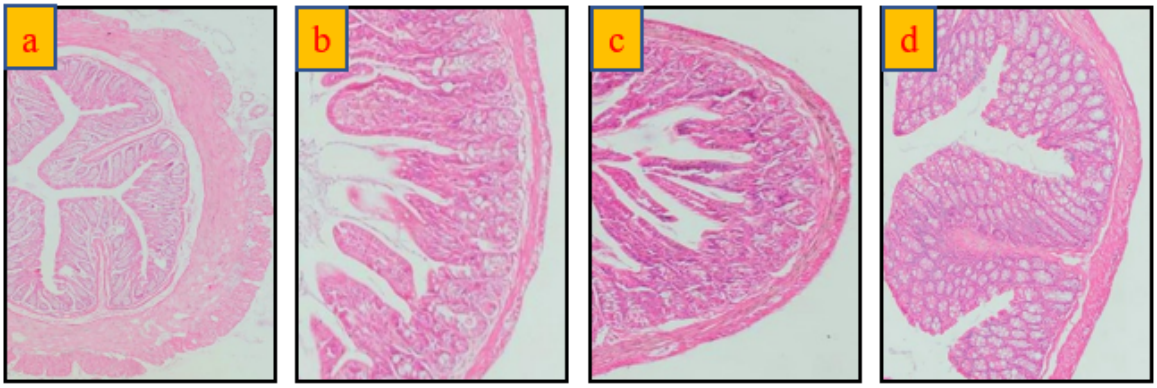


Figure 3

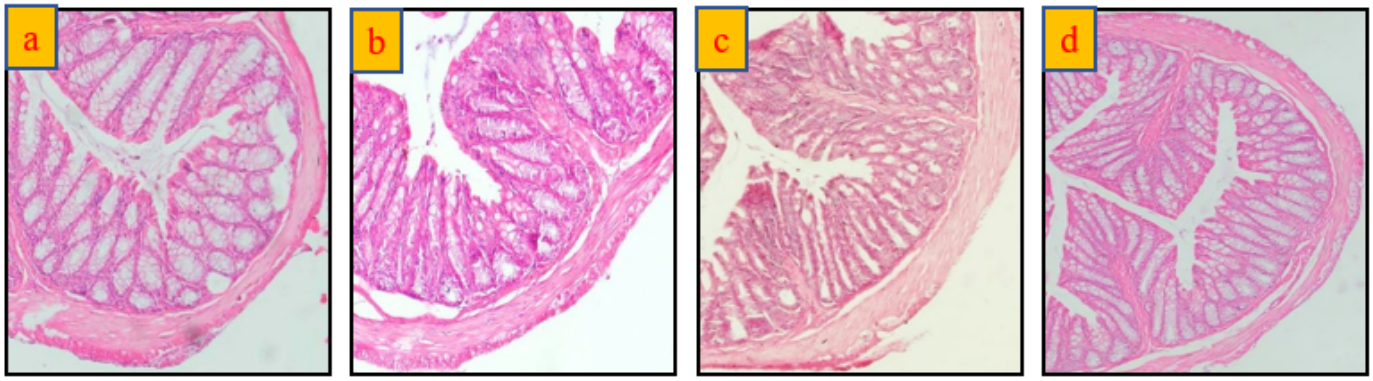
Effects of *L. plantarum* DLPT4 oral administration on the body weight (A) and thymus index(B) of CTX-induced BALB/c mice. NG, normal group; MG, model group; DLPT4-L, Low concentration group (10^7 CFU/mL); DLPT4-H, High concentration group (10^9 CFU/mL)



(A)



(B)



(C)

Figure 4

Effects of *L. plantarum* DLPT4 oral administration on the changing of the major organs. (A) Histological Observation for BALB/c mice's thymus, (B) Histological observation on ileum and colon (C) (HE, 100 ×). a, NG, normal group; b, MG, model group; c, DLPT4-L, Low concentration group (10^7 CFU/mL); d, DLPT4-H, High concentration group (10^9 CFU/mL)

