Front. Med. 2019, 13(4): 451–460
https://doi.org/10.1007/s11684-018-0675-3

RESEARCH ARTICLE

Structural shifts in the intestinal microbiota of rats treated with cyclosporine A after orthotropic liver transplantation

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Abstract **Understanding the effect of immunosuppressive agents on intestinal microbiota is important to reduce the mortality and morbidity from orthotopic liver transplantation (OLT). We investigated the relationship between the commonly used immunosuppressive agent cyclosporine A (CSA) and the intestinal microbial variation in an OLT model. The rat samples were divided as follows: (1) N group (normal control); (2) I group (isograft LT, Brown Norway [BN] rat to BN); (3) R group (allograft LT, Lewis to BN rat); and (4) CSA group (R group treated with CSA). The intestinal microbiota was assayed by denaturing gradient gel electrophoresis profiles and by using real-time polymerase chain reaction. The liver histopathology and the alanine/aspartate aminotransferase ratio after LT were both ameliorated by CSA. In the CSA group, the numbers of rDNA gene copies of *Clostridium* cluster I, *Clostridium* cluster XIV, and Enterobacteriaceae decreased, whereas those of *Faecalibacterium prausnitzii* increased compared with the R group. Cluster analysis indicated that the samples from the N, I, and CSA groups were clustered, whereas the other clusters contained the samples from the R group. Hence, CSA ameliorates hepatic graft injury and partially restores gut microbiota following LT, and these may benefit hepatic graft rejection.**

Keywords microbial community; liver transplantation; immunosuppressive agents; cyclosporine A

Introduction

Orthotopic liver transplantation (OLT) is a conventional life-saving treatment for patients with non-reversible liver diseases, such as hepatic failure, cirrhosis, and malignancy, as well as some metabolic diseases. Survival outcomes after LT have constantly improved using upgraded immunosuppressive agents (ISAs) [1]. However, the mortality and morbidity secondary to infectious complications and chronic rejection after transplantation remain a problem [2,3].

The intestinal microbiota forms a symbiotic ecosystem, which is a major metabolic "organ" that is responsible for the homeostatic balance in the human body. A total of 10–100 trillion microorganisms in the human digestive tract are important for health promotion [4–6]. However, due to the existence of "gut–liver axis," this intestinal equilibrium could be disrupted by pathological conditions that are associated with immune disorders, such as liver cirrhosis, hepatic encephalopathy, inflammatory bowel disease, autoimmune encephalomyelitis, type 1 diabetes, rheumatoid arthritis, and allergic diseases [7–9]. Acute rejection after OLT may cause structural shifts in the gut microbiota in rats, and may be related with host immunity [10]. Most OLT recipients need long-term immunosuppression, and this long-term exposure to ISAs causes pathophysiological changes in the immune system and the intestinal barrier [11–13]. Thus, understanding the relationship between ISAs and intestinal microbiota is extremely important in OLT research.

Previous research by using traditional cultivation

Received January 31, 2018; accepted October 27, 2018

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methods have limited our comprehension of the complexity of the intestinal microbiota because most of the bacteria cannot be cultured [10,14]. Culture-independent techniques, such as denaturing gradient gel electrophoresis (DGGE) and quantitative real-time polymerase chain reaction (qRT-PCR), have facilitated the investigation of the characteristics of gut microbiota with increased broadness and decreased bias [15,16]. Moreover, DGGE, a type of DNA fingerprinting technique, can excise and purify PCR products from the gel for sequencing. PCR-DGGE is a fast, practical method for recognizing the majority of microbiota by using visual fingerprints. Furthermore, qRT-PCR can be used to analyze the population in a sensitive and quantitative manner. Therefore, these techniques are preferred for analyzing the composition, structure, and variation of microbial biodiversity.

Many factors in LT research, such as surgery, antibiotics, probiotics, ISAs, and chemotherapy or other procedures, can alter the composition of gut microbiota and immune balance [17]. ISAs affect the fecal microbiome of renal transplant recipients, suggesting that the gut microbial community analysis may become a new tool for evaluating the therapeutic effect of ISAs [18]. However, the effect of the commonly used immunosuppressive agent, cyclosporine A (CSA), on the intestinal microbiota in LT has not been reported. Our previous work found that ischemic preconditioning improves the intestinal microbiota after OLT [19]. This study aimed to evaluate the role of CSA on gut microbial variation in rats based on an OLT model.

Materials and methods

Rat LT models inbred

Male Brown Norway (BN, $n = 32$) and Lewis ($n = 32$) rats were obtained from the Vital River Laboratories (Beijing, China), each weighing 200–250 g. All rats were nourished by feeding commercial rat chow pellets and fostering in a specific-pathogen-free facility with constant humidity at 22 °C. The experimental animals were divided into four groups: (1) N group (normal control, $n = 8$): normal BN rats with no operations; (2) I group (isograft LT, BN to BN, $n = 8$): recipients and donors were both BN rats; (3) R group (allograft LT, Lewis to BN, $n = 8$): recipients were BN rats and donors were Lewis rats; and (4) CSA group (R group treated with CSA, $n = 8$). OLT was performed as described previously in our centers [19]. All procedures and techniques in the study were authorized by the Ethics Committee for the Use of Experimental Animals in Zhejiang University. CSA (2 mg/kg, diluted in 0.5 mL saline) was administered intragastrically twice daily for 28 days after OLT. Antibiotics, ISAs, or blood transfusion were not administered in the allograft and isograft groups.

