

Effect of Intestinal Microbiota Alteration on Hepatic Damage in Rats with Acute Rejection After Liver Transplantation

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Abstract The previous studies all focus on the effect of probiotics and antibiotics on infection after liver transplantation. Here, we focus on the effect of gut microbiota alteration caused by probiotics and antibiotics on hepatic damage after allograft liver transplantation. Brown-Norway rats received saline, probiotics, or antibiotics via daily gavage for 3 weeks. Orthotopic liver transplantation (OLT) was carried out after 1 week of gavage. Alteration of the intestinal microbiota, liver function and histopathology, serum and liver cytokines, and T cells in peripheral blood and Peyer's patch were evaluated. Distinct segregation of fecal bacterial diversity was observed in the probiotic group and antibiotic group when compared with the allograft group. As for diversity of intestinal mucosal microbiota and pathology of intestine at 2 weeks after OLT, antibiotics and probiotics had a significant effect on ileum and colon. The population of *Lactobacillus* and *Bifidobacterium*

in the probiotic group was significantly greater than the antibiotic group and the allograft group. The liver injury was significantly reduced in the antibiotic group and the probiotic group compared with the allograft group. The CD4/CD8 and Treg cells in Peyer's patch were decreased in the antibiotic group. The intestinal Treg cell and serum and liver TGF- β were increased markedly while CD4/CD8 ratio was significantly decreased in the probiotic group. It suggested that probiotics mediate their beneficial effects through increase of Treg cells and TGF- β and deduction of CD4/CD8 in rats with acute rejection (AR) after OLT.

Abbreviations

OLT Orthotopic liver transplantation
AR Acute rejection

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Introduction

Orthotopic liver transplantation (OLT) is a life-saving procedure for patients with end-stage liver disease such as acute or chronic hepatic failure and cirrhosis [1]. Nevertheless, morbidity and mortality due to rejection and infectious complications remain as major problem following transplantation. Advances in surgical procedure, immunosuppressive therapies, prophylactic strategies, critical care, and donor and recipient screening have contributed to the evolving microbiology and epidemiology of infectious complications after liver transplantation [2]. Although decreased overall, infections continue to be a major contributor to graft loss and morbidity [3]. Meanwhile, with the use of prophylactic antibiotics, the emergence of antibiotic resistance has increased significantly [4]. Strategies in preparing patients for liver transplantation besides mechanical bowel preparation and administration of antibiotics must be considered. Many of the observed

infections are gut derived, and bacterial translocation has been considered an important contributor to the development of infection and sepsis in surgical patients [5]. These suggest that an alternative approach of infection treatment is to restore the damaged microbial intestinal communities.

In the past, several published papers confirmed the positive influence of probiotics administration on infection after liver transplantation [6, 7]. The previous studies all focus on the effect of probiotics and antibiotics on infection after liver transplantation. To date, there are few studies examining the relationship between gut microbiota and acute rejection after liver transplantation [8, 9]. We hypothesized that alteration in gut microbiota after using probiotics and antibiotics could have an effect on liver injury after OLT. So, we studied if and how the intestinal microbiota alteration plays a role in the pathogenesis of liver injury in rats with acute rejection (AR) after OLT. To achieve this aim, the alteration of gut microbiota was induced through oral supplementation of probiotics and antibiotics. Alteration of the intestinal microbiota, mucosal histology and ultrastructure, liver function (Table 1), hepatic histopathology, serum and liver cytokines, CD4⁺ T cells, CD8⁺ T cells, and Treg cells in peripheral blood and Peyer's patch were evaluated.

Materials and Methods

Animal and Liver Transplantation Model

Inbred male Lewis ($n=24$) and Brown-Norway (BN, $n=24$) rats weighing 200–250 g were purchased from the Vital River Laboratories (Beijing, China). All animals were fed with commercial rat chow pellets and housed under conditions of constant temperature (22 °C) and humidity in a specific-pathogen-free facility. Allograft, antibiotic, and probiotic models were studied. The allograft group (BN, $n=8$) received

2 ml/day of physiologic saline by daily gavage for 3 weeks. The antibiotic group (BN, $n=8$) received 2 ml/day of gentamicin solution (4 U, Zhejiang RuiXin Pharmaceutical, Hangzhou, China) by daily gavage for 3 weeks. The probiotic group (BN, $n=8$) received 2 ml/day of Peifeikang (2.0×10^8 CFU/ml of living combined *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*, Shanghai XinYi Pharmaceutical, Shanghai, China) for 3 weeks. Orthotopic liver transplantation (OLT) was carried out from Lewis to BN rats after 1 week of gavage. OLT was performed according to the technique as previously described by Kamada and Calne [10]. The transplantation procedure required less than 60 min, during which the portal vein was clamped for 13 to 15 min. No blood transfusion or immunosuppressive regimen was administered. All procedures in this study were performed according to the guidelines of the Council of Animal Care at Zhejiang University. Six recipients (two in each group) who died within 2 weeks after the transplantation were excluded from the study. One in the probiotic group died at the 3rd day after the OLT because of infrahepatic vena cava thrombosis. The remaining five recipients died within 2 weeks after the OLT because of biliary obstruction or rejection. There were six recipients alive in each group on the 15th day after the OLT.

Sample Collection

Fresh feces were collected at 1 day before OLT (T1) and 1 week (T2) and 2 weeks (T3) after the operation, respectively, and stored at -20 °C for further analysis. On the 15th day after the OLT, all animals were sacrificed after halothane anesthesia. Blood was collected from the portal vein for measurement of liver function and cytokines. The mesenteric lymph nodes from the ileocecal area and portal vein blood were taken for flow cytometric analyses. Tissue samples from the left lobe of the liver were taken for histological and cytokine analysis. Jejunum, ileum, and colon samples were taken for microbiological and histological study.

Table 1 Comparison of liver function between the experiment groups

	Allograft	Antibiotic	Probiotic
ALB (g/l)	17.47±1.37	16.52±2.75	15.87±0.92
ALT (U/l)	268.67±143.27	149.4±103.59	187.273±54.43
AST (U/l)	1,207.50±386.48	638.4±223.30*	788.27±148.30*
TB (μmol/l)	149.48±27.94	126.23±32.48	148.17±20.34
DB (μmol/l)	101.80±19.49	84.67±21.60	107.77±15.55
AKP (U/l)	373.60±42.45	287.5±71.10*	366.5±78.83
GGT (U/l)	11.40±2.24	11.50±3.02	12.77±1.32

Values were expressed as mean±SD

ALB serum albumin, ALT alanine transaminase, AST aspartate aminotransferase, TB total bilirubin, DB direct bilirubin, AKP alkaline phosphatase, GGT glutamyl peptidase

* $p<0.05$ compared with the allograft group

Liver Function

Blood samples collected at T3 were centrifuged at 3,000g for 10 min at room temperature to separate the serum for liver function analysis by Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan).

DNA Extraction

The bacterial DNA was extracted from the fecal, jejunum, ileum, and colon samples using QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions with minor modification as described [8, 11]. All DNA was stored at -20 °C before further analysis.

PCR-DGGE Analysis of the 16S rRNA Gene V3 Region

For amplification of bacterial DNA, universal bacterial primers 341F and 534R for the V3 regions of 16S rRNA genes and the reaction conditions were performed as described in our previous studies [8, 11]. Denaturing gradient gel electrophoresis (DGGE) of the PCR products were performed with 25 to 50 % gradient, using a D-Code system (Bio-Rad) as described [12, 13].

Real-Time Quantitative PCR

16S rRNA gene-targeted quantitative PCR (qPCR) for *Lactobacillus*, *Clostridium leptum*, *Bacteroides*, *Bifidobacterium* spp., and Enterobacteriaceae was performed with a Power SYBR Green PCR Master Mix (Takara, Dalian, China) on an CFX96 Real-Time PCR Detection System according to the manufacturer's instructions (Bio-Rad, Hercules, CA). The primer and reaction conditions were performed as described in our previous studies [8, 11].

Histological Evaluation

Samples from the left lobe of the liver, jejunum, ileum, and colon were fixed, paraffin-embedded, cut into 2- μ m sections, and then examined under light microscopy after staining with hematoxylin and eosin. At least three slides were studied from each specimen in a blinded fashion. Ileum samples were fixed and then embedded as described [14]. The ultrastructure of the intestinal mucosa was analyzed using a Philips Tecnai 10 electron microscope (Philips, Eindhoven, Netherlands).

Flow Cytometric Analysis of Blood and Peyer's Patch Samples

Abs against the markers anti-rat CD3-PE, anti-rat CD8a-PE-Cy7, anti-rat CD4-FITC, anti-rat CD25-PE, and anti-mouse/rat Foxp3-PE-Cy5 with corresponding isotype-matched controls were purchased from eBioscience (San Diego, CA, USA). For CD4⁺ T cells and CD8⁺ T cells, red blood cells were eliminated by incubation for 10 min with lysing buffer and then were centrifuged at 250g for 5 min. Peyer's patch isolation was performed as described [15]. In each flow cytometry tube, 200- μ l blood or Peyer's patch cell (2×10^6) suspensions were incubated with 10 μ l CD4-FITC, 5 μ l CD3-PE, and 10 μ l CD8a-PE-Cy7 for 20 min at 4 °C in the dark. Cell pellet were resuspended and washed with 2 ml phosphate buffer saline (PBS) by 250 g for 5 min. Washed cell pellets were then resuspended in 200 μ l PBS containing 1 % paraformaldehyde for analysis. For Treg cells, 200- μ l blood or Peyer's patch cell (2×10^6) suspensions were incubated with 0.5 μ l CD4-FITC and 0.6 μ l CD25-PE for 20 min at 4 °C in the dark. Then, 2 ml flow cytometry staining buffer

was added. The mixture was then centrifuged at 250g for 5 min. The suspensions incubated with Fixation/Permeabilization Concentrate for 40 min at 4 °C in the dark. Cell pellet were resuspended and washed with 2 ml permeabilization buffer by 250 g or 5 min. Then, the suspensions were incubated with FoxP3-PE 5 μ l for 30 min at 4 °C in the dark and then washed by permeabilization buffer. Washed cell pellets were then resuspended in 200 μ l flow cytometry staining buffer for analysis.

Cytokine Analysis

The effector serum cytokines levels of Th1 (IL-2, IFN- γ , TNF- α), Th2(IL-4, IL-5, IL-13), Treg (TGF- β 1), and Th17 (IL-17) were determined using a multiplex reader (BioRad Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's recommended protocol. Data were analyzed using Bio-Plex manager software. Rat liver-extracted above cytokines were assayed using ELISA adapting the procedures recommended by the manufacturer (R&D Systems, China).

Statistics Analysis

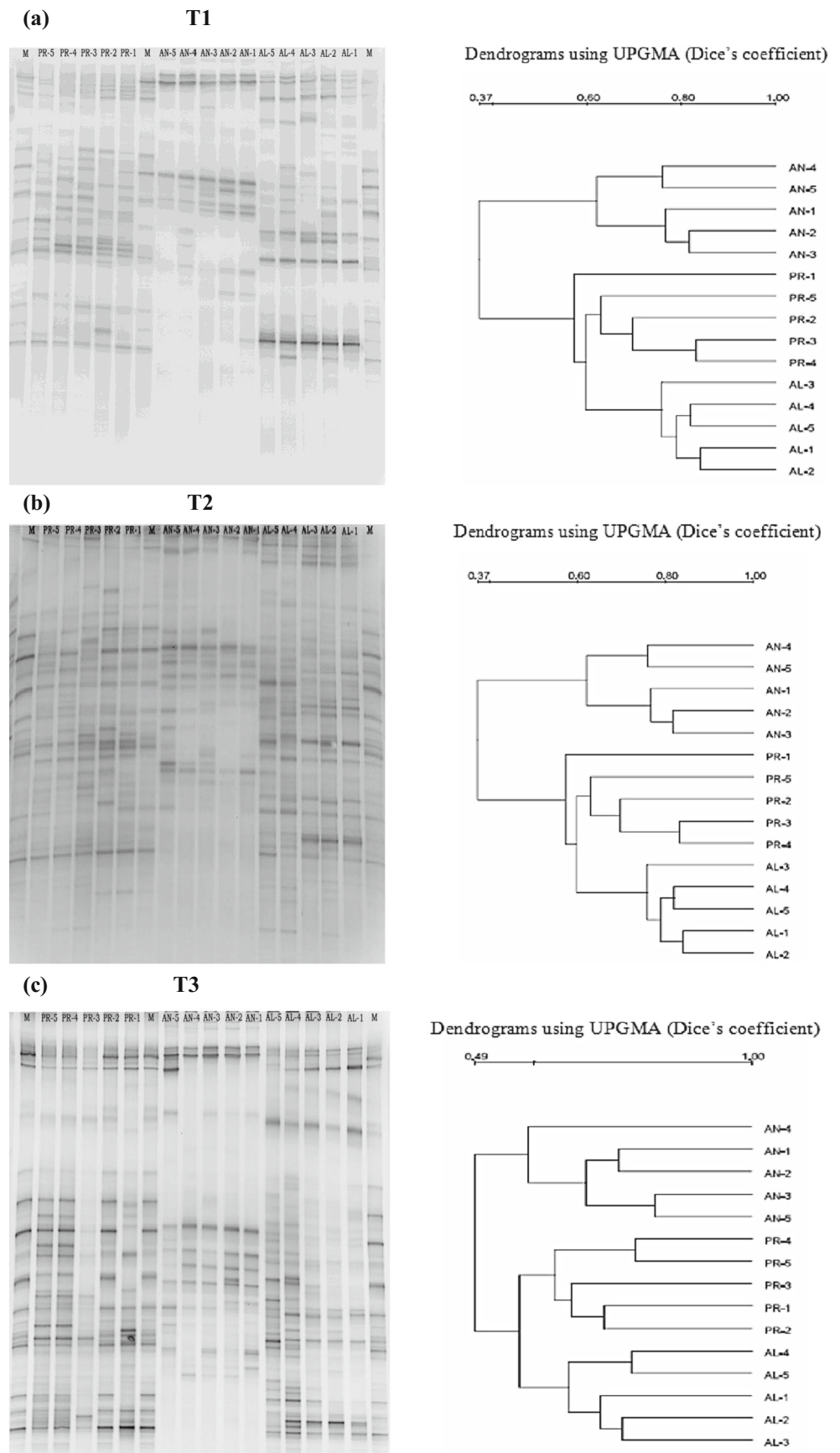
The similarities of PCR-DGGE DNA profiles were analyzed with Quantity One[®] 1-D Analysis software (version 4.6.2; Bio-Rad Laboratory, Hercules, CA, USA). A similarity matrix was constructed using Dice's similarity coefficient. Dendrograms were constructed by the unweighted pair group method, using arithmetic averages (UPGMA). Quantitative data were expressed as means \pm standard deviation (SD). Statistical analyses were performed with SPSS 13.0 for windows (SPSS Inc., Chicago, IL, USA) using Fisher's exact test for categorical data and one-way ANOVA for quantitative data.

Results

Dendrograms Using UPGMA Based on PCR-DGGE Fingerprint

PCR-DGGE fingerprint analysis for predominant bacteria was used to capture the structural response in gut microbiota before and after the operation in the three groups (Fig. 1a–c). Dendrograms using UPGMA based on the PCR-DGGE fingerprint indicated that fecal microbiota structures of the three groups showed significant difference. The bacterial diversity reduced significantly in the antibiotic group compared with the allograft group and the probiotic group at T1, T2, and T3 (Fig. 1a–c, the similarity indices were 41, 42, and 45 %, respectively). A very distinct segregation was also observed in

Fig. 1 Structural analysis of gut microbiota in rats of allograft, probiotic, and antibiotic groups. **a–c** 16S rRNA gene V3 region PCR-DGGE profiles and UPGMA dendrograms of fecal samples obtained from allograft group (*AL* group), probiotic group (*PR* group), and antibiotic group (*AN* group) at 1 day (*T1*) before the OLT and 1 week (*T2*) and 2 weeks (*T3*) after the OLT; *M* represents the marker for DGGE analysis

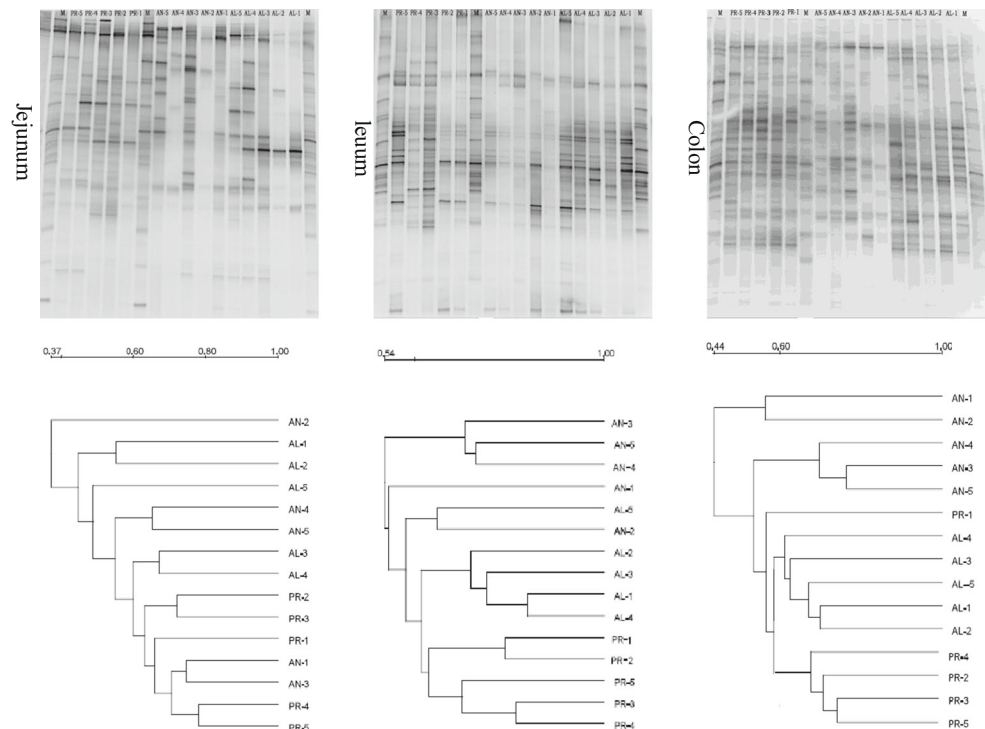


the probiotic group compared with the allograft group at T1, T2, and T3 (Fig. 1a–c, the similarity indices were 51, 60, and 61 %, respectively). As for diversity of intestinal mucosal microbiota, it was similar between the three groups in jejunum at T3. However, a very distinct segregation was observed in ileum and colon between the three groups at T3 (Fig. 2, the similarity indices were 54 and 44 %, respectively).

Comparison of Predominant Bacteria by Real-Time qPCR

Since drastic changes were observed in bacterial community diversity between the groups, real-time qPCR was used to quantify predominant bacteria of fecal samples. The mean log₁₀ 16S ribosomal DNA (rDNA) gene copy number, standard deviation, and *p* value probabilities at T1, T2, and T3 were given in Table 2. Considering the number of 16S rDNA genes of the *Bacteroides*, no significant difference was found between the three groups at T1, T2, and T3 ($p > 0.05$). The rats in the allograft group had significantly greater population sizes of the Enterobacteriaceae than the antibiotic group at T2 ($p < 0.05$). The rats in the probiotic group had significantly greater population sizes of the *Lactobacillus* and *C. leptum* than the antibiotic group at T3 ($p < 0.05$). As for *Bifidobacterium* spp., there was a significant increase in the probiotic group when compared with the antibiotic group at T1, T2, and T3 ($p < 0.05$). There was a significant increase in *Lactobacillus* and *Bifidobacterium* spp. gene copies in the probiotic group when compared with the allograft group at T3 ($p < 0.05$).

Fig. 2 Structural analysis of intestinal microbiota in rats of the allograft, probiotic, and antibiotic groups. 16S rRNA gene V3 region PCR-DGGE profiles and UPGMA dendrograms of intestinal mucosa samples obtained from allograft group (AL group), probiotic group (PR group), and antibiotic group (AN group) at 2 weeks after the OLT; M represents the marker for DGGE analysis



Histopathology and Ultrastructure of the Intestinal Mucosa

Electron microscopy revealed that the microvilli of the ileum epithelial cells in the probiotic group were homogeneously distributed. In the allograft group, the microvilli were sparse and stunted. However, in the antibiotic group, they were ruptured, stunted, and more sparsely distributed than the allograft group (Fig. 3). In the allograft group, histological macroscopic examination showed neutrophil and lymphocyte infiltration, hyperemia, and few fluid accumulation within the lumen of the jejunum. The similar observations were showed in rats subjected to the antibiotic and the probiotic group. Intestinal inflammation was semiquantitatively graded as follows: 1 (normal mucosa), 2 (slight inflammation), 3 (intermediate inflammation), 4 (severe inflammation), and 5 (fulminant inflammation) according to Danielsson et al. [16, 17]. There was no significant difference between the three groups in the jejunum (2.67 ± 0.51 , 2.50 ± 0.54 , and 2.50 ± 0.54 , respectively, $p > 0.05$). The inflammation score of the ileum in the antibiotic group (2.17 ± 0.41) and the probiotic group (2.30 ± 0.52) showed less than the allograft group (3.00 ± 0.63 , $p < 0.05$). The colonic inflammation score of the Allograft group (3.50 ± 0.55) was significantly higher than the antibiotic group (2.33 ± 0.52) and the probiotic group (2.50 ± 0.84 , $p < 0.05$) (Fig. 4).

Hepatic Histopathology

Under light microscope, the allograft group showed prominent portal, periportal, and perivenular necrosis, with marked

Table 2 Quantity of predominant bacteria in feces by real-time qPCR

Phylogenetic group		Bacterial counts (copies/g) ^a			<i>p</i> value one-way ANOVA		
		Allograft Mean±SD	Antibiotic Mean±SD	Probiotic Mean±SD	Allograft-gentamicin	Allograft-Probiotic	Antibiotic-Probiotic
<i>Bacteroides</i>	T1	6.84±0.86	6.85±1.82	7.95±0.23	0.997	0.121	0.121
	T2	7.75±0.41	7.71±0.95	7.61±0.57	0.925	0.730	0.801
	T3	8.20±0.30	7.26±1.09	7.42±0.83	0.065	0.118	0.741
<i>Bifidobacterium</i> spp.	T1	3.94±0.78	2.99±0.76	4.60±0.27	0.158	0.243	0.007*
	T2	3.55±1.14	3.50±0.49	4.73±0.64	1.000	0.151	0.013*
	T3	3.71±0.78	4.46±0.71	4.45±0.75	0.579	0.035*	0.037*
<i>Clostridium leptum</i>	T1	5.30±0.46	5.40±0.81	5.27±0.67	0.799	0.939	0.741
	T2	5.04±0.49	5.10±0.63	5.02±0.97	0.886	0.972	0.858
	T3	5.36±0.77	4.69±0.59	6.23±0.27	0.306	0.102	0.002*
<i>Lactobacillus</i>	T1	5.02±0.21	5.16±1.32	4.87±1.19	0.819	0.809	0.638
	T2	5.48±0.96	5.69±1.18	5.96±1.08	0.742	0.453	0.670
	T3	5.08±0.79	4.93±0.73	6.06±1.00	0.248	0.048*	0.035*
Enterobacteriaceae	T1	6.31±0.75	6.31±0.62	6.28±0.63	0.996	0.935	0.939
	T2	6.51±0.33	6.01±0.22	6.07±0.48	0.030*	0.052	0.781
	T3	6.32±0.60	5.76±0.47	5.73±0.46	0.080	0.064	0.906

Comparison of predominant bacteria in feces between the allograft group, antibiotic group, and probiotic group at T1, T2, and T3 by real-time qPCR. Values were shown as mean±standard errors (SD). Statistical differences were calculated by one-way ANOVA

* $p < 0.05$ (statistical significance between groups)

^aLog10 no. of 16S rDNA gene copies per gram (wet weight)

patchy edema. Extensive mononuclear inflammatory infiltrate was seen in the portal spaces with lobular expansion. The lobular architecture was distorted. These pathological changes were shown with arrows in Fig. 5. Necrosis or inflammation of hepatic parenchyma was semiquantitatively graded as follows: 0 (absent), 1 (mild), 2 (moderate), and 3 (extensive). The necrosis or inflammation of hepatic parenchyma in the antibiotic group (1.37 ± 0.51) and the probiotic group (1.25 ± 0.46)

showed less than the allograft group (2.87 ± 0.35 , $p < 0.05$) (Fig. 5).

Comparison of Liver Function

The liver function level of the rats from the three groups was listed in Table 1. There was a statistically significant decrease of AST in the antibiotic group and the probiotic group when

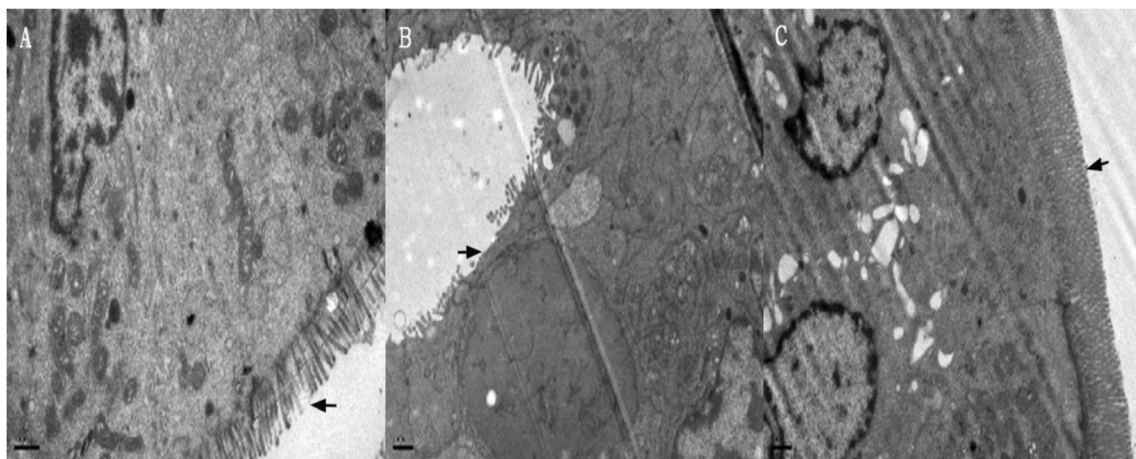
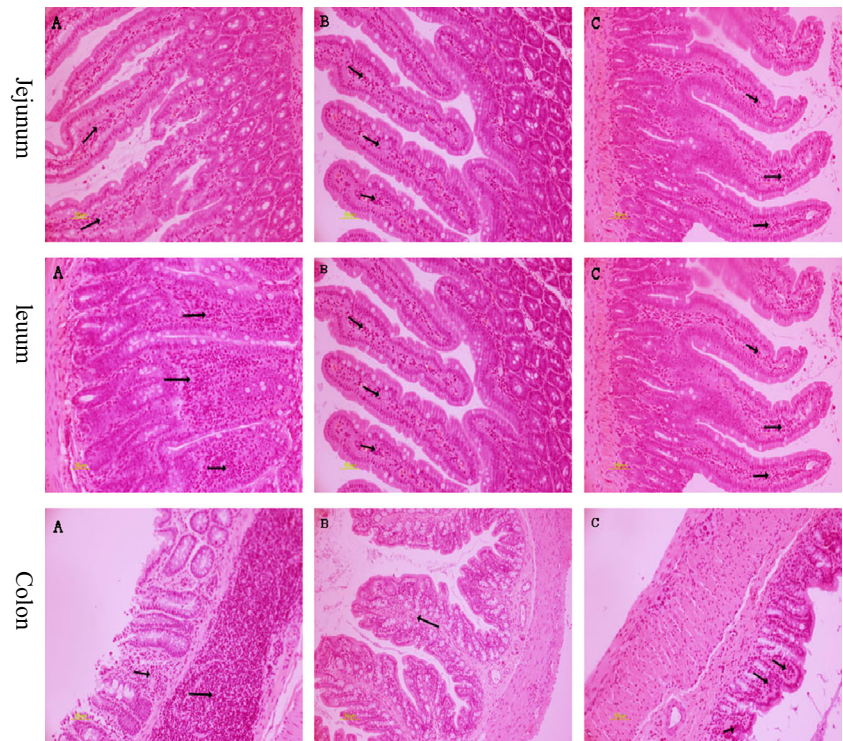


Fig. 3 Transmission electron micrograph of ileal mucosal structure ($\times 10,000$, $bar = 1 \mu m$). **a** Allograft group, **b** antibiotic group, and **c** probiotic group (Black arrow, microvilli)

Fig. 4 Light micrograph of the jejunum, ileum, and colon stained with hematoxylin and eosin (HE, $\times 200$). **a** Allograft group, **b** antibiotic group, and **c** probiotic group. *Black arrow*, example of neutrophil and lymphocyte infiltration



compared with the allograft group ($p < 0.05$). AKP of the antibiotic group was lower than the allograft group ($p < 0.05$). There was no significant increase of other liver function index (ALB, ALT, TB, DB, GGT) in the allograft group when compared with the antibiotic group and the probiotic group ($p > 0.05$).

T Cells and Cytokines

Compared with the allograft group, the CD4/CD8 in patch was decreased in the antibiotic group and the probiotic group.

The Treg cells in Peyer’s patch were decreased in the antibiotic group, but increased in the probiotic group when compared with the allograft group (Fig. 6). In contrast to the cell populations identified in the Peyer’s patch, there was no significant difference of CD4/CD8 and Treg cells in the blood between the three groups (Fig. 7). As for serum and liver cytokines (IFN- γ , TNF- α , IL-4, IL-5, IL-13, and IL-17), there was no significant statistical difference between the three groups. The concentrations of serum IL-2 was significantly decreased while TGF- β 1 was increased in the probiotic group compared with the allograft group ($p < 0.05$). The TGF- β 1

Fig. 5 Light micrograph of hepatic histopathology stained with hematoxylin and eosin (HE, $\times 200$). **a** Allograft group, **b** antibiotic group, and **c** probiotic group. *Black arrow*, mononuclear inflammatory infiltration; *red arrow*, necrosis; and *green arrow*, hyperemia

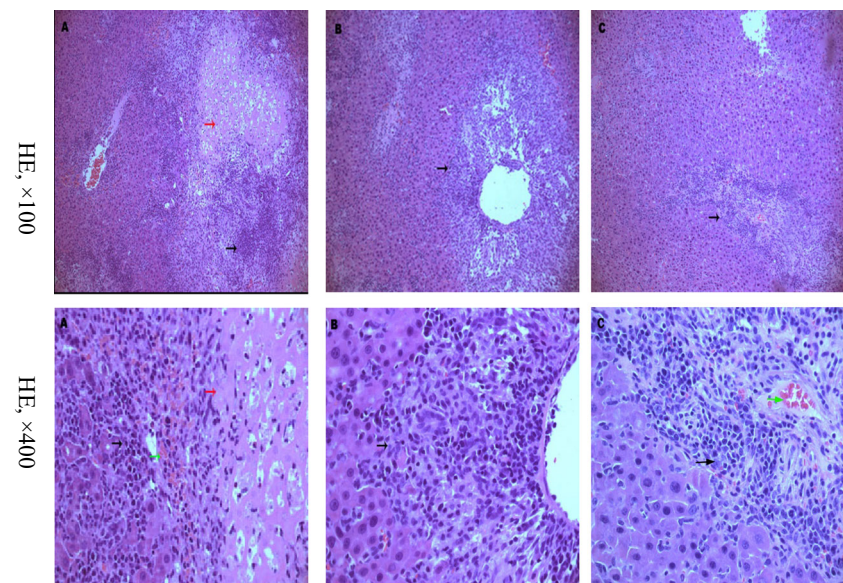
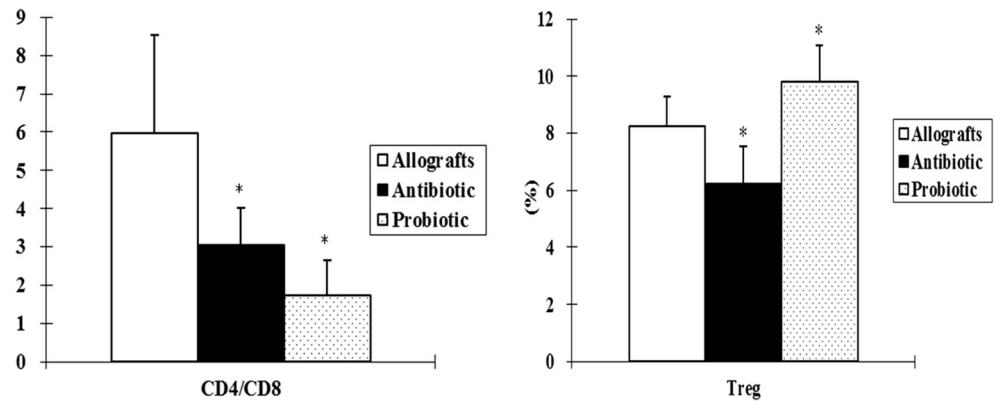


Fig. 6 Comparison of CD4/CD8 and Treg cells in Peyer's patch between the experiment groups. * $p < 0.05$ compared with the allograft group



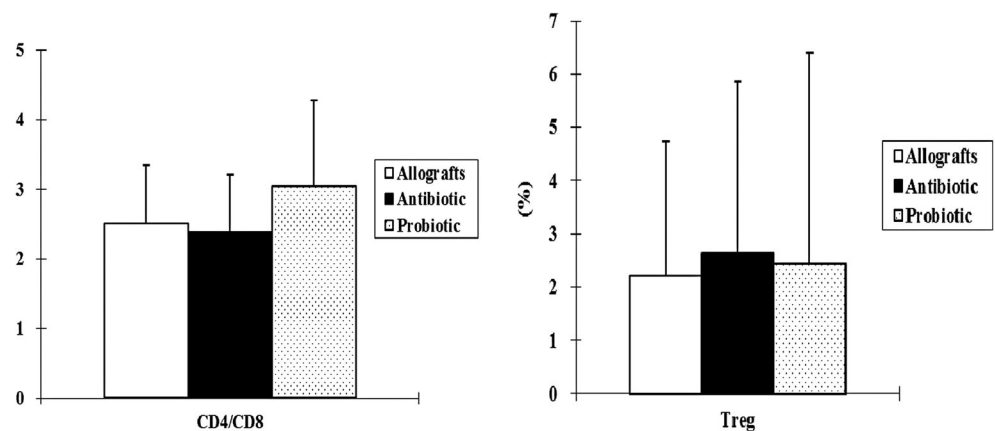
level in liver tissues was significantly higher in the probiotic group compared with the allograft group ($p < 0.05$) (Fig. 8).

Discussion

OLT has become a routine operation for patients with end-stage liver disease. Nevertheless, infectious complications remain as major problem following transplantation. With the development of broad-spectrum antibiotics, a novel concept was designed to eliminate potential pathogenic bacteria using selective digestive decontamination (SDD). However, extensive use of antibiotics poses the patient at risk of *Clostridium difficile* or fungal infection and antimicrobial resistance [18]. Recently, it demonstrated that probiotics were useful for the prevention of bacterial translocation and gut-derived bacterial infections. Randomized controlled trials (RCTs) in patients undergoing liver transplantation proved that probiotics reduced the incidence of infections, the amount of antibiotics administered, and the length of hospital stay [6, 7]. The previous studies confirmed the positive influence of probiotics and antibiotics on infection after liver transplantation. Here, we focus on the effect of gut microbiota alteration on hepatic damage after allograft liver transplantation.

The bacterial diversity alteration in the gut after using probiotics and antibiotics was proved by PCR-DGGE. The fecal bacterial diversity reduced significantly in the antibiotic group compared with the allograft group and the probiotic group at T1, T2, and T3. A very distinct segregation was also observed in the probiotic group compared with the allograft group. It reported that the dominant species differ between the mucosa-associated and fecal microbiota, but it is relatively stable along the distal digestive tract in human [19, 20]. So, we also study the effect of antibiotics and probiotics on intestinal mucosa-associated bacterial diversity. It found that antibiotics and probiotics had significant effect on microbiota diversity of ileum and colon at T3. As for diversity of intestinal mucosal microbiota at T3, there was no significant alteration of intestinal microbiota diversity in the jejunum. These suggested that the effect of antibiotics and probiotics on intestinal bacterial diversity was site dependent and the diversity of microbiota significantly reduced in the antibiotic group. It reported that the class of antibiotic significantly shaped the microbiota on the basis of the antimicrobial effect (bactericidal or bacteriostatic) and the mode of action. Protein synthesis inhibitor (clindamycin) introduced higher variance in microbiota composition than cell replication inhibitor (moxifloxacin) and cell envelop synthesis inhibitor (amoxicillin), giving way to a

Fig. 7 Comparison of CD4/CD8 and Treg cells in the blood between the experiment groups.



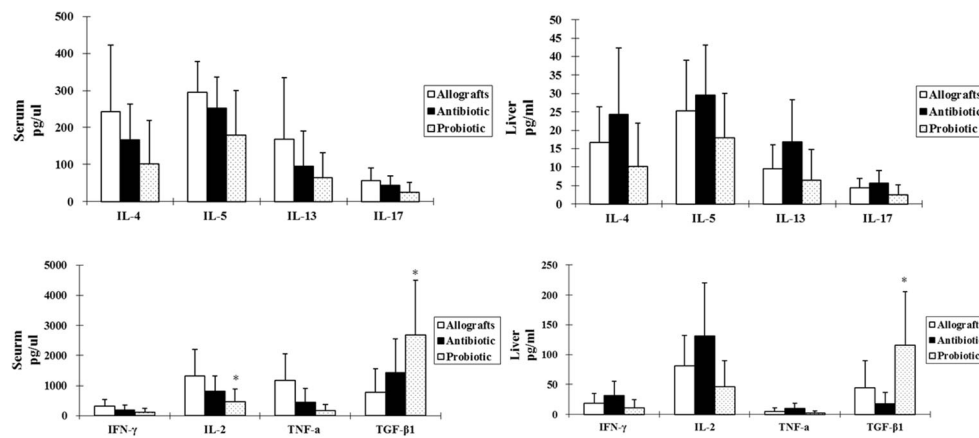


Fig. 8 Comparison of cytokines between the experiment groups. * $p < 0.05$ compared with the allograft group

different bacterial community structure [21, 22]. This may explain why the fecal bacterial diversity reduced significantly in the antibiotic gentamicin (protein synthesis inhibitor) in this study. The pathology of intestine showed that antibiotics reduced the inflammation of ileum and colon, but the microvilli of the ileum epithelial cells were destroyed severely. These suggest that antibiotics destruct the mucosal barrier and disturb the ecological balance between the host and the normal microbiota. However, the probiotic can reduce the inflammation of ileum and colon and protect the microvilli of the ileum epithelial cells from destruction. The inflammation of hepatic parenchyma and liver function injury in the antibiotic group and probiotic group was lighter than the allograft group. So in order to define the possible mechanisms involved, we examine the changes of immune function after disturbance of gut flora by antibiotics and probiotics under condition of acute rejection.

CD4/CD8 ratio is a sensitive indicator of clinical diagnosis to determine immune dysfunction. The increase of this ratio indicates cellular immune function in a “hyperactive” state, such as organ transplant rejection, rheumatoid arthritis, and so on. It reports that Tregs could downregulate immune responses to prevent AR and there is a functional imbalance between Th17 cells and Treg in tolerance and rejection after OLT [23]. Here, the results showed that the intestinal Treg cell and serum and liver TGF- β were increased markedly while CD4/CD8 ratio significantly decreased in the probiotic group. It suggests that the probiotics may reduce liver injury by increase of Tregs and TGF- β and deduction of CD4/CD8. The rats in the probiotic group had greater population of *Lactobacillus* and *Bifidobacterium* than the allograft group at T3. The evidence from in vitro and in vivo studies also suggests that probiotics may mediate their beneficial effects through induction of regulatory T cells. The Treg cells bearing surface TGF- β increased in animal models of colitis following administration of probiotics VSL#3 (strains of *Lactobacilli*, *Bifidobacteria*, and *Streptococcus thermophilus*). The Treg

cells were effective in conferring protection against colitis in a cell-transfer system. Importantly, the protective effect was dependent on TGF- β and IL-10 and was abolished by appropriate neutralizing antibodies [24]. Similarly, administration of VSL#3 resulted in the expansion of colonic Foxp3+Tregs in conjunction with significantly reduced pouchitis disease activity scores [25]. Furthermore, the supplementation with *Lactobacillus* was associated with an increased frequency of peripheral Tregs in IBD patients [25, 26]. It reported that an overall reduction in gut microbiota because of orally administered gentamicin resulted in protection from acute liver injury [27]. However, the role of antibiotics in liver homeostasis remains controversial [27, 28]. CD4/CD8 ratio and Treg cells in Peyer’s patch were decreased in rats using antibiotics. The population of *Lactobacillus*, *C. leptum*, and *Bifidobacterium* in the antibiotic group was significantly less than the probiotic group at T3. A study reported that changes in the intestinal microbiota composition caused by antibiotic usage could lead to differential regulation of the Th17/Treg balance affecting intestinal immune responses [29, 30]. The antibiotics may protect liver injury through mechanism different with probiotic. More research is needed to study the reason why antibiotic can reduce the liver injury caused by acute rejection after OLT.

In conclusion, the antibiotics and probiotics can reduce the liver injury in rats with acute rejection after liver transplantation. Probiotics may mediate their beneficial effects through induction of Treg cells and TGF- β and deduction of CD4/CD8 in rats with AR after OLT. More research is needed to study the mechanism of antibiotics reducing liver injury caused by AR after OLT. The intestinal microbiota is considered an important and complex immune “organ” of the host, and further studies are needed for more detailed understanding of the immunity interactions between intestinal microbiota and rejection process after OLT.