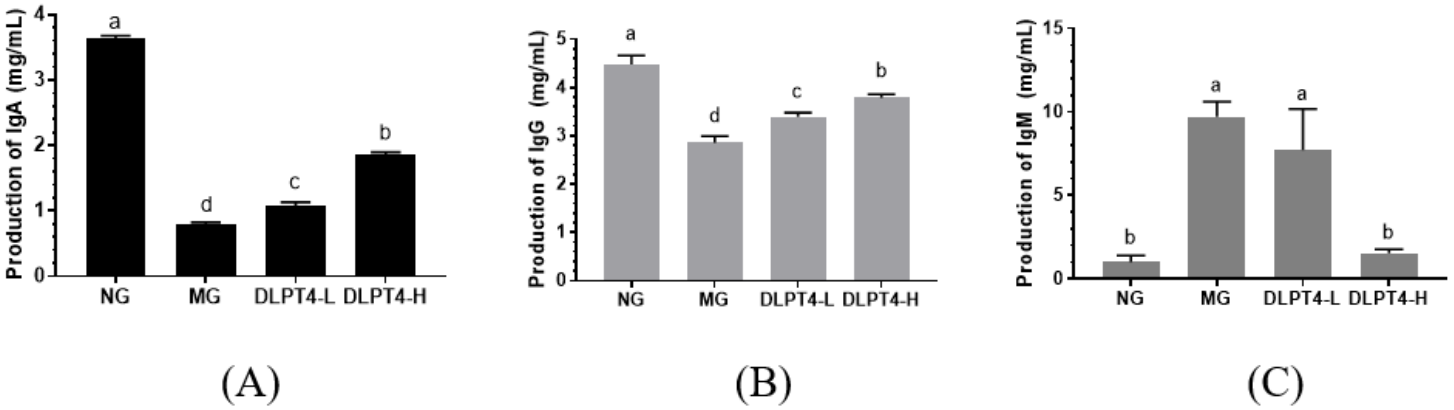


Figure 5

Effects of *L. plantarum* DLPT4 oral administration on the production of IgA (A), IgG (B), IgM (C) in BALB/c mice. Labeling different lowercase letters indicates significant differences ($p < 0.05$). NG, normal group; MG, model group; DLPT4-L, Low concentration group (10^7 CFU/mL); DLPT4-H, High concentration group (10^9 CFU/mL).

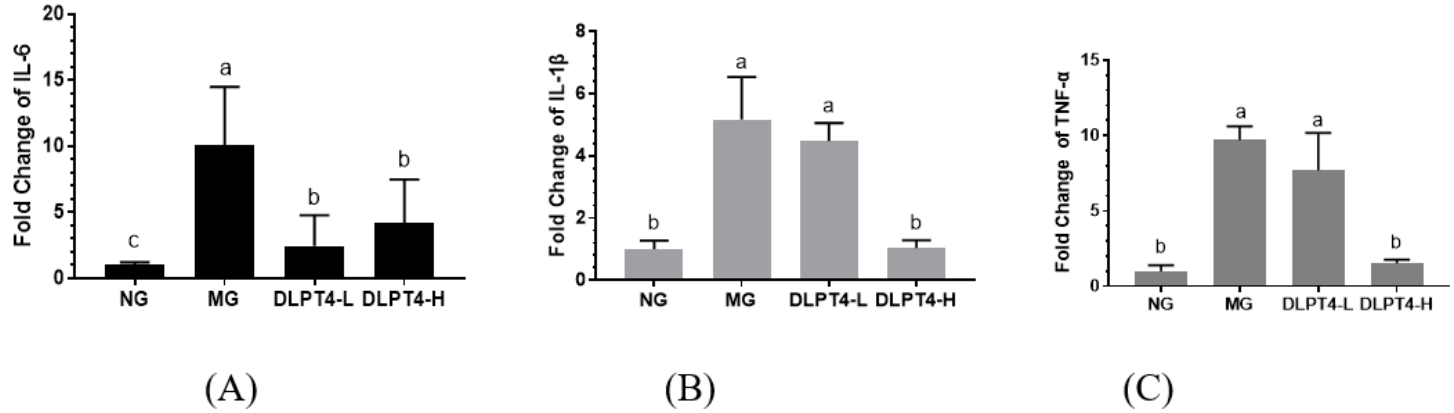


Figure 6

L. plantarum DLPT4 alleviated intestinal inflammation in CTX-treated mice. The gene expression level of IL-6 (A), IL-1β (B), TNF-α(C). Labeling different lowercase letters indicates significant differences ($p<0.05$). NG, normal group; MG, model group; DLPT4-L, Low concentration group (10^7 CFU/mL); DLPT4-H, High concentration group (10^9 CFU/mL).