Sample collection

All animals were anesthetized with 4% chloral hydrate and killed 28 days after OLT. Blood samples were taken from the portal vein for the analysis of liver function. Hepatic tissue samples were fixed in 10% neutral formalin for further histological analysis through light microscopy. The ileocecum was removed, and fresh feces were collected and preserved at −80°C for later analysis.

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels

The serum was separated at room temperature by centrifuging blood samples at 3000× *g* for 10 min, and the AST and ALT levels were analyzed using a Hitachi 7600 automatic analyzer (Tokyo, Japan).

Primers and qRT-PCR

16S rRNA gene-targeted qRT-PCR was performed using RT-PCR Detection System according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The primers of the dominating bacteria are shown in Table 1 and the details of PCR settings and data analysis were based in our previous studies [20].

Table 1 The primers of the dominating bacteria

Target	Sequence (5'–3')	Sequence (3'–5')	Annealing temperature (°C)
Faecalibacterium prausnitzii	GATGGCCTCGCGTCCGATTAG	CCGAAGACCTTCTTCCTCC	58
Enterococcus	AACCTACCCATCAGAGGG	GACGTTCAGTTACTAACG	57
Bifidobacterium spp.	GGGTGGTAATGCCGGATG	TAAGCCATGGACTTTCACACC	59
Lactobacillus	AGCAGTAGGGAATCTTCCA	ATTYCACCGCTACACATG	58
Enterobacteriaceae	CATTGACGTTACCCGCAGA-AGAAGC	CTCTACGAGACTCAAGCTTGC	63
Bacteroides	GAAGGTCCCCCACATTG	CAATCGGAGTTCTTCGTG	56
Clostridium cluster XIVab (CG3)	GAWGAAGTATYTCGGTATGT	CTACGCWCCCTTTACAC	54
Clostridium clusters XI (CG2)	ACGCTACTTGAGGAGGA	GAGCCGTAGCCTTTCACT	58
Clostridium clusters I (CG1)	TACCHRAGGAGGAAGCCAC	GTTCTTCCTAATCTCTACGCAT	63

DGGE profiles and sequence of DGGE bands

Bio-Rad D-code System facilities were used for DGGE analysis. The PCR products (200 ng) were separated on 8% (*w*/*v*) polyacrylamide gels. Electrophoresis was performed in 1× Tris-acetate EDTA buffer at 200 V (identical voltage, 60 °C) for 4 h. The gels were dyed by using the SYBR Green I (Sigma, St. Louis, MO, USA) and images were taken by using UVI Gel Documentation System (UVItec, Cambridge, UK). BioNumerics (version 6.01, Applied Maths, Sint-Martens-Latem, Belgium) was used to process the DGGE profiles. The important DGGE bands were resected and sequenced. The sequencing procedure for the DGGE bands was based on a previous study [19]. The sequences of predominant DGGE bands and the existing 16S rRNA genes preserved in GenBank were compared using BLAST and then categorized according to their affinity to the closest neighbor. Based on evolutionary distances, MEGA 5.0 was used to construct the phylogenetic tree through neighbor-joining method.

Statistical analysis

The affinities of PCR-DGGE DNA profiles and the similarity matrix were constructed using Quantity One® 1-D Analysis (version 4.6.2; Bio-Rad) and Dice's similarity coefficient. By using unweighted pair-group method with arithmetic means (UPGMA), dendrograms were constructed using the unweighted pair group method. Leave-one-out cross-validation method was used to assess the partial least squares discriminant analysis models, especially the correct classification rate. One-way analysis of variance (ANOVA) was conducted, followed by Martens' uncertainty test to sort stable and significant X variables. Quantitative data were expressed as mean ± SEM. Statistical analyses were conducted using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). For the analysis of parametric data, group statistical significance was analyzed using one-way ANOVA and post hoc Bonferroni's multiple comparison tests. For the analysis of nonparametric data, Kruskal–Wallis and Dunn's multiple comparison tests were conducted. Statistical significance was set at $P < 0.05$.

Results

CSA improved the liver histopathology after LT

Congestion, necrosis, or inflammation seldom occurred in the liver tissues of the N and I groups (Fig. 1). The congestion in the sinusoids and central vein (white arrow head) and hepatocyte necrosis (arrow) were evident in the R group. Central vein congestion and tissue damage hardly occurred in the liver lobules in CSA compared with the R group. Results demonstrated that CSA improved the liver histology in this rat LT model.

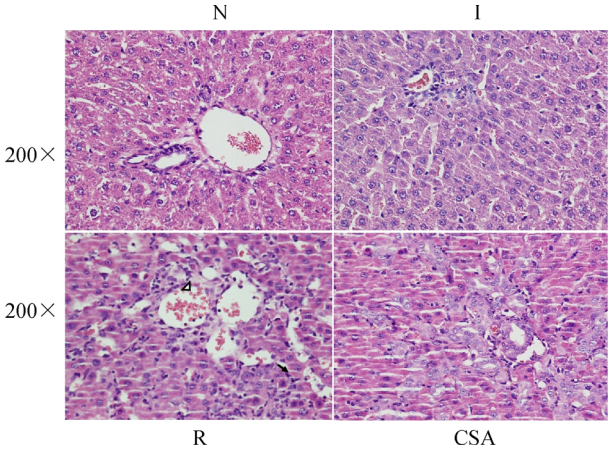

Fig. 1 Liver histopathology (hematoxylin and eosin stain, original magnification 200×).

CSA decreased the serum ALT and AST level after LT

Compared with the N group, all the other groups showed an increased ALT and AST, especially the R ($P < 0.05$) and CSA ($P < 0.05$) groups (Fig. 2). Compared with the I group, ALT and AST were elevated in the R group ($P < 0.05$). CSA significantly reduced the serum levels of AST and ALT compared with the R group ($P < 0.05$). Results show that CSA attenuated liver injury by decreasing the serum ALT/AST levels after LT.

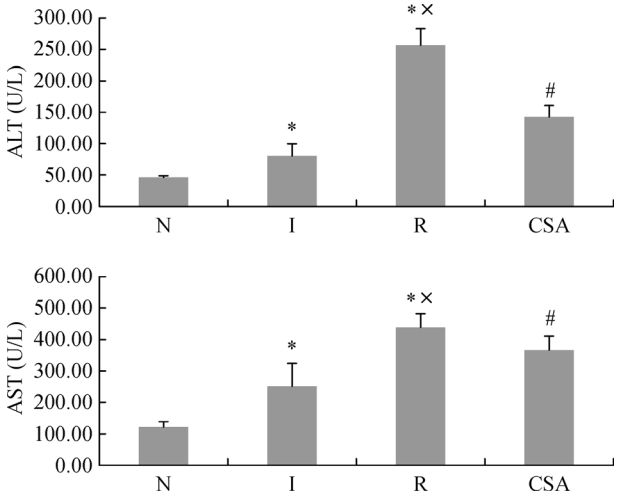

Fig. 2 ALT and AST levels in the N, I, R, and CSA groups ($n = 6$ per group). Values are expressed as mean ± SEM. $^{*}P < 0.05$ versus N, $^{\times}P < 0.05$ versus I group, $^{\#}P < 0.05$ versus R group.

Quantitative analysis of fecal-dominating bacteria through qRT-PCR

To evaluate the effect of CSA on the gut microflora in rats after LT, nine predominant microbiotas were assessed through qPCR at the genetic level (Fig. 3). In comparison with the N group, the numbers of 16S rDNA gene copies of *Enterococcus* spp. (ENCO) in the CSA group and *Clostridium* cluster I (CG1) and Enterobacteriaceae (ECO) in the R group were markedly increased, whereas those of *Faecalibacterium prausnitzii* (FPRA), *Clostridium* cluster XI (CG2), and *Clostridium* cluster XIV (CG3) in the CSA and R groups were markedly decreased. Compared with the R group, the numbers of 16S rDNA gene copies of CG3, CG1, and ECO in the CSA group decreased, whereas those of FPRA increased. In other words, CG1 and ECO were dramatically increased in the R group compared with the N group but decreased to a nearly normal level in the CSA group.

CSA improved the intestinal microbiota in DGGE profiles

Microbial diversity analysis

The DGGE profiles of fecal microbiota showed alterations in the composition of the intestinal microbial flora (Fig. 4). The gray density of each band in each lane was analyzed using Gel-Pro Analyzer, and Past was used to analyze microbial diversity. The Shannon's diversity index in the CSA group (3.26 ± 0.17) was markedly increased compared with that in the I group (3.04 ± 0.16, $P = 0.046$). The Shannon's diversity index did not differ significantly among the other groups. Hence, CSA improved the diversity of intestinal microbiota and the richness of species after LT in rats.

DGGE profile cluster analysis

To assess the features of DGGE profiles of various groups, the Dice coefficient and UPGMA were utilized to show similarities in band pattern (Fig. 5). One cluster contained the samples from the N, I, and CSA groups, while the other clusters included samples from the R group. The total similarity of the cluster was 79.7%. Alterations among the different groups of DGGE profiles were also proved by multidimensional scaling (MDS) (Fig. 5B) and principal component analysis (PCA) (Fig. 5C). The distance between two data points represents the extent of difference between the gut microbial compositions. Microbial compositions of rats from the R group were clustered,

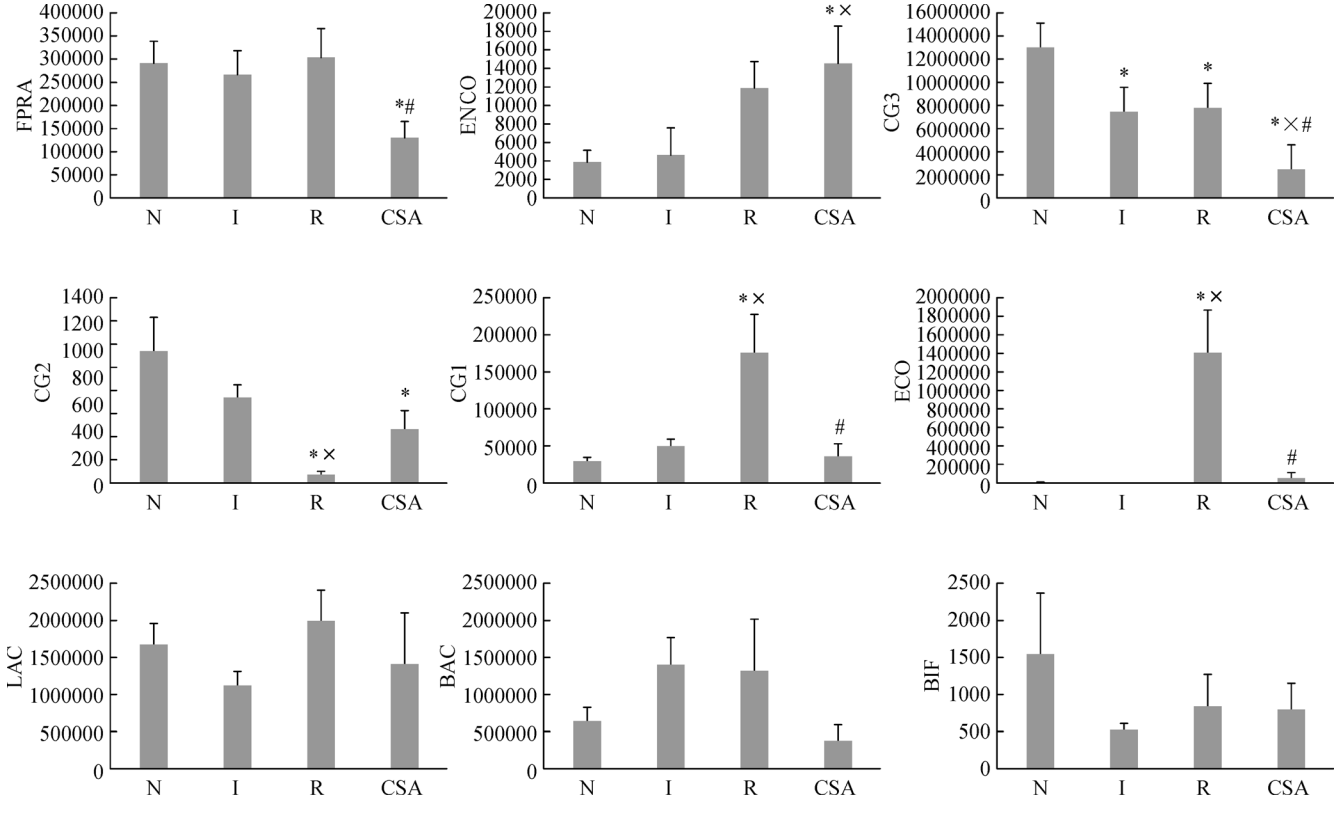

Fig. 3 Numbers of fecal-dominating bacteria in the N, I, R, and CSA groups ($n = 8$ per group). Values are expressed as mean ± SEM. *$P < 0.05$ versus N group, $^{\times}P < 0.05$ versus I group, $^{\#}P < 0.05$ versus R group. FPRA, *Faecalibacterium prausnitzii*; ENCO, *Enterococcus* spp.; CG1, *Clostridium* cluster I; CG2, *Clostridium* cluster XI; CG3, *Clostridium* cluster XIV; ECO, Enterobacteriaceae; LAC, *Lactobacillus* spp.; BAC, *Bacteroides–Prevotella* group; BIF, *Bifidobacterium* spp.

