Lactobacillus Paracasei R3 Protects Against Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice via Regulating Th17/Treg Cell Balance

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Abstract

Inflammatory bowel diseases (IBD), mainly comprising ulcerative colitis (UC) and Crohn's Disease, are most often a polygenic disorder with contributions from the intestinal microbiome, defects in barrier function, and dysregulated host responses to microbial stimulation. Strategies that target the microbiota have emerged as potential therapies and, of these, probiotics have gained the greatest attention. Herein, we isolated a strain of *Lactobacillus paracasei* R3 (*L.p* R3) with strong biofilm formation ability from infant feces. Interestingly, we also found *L.p* R3 strain can ameliorate the general symptoms of murine colitis, alleviate inflammatory cell infiltration and inhibit Th17 while promote Treg function in murine DSS-induced colitis. Overall, this study suggested that *L.p* R3 strain significantly improves the symptoms and the pathological damage of mice with colitis and influences the immune function by regulating Th17/Treg cell balance in DSS-induced colitis in mice.

1 Introduction

Inflammatory bowel diseases (IBD), mainly comprising ulcerative colitis (UC) and crohn's disease, are most often a polygenic disorder with contributions from the intestinal microbiome, defects in barrier function, and dysregulated host responses to microbial stimulation. Strategies that target the microbiota have emerged as potential therapies for IBD and, of these, probiotics have gained the greatest attention.

From discovery to clinical application, probiotics have been developed and explored for many years. The category and mechanism of probiotics are gradually clear, and the clinical application is more and more extensive, and their functions mainly include intestinal protection, immune regulation, antibacterial effect, nutrition, anti-tumor, protecting liver, reducing blood lipid and so on[1–7]. Intestinal probiotics, as a kind of active microorganisms, need to be colonized in human intestinal epithelial cells, and then communicate with the intestinal mucosa layer to play an important role in the metabolic, immune and intestinal health protection function of host individuals[1].

Supplementation of probiotics can be used as an treatment IBD. However, due to the complex pathogenesis of IBD and the influence of individual probiotics, there are many probiotics in the literature, but few strains are used for actual transformation. Therefore, it is still necessary to provide scientific and detailed evidence for the beneficial effects of probiotics. *Escherichia coli Nissle 1917* is a non-pathogenic probiotic that can relieve clinical symptoms in patients with UC with the same efficacy and safety as mesalazine (MSLZ)[8–10]. The synergistic effect of probiotics and 5-ASA in patients with mild to moderate UC promotes clinical remission. Studies have shown that probiotics could reduce proinflammatory cytokines (such as TNF-α and IL-1β) and increase anti-inflammatory factors(IL-10) via inhibiting TLR4/NF-κB signaling pathway and PI3K/Akt/NF-κB signaling pathway to maintaining UC remission[11]. Administration of *Lactobacillus paracasei* strains also improves immunomodulation and changes the composition of gut microbiota leading to improvement of colitis in mice[12, 13]. Herein, we isolated a strain of *Lactobacillus paracasei* R3 (*L.p* R3) with strong biofilm formation ability from infant feces. Interestingly, we also found *L.p* R3 strain can ameliorate the general symptoms of murine colitis, alleviate inflammatory cell infiltration and inhibit Th17 while promote Treg function in murine DSS-induced colitis.

2 Materials And Methods

2.1 Probiotics isolation

L.p R3 were isolated from baby feces and identified by API 50CH strips (BioMerieux) and 16S rDNA sequence in our laboratory. The strain was grown in Man, Rogosa, Sharpe (MRS) broth (Oxoid, Cambridge, UK) and on MRS agar (Oxoid,Cambridge, UK). Inoculated plates were incubated for 48 h at 35℃under anaerobic conditions. *L.p* R3 was incubated overnight at 35℃ in MRS broth.

2.2 Detection of growth curve

Preparation of fresh bacterial suspensions for growth curves, gastrointestinal tolerance and biofilm formation experiments. MRS broth was used to activate *L.p* R3 strain, strain cells were collected by centrifugation at 4,000g × g for 10 min, washed twice with PBS, and resuspended at 1.5×10^8 cfu/ml. The fresh suspension was inoculated into 200 ml MRS liquid medium according to 2%(v/v)inoculation and 35℃culture for 24 h. From 0h to 48h, sampling every 2h, measuring the OD_{630} value. Each time point is repeated 3 times, taking the average value. MRS broth was a blank control. And the growth curve was drawn with culture time as horizontal coordinate and absorbance as vertical coordinate.

2.3 Gastrointestinal fluid tolerance

Equipped with artificial intestinal fluid and PH = 2, 3, 4, 7 gastric juice. The fresh suspension was inoculated into 20 ml MRS liquid medium according to 2% (v/v) inoculation and 35℃culture for 16h. Cultures were collected by centrifugation at 4,000g × g for 10 min, washed twice with PBS, discard supernatant. First, the bacterial cells were treated with 4 kinds of gastric juice, 35℃ and 50 r/min, for 3 h,count the number of live bacteria at 30 min, 90 min and 180 min time point. After 3 hours of gastric juice treatment, centrifuge to discard gastric juice, then add the same volume of intestinal fluid, 35℃ and 50 r/min, for 3 h, count the number of live bacteria at 270 min and 360 min time point.The concentration of bacterium was determined by colony plate counting method. Each group was set up 3 parallel and repeated 3 times. Survival rate =(number of live bacteria per time point (cfu/ml)/1.5×10^8 cfu/ml)×100%.

2.4 Biofilm formation

Semi-quantitative determination of biofilm formation ability of strain by micro-pore plate.The fresh suspension was inoculated into 200 ml MRS liquid medium according to 2%(v/v)inoculation, mix well, add to 96 hole plate, each hole 200ul, 35℃static culture for 48 h, determine the OD_{630} value by enzyme labeling instrument, recorded as A1, MRS broth was a blank control.Discard excess medium, washed twice with PBS to remove residual media and surface plankton bacteria, with 200uLmethanol fixed for 10 min in each hole, natural drying, with100ul 1% crystal violet solution to dye 30 min, washed twice with PBS

to remove excess dyes. When dried naturally, 200 ul 95% of ethanol were added to each hole to dissolve the crystal violet completely. The OD_{630} value was measured, recorded as A2. Each group was set up 3 parallel and repeated 3 times. The adhesion rate is used to express the film forming ability, recorded as B, B = A2-A2C/A1 − A1C, A1C and A2C are the blank control absorbance value when measuring A1 and A2, respectively. The B < 0.1 is non-adhesion, 0.1 ≦ B < 0.5 low-level film formation, 0.5 ≦ B < 1 medium-level film formation and B ≧ 1 strong adhesion film formation.

2.5 whole genome sequencing analysis

Sequencing and bioinformatics analysis commissioned by Guangdong Nanxin Medical Technology Co., Ltd. Using a Illumina platform, the operation is mainly divided into five steps: extraction of genome DNA, library preparation, high-throughput sequencing, genome assembly, genome fine mapping and biological information analysis (including three aspects of gene prediction, gene annotation and genome comparative analysis). In gene function annotation analysis, a total of 11 databases were used, including ARDB, CARD, CAZY, GO, COG, KEGG, T3SS, NR,DBCAN, SWISSPROT and IPR .

2.6 Safety evaluation of *L.p* R3 strain

Acute toxicity test: 12 Balb/c mice, after 1 week of adaptive feeding, randomly divided into 2 groups including control group and *L.p* R3 group,six mice per group. MRS broth was used to activate *L.p* R3 strain, strain cells were collected by centrifugation at 4,000g × g for 10 min,washed twice with PBS, and resuspended at 1×10^{12} cfu/ml. The experimental group was given 200ul bacteria suspension each time, and the control group was given 200ul saline each time, once a day, for 30 days. Record the general signs of the mice daily, such as movement, hair, weight, death or not. On the 31st day, Cervical dislocation method was used to kill and dissect mice to take their organs. To observe the morphological changes of heart, liver, spleen, lung and kidney, and to measure organ quality, Calculate organ index = m1/m2(m1 is organ quality (g), m2 was weight quality in mice (g)).

2.7 Design of animal experiments

Six-week-old C57BL/6 mice(weighing 20.0 + 2.0 g, from Guangdong Medical Laboratory Animal Center, Foshan, Guangdong Province, China) were housed in plastic cages (at 22 ± 2°Cand 50 ~ 60% relative humidity with a 12 h dark/12 h light cycle) with free access to diet and water. Acute colitis was induced by administering 3% (w/v) DSS (molecular mass 36,000–50,000 Da; MP Biochemicals) in drinking water. The concentrations of *L.p* R3 strain and mesalazine (MSLZ) used in the experiment were 1.0×10^{9}CFU/mL and 52mg/mL, respectively.C57BL/6 mice were randomly divided into five groups (n = 6), including Normal group, DSS group,MSLZ group,*L.p* R3 group and *L.p* R3 combined with MSLZ group(*L.p* R3 + MSLZ group). For 14 days, each mouse was given 200 uL/d by gavage at a fixed daily time. From 8–14 days, the normal group was free to drink double distilled water, and the other 4 groups of mice were free to drink 3% DSS solution for 7 days to establish an acute UC model. Observe and record the daily condition of mice during the test. After the experiment, serum and colon samples from mice were collected for study. The experiment was approved by and performed in accordance with the guidelines of the ethics committee of Guangdong Medical University.

2.8 Determination of disease activity index(DAI)

The disease activity index (DAI) was determined by scoring the degree of weight loss, stool stiffness, and the presence of occult or whole blood (Table 1). Occult blood in faecals was evaluated with a Faecal Occult Blood Test Kit (Jiancheng Biotech, Nanjing,china P.R).

Table 1
Disease activity index score

Score	Weight loss	Stool cinsistency	Gross bleeding
0	None	Normal	negative
1	1–5%		
2	6–10%	Losse stool	Haemoccult
3	11–15%		
4	> 15%	Diarrhea	Bleeding(visible)
The disease activity index=(combined score of weight loss, stool consistency and bleeding)/3.			

2.9 Histological assessment

Colon length was measured as described above to assess colon shortening. 1 cm distal colon was fixed with PBS-buffered 10%(v/v) formalin buffer for 48 hours, then embedded in paraffin, cut into 5-um thick sections, hematoxylin and eosin (H&E)Staining and inspection with light microscope. A blinded histopathologic analysis was used to score the stage of colitis based on morphological criteria Ringed (Table 2). Randomly select 3 sections of each sample and examine 10 random fields (magnification 100x) in each section. The average value for each part is calculated by the average level.

Table 2
Histological disease scoring system

Score	Histological features
0	Normal colonic mucosa
1	Loss of one-third of the crypts
2	Loss of two-third of the crypts
3	The lamina propria is covered with a single lay inflammatory cell infiltration is present
4	Erosions and marked inflammatory cell infiltra

2.10 Immunohistochemistry

Immunohistochemistry was performed on 4 um thick paraffinembedded sections of the colon. The with incubated were sections anti-FOXP3(1:200), anti-CD25(1:200), anti-IL-17A(1:20 0) at 4°C overnight.VECTASTAIN ABC Kit (Vector, CA)was used as secondary antibody. Immunoreactivity was analyzed with a microscope (Olympus AX70, Olympus Optical Corp., Tokyo, Japan). The total number of CD25, FOXP3, IL-17A and IL-10 positve cells in each of four nonadjacent fields of view was counted per colon sample(magnification, 100×).

2.11 Western blot

The tissue was homogenized with lysis buffer, an equal amount of protein (50 ug) was analyzed by 12% SDS-polyacrylamide gel electrophoresis, and the blotted membrane was blocked with 5% BSA in TBST for 60 mins at room temperature, anti-FOXP3 (1:1000, Abcam), anti-CD25 (1:1000, Affinity Biosciences), anti-IL-17A (1:1000, Affinity Biosciences), anti-L-10 (1:1000, bs-6761R, Bioss) and FN-y (1:1000, Affinity Biosciences) antibody overnight at 4C.GAPDH (1:1000.Sigma) was used as a loading control. The HRP-conjugated anti-rabbit or anti-mouse IgG(1:2000, Cell Signaling) was used as a secondary antibody and the membrane was developed using an enhanced chemiluminescence system(Amersham Pharmacia Biotech).

2.12 Measurement of cytokine

Cytokines such as IL-10, IFN-y, IL-17A and IL-17F were measured in duplicate with Multi-Analyte Flow Assay Kit(Biolegend).

2.13 Statistical analysis

Figures and statistics were performed using Prism 7.0 a software (Graphpad Inc.). All data represent mean ± SEM. Data that was not successfully distributed was log converted and evaluated for distribution. Using Student's t-test or ANOVA with Tukey test to determine significant differences between two groups or analyze significant differences between multiple tests Group.

