Microbiota transplants from feces or gut content attenuated portal hypertension and portosystemic collaterals in cirrhotic rats

Running title: FMT attenuated portal hypertension

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Abbreviations:

α-SMA, α-smooth muscle actin; ALT, alanine transaminase; AST, aspartate transaminase; AVP, arginine vasopressin; BDL, bile duct ligation; eNOS, endothelial nitric-oxide synthase; FGF, fibroblast growth factors; FMT, fecal microbiota transplantation; FXR, farnesoid X receptor; GCH, GTP cyclohydrolase; GMT, gut microbiota transplantation; LDA, linear discriminant analysis; SHP, small heterodimer partner; SMA, superior mesenteric artery; SRS, splenorenal shunt; TGFβ, transforming growth factor β; TIMP, tissue inhibitors of metalloproteinases; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor
Clinical Perspectives

- Microbiota transplantation in BDL rats improved gut dysbiosis that is consistent with the finding in cirrhotic patients, showing that microbiota transplantation is reproducible in BDL-cirrhosis model.
- Microbiota transplantation ameliorated portosystemic collaterals without affecting severity of liver fibrosis.
- Collaterals-related complications, such as hepatic encephalopathy and gastroesophageal variceal hemorrhage, may be reasonable treatment targets of microbiota transplantation which deserve further investigation.
Abstract

Liver cirrhosis and portal hypertension is the end of chronic liver injury with hepatic, splanchnic and portosystemic collateral systems dysregulation. Liver injury is accompanied by gut dysbiosis whereas dysbiosis induces liver fibrosis, splanchnic angiogenesis and dysregulated vascular tones vice versa, making portal hypertension aggravated. It has been proved that intestinal microbiota transplantation alleviates dysbiosis. Nevertheless, the influences of microbiota transplantation on cirrhosis related portal hypertension are not so clear. Liver cirrhosis with portal hypertension was induced by bile duct ligation in rats. Sham rats were surgical controls. Rats randomly received vehicle, fecal or gut (terminal ileum) material transplantation. The results showed that microbiota transplantation from feces or gut material significantly reduced portal pressure in cirrhotic rats ($P = .010, .044$). Hepatic resistance, vascular contractility, fibrosis and relevant protein expressions were not significantly different among cirrhotic rats. However, microbiota transplantation ameliorated splanchnic hyperdynamic flow and vasodilatation. Mesenteric angiogenesis, defined by whole mesenteric window vascular density, decreased in both transplantation groups and phosphorylated eNOS was downregulated. Portosystemic shunts determined by splenorenal shunt flow decreased in both transplantation groups ($P = .037, .032$). Shunting severity assessed by microsphere distribution method showed consistent results. Compared to sham rats, cirrhotic rats lacked Lachnospiraceae. Both microbiota transplants increased Bifidobacterium. In conclusion, microbiota transplantation in cirrhotic rats reduced portal pressure, alleviated splanchnic hyperdynamic circulation and portosystemic shunts. The main beneficial effects may be focused on portosystemic collaterals-related events, such as
hepatic encephalopathy and gastroesophageal variceal hemorrhage. Further clinical investigations are mandatory.

**Keywords:** cirrhosis; portal hypertension; microbiota transplantation; portosystemic shunts; angiogenesis
Introduction

Portal hypertension is a pathological condition linked to liver damages and most commonly, liver cirrhosis. Along with the elevation of portal pressure, lethal complications develop with high morbidity and mortality [1]. Portal pressure is determined by hepatic, splanchnic and collateral vascular systems. During the progression of liver cirrhosis, abnormal collagen fiber deposition in the liver interferes with the outflow of portal blood flow. The dysregulation of hepatic vascular contractility further enhances hepatic vascular resistance. Furthermore, the splanchnic inflow increases due to pathological splanchnic vasodilatation and angiogenesis. Both of them increase portal venous inflow and further contribute to a high pressure in the portal system [2]. To divert the portal blood flow, portosystemic collateral vessels and complications develop. A recent human study from Baveno VI-SPSS group showed that large spontaneous porto-systemic shunts correlated with overt hepatic encephalopathy and poor 1-year survival [3].

Gut microbiota consists of 10-100 trillion bacteria and is considered an extremely complex system [4]. The microbiome and liver orchestrate to maintain the body homeostasis. However, the gut-liver mutually beneficial relationship becomes unbalanced when dysbiosis develops. The break down leads to the aggravation of cirrhosis and portal hypertension though multiple aspects [5]. In the liver, gut dysbiosis enhances inflammation, fibrogenesis and even vascular resistance [6]. In splanchnic system, dysbiosis promotes angiogenesis [7]. Reversion of dysbiosis is thus a conceivable way to control portal hypertension.

Fecal microbiota transplantation (FMT) is the transferring of fecal microbiota from healthy individuals. It has been noted that in Clostridium difficile-associated disease, FMT alleviated dysbiosis and restored normal flora in patients with dysbiosis
Regarding the relevant study in liver diseases, a phase 1 study showed that FMT significantly improved hepatic encephalopathy. Another phase 1, randomized, placebo-controlled trial performed in decompensated cirrhotic patients revealed that FMT alleviated dysbiosis and hepatic encephalopathy. However, in both studies, liver function as assessed by model for end-stage liver disