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***Clinical Trials Study***

**Characteristics of fecal microbial communities in patients with non-anastomotic biliary strictures after liver transplantation**

Zhang J *et al*. Fecal microbial communities in NAS patients

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**Abstract**

***AIM***

to explore the possible relationship between fecal microbial communities and non-anastomotic stricture (NAS) after liver transplantation (LT).

***Methods***

A total of 30 subjects including 10 patients with NAS, 10 patients with no complications after LT, and 10 non-LT healthy individuals were enrolled. Fecal microbial communities were assessed by the 16S rRNA gene sequencing technology.

***Results***

Different from the uncomplicated and healthy groups, unbalanced fecal bacterium ratio existed in patients with non-anastomotic biliary strictures after liver transplantation. The results showed NAS patients were associated with decreases of *Firmicutes, Bacteroidetes* and increases of *Proteobacteria* at the phylum level, with the proportion-ratio imbalance between potential pathogenic families including *Enterococcaceae, Streptococcaceae, Enterobacteriaceae,* *Pseudomonadaceae* and dominant families including *Bacteroidaceae*.

***Conclusion***

These compositional shifts of the increase of potential pathogenic bacterium as well as the decrease of dominant bacterium might contribute to the incidence of NAS.

**Key words:** Non-anastomotic stricture; orthotopic liver transplantation; fecal microbiota; dysbacteriosis; ischemia-reperfusion injury

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**C****ore tip:** This study is the first attempt to investigate the possible relationship between gut microbiota and post-liver transplantation (LT) biliary complication based on the 16S rRNA sequencing technology. Our results showed unbalanced ratio of pathogenic bacterium to dominant bacterium really existed in patients with non-anastomotic stricture after LT. Indicated the shifts of fecal microbial communities may involve or exacerbate the process of bile duct’s injury, which may contribute to the mechanism research and prevention in future.

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**Introduction**

As Thomas Starzl performed the first human [liver transplant](https://en.wikipedia.org/wiki/Liver_transplant)ation in 1963, orthotopic liver transplantation (OLT) has been regarded as the standard therapy for patients with end-stage liver diseases. In the past three decades, the post-operative complications decreased markedly due to the improvement of surgical techniques and immunosuppressive treatment[[1](#_ENREF_1),[2](#_ENREF_2)]. However, the morbidity of biliary stricture after OLT is still high, ranging from 5% to 20%[[3](#_ENREF_3)]. Non-anastomotic stricture, also known as ischemic type biliary stricture, is a lethal complication for recipients and severely affects their long-term prognosis[[4](#_ENREF_4)]. Factors including poor liver graft, ABO-incompatibility, cytomegalovirus (CMV) infection. *etc*., may contribute to the development of non-anastomotic stricture (NAS), and ischemic reperfusion related inflammatory injury is commonly regarded as an inducer of this pathologic process[[5-8](#_ENREF_5)]. But up to date, the definite mechanisms of NAS remain unknown.

Gut microbiota is the general term for all microorganisms (mainly for bacterium) living in human intestine, with a microbial density larger than 1014 cells/g, containing 100 times more genes than human’s[[9](#_ENREF_9),[10](#_ENREF_10)]. Current researches have titled the gut bacteria as human’s another organ for its enormous influences in human’s metabolic activity, barrier function and immunity development. However, endotoxemia caused by dysbacteriosis was also connected to obesity, diabetes, nonalcoholic fatty liver diseases (NAFLD), autoimmune disorders[[11](#_ENREF_11),[12](#_ENREF_12)], and even played a key role in ischemic reperfusion injury[[13](#_ENREF_13)]. While for patients underwent liver transplantation, complex factors like portal vein blocking, ischemic reperfusion injury, antibiotics or immunosuppression use can seriously impair recipient’s immune function, destroy intestinal barrier, finally increase the risk of dysbacteriosis, these changes of microbiota may directly injury host’s liver parenchyma through the “gut-liver” axis[[14](#_ENREF_14)]. Actually, the relationship between dysbacteriosis and postoperative complications including acute rejection, early-stage infection and graft loss are under investigation[[15](#_ENREF_15),[16](#_ENREF_16)]. Account for all of these, we hypothesized that quantitative or qualitative alterations of gut microbiota may involve or exacerbate graft’s ischemic reperfusion injury, which eventually lead to the NAS. But so far, the detailed relationship between them has never been explored. Furthermore, whether the changes of gut microbiota contribute to the occurrence of NAS after OLT is still obscure.

In this study, we explored the potential relationship between gut microbiota and NAS by investigating the microbial communities’ changes in patients diagnosed with NAS.

**Materials and methods**

***Patients enrollment***

All subjects in this study came from the first affiliated hospital of Xi’an Jiaotong University, with no history of systemic antibiotics or probiotics within previous 3 mo. We excluded patients accompanied by other digestive comorbidities, autoimmune disorders, NAFLD, obesity or diabetes mellitus, and those suffered from diarrhea or constipation within 1 month were not included either. Patients with NAS were defined as suffering from repeated cholangitis, the magnetic resonance cholangio-pancreatography (MRCP) or endoscopic retrograde cholangio-pancreatography (ERCP) results suggested multiple strictures located in donor biliary system with/without anastomotic stricture. To eliminate arterial factors, those accompanied with hepatic artery thrombosis were not included. For patients in uncomplicated group, they had no obvious complications after OLT, the regular reexaminations (symptoms, physical examinations, B-ultra sound, CT scan, biochemical test, plasma concentration of immunosuppressive drugs) were normal. The healthy controls were those non-LT individuals who came to hospital for a routine health examination, with no digestive diseases or surgical history and their routine tests indexes were in normal ranges. Finally a total of 30 patients meeting inclusion criteria were enrolled, including 20 post-LT patients (10 in NAS group and 10 in uncomplicated group）and 10 healthy controls.

All participants were totally informed of the related matters prior to entering in and signed the informed consent. This study was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board of the first affiliated hospital of Xi’an Jiaotong university.

***Surgical procedure***

All post-LT Patients underwent OLT in the first affiliated hospital of Xi’an Jiaotong University. Organ donation or transplantation in this study was strictly implemented under the regulation of the China Organ Donation Committee (CODC), Organ Transplant Committee (OTC), and the Declaration of Helsinki. Recipients were carefully evaluated before operation, while candidates diagnosed with hepatocellular carcinoma (HCC) totally accorded with the Milan criteria[[17](#_ENREF_17)]. Operations were performed with an ABO-compatible liver graft by the same group of doctors. All grafts derived from donors of cardiac death (DCD) and preserved in 4℃ UW liquids before LT. During the operation, graft’s common bile duct bonded to recipient’s by means of duct to duct anastomosis, interruptedly suturing for anterior wall and continuously for posterior with 6-0 absorbable string. A T-tube was applied just as necessarily required. After operation, they were given the triple regimen anti-rejection therapy by tacrolimus, mycophenolate mofetil and methylprednisone.

***Comparing variables***

We documented individual’s basic characteristics, including age, gender, body mass index (BMI), current state of smoking or drinking, blood routine test and liver function indexes within 48h before sample collecting. For post-LT patients, graft related factors (warm and cold ischemic time) and perioperative characteristics (including Child-Pugh classification, total duration of operation, anhepatic phase, bleeding volume, T-tube inserted or not) were reviewed. The duration from LT to diagnosis and the duration from LT to sample collecting were also respectively recorded.

***Sampling collection***

All fecal samples were carefully collected avoiding the pollution of urine, then accurately weighed and sub packaged into a 2 mL micro centrifuge tube (180-200 mg per tube), then immediately stored at -80 ℃ before analysis. All these stages were finished within 30 minutes.

***DNA extraction***

The fecal DNA were extracted according to the manufacturer’s instruction of testing kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA, United States). For one aliquot, scraped bits of stool into a 2 ml microcentrifuge tube on ice, added in 1.4 mL buffer ASL (from the QIAamp DNA Stool Mini Kit) before the sample thawed, vortexed continuously for 1 min until the sample was thoroughly homogenized. Water-bathed for 5 min at 70 ℃, then vortexed for 15 s and centrifuged the sample at a speed of 2000 *g* for 1 min. Discarded the sediment, pipetted 1.2 mL of the supernatant into a mew 2 mL microcentrifuge tube and added in 1 inhibitEX tablet (from the Kit), vortexed for 1 min until the tablet completely suspended. Incubated suspension for 1min at room temperature, then centrifuged for 3 min. Pipetted all the supernatant into a new 1.5 mL microcentrifuge tube and centrifuged for 3 min. Pipetted above 200 μL supernatant into a new 1.5 mL microcentrifuge tube which already had contained 15 μL proteinase K. Added 200 uL Buffer AL (from the Kit) and vortexed for 15 s, incubated at 70 ℃ for 10 min. Added 200 μL anhydrous ethanol to the lysate and vortexed thoroughly. Carefully applied the lysate to the QIAamp spin column and centrifuged for 1 min. Transferred the QIAamp spin column into a new 2 mL collection tube and discarded the tube containing filtrate. Added in 500 μL Buffer AW1 (from the Kit), centrifuged for 1 min and discarded the filtrate. Opened the QIAamp spin column, added 500 μL Buffer AW2 (from the Kit), centrifuged for 3 min and placed the spin column into a new 2 mL collection tube, then centrifuged for 1min. Transferred the spin column into a new 1.5 mL tube and pipetted 200 uL Buffer AE onto the QIAamp membrane. Incubated at room temperature for 1min, then centrifuged for 1 min to elute DNA. Finally, stored the filtrate (containing DNA) at -20 ℃.

***PCR and sequencing***

Use the DNA isolated from fecal samples as a template for the ampliﬁcation of the 16S rRNA V3-V4 region, the universal primer was F (5’-NNNNNNN ACTCCTACGGGAGGCAGCA-3’) and R (5’NNNNNNN GGACTACVSGGGTATCTAAT-30), the NNNNNNN were unique seven-base barcode used to tag each PCR product. The PCR was finished according to the touchdown protocol reaction[[18](#_ENREF_18)]. Reaction conditions were as follows: 5.0 μL 5 × reaction buffer (TaKaRa, Dalian, China), 5.0 μL 5 × high GC buffer (TaKaRa, Dalian, China), 0.5 μL dNTP (10 mmol/L) mixture , 1.0 uL forward primer (10 μmol/L), 1.0 uL reverse primer (10 μmol/L), 0.25 μL Q5 high-fidelity DNA polymerase (5U/uL, TaKaRa, Dalian, China), 1 uL DNA template in a total of reaction volume of 25 μL. Each PCR product was gel puriﬁed on 2% agarose gel electrophoresis. DNA was isolated using the Axygen Axy Prep DNA Gel Extraction kit (Axygen, Shanghai, China). The sequencing was finished with the help of the Illumina Miseq System (Illumina) .

***Bioinformatics analysis***

The sequencing data of samples were analyzed using pyrosequencing pipeline tools at RDP 10 (<http://pyro.cme.msu.edu/>). Bacterial diversity was determined by sampling-based analysis of operational taxonomic units (OTUs), α-diversity index (including rarefaction curves, Chao1 index, ACE index, Shannon index and Simpson index, estimated at a distance of 5%), as well as principal component analysis (PCA). Of them, the OTU is an operational definition referred to those closely related individuals, in the system of [biological classification](https://en.wikipedia.org/wiki/Biological_classification), it is defined based on a similarity threshold to classify microbial [species](https://en.wikipedia.org/wiki/Species) into different taxonomic levels (97% similarity equal to the level of species)[[19](#_ENREF_19),[20](#_ENREF_20)]. Species accumulation curves is applied to assess [species richness](https://en.wikipedia.org/wiki/Species_richness) based on the results of species and individual sampling. It can only be compared when the species richness has reached a clear [asymptote](https://en.wikipedia.org/wiki/Asymptote)[[21](#_ENREF_21)]. PCA is mathematically defined as an [orthogonal](https://en.wikipedia.org/wiki/Orthogonal_transformation) [linear transformation](https://en.wikipedia.org/wiki/Linear_transformation) which transforms the original data to a new [system](https://en.wikipedia.org/wiki/Coordinate_system) defined as principal component. Hence, the greatest variances by some projection of the data comes to lie on the corresponding principal component, which makes it easier to investigate the correlation between multiple variables[[22](#_ENREF_22)].

***Data analysis***

Diversity indexes and the species accumulation curve were calculated by QIIME. PCA plots of the bacterial communities were created using pcaMethods (Stacklies *et al*, 2007) in R (R Development Core Team, 2012). Differences of categorical variables among groups were analyzed by Chi-square or Fisher’s exact test, final results were expressed as percentage (%). For continuous variables, ANOVA test was used if data met the normal distribution or Mann-Whitney test if not, corresponding results were expressed as mean ± SD or median (range). Statistical analyses were conducted by SPSS version 18.0 (SPSS Inc., Chicago, IL, United States). P value <0.05 was considered statistically significant.

**Results**

***Patients‘ basic characteristics***

As Tab.1 showed, patients in 3 groups shared the similar age distribution, gender proportion, and BMI in our study (all *P* > 0.05). Results of blood routine tests were generally in normal ranges and of no differences among each other (*P* > 0.05, Table 1). While for liver function, all median or mean values were obviously abnormal for patients diagnosed with NAS, but no differences existed between the uncomplicated and healthy control group. Notably, for patients with NAS, biliary tract associated indexes like ALP and GGT, were elevated as nearly 4 times as healthy controls’ (*P* < 0.05), while ALB level was seriously decreased with a mean value of 34.14 g/L (41.1 g/L for healthy and 41.9 g/L for uncomplicated, *P* < 0.05).

For all patients underwent LT, the main inducers were HBV-related cirrhosis (80.00% *vs* 80.00%, *P* = 0.568, Table 2), others including subacute liver failure (SALF), hepatocellular carcinoma (HCC) and drug-induced liver injury (DILI) were relatively few in this study. Distributions of preoperative Child-Pugh scores between two groups were similar also, with the percentage of patients attributing to Child-Pugh A or B were 50% *vs* 50% (*P* = 0.834, Table 2). In addition, other factors such as liver grafts’ ischemic time, the mean duration of anhepatic phase, total operation duration, intraoperative bleeding volume, and the proportion of T-tube application were all equally distributed, (all *P* > 0.05, Table 2). The median duration from LT to final diagnosis of NAS was 9 months, from LT to sample collecting in two post-LT groups were 21 and 15 months respectively (*P* = 0.129).

***DNA sequencing results***

According to the samples number and species OTUs, we calculated the species accumulation curve of all participants (Figure 1). In this study, the curve had reached a plateau, the species had no more obvious increase as the samples number increased, which indicated that the sample volume in our study was relatively large enough to reflect the species richness.

***Microbiota diversity characteristics***

To ensure the validity, we excluded those rare OTUs of which the richness was less than 0.001% of the total, and also took a flattening process to eliminate the bias of sequencing depth. Finally, we got a total of 1,494,713 valid sequences, with an average sequence length of 468 bps. For these three groups, the mean valid sequences number were 52,222, 49,947, 47,302 respectively (*P* > 0.05, Figures 2 and 3).

As for the microbial community diversity, the OTUs number of phylum level in healthy control group was 969 ± 43, while in two post-LT groups, the numbers were 443 ± 75 and 568 ± 122 respectively, obviously smaller than healthy controls (both *P* < 0.05, Table 3). It seemed that there were more OTUs in NAS than uncomplicated group, but the difference was not significant. Similarly, these manifestations were also applicable to the OTUs distributions of order/family/genus/species levels (Table 3). Meanwhile, both two post-LT groups showed smaller α-diversity index (including Chao1, ACE, Simpson and Shannon index) than the healthy controls (*P* < 0.01, Table 4). All of these indicated that patients underwent LT had a lower gut microbiota diversity (including richness and species number) than healthy controls. Furthermore, though of no significant differences, gut microbiota of patients with NAS after liver transplantation was more diverse than those of uncomplicated group, we thought it was mainly due to the increases of potentially pathogenic bacterium (details were described later).

About the Principal Component Analysis (PCA) of different groups, the healthy controls were shown well aggregating and not overlapped with the two post-LT groups. Post-LT individuals of two groups were partially overlapped, but they still had their own trend to aggregate separately, so we can still distinguish NAS cluster from the group of uncomplicated (Figure 4). In summary, we can conclude that the variation among groups were larger than within groups, clustering in our study were actually feasible, (PC1 = 24.08%, PC2 = 17.12%).

***Distribution of gut bacteria***

As Fig.5 shown, gut microbiota in this study were mainly composed of six phyla, including *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Acidobacteria* and [*Verrucomicrobia*](javascript:gg('p__Verrucomicrobia');). *Firmicutes* and *Bacteroidetes*, as the main bacterium coexisting in human’s intestine, contributed to 92.32% of the total microbiota in healthy controls group, while the proportions were 77.11% in uncomplicated group, 57.40% in NAS, obviously smaller than healthy controls (both *P* < 0.05). Specifically, the changes of *Firmicutes* in post-LT patients was mostly due to the decreases of *Lachnospiraceae* and *Ruminococcaceae* at family level, accompanied by the increases of *Enterococcaceae* and *Streptococcaceae* (all owned to *Bacilli* class, Table 5). Especially for NAS group, the proportions of later two were significantly larger than uncomplicated group’s, (2.60% *vs* 1.20%, 8.60% *vs* 3.90%, both *P* < 0.05, Table 5). For *Bacteroidetes,* uncomplicated patients after LT share the similar proportions to healthy group (*P* > 0.05). While further analyzing, this phenomenon was caused by the increase of *Bacteroidaceae* and equivalent decrease of *Prevotellaceae* at family level. However, phylum of *Bacteroidetes* was substantially decreased in NAS group, with a constituent ratio of only 5.11%, nearly one fifth of the healthy group’s (*P* < 0.05, Table 5). Of them, the decrease of *Bacteroidaceae* and *Prevotellaceae* at family level played the inducing role in this change, from the normal 11.60%, 11.60% to 2.70%, 0.70% (both *P* < 0.05, Table 5). As for the phylum of *Proteobacteria*, it increased obviously in both two post-LT groups, especially for patients with NAS, proportion of *Proteobacteria* was up to as nearly as 30 times than healthy group’s (32.44% ± 7.32% *vs* 1.99% ± 0.25%, *P* < 0.05, Figure 5), while proportions of family of *Enterobacteriaceae* in three groups were 0.70%, 12.80%, 27.60%, *Pseudomonadaceae* were 0.00%, 0.00% and 5.90% (all *P* value between three groups were less than 0.05, Tab.4). Similarly, phylum of [*Verrucomicrobia*](javascript:gg('p__Verrucomicrobia');) also increased in post-LT patients (*P* < 0.05, Figure 4). Besides these, the proportions of *Actinobacteria* and *Acidobacteria* were relatively balanced, no significant differences existed among three groups.

**Discussion**

Nowadays, more and more researches have suggested the potential relationship between gut microbiota and liver diseases. Bacterial overgrowth or dysbacteriosis has also been proved contributing to recipient’s post-LT complications[[23](#_ENREF_23)]. In this study, we investigated the fecal microbial communities of patients diagnosed with NAS depending second-pyrosequencing of the16S rRNA V3-V4 region, taking the well-recovery recipients (uncomplicated) after OLT as negative controls, normal non-LT individuals as healthy controls, to explore the possible relationship between post-LT biliary complications and host’s gut microbiota.

According to our result, structural change of fecal microbial communities was observed in patients underwent liver transplantation, especially for those diagnosed with NAS. As α-diversity indexes reflected, post-LT patients presented with significant lower gut microbial diversity than healthy individuals, with the decreases of *Firmicutes, Bacteroidetes* and increases of *Proteobacteria,* [*Verrucomicrobia*](javascript:gg('p__Verrucomicrobia');) at the phylum level. While *Firmicutes* and *Bacteroidetes* were intestinal dominant bacterium, playing the key role in maintaining host’s intestinal homeostasis. Decreases of these two bacterium always indicated the destruction of intestinal barrier function and increased the risk of bacterial translocation[[24](#_ENREF_24)]. In fact, the decreases of these two phyla were partially attributed to the increases of *Proteobacteria* and [*Verrucomicrobia*](javascript:gg('p__Verrucomicrobia');), which usually contributed to a very small portion for human gut microbiota[[25](#_ENREF_25),[26](#_ENREF_26)]. Similar changes had also been reported in cirrhosis patients waiting for OLT[[27](#_ENREF_27)]. However, the shifts in our study were more obvious. At the family level, we found proportions of *Prevotellaceae*, *Bacteroidaceae, Lachnospiraceae* and *Ruminococcaceae* were lower in post-LT patients, accompanied with the increases of *Enterococcaceae*, *Streptococcaceae, Enterobacteriaceae* and *Pseudomonadaceae*. In previous studies, families of *Lachnospiraceae* and *Ruminococcaceae* were suggested participating in the metabolism of short-chain fatty acids (SCFAs), while SCFAs has been regarded as molecular link between the microbiota and the inflammation by acting on their specific G protein-coupled receptors 43 (GPR 43). Exogenous supplement of SCFAs can inhibit oxidative stress and inflammatory response induced by high glucose and bacterial endotoxins (LPS)[[28-30](#_ENREF_28)]. Therefore, loss of these potential beneficial bacterium during perioperative period may aggravate systemic inflammatory reaction and finally lead to liver injury[[31](#_ENREF_31)]. Meanwhile, families of *Enterococcaceae, Streptococcaceae, Enterobacteriaceae* and *Pseudomonadaceae* were commonly attributed to the pathogenic bacterium, their overgrowth has been found participating in various kinds of human diseases, even linearly correlated to patient’s Child-Pugh score[[27](#_ENREF_27),[32-34](#_ENREF_32)]. Moreover, bacterial translocation and elevation of LPS have been estimated in rats with liver’s ischemia-reperfusion injury or post-LT acute rejection[[35-37](#_ENREF_35)]. While Ren also found that liver ischemic preconditioning can improve intestinal barrier function and promote the restorations of intestinal microbiota following OLT[[38](#_ENREF_38)].

Compared with patients of no complications after liver transplantation, patients diagnosed with NAS in our study showed more significant decrease of *Bacteroidetes* and increase of *Proteobacteria* at phylum level, with higher proportions of *Enterococcaceae, Streptococcaceae, Enterobacteriaceae* and *Pseudomonadaceae*. This dramatic shift in the ratio between phyla or the expansion of *Proteobacteria* is often referred to as dysbacteriosis. Outgrowth of *Enterococcaceae, Streptococcaceae, Enterobacteriaceae* and *Pseudomonadaceae* will lead to large release of LPS and peptidoglycan. When recognized by human immune system *via* Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain like receptors (NLRs), would trigger the proinflammatory cascade NF-κB and directly stimulate hepatic stellate cells, which finally contributed to liver damage and liver disease progression[14,[39,40](#_ENREF_39)]. For patients underwent hepatic inflow occlusion and immunosuppressive treatment during or after OLT, these overgrowed pathogenic bacterium may easily penetrate through the intestinal barrier and translocate in the bloodstream, finally aggravated the ischemic reperfusion injury. While bile ducts are susceptible to inflammatory damage, so serious gut dysbacteriosis may exacerbate the cholangiocyte apoptosis and eventually progress into the strictures of bile duct[[41](#_ENREF_42),[42](#_ENREF_43)]. Whereas, proportions of *Lachnospiraceae* and *Ruminococcaceae* were similar between NAS group and uncomplicated group, indicating the overgrowth of former four pathogenic bacterium contributed more effect to the pathologic process. Nevertheless, the detailed relationship between bacterial shifts and NAS was not clear.

NAS is a serious and progressive complication after OLT, while grafts associated factors were commonly uncontrollable, seeking new breakthrough from recipients themselves is quite important for its prevention. Interestingly, adjustment of microbial structure has been recommended in the treatment of inflammatory bowel disease and metabolic diseases[[43](#_ENREF_44)]. Inhibition of pathogenic bacterium by antibiotics or probiotics has also been proved improving  cirrhosis patient’s prognosis, preventing the early-stage infection and acute rejection after OLT[[44-46](#_ENREF_45)]. Therefore, targeted interventions to microbial compositional shift in NAS may contribute to its treatment in future.

As we know, this study is the first attempt to investigate the possible relationship between gut microbiota and post-LT biliary complication**.** With all possible influencing factor including preoperative characteristics and postoperative intervention equally distributed between all objectives, unbalanced ratio between pathogenic bacterium to dominant bacterium existed in patients with non-anastomotic biliary strictures after liver transplantation. This finding might indicate the shifts of fecal microbial communities involve or exacerbate the process of bile duct’s injury. However, we admitted it’s a small-volume study from a single-center experience, and how gut microbial changes related to NAS remains obscure. To verify the possible mechanisms, larger-scale, multicenter studies are necessary in future.

In conclusion, our findings showed fecal microbial composition of patients with nonanastomotic biliary stricture was distinct from those with no complications after orthotopic liver transplantation. These compositional shifts of the increase of potential pathogenic bacterium (e.g., including *Enterococcaceae, Streptococcaceae, Enterobacteriaceae and Pseudomonadaceae*) as well as the decrease of dominant bacterium (e.g., Bacteroidaceae ), might contribute to the incidence of NAS. However, the underlying mechanism warrants further investigation.

**ARTICLE HIGHLIGHTS**

***Research background***

The background, present status and significance of the study should be described in detail. Non-anastomotic biliary stricture (NAS) is a lethal disorder after liver transplantation (LT), but the mechanisms are still obscure. Gut microbiota has been shown to participate in the pathogenesis of some post-LT complications, while the characteristics of microbial communities in patients with NAS have never been investigated.

***Research motivation***

The main topics, the key problems to be solved and the significance of solving these problems for future research in this field should be described in detail.

The purpose of this study was to explore the possible relationship between fecal microbial communities and NAS after OLT.

***Research objectives***

The main objectives, the objectives that were realized, and the significance of realizing these objectives for future research in this field should be described in detail.It may contribute to the possible mechanism research about NAS after LT, also shed some light on its prevention in future.

***Research methods***

The research methods (*e.g.*, experiments, data analysis, surveys and clinical trials) adopted to realize the objectives as well as the characteristics and novelty of these research methods should be described in detail. A total of 30 subjects including 10 patients with NAS, 10 patients with no complications after LT, and 10 non-LT healthy individuals were enrolled. Fecal microbial communities were assessed by the 16S rRNA gene sequencing technology. Diversity indexes and the species accumulation curve were calculated by QIIME. PCA plots of the bacterial communities were created using pcaMethods. Other data analysis were finished by Chi-square or Fisher’s exact test or ANOVA test by SPSS.

***Research results***

The research findings, their contributions to the research in this field, and the problems that remain to be solved should be described in detail. Different from the uncomplicated and healthy groups, unbalanced fecal bacterium ratio existed in patients with non-anastomotic biliary strictures after liver transplantation. The results showed NAS patients were associated with decreases of Firmicutes, Bacteroidetes and increases of Proteobacteria at the phylum level, with the proportion-ratio imbalance between potential pathogenic families including *Enterococcaceae*, *Streptococcaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and dominant families including *Bacteroidaceae*.

***Research conclusions***

These compositional shifts of the increase of potential pathogenic bacterium as well as the decrease of dominant bacterium might contribute to the incidence of NAS. Gut microbiota may involve the pathological process of NAS. Factors including poor liver graft, ABO-incompatibility, cytomegalovirus(CMV) infection contribute to the development of NAS.

Dysbacteriosis may be another inducer contribute to the development of NAS. The shifts of fecal microbial communities may involve or exacerbate the process of bile duct’s injury. Unbalanced ratio of pathogenic bacterium to dominant bacterium really existed in patients with NAS after liver transplantation. Shifts of fecal microbial communities really existed in patients with NAS after liver transplantation. What are the implications of this? Bacterial intervention may be a new therapy for preventing the [occurences](http://dict.youdao.com/search?q=disease%20progression%0D%0A&keyfrom=fanyi.smartResult) of NAS.

***Research perspectives***

According to our study, shifts of fecal microbial communities may involve or exacerbate the process of bile duct’s inflammation. This might be helpful for NAS’s prevention. While the definite relationships were obscure, more mechanisms research about how microbiota affected the pathological process should be carried out in future. To learn more interaction relationship between microbiota and biliary inflammatory injury, technology based on [functional genomics](http://dict.youdao.com/w/functional%20genomics/#keyfrom=E2Ctranslation) may be implied for future research.

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**Table 1 Basic characteristics of subjects *n* (%)**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Healthy (*n* = 10)** | **Post-LT** | |
| **Uncomplicated (*n* = 10)** | **NAS (*n* = 10)** |
| Age (yr) | 38 ± 12 | 43 ± 11 | 42 ± 9 |
| Male | 9 (90.0) | 8 (80.0) | 8 (80.0) |
| BMI (kg/m2) | 23.3 ± 2.5 | 22.1 ± 2.6 | 22.4 ± 2.7 |
| Current smoking | 3 (30.0) | 2 (10.0) | 0 |
| Current drinking | 2 (20.0) | 0 | 0 |
| Blood routine test |  |  |  |
| HB (g/L) | 122.5 ± 12.7 | 129.0 ± 20.0 | 127.4 ± 9.0 |
| WBC (× 109) | 6.0 ± 1.7 | 5.1 ± 2.2 | 5.2 ± 2.5 |
| Neu (%) | 59.6 ± 14.8 | 66.4 ± 16.4 | 64.3 ± 20.0 |
| Liver function |  |  |  |
| AST (U/L) | 21.3 (7.9-39.6) | 41.0 (13.0-93.0) | 57.1 (17.0-107.0)a,c |
| ALT (U/L) | 20.1 (14.6-34.4) | 49.3 (12.0-89.1) | 57.3 (18.0-111.0)a,c |
| ALP (U/L) | 77.3 ± 31.7 | 93.9 ± 17.2 | 332.8 ± 52.4a,c |
| GGT (U/L) | 27.2 ± 8.2 | 53.3 ± 35.6 | 226.4 ± 83.4a,c |
| TB (umol/L) | 13.7 ± 6.7 | 27.4 ± 17.6 | 104.43 ± 47.8a,c |
| DB (umol/L) | 5.4 ± 3.1 | 12.5 ± 8.6 | 43.8 ± 6.8a,c |
| ALB (g/L) | 41.1 ± 2.9 | 41.9 ± 5.3 | 34.1 ± 5.0a,c |

Healthy: healthy non-LT individuals, *n* = 10; NAS: patients diagnosed with non-anastomotic biliary strictures after liver transplantation, *n* = 10; Uncomplicated: patients with no complications after liver transplantation, *n* = 10; Blood routine tests and liver function indexes were with 48 h before sample collecting. Data were presented as mean standard ± deviation, median (range) or percentage where appropriate. a*P* < 0.05 *vs* healthy control group, c*P* < 0.05 *vs* uncomplicated group. BMI: body mass index; HGB: hemoglobin, WBC: white blood cell; Neu%: neutrophil ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyltransferase; ALB: albumin; TBIL: total bilirubin; DBIL: direct bilirubin; LT: liver transplantation.

**Table 2 Operative characterictics of post-liver transplantation patients *n* (%)**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Uncomplicated (*n* = 10)** | **NAS (*n* = 10)** | ***P* value** |
| Primary disease |  |  |  |
| HBV cirrhosis | 8 (80.0) | 8 (80.0) | 0.568 |
| HBV SALF | 0 (0.0) | 1 (10.0) |
| HCC | 1 (10.0) | 1 (10.0) |
| DILI | 1 (10.0) | 0 (0.0) |
| Child-Pugh classification |  |  |  |
| A | 1 (10.0) | 1 (10.0) | 0.834 |
| B | 4 (40.0) | 4 (40.0) |
| C | 5 (50.0) | 5 (50.0) |
| WIT (min) | 7 ± 2 | 8 ± 0 | 0.108 |
| CIT (h) | 7 ± 1 | 6 ± 1 | 0.291 |
| Total operation duration (min) | 366 ± 80 | 377 ± 62 | 0.893 |
| [Anhepatic phase](http://www.baidu.com/link?url=pwSyX6o46wYNmV48ZjiFgX1u1VeL1eWwfOJj5x9CW7g9z5fPKoVLbyE_3UGCwbXSvqInAjMvr8BZtsqogOdrsyym0I3LF8BS6vOrl4hPV1vv0dzYr66AZMdfS-mavkt3) (min) | 46 ± 10 | 49 ± 7 | 0.513 |
| Bleeding Volume (mL) | 1760 ± 347 | 1311 ± 268 | 0.329 |
| T-tube insertion | 8 (80.00) | 7 (70.00) | 0.906 |
| Median time from LT to NAS (m) | - | 9 (5-13) | - |
| Median time from LT to SC (m) | 15 (6-36) | 21 (13-32) | 0.129 |

a*P* < 0.05 *vs* healthy control group, c*P* < 0.05 *vs* uncomplicated group. SALF: subacute liver failure; HCC: Hepatocellular carcinoma; DILI: Drug-induced liver injury; WIT: warm ischemia time; CIT: cold ischemia time; SC: sample collecting; LT: liver transplantation.