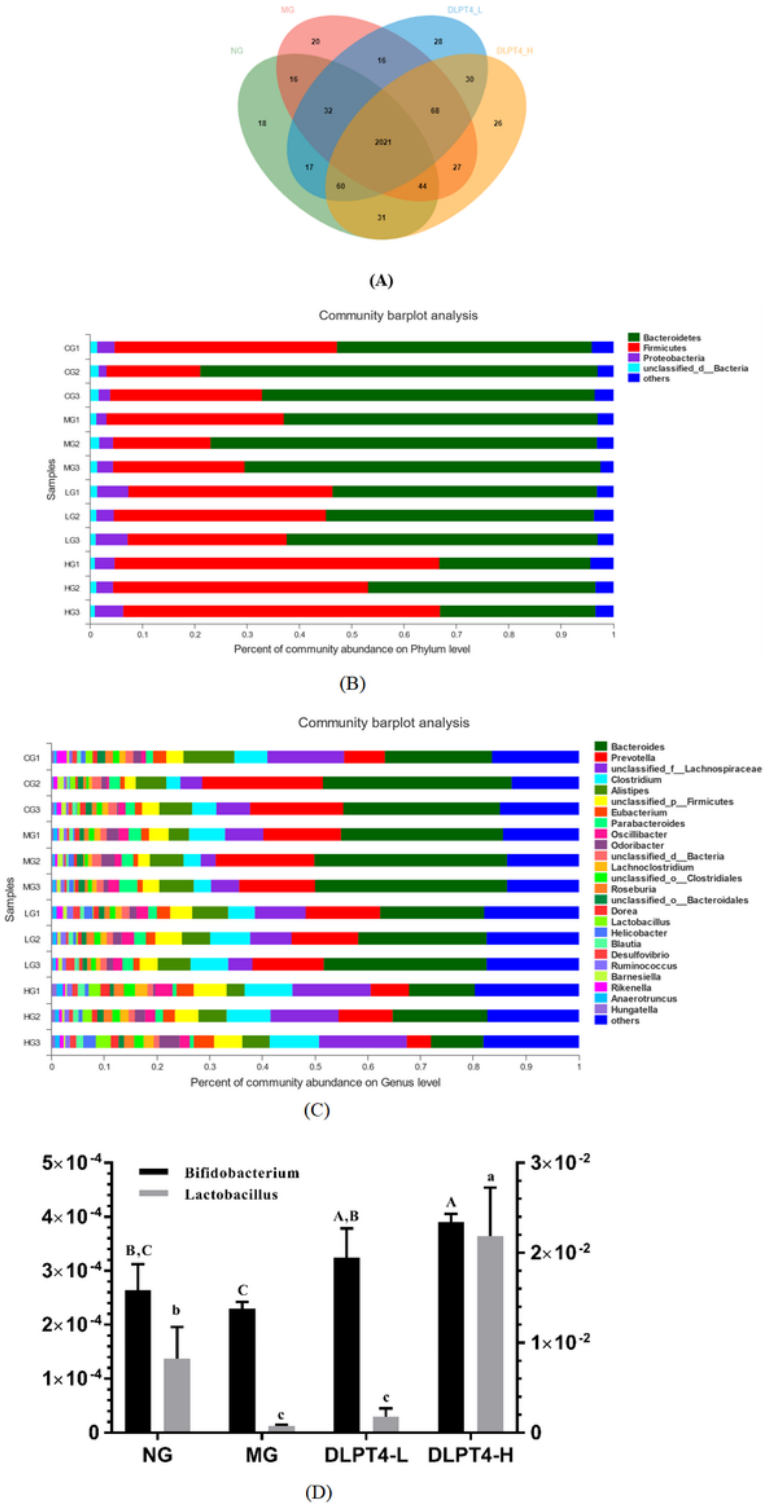


Figure 7

Effects of *L. plantarum* DLPT4 on microbial diversity. Venn diagram of intestinal flora in each group of mice (A). Composition and abundance of the feces samples from BALB/c mice at phylum (B), genus (C) levels and the ratio of *Bifidobacterium* and *Lactobacillus* (D) in various groups. Labeling different lowercase letters indicates significant differences ($p < 0.05$). NG, normal group; MG, model group; DLPT4-L, Low concentration group (10^7 CFU/mL); DLPT4-H, High concentration group (10^9 CFU/mL).