3 Results

3.1 Biological properties of *L.p* R3

L.p R3 were isolated from infant feces and identified by API 50CH Biochemical reaction system, named and preserved in our laboratory. The dynamic growth curve in vitro showed that *L.p* R3 strain had a logarithmic phase at 4h ~ 18h and then entered the stationary phase, no apparent death phase (Fig. 1A). Under successive effects of artificial gastric and intestinal fluids, the survival rate of *L.p* R3 was above 90% and 70% in PH = 3.0, 4.0 and 7.0 gastric and intestinal fluids, respectively. The survival rate of *L.p* R3 reached (58.76 ± 1.32)% and (43.08 ± 0.27)% after 3 h in PH 2.0 artificial gastric juice and intestinal fluids treatment(Fig. 1B). These results indicate that *L.p* R3 has good tolerance to gastrointestinal fluid and can survive and maintain activity under adverse gastrointestinal conditions. Biofilm formation rate of *L.p* R3 strain was more than 0.6 in the experiment of semi-quantitative determination of biofilm formation ability, showed that the strain had better ability of biofilm formation(Fig. 1C□D) .

3.2 Whole genome sequencing analysis of *L.p* R3

To look for other possible characteristics of *L.p* R3, its entire genome was sequenced(Fig. 2A). The main features of *L.p* R3 genome are reported in Table 3.The assembling of reads generated 92 contigs, giving a genome size of 3.095 Mb with a GC content of 46.17%. Using multiple databases to analyze the gene function of the strain, gene prediction and annotation indicated the total number of 3171 predicted protein-coding sequences (CDSs) and showed 60 structural RNAs. The strain had no plasmids on its chromosomes and no virulence genes were detected. It is noteworthy that the strain genes involve antibiotic resistance genes and TXSS systems associated with pathogenic mechanisms of Gram-negative bacteria. However, the similarities between the detected resistance gene locus and the corresponding antibiotic resistance gene sequences were less than 80%. And its did not evidence any acquired resistance gene in *L.p* R3, hence this strain can be considered safe regarding possible transmissible antibiotic resistances. As for the existence of TXSS system, whether the strain has the risk of infection needs further study. COG database annotations were shown in Fig. 2B. The largest part of this subsystem is allocated to the Carbohydrate transport and metabolism(13.01%), General function prediction only(9.32%), Translation, ribosomal structure and biogenesis(9.28%), Amino acid transport and metabolism(8.90%),Transcription(6.48%), Inorganic ion transport and metabolism(5.16%) and Cell wall/membrane/envelope biogenesis(5.06%), respectively. It is noteworthy that the strain contains proteins related to Lipid transport and metabolism(3.27%) and Mobilome: prophages, transposons (1.66%).

Table 3
Genome feature of L.p R3

Attribute	Values
Genome size	3,095,229 bp
G + C content (%)	46.17
Contig N50	89,515bp
Contig L50	11
Number of contigs	92
Number of protein conding sequence (CDSs)	3,171
Number of rRNAs	3
Number of tRNAs	57

3.3 Safety evaluation of *L.p* R3

L.p R3 did not produce hemolysis using colombian blood agar plate by anaerobic culture for 48 h. By feeding *L.p* R3(concentration of 1×10^{12} cfu/ml) to mice for 7day and 30 day, there was no significant difference in body weight, organ index, and blood glucose compared with the control group (Fig. 3). Experimental results showed that the activity and mental state of mice were normal, eating and drinking water were stable, the fur was bright, the fecal state was normal, no death occurred and no lesions were found in Heart, liver, spleen, lung and kidney organ. And Body weight and major organ index of mice were not significantly different compared with the control group ($p > 0.05$)(Fig. 3).The results suggest that the strain had no risk of infection and did not affected the normal physiology of mice.

3.4 *L.p* R3 strain ameliorates the general symptoms of murine colitis

In DSS-induced UC model, normal group mice showed normal activity, mental state, normal defecation,bright hair and normal weight gain. And other four groups mice began to develop different degrees of UC symptoms on the third day of free drinking of 3% DSS solution.These results show that the model is successful. Especially, the symptoms of mice in the DSS group are becoming more and more serious, with poor spirit, obvious blood stool and hair removal symptoms. Compared with the treatment group,the weight ratio of the DSS group decreased significantly ($p < 0.05$) (Fig. 4A). The DAI score, which is an indicator of the severity of colitis, including the extent of body weight loss, haemoccult positivity or gross bleeding and stool consistency, was evaluated for each animal (Table 1). As shown in Fig. 4B, according to normal group, the order of DAI score from large to small is DSS group > MSLZ group ≥ LP R3 group > LP R3 + MSLZ group. DSS group began to show significantly higher DAI values compared to the control group ($P < 0.05$). The DAI values of MSLZ + Lp R3 groups were significantly lower ($p < 0.05$) than DSS group.The results suggesting that L.p R3 can alleviate weight loss and UC symptoms in mice caused by DSS.

3.5 L.p R3 strain alleviates inflammatory cell infiltration in murine DSS-induced colitis

We studied the colon of experimental mice and the results were shown in Fig. 4C and 4E. During colonic inflammation, the length of the mouse colon is shortened by ulceration.This is accompanied by adhesions of colonic tissue. In DSS group, colon length was significantly shorter than in the control group ($p < 0.05$). The colon length was recovered to different degrees in the three treated-groups compared with DSS group, and the recovery was most obvious in LP R3 + MSLZ group ($p < 0.001$). In further colon histopathological experiments, mice colonic structure damage, glands incomplete, a large number of inflammatory cell infiltration, typical inflammatory changes in DSS group. And the histological injury score of DSS group was significantly higher than that of other 4 groups ($p < 0.001$). while the *L.p* R3 administered mice exhibited significantly less histological damage.There was less loss of crypt and goblet cells and less infiltration of various immune cells in the *L*.pR3 administered mice(Fig. 4E). Similarly,the systemic inflammatory response, assessed by splenomegaly, is shown in Fig. 4D. Spleen index were also significantly higher in DSS mice than in controls ($p < 0.05$). In mice fed with *L.p* R3, the mean spleen index was lower than in DSS animals ($p < 0.05$) and more similar to control animals. The results confirmed that the strain can relieve inflammation and pathological damage of the colon by DSS-induced.