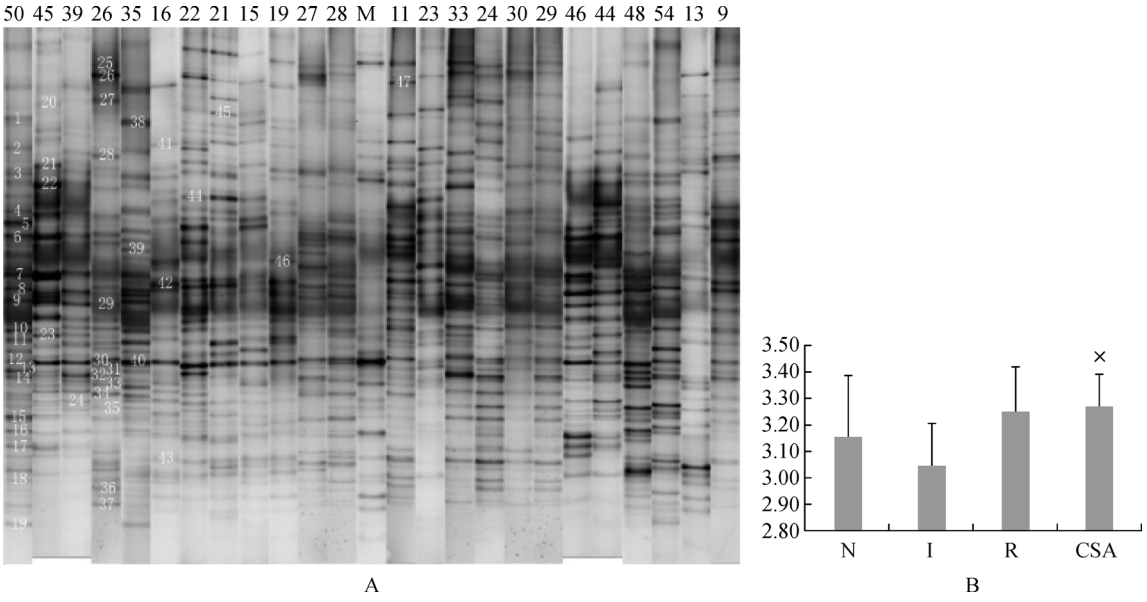

Fig. 4 CSA improved the intestinal microbiota in rats after LT as shown in the DGGE profiles. (A) DGGE profiles of intestinal bacteria from rats. Numbers of sample above the lanes represent rats from various groups. 50, 45, 39, 26, 35, and 16 samples were from N group; 22, 21, 15, 19, 27, and 28 samples were from I group; 11, 23, 33, 24, 30, and 29 samples were from CSA group; and 46, 44, 48, 54, 13, and 9 samples were from the R group. Gel-to-gel comparison is marked by different marker lanes. Each bacterial clone indicates a band. Numbers of each band (corresponding to Fig. 6 band classes) expressed the position of bands abscised for analyses (e.g., 8 means band class 8). (B) Diversity of fecal microbial comparison (Shannon's diversity index). Values are expressed as mean ± SEM. $^{\times}P < 0.05$ versus I group.

showing a separation from the other groups as determined by MDS and the PCA of the *x*, *y,* and *z* axes with contribution rates of 34.7%, 16.1%, and 12.2%, respectively. These data indicate that the composition of the intestinal microbiota from rats in the CSA and N groups was uniform and clustered.

Analysis of sequences from the DGGE profiles: phylogenetic tree

To assess the phylogenetic relativity of the intestinal microbial species and investigate the predominant bacteria in the intestinal microbiota as induced by CSA in rats after LT, we analyzed the phylogenetic tree of the DGGE bands (Fig. 6). In the 47 bands of PCR-DGGE, 43 band classes were affirmed. At least two different DNA samples were extracted, sequenced, and assigned to a species or phylotype of the bacteria based on the highest sequence similarity match (90%–100%) to the GenBank sequences obtained from the BLAST analysis.

Fig.6 shows that nearly all matched bacteria in DGGE bands were classified into 3 phyla: Firmicutes (47.7%), Bacteroidetes (47.7%), and Proteobacteria (4.5%). We analyzed each band of the different groups based on the gray scale. Twenty-nine band classes were found with slight alteration of intensity among the different groups. The band intensities of classes 15, 25, 26, 46, and 36 in the CSA group and 21, 23, and 37 in the R group were significantly increased compared with those in the N group, while band classes 8, 9, and 10 in the CSA group were decreased. The intensities of band classes 26, 46, and 36 were apparently improved in the CSA group compared with the R group, while band classes 21, 8, 33, 23, 14, 16, and 37 were decreased. The intensities of band classes 21, 23, and 37 were significantly improved in the R group compared with the N group and was reduced to the normal level in the CSA group. The most relevant matched species of the 14 predominant classes of microbial composition alteration were detected in the phylogenetic tree (Fig. 7). In these predominant bacteria, 50% (7/14) were classified as Bacteroidetes, 50% (7/14) as Firmicutes, and none as Proteobacteria.

Discussion

Intestinal microbiota is a dominating metabolic "organ" that is responsible for the symbiotic balance in the body [21,22]. Approximately 10–100 trillion microorganisms, whose collective genome contains 100-fold more genes than the entire human genome, comprise 400 different types of bacteria in the human intestinal tract [21,23]. Their

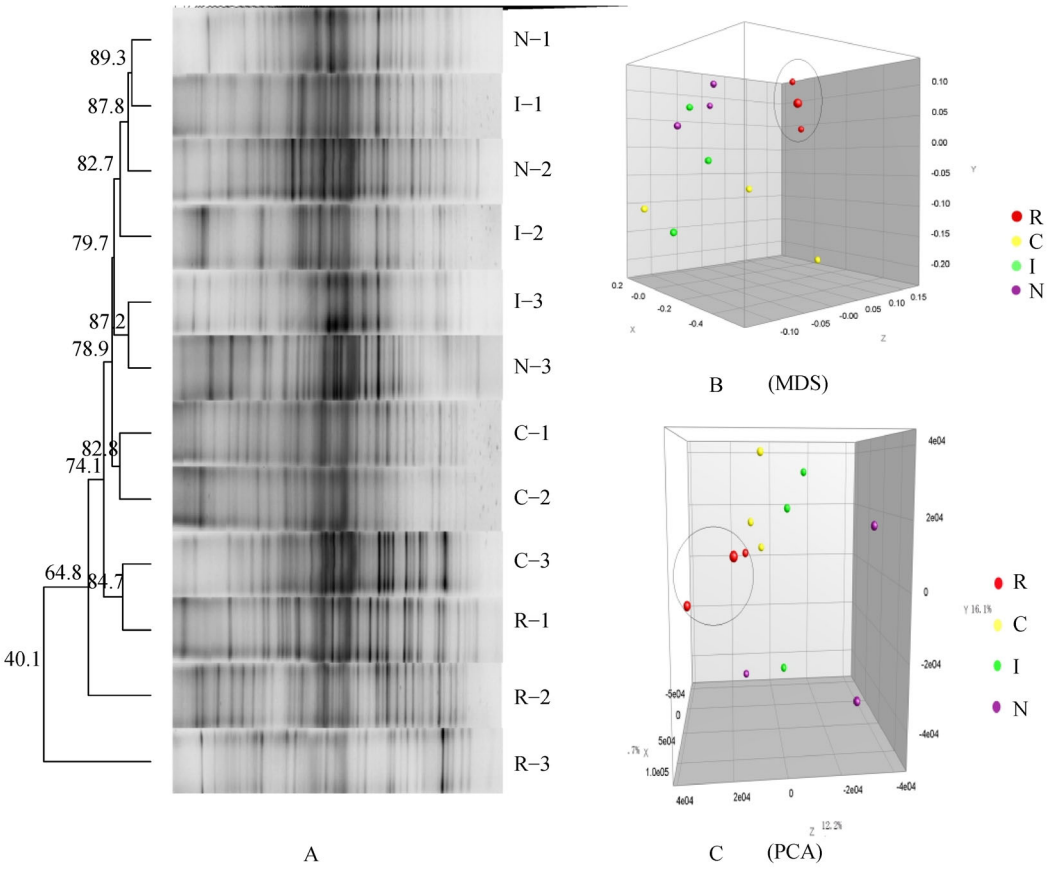

Fig. 5 DGGE profile cluster analysis assessed with universal primers V3, the utilizing Dice's coefficient and UPGMA. (A) DGGE profiles cluster analysis from the different groups. Metric scale expresses the degree of similarity. (B) Cluster MDS analysis shown in (A). The plot shows an optimized three-dimensional expression of the similarity matrix obtained from the BioNumerics software; the *x*, *y* and *z* axes express three different dimensions separately: Dim 1, 2, and 3. Euclidean distance between 2 points reflects similarity. (C) Fecal microbial PCA of DGGE fingerprinting shown in (A). Reoriented plots maximize the variation among different lanes along the first 3 principal components with contributions of 34.7%, 16.1%, and 12.2% as obtained from the BioNumerics software.

significance in resisting enteric pathogen invasion and in the maturity of the initial immune system have been demonstrated [5,24]. They are also associated with liver diseases, including hepatic ischemia/reperfusion injury [19,25], alcoholic steatohepatitis [26], liver cirrhosis [7], and hepatocellular carcinoma [27] and with rejection after OLT [10]. Usually, individual infectious risk is determined by the balance between pathogenic exposure and the overall immunosuppression [28]. Patients with a serious reduction in intestinal microbiota now have an increased rate of postoperative infection.

For most LT patients, ISAs are used conventionally, but they deal adverse effects on recipients, especially infections and neoplastic alterations. CSA is a highly selective inhibitor of T cell activation that can significantly improve survival rate and decrease toxic effects compared with previous ISAs. CSA is among the most important ISAs administered currently [29]. The infectious risk after OLT is determined integrally by intestinal microbial disturbances, antibiotic agents, and the overall immunosuppression level. Thus, understanding the compositional shifts in the intestinal microbiota as induced by immunosuppression after OLT is important in preventing postoperative infection.

Our group has demonstrated that abundant gut microbiota was dramatically altered in patients with cirrhosis, ischemia/reperfusion injury, or liver transplantation [7,19]. We have also investigated the relationship between predominant fecal microbial composition and postoperative infection and discovered that an empirical antimicrobial strategy does not reduce the risk of postoperative infection due to the disturbance of gut microecological balance [28]. In our previous work, rats with acute rejection (R group in this study) showed compositional alterations in the gut microbiota. Overgrowth was observed on *Bacteroides* and *Ruminococcus*, which is accompanied by increased plasma endotoxin and rate of bacterial translocation [10]. Similar results were found in the R