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Conflict of Interest The authors declare no conflict of interest.

References

- Barkholt L, Ericzon BG, Tollema J, Malmberg AS, Ehrnst A, Wilczek H, Andersson J (1993) Infections in human liver recipients: different patterns early and late after transplantation. *Transpl Int* 6: 77–84
- Roberts MS, Angus DC, Bryce CL, Valenta Z, Weissfeld L (2004) Survival after liver transplantation in the United States: a disease-specific analysis of the UNOS database. *Liver Transpl* 10:886–897
- Romero FA, Razonable RR (2011) Infections in liver transplant recipients. *World J Hepatol* 3:83–92
- So AD, Gupta N, Cars O (2010) Tackling antibiotic resistance. *BMJ* 340:c2071
- Guarner F, Malagelada JR (2003) Gut flora in health and disease. *Lancet* 361:512–519
- Rayes N, Seehofer D, Theruvath T, Schiller RA, Langrehr JM, Jonas S, Bengmark S, Neuhaus P (2005) Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation—a randomized, double-blind trial. *Am J Transplant* 5:125–130
- Rayes N, Seehofer D, Hansen S, Boucsein K, Muller AR, Serke S, Bengmark S, Neuhaus P (2002) Early enteral supply of lactobacillus and fiber versus selective bowel decontamination: a controlled trial in liver transplant recipients. *Transplantation* 74:123–127
- Xie YR, Luo ZB, Li ZF, Deng M, Liu H, Zhu B, Ruan B, Li LJ (2012) Structural shifts of fecal microbial communities in rats with acute rejection after liver transplantation. *Microb Ecol* 64:546–554
- Wu ZW, Ling ZX, Lu HF, Zuo J, Sheng JF, Zheng SS, Li LJ (2012) Changes of gut bacteria and immune parameters in liver transplant recipients. *Hepatobiliary Pancreat Dis Int* 11:40–50
- Kamada N, Calne RY (1983) A surgical experience with five hundred thirty liver transplants in the rat. *Surgery* 93:64–69
- Xie YR, Liu SL, Liu X, Luo ZB, Zhu B, Li ZF, Li LJ, He Y, Jiang L, Li H, Ruan B (2011) Intestinal microbiota and innate immunity-related gene alteration in cirrhotic rats with liver transplantation. *Transplant Proc* 43:3973–3979
- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700
- Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 70:4800–4806
- Xing HC, Li LJ, Xu KJ, Shen T, Chen YB, Sheng JF, Chen Y, Fu SZ, Chen CL, Wang JG, Yan D, Dai FW, Zheng SS (2006) Protective role of supplement with foreign Bifidobacterium and Lactobacillus in experimental hepatic ischemia-reperfusion injury. *J Gastroenterol Hepatol* 21:647–656
- Lefrancois L, Lycke N (2001) Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer's patch, and lamina propria cells. *Curr Protoc Immunol* Chapter 3: Unit 3.19
- Danielsson A, Hellers G, Lyrenas E, Lofberg R, Nilsson A, Olsson O, Olsson SA, Persson T, Salde L, Naesdal J et al (1987) A controlled randomized trial of budesonide versus prednisolone retention enemas in active distal ulcerative colitis. *Scand J Gastroenterol* 22:987–992
- Geboes K, Dalle I (2002) Influence of treatment on morphological features of mucosal inflammation. *Gut* 50(Suppl 3): III37–III42
- Safdar N, Said A, Lucey MR (2004) The role of selective digestive decontamination for reducing infection in patients undergoing liver transplantation: a systematic review and meta-analysis. *Liver Transpl* 10:817–827
- Lepage P, Seksik P, Sutren M, de la Cochetiere MF, Jian R, Marteau P, Dore J (2005) Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflamm Bowel Dis* 11:473–480
- Seksik P, Lepage P, de la Cochetiere MF, Bourreille A, Sutren M, Galmiche JP, Dore J, Marteau P (2005) Search for localized dysbiosis in Crohn's disease ulcerations by temporal temperature gradient gel electrophoresis of 16S rRNA. *J Clin Microbiol* 43:4654–4658. doi: 10.1128/JCM.43.9.4654-4658.2005
- Perez-Cobas AE, Artacho A, Knecht H, Ferrus ML, Friedrichs A, Ott SJ, Moya A, Latorre A, Gosalbes MJ (2013) Differential effects of antibiotic therapy on the structure and function of human gut microbiota. *PLoS One* 8:e80201. doi:10.1371/journal.pone.0080201
- Quigley EM, Monsour HP (2013) The gut microbiota and the liver: implications for clinical practice. *Expert Rev Gastroenterol Hepatol* 7:723–732. doi:10.1586/17474124.2013.848167
- Gill H, Prasad J (2008) Probiotics, immunomodulation, and health benefits. *Adv Exp Med Biol* 606:423–454
- Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M (2005) Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. *J Immunol* 174:3237–3246
- Pronio A, Montesani C, Butteroni C, Vecchione S, Mumolo G, Vestri A, Vitolo D, Boirivant M (2008) Probiotic administration in patients with ileal pouch-anal anastomosis for ulcerative colitis is associated with expansion of mucosal regulatory cells. *Inflamm Bowel Dis* 14: 662–668
- Lorea Baroja M, Kirjavainen PV, Hekmat S, Reid G (2007) Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients. *Clin Exp Immunol* 149:470–479
- Li YT, Wang L, Chen Y, Chen YB, Wang HY, Wu ZW, Li LJ (2010) Effects of gut microflora on hepatic damage after acute liver injury in rats. *J Trauma* 68:76–83
- Marshall JC, Nathens AB (1996) The gut in critical illness: evidence from human studies. *Shock* 6(Suppl 1):S10–S16
- Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4: 337–349
- Li J, Lai X, Liao W, He Y, Liu Y, Gong J (2011) The dynamic changes of Th17/Treg cytokines in rat liver transplant rejection and tolerance. *Int Immunopharmacol* 11:962–967