3.6 *L.p* R3 strain reverses inflammatory cytokines IL-17A/IL-10 rates

Determination immune factors of IFN-γ, IL-10, IL-17A and IL-17F in peripheral serum of mice by liquid-phase chip method, to explore the immunomodulatory mechanism of L.p R3 strain. As shown in the Fig. 5, four cytokines have different levels in peripheral blood of each groups. Normal group as reference,the contents of IFN-γ, IL-17A, IL-17F and IL-10 were increased in DSS group, except for IL-10, there were significant differences($p < 0.05$). Interestingly, the three treatment groups were just the opposite of the model group.The levels of peripheral blood IFN-γ, IL-17A and IL-17F decreased significantly and IL-10 increased significantly in three treatment groups($p < 0.05$). Moreover, the IL-17A/IL-10 value of NS group was set to 1, and the IL-17A/IL-10 values of each group were compared to evaluate the changes of Th17/Tregs cell balance in mice after treatment. The results showed that the IL-17A/IL-10 value of DSS group was significantly higher than that of the other four groups($p < 0.05$). This indicates that Th17 cells infiltrate and secrete the pro-inflammatory factor IL-17A during inflammation. After treatment with L.p R3 or/and MSLZ, the anti-inflammatory factor IL-10 increases and the IL-17A/IL-10 value reverses.

3.7 *L.p R3* strain inhibits Th17 and promotes Treg function in murine DSS-induced colitis

Th17 cell is a T cell subset that can secrete IL-17. Tregs cells are characterized by the expression of Foxp3, CD25 and CD4. Therefore, the expression of cells in tissues can be reflected by detecting Th17 and Tregs related cell markers. The expression of Foxp3, IL-10, IL-17A and IFN-γ in colonic tissues of mice in each group was detected by immunohistochemistry as shown in Fig. 6A and 6B. Normal group as reference, the expression of Foxp3, IL-10, IL-17A and IFN-γ increased to varying degrees in DSS group and 3 treated groups. And compared with DSS group, in the treatment groups of MSLZ, LP R3 and LP R3 + MSLZ, Foxp3 and IL-10 expression increased in turn, while IL-17A and IFN-γ expression decreased in turn, they were statistically different ($p < 0.05$).The expression of CD4, CD25, Foxp3, IL-10, IL-17A and IFN-γ in colon of mice in each group was further examined by Western blotting as shown in Fig. 6C. Normal group as reference,the CD4, CD25 and Foxp3 expression of the three treatment groups was higher than that of the DSS group, and the differences were statistically significant ($p < 0.05$). And the expression of IL-17A and IFN-γ in treatment group was significantly lower than that in DSS group($p < 0.05$). IL-10 not well detected, probably because protein expression is affected by a variety of factors. The above results suggest *L.p* R3 could promote the expression of Tregs cell markers and inhibits the expression of Th17 cell markers. Treg/Th17 cell imbalance in inflammation caused by DSS was restored after treatment.

4 Discussion

L.p R3 strain, as a member of traditional probiotics, was isolated from infant faeces by our team. We studied the survival ability of the strain in vitro. The dynamic growth curve in vitro showed that *L.p* R3 strain had a logarithmic phase at 4 h ~ 18h and then entered the stationary phase, no apparent death phase.The strain had good characteristics with gastrointestinal fluid tolerance and strong biofilm formation ability. Probiotics are often used as active microorganisms, which need to withstand the persecution of gastrointestinal fluid and reach intestinal colonization with a high number of live bacteria, and to play a role in promoting the health of the body. Colonization is the premise of growth and development of probiotics and a physiological function under adhesion. The colonized bacteria can form biofilm on the surface of adhesion membrane to prevent the invasion of foreign bacteria and protect the health of intestinal mucosa. Therefore, acid tolerance, bile salt tolerance and intestinal mucosal adhesion all used as criteria for screening probiotics[14].

L.p as an important member of *Lactobacillus*, is Gram-positive bacterium widely found in human oral cavity, intestinal tract and fermented foods. *L.p* has the function of regulating intestinal flora balance, enhancing human immunity and anti-tumor[15–17]. At present, it has been used in food, health care and industrial production, which has attracted wide attention at home and abroad. *Lactobacillus paracasei subsp. paracasei* NTU 101 (NTU 101) is a multifunctional strain that has been shown in previous studies to possess anti-inflammatory properties and to exert a modulatory effect on intestinal bacteria associated with certain pathogenic mechanisms of IBD. The probiotic strains could reduce oxidative stress and the inflammatory response in DSS-treated mice by enhancing the antioxidative capacity of total antioxidants capacity (GR, GSH, CAT, SOD, MDA), and hindering the secretion of proin-inflammatory cytokines (such as TNF-α, IL-6, IFN-γ, and IL-12) [12]. *Lactobacillus paracasei ssp. paracasei* YBJ01 reduced d-galactose-induced oxidation in male Kuming mice. Strain LPSP-YBJ01 significantly increased serum superoxide dismutase (SOD), glutathione peroxidase, and total-antioxidant capability, and inhibited generation of malondialdehyde in a dose-dependent manner[18]. In addition, strain LPSP-YBJ01 also increased the hepatic and splenic protein expressions of some antioxidant enzymes such as catalase, Cu/Zn-SOD, and Mn-SOD in mice treated with d-galactose[18].

The safety of probiotic strains is also important, including potential pathogenic virulence factors and resistance. At present, the probiotic products in China only list the name of bacteria, there is no specific strain name and related genetic background information, but the probiotic and safety of probiotics are strain specific. Genomics studies on the probiotic and safety of probiotics are essential to obtain lactic acid bacteria that can be used to develop probiotics. This method can fully reveal the genetic information of lactic acid bacteria, systematically explain the physiological function and metabolic mechanism of lactic acid bacteria, lay the foundation for the systematic classification and genetic evolution of lactic acid bacteria, and provide the basis for breeding excellent strains.In this study,the whole genome sequencing showed that the genome size of *L.p*R3 strain is 3.095 Mb with a GC content of 46.17%, and possesses 3171 protein-coding sequences. COG annotation data of *L.p* R3 shown that the protein coding sequence mainly involves the basic physiological activities of the strain,such as Carbohydrate transport and metabolism(13.01%), General function prediction only(9.32%), Translation, ribosomal structure and biogenesis(9.28%), Amino acid transport and metabolism(8.90%), Transcription(6.48%), Inorganic ion transport and metabolism(5.16%) and Cell wall/membrane/envelope biogenesis(5.06%). It is noteworthy that the strain contains proteins related to Lipid transport and metabolism(3.27%). *L.p* R3 may have lipid-lowering potential, it is valuable to be researched fartherly. Fortunately,the strain had no plasmids on its chromosomes and no virulence genes were detected. It was safe for antibiotic resistance gene delivery and virulence gene. To further evaluate the safety of *L.p* R3, we also examined its hemolysis on the blood plate and its infection risk in mice. The strain did not produce hemolytic rings in vitro, nor did it cause infection in mice fed with high concentration of live bacteria, and did not affect mice normal physiological activities.