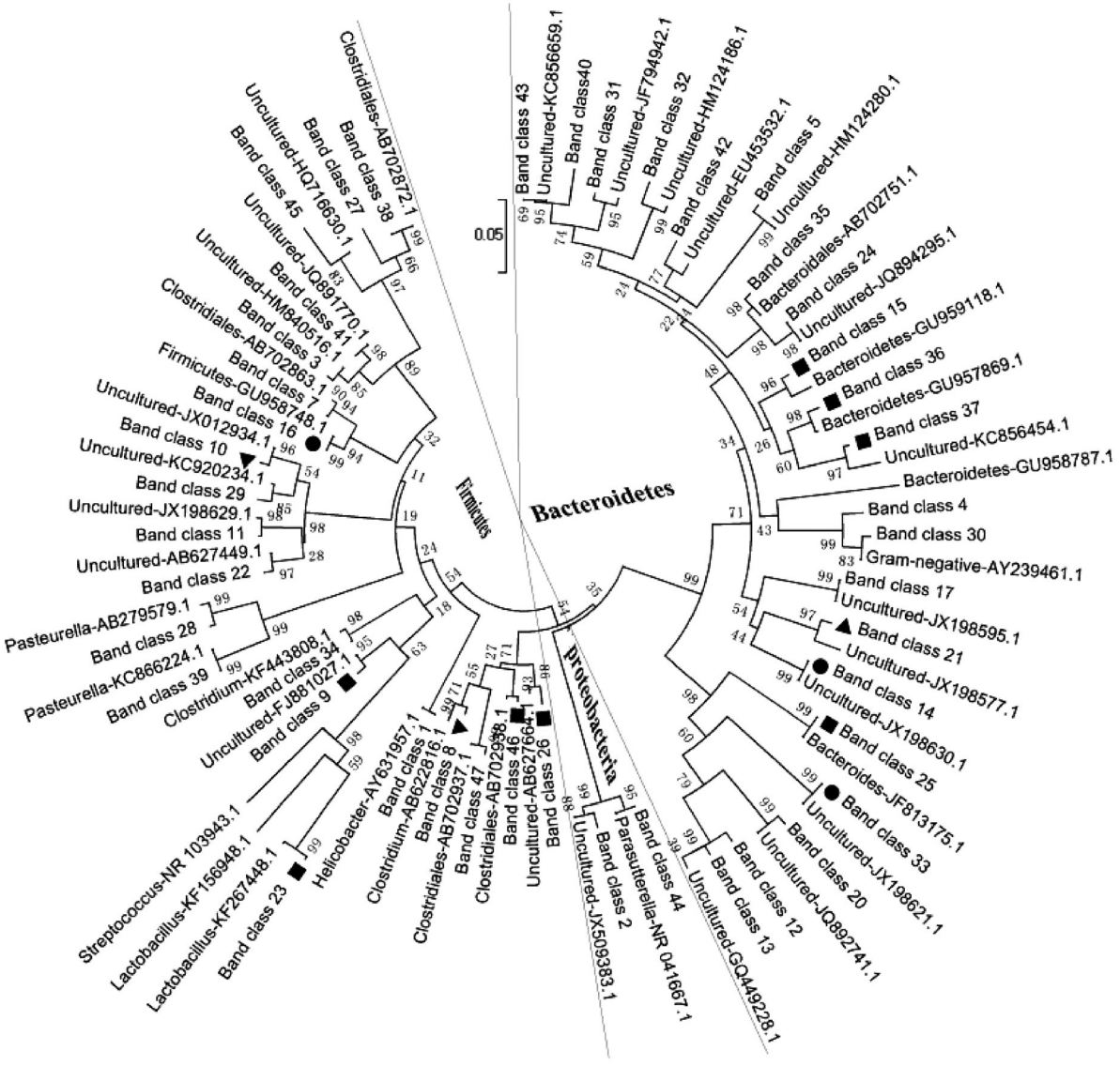

Fig. 6 Neighbor-joining method was used for phylogenetic tree sequencing. Black squares indicate band classes with increased intensity versus the N group; black triangles indicate band classes with decreased intensity versus the N group; black diamonds indicate band classes with increased intensity versus the R group; and black spots indicate band classes with decreased intensity versus the R group. The plot was generated from MEGA5 software.

group in the present study. Results show that graft rejection (R group) impaired the liver morphology and function and microbiological function of the gut ecosystem compared with the N and I groups. However, CSA improved the liver morphology, ALT/AST ratio, and the variation in the intestinal microbiome. Cluster analysis shows that the N and CSA groups were clustered with 74.1% similarity, representing a high similarity in terms of composition between the groups. This result agrees with previous findings on liver morphology and function.

Our results show restored liver graft injury and partially improved gut microbiota in the CSA group. In addition, CSA elevated the gut microbial richness and species diversity, which was similar to changes in the profile of the N group. Cluster analysis also shows similarity in the DGGE profiles between the CSA and N groups, which was confirmed by the MDS and PCA results. Additionally, the phylogenetic analysis of the sequences indicates that CSA partially restored the intestinal microbiota compared with that in the R group. The intensities of band classes 21, 23, and 37 were apparently elevated in the R group compared with the N group but was reduced to the normal level in the CSA group. Key bacteria corresponding to the 14 predominant band classes of microbial compositional alteration were identified in the phylogenetic tree, which were assigned to phylum Bacteroidetes or Firmicutes.

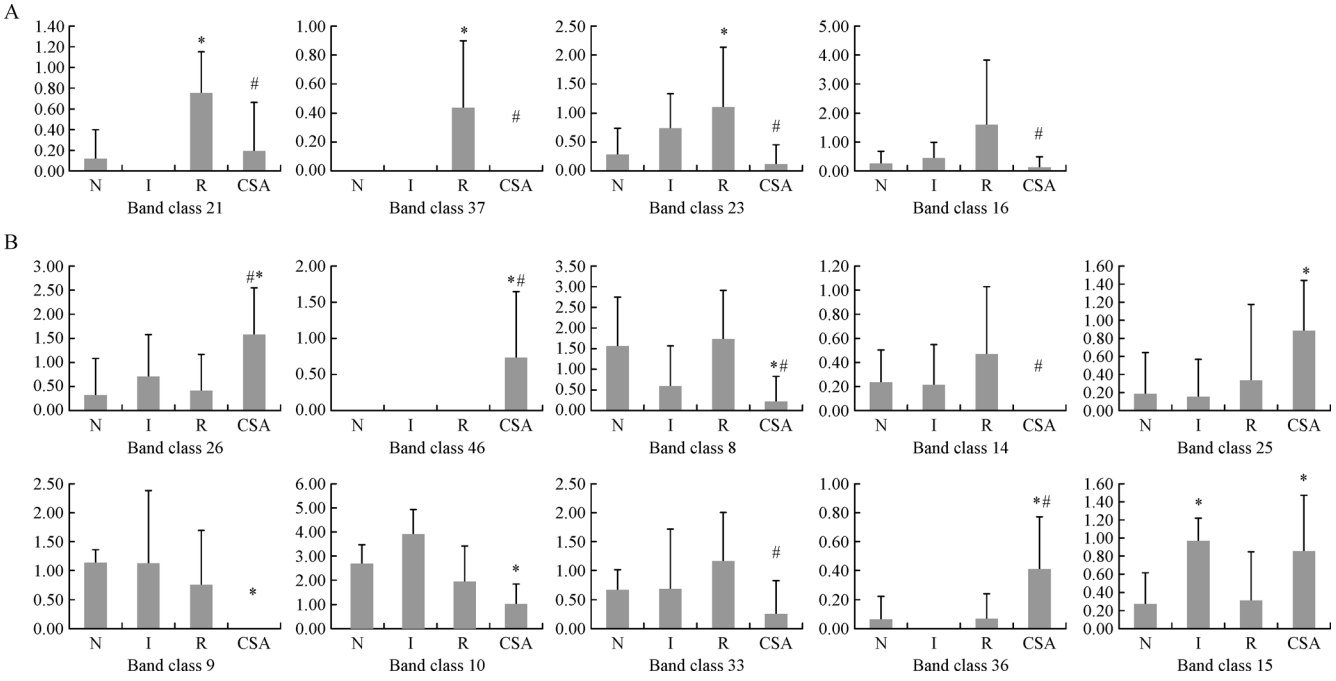

Fig. 7 The key band changed among the groups. (A) The R group increased the band intensity, but CSA restored it to the N group level. (B) The key bands changed in the CSA group compared with the N group, while the R group maintained a normal level ($n = 6$ in each group). Values are expressed as mean ± SEM. $^{*}P < 0.05$ versus N group, $^{\#}P < 0.05$ versus R group.

Subsequently, these changes after CSA treatment enhanced the balance and stability of the intestinal microbiota and indicated the protective effect of CSA on intestinal microbiota in LT.

The intestinal microbiome has a complex ecological structure. In liver surgery, alteration of the gut microbiota is responsible for or contributes to hepatic injury or its recovery [22]. Otherwise, liver injury always results in a compositional shift in intestinal microbiota as a result of the gut–liver axis [26]. In other words, liver function recovery could restore the intestinal microbial ecology [19], implying that microbial profiling is a latent biomarker for liver injury. Several studies have suggested microbiota transplantation as a potential therapy for inflammatory bowel diseases [30], chronic gastrointestinal infections [31], and metabolic and autoimmune diseases [32]. A study shows that fecal transplantation is more effective than vancomycin for *Clostridium difficile* infection [33]. Thus, the manipulation of the intestinal microbiota is a possible safe and effective treatment for human diseases.

To the best of our knowledge, this study is the first to investigate the intestinal microbiota in rats treated with CSA after LT. Results indicate the protective effect of CSA in LT in a microbial pathway, which may provide means to evaluate the efficacy of emerging ISAs in the future. However, this study had some limitations. Aside from focusing on changes in the intestinal microbiota in rats with CSA after OLT, we should consider normal rats treated with CSA only and the baseline data in all groups at day 0. Otherwise, many clinical samples would be needed to confirm that the conclusions are also applicable to humans. Moreover, aside from the simply usage of CSA, several protective treatments including probiotics, antibiotics, and prebiotics are being currently used. Further research is needed, considering the influence of all these factors.

In conclusion, our study indicates that CSA could ameliorate hepatic graft injury and partially restore the gut microbiota after LT, which may benefit hepatic graft rejection.

Acknowledgements

This work was supported by the China Postdoctoral Science Foundation (No. 2017M610374), Zhejiang Medical Health Technology Project (No. 2019313269), Innovative Research Groups of the National Natural Science Foundation of China (No. 81421062), National Natural Science Foundation of China (No. 81470891), and Science and Technology Bureau of Zhejiang Province, China (No. 2016C33145).

Compliance with ethics guidelines

Junjun Jia, Xinyao Tian, Jianwen Jiang, Zhigang Ren, Haifeng Lu, Ning He, Haiyang Xie, Lin Zhou, and Shusen Zheng declare that they have no conflicts of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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