**Table 3 OTUs distribution in 3 groups of different levels**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Healthy (*n* = 10)** | **Post-LT** | |
| **Uncomplicated (*n* = 10)** | **NAS (*n* = 10)** |
| Phylum | 969 ± 43 | 443 ± 75a | 568 ± 122a |
| Class | 969 ± 43 | 443 ± 75a | 568 ± 122a |
| Order | 969 ± 43 | 443 ± 75a | 567 ± 122a |
| Family | 889 ± 37 | 413 ± 68a | 525 ± 110a |
| Genus | 414 ± 14 | 254 ± 35a | 261 ± 44a |
| Species | 129 ± 7 | 88 ± 9a | 81 ± 11a |

a*P* < 0.05 *vs* healthy control group. LT: liver transplantation.

**Table 4 α-diversity indexes of 3 groups**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Healthy (*n* = 10)** | **Post-LT** | |
| **Uncomplicated (*n* = 10)** | **NAS (*n* = 10)** |
| Chao1 Index | 649.30 ± 34.76 | 269.70 ± 45.09a | 303.44 ± 76.86a |
| ACE | 834.03 ± 59.10 | 346.72 ± 67.73a | 413.30 ± 88.68a |
| Simpson | 0.91 ± 0.01 | 0.81 ± 0.02a | 0.75 ± 0.04a |
| Shannon | 5.71 ± 0.26 | 3.73 ± 0.33a | 3.65 ± 0.50a |

a*P* < 0.05 *vs* healthy control group. LT: liver transplantation.

**Table 5 Main bacterial families attributing to the microbial community’s changes**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Phylum** | **Class** | **Family** | **Healthy (*n* = 10)** | **Post-LT** | |
| **Uncomplicated (*n* = 10)** | **NAS (*n* = 10)** |
| Bacteroidetes | Bacteroidia | *Bacteroidaceae* | 11.60% ± 5.33% | 16.20% ± 3.20% | 2.70% ± 0.97%a,c |
| *Prevotellaceae* | 11.60% ± 4.56% | 0.00% ± 0.00%a | 0.70% ± 0.08%a |
| Firmicutes | Bacilli | *Enterococcaceae* | 0.00% ± 0.00% | 1.20% ± 0.45%a | 2.60% ± 0.87%a,c |
| *Leuconostocaceae* | 0.00% ± 0.00% | 0.70% ± 0.20% | 0.40% ± 0.05% |
| *Streptococcaceae* | 0.30% ± 0.11% | 3.90% ± 1.05%a | 8.60% ± 4.10%a,c |
| *Lachnospiraceae* | 21.50% ± 6.78% | 9.80% ± 2.45%a | 10.50% ± 3.44%a |
| *Ruminococcaceae* | 30.90% ± 6.78% | 7.00% ± 3.16%a | 11.20% ± 2.33%a |
| Proteobacteria | γ-proteobacteria | *Enterobacteriaceae* | 0.70% ± 0.35% | 12.80% ± 2.56%a | 27.60% ± 7.06%a,c |
| *Pseudomonadaceae* | 0.00% ± 0.00% | 0.00% ± 0.00% | 5.90% ± 3.16%a,c |
| Verrucomicrobia | Verrucomicrobiae | *Verrucomicrobiaceae* | 0.10% ± 0.09% | 0.40% ± 0.16%a | 0.40% ± 0.05%a |

a*P* < 0.05 *vs* healthy control group, c*P* < 0.05 *vs* uncomplicated group. LT: liver transplantation.

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**Figure 1 Species accumulation curves.**

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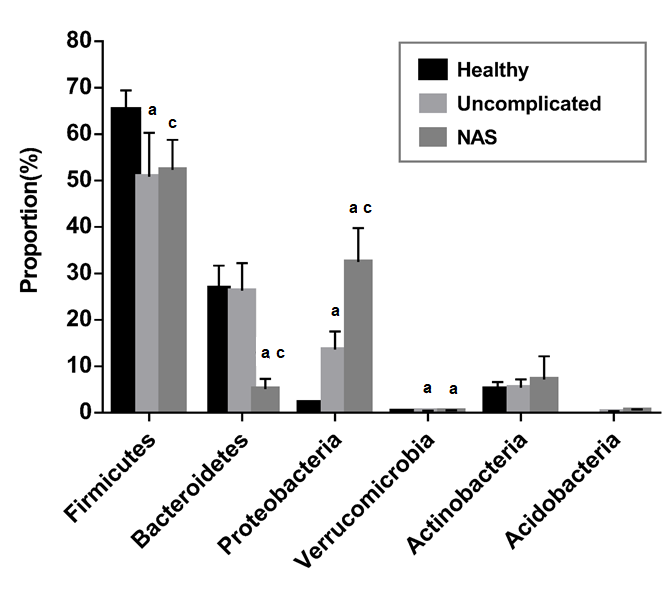
**Figure 2 The distributions of sequence length of all patients.**

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**Figure 3 Sequences number in 3 groups.**

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**Figure 4 Principal component analysis.**



**Figure 5 Distribution of bacterium at different phyla.** a*P* < 0.05 *vs* healthy controls group, c*P* < 0.05 *vs* uncomplicated group.