L.p R3, as a probiotic strain, were specially studied on the function of intestinal protection in our laboratory. The mouse model of ulcerative colitis was successfully induced by DSS. The treatment of UC model mice by intragastric administration of *L.p* R3 significantly relieved UC symptoms and pathological damage of colon tissue. During our study, UC model mice were significant the body weight loss, poor mental state, hematochezia and hair loss,and their colon pathology showed mucosal tissue damage and extensive inflammatory cell infiltration. Interestingly, the above-mentioned conditions in mice treated with MSLZ or *L.p* R3 improved significantly. DAI and histological damage scores were used to quantify the extent of damage in each group mice.The values of two indicators are in the same order, and the order from large to small is DSS group > MSLZ group ≥ LP R3 group > LP R3 + MSLZ group. This study showed that MSLZ and *L.p* R3 had a therapeutic effect on UC, and the combination of probiotics and MSLZ had a better therapeutic effect.

UC is a refractory inflammatory disease that mainly affects the intestine, and is one of the chronic diseases that endanger human health. Global prevalence is projected to affect up to 30 million individuals by 2025[19]. The incidence of UC continues to increase, but so far, the specific pathogenesis of UC is unknown. It is generally believed that the pathogenesis of UC involves the results of multiple interactions including genetics, microorganisms, and the body's immune system[20]. In recent years, the pathogenesis of UC has been continuously studied through UC patients and animal models of UC, and it has been found that the occurrence and development of UC are closely related to the balance of Foxp3 + Tregs cells and Th17 cells (producing IL-17A and IL-17F)in vivo[21–23]. Regulatory T cells(Tregs) are a subset of T cells with negative immune regulation. Tregs play an important role in maintaining immune balance and forming peripheral immune tolerance. Tregs can participate in the inflammatory response in vitro through a cell contact-dependent mechanism. Tregs can also

participate in the pathogenesis of UC by regulating the release of inhibitory cytokines (IL-10, etc.), and their low number or dysfunction can lead to the occurrence of the disease[23–25]. T helper cell 17(Th17) is a subset of T cells capable of secreting IL-17. IL-17 is a pro-inflammatory cytokine with pro-inflammatory activity that plays diverse roles in different diseases. The level of IL-17A was significantly increased in UC patients compared with normal subjects, and similarly, in a mouse model of ulcerative colitis, the content of IL-17A was also significantly higher[26, 27].

In this study, we found that Tregs and Th17 cells are keep balance under normal conditions. But in the inflammatory state, Th17 cells migrate from the circulatory system to local inflammatory sites in the intestine and are enriched at the site of inflammatory injury, resulting in a highly activated mucosa of the digestive tract, inducing intestinal immune response, increased release of injurious cytokines, leading to mucosal injury, and compensatory increase of Treg cells, but their increased levels cannot counter the increased levels of pro-inflammatory factors, resulting in an inflammatory response. The expression of Th17 and Tregs cell markers in colon tissues was detected by immunohistochemistry and Western blotting. In the results of immunohistochemistry,normal group as reference, the expression of Foxp3, IL-10, IL-17A and IFN-γ increased to varying degrees in DSS group and 3 treated groups. Compared with DSS group, *L.p* R3 had the same effect as MSLZ. The strain increased Foxp3 and IL-10 expression, but decreased IL-17A and IFN-γ expression, they were statistically different ($p < 0.05$). Again, in the results of Western blotting, the CD4,CD25 and Foxp3 expression of the three treatment groups was higher than that of the DSS group, while the expression of IL-17A and IFN-γ in treatment group was significantly lower than that in DSS group($p < 0.05$). Obviously, L.p R3 could promote the expression of Tregs cell markers and inhibits the expression of Th17 cell markers. the Treg/Th17 cell imbalance in inflammation caused by DSS was restored after treatment.

Detection of cytokines in serum of experimental mice and further analysis of anti-inflammatory mechanism of *L.p* R3.The contents of IFN-γ, IL-17A, IL-17F and IL-10 were increased in DSS group,except for IL-10, there were significant differences($p < 0.05$). Interestingly, the three treatment groups were just the opposite of the model group. The levels of peripheral blood IFN-γ, IL-17A and IL-17F decreased significantly and IL-10 increased significantly in three treatment groups($p < 0.05$). Moreover, the IL-17A/IL-10 value of DSS group was significantly higher than that of the other four groups($p < 0.05$). This indicates that Th17 cells infiltrate and secrete pro-inflammatory factors IL-17A during inflammation. With *L.p* R3 or/ and MSLZ treatment, the IL-10 of ant-inflammatory factors increased, the value of IL-17A/IL-10 reversed. To further confirm that *L.p* R3 has a good therapeutic effect on UC by regulating Th17/Treg cell balance.

Therefore, we speculated that the supplementation of *L.p* R3 improved the imbalance of intestinal microorganism, down-regulated the activity of Th17 cells, decreased the secretion of pro-inflammatory cytokines and induced the production of Tregs, and achieved a new balance of intestinal pro-inflammatory and anti-inflammatory cytokines, thereby improving intestinal inflammatory response and mucosal injury, which may be one of the important mechanisms of *L.p* R3 treatment of UC. In this study, the therapeutic effect of *L.p* R3 was similar to that of mesalazine, and the combination therapy was better, providing a new choice for clinical treatment.

Conclusions

In summary, *L.p* R3 significantly improves the symptoms and the pathological damage of mice with colitis and influences the immune function by regulating Treg/Th17 cell balance in DSS-induced colitis in mice. And *L.p* R3 had the advantages of high safety, good gastrointestinal fluid tolerance and strong biofilm formation ability.These results provide a good basis for commercial production. However, whether the clinical application of *L.p* R3 bacteria could achieve the same therapeutic effect, considering the complexity between human body and experimental animals, needed further study.

Abbreviations

L.p
Lactobacillus paracasei; UC:ulcerative colitis; IBD:Inflammatory bowel diseases; DSS:Dextran sulfate sodium; DAI:disease activity index; MSLZ:mesalazine;

Declarations

Ethics approval and consent to participate

This study was approved by and performed in accordance with the guidelines of the ethics committee of Guangdong Medical University.

Consent for publication

Consents for publication were obtained from all participants.

Competing interests

The authors declare no competing interests.