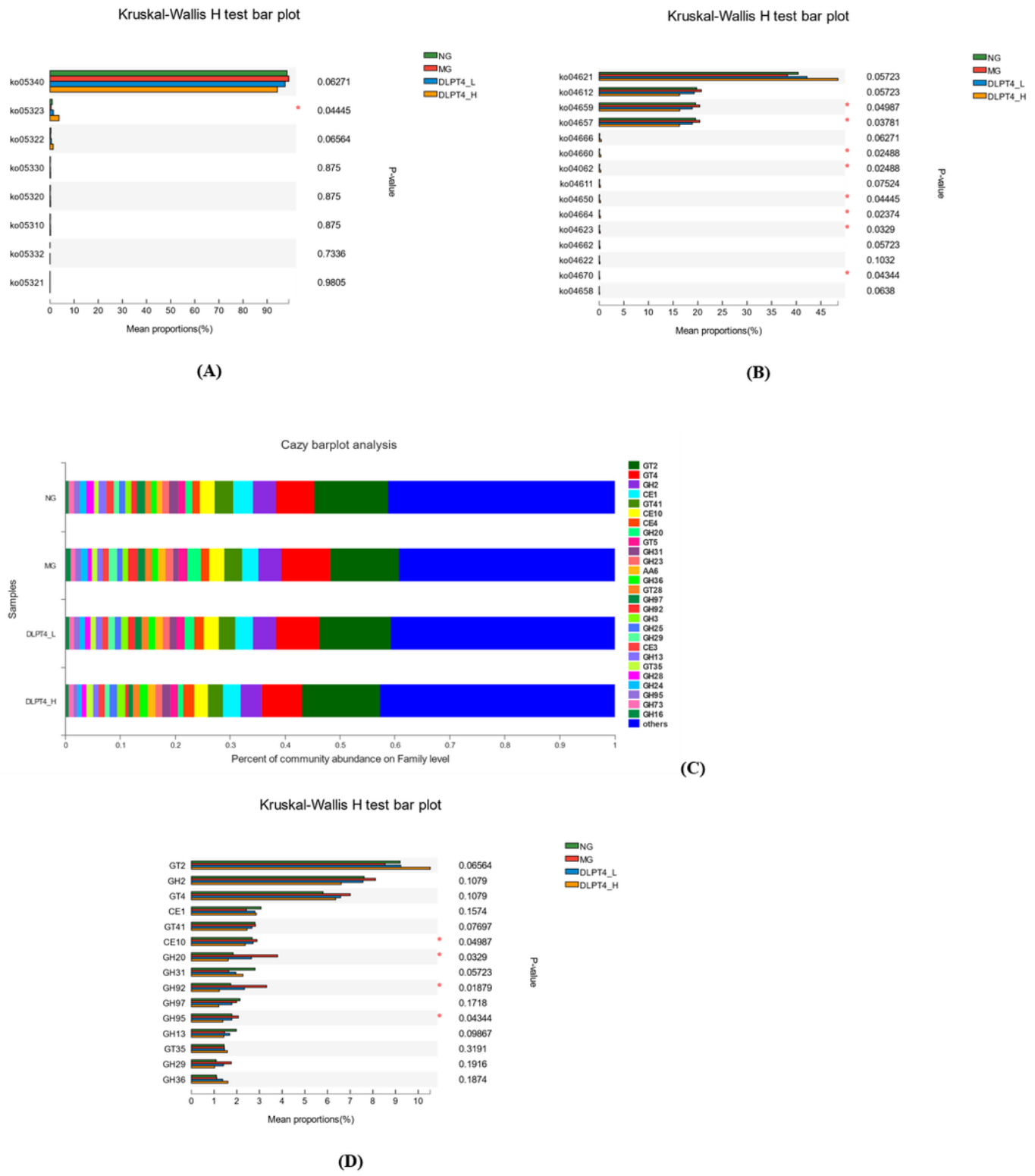


Figure 8

Function differences of intestinal microbes. (A) immune diseases of Pathway Level 2 (B) immune system of Pathways Level 2, (C) CAZyme family level, (D) difference analysis of CAZyme family level. NG, normal group; MG, model group; DLPT4-L, Low concentration group (10^7 CFU/mL); DLPT4-H, High concentration group (10^9 CFU/mL).

Supplementary Files

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- Supplementaryinformation1.xls
- GraphicalABstract.png