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Author Contributions

J.C.Z, W.Q.Y, J,H, and Z.Y.Y conceived, designed and supervised the overall study. J,H, and Z.Y.Y, Y.Y.L collected and verified the mycobacterial strains. X.X.C, Y.F.L, B.H.L, S.B.Z, Z.P.C, H.L.Z, and X.Y.Z processed the samples and performed the experiments. Z.Z T.C J.H and Z.Y.Y analysed the data. J,H, Z.Y.Y, W.J.W and J.C.Z wrote the paper. All authors read and approved the final manuscript.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article.

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Figures

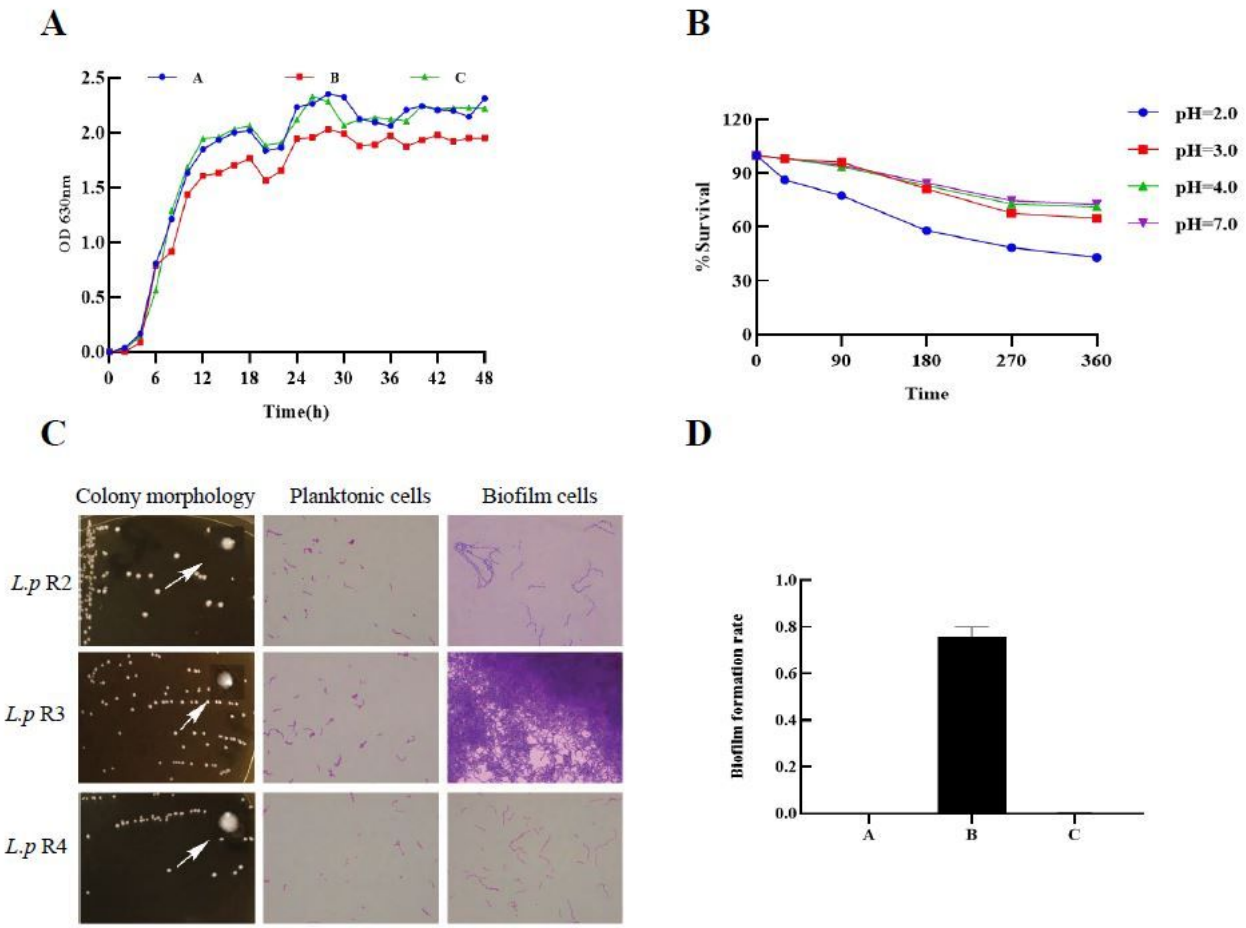

Figure 1

Biological properties of L.p R3 strain. (A)Growth curves of L.pR2, R3 and R4 strains were detetcted. (B) Gastrointestinal tract tolerance of L.p R3 strain were examined. (C and D) Biofilm formation ability of L. p R2, R3 and R4 strains were measured.

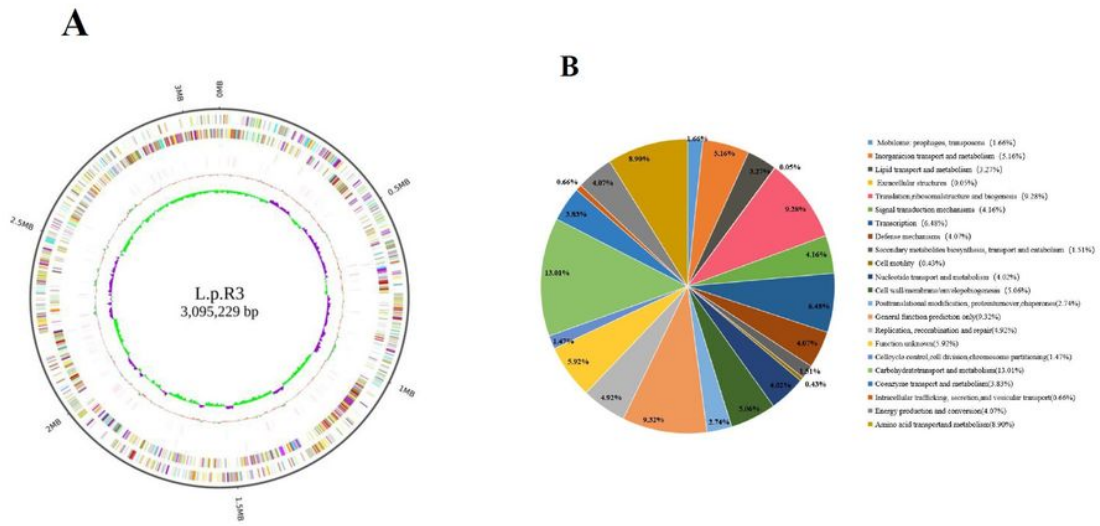

Figure 2

whole genome sequencing analysis of L.p R3 strain. (A)The genome cycle graph of L.p R3. (B)COG Function Classification

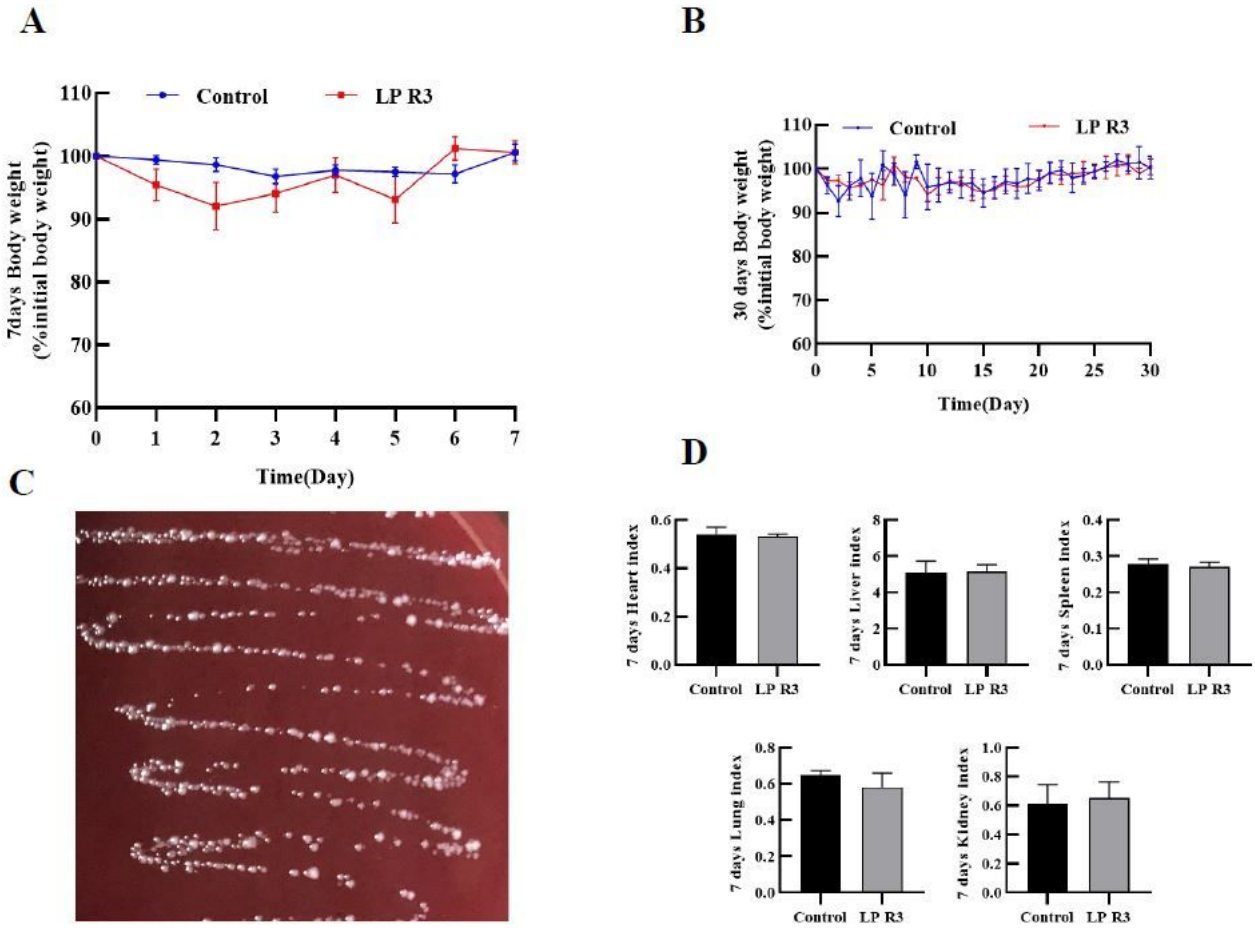

Figure 3

Safety evaluation of L.p R3 (A) Weight changes in mice fed for 7 days. (B)Weight changes in mice fed for 30 days. (C)Experimental results of hemolysis. (D)Organ index of mice.

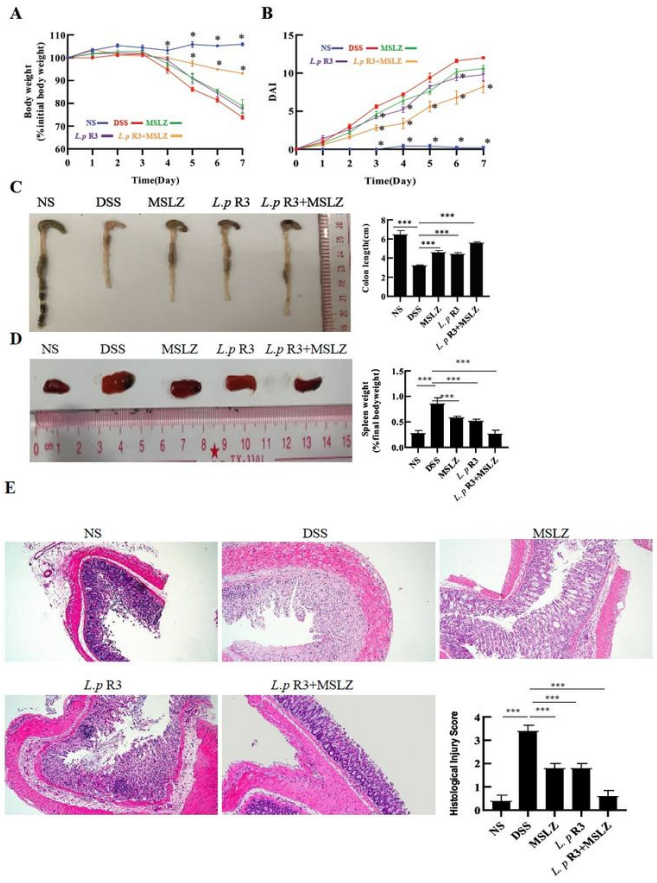

Figure 4

L.p R3 strain ameliorates the general symptoms of murine colitis (A) Percentage change in body weight of mice after induction of colitis.(B) Daily disease activity index (DAI) of mice after induction of colitis.(C) Representative images showing the colon lengths on days 14. (D) Representative images of the spleen and SI on days 14. (E)Histological analysis of the severity of colon sections in DSS-induced colitis mouse model; Colon tissue was paraffin-embedded and stained with hematoxylin and eosin. Cellular infiltration, crypt distortion, and goblet cell loss were analyzed on days 14.H&E stain scale: 100 mm. Values are means ± SEMs (n = 6). Statistical analysis was performed by two-way ANOVA with a NewmaneKeuls post-hoc test.*P < 0.05, compared with DSS group.

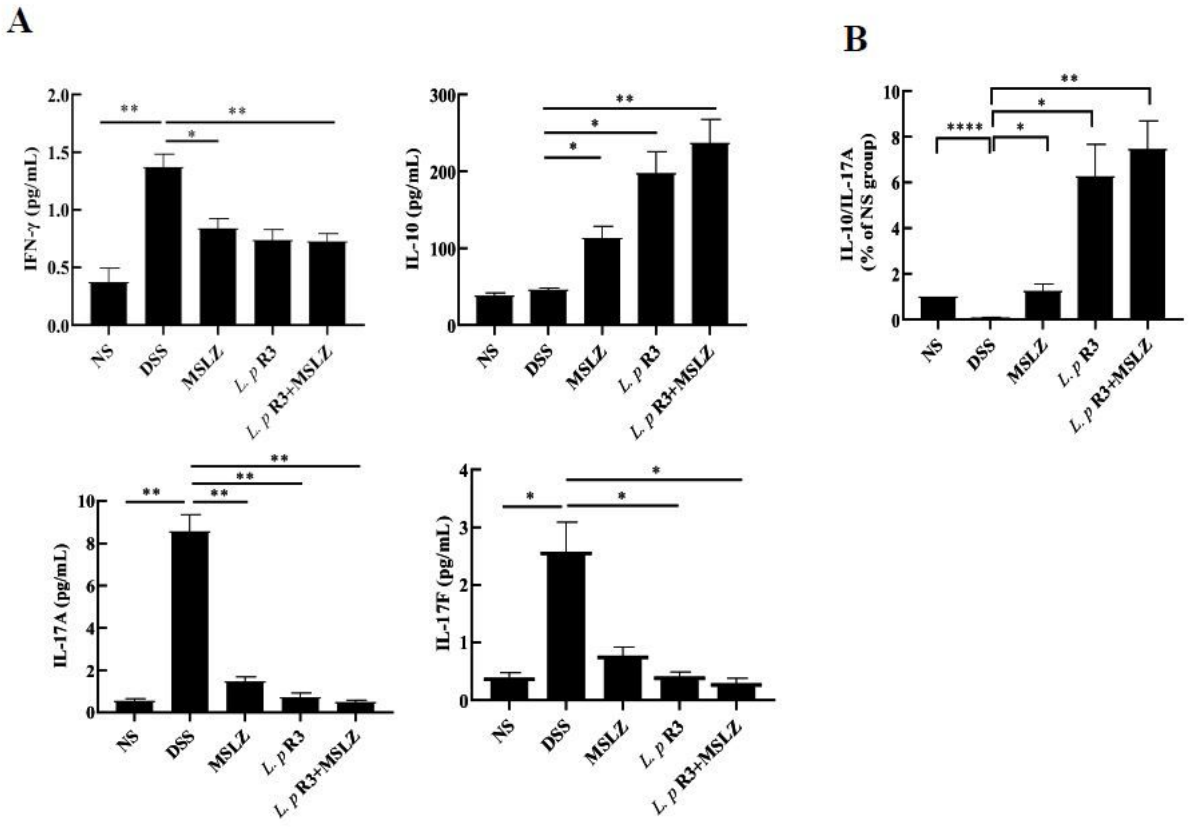

Figure 5

Detection of serum immune cytokines by liquid-phase chip method. (A)The contents of IFN-γ, IL-17A, IL-17F and IL-10 in peripheral serum.(B)IL-17A/IL-10 in peripheral serum of each group mice.Statistical analysis was performed by two-way ANOVA with a NewmaneKeuls post-hoc test.*P < 0.05, compared with DSS group.

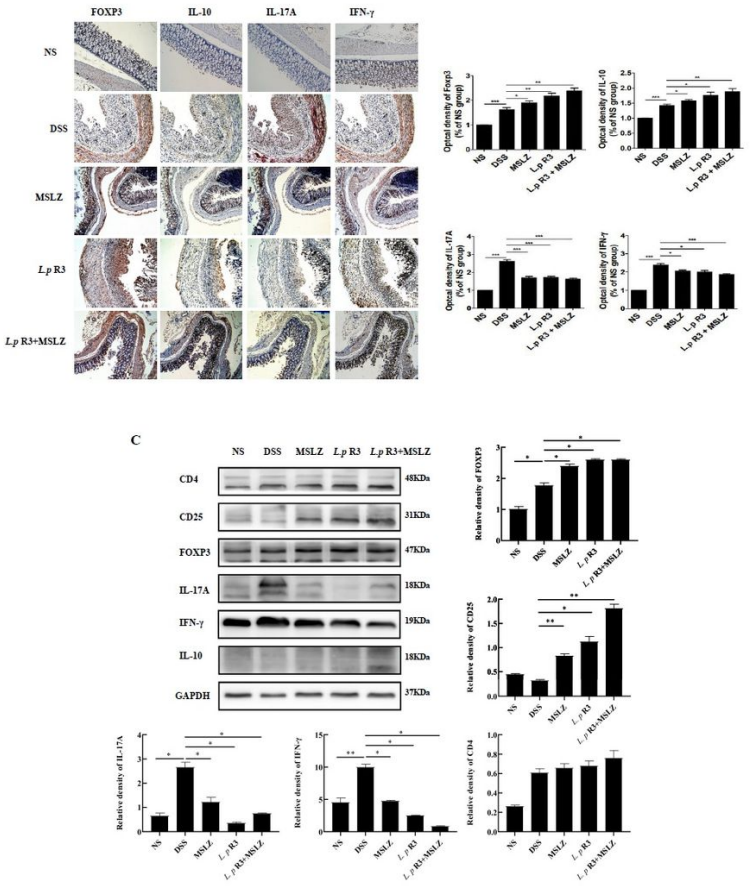

Figure 6

L.p R3 attenuates colonic inflammatory responses but induces Treg responses in DSS-induced experimental murine colitis. (A)Foxp3, IL-10, IL-17A and IFN-γwere detected in the colon by the immunohistochemical method. (B)Positive score of colon Foxp3, IL-10, IL-17A and IFN-γpathology in each group. (C) Release of CD4, CD25, FOXP3, IL-17A, IFN-γ, IL-10 proteins in the colonic tissues was assessed by immunoblotting and the realative density of CD4, CD25, FOXP3, IL-17A, IFN-γproteins in the colonic tissues was assayed by Image-Pro Plus*,P < 0.05; **,P < 0.01; ***,P < 0.001.The results are representative of at least three independent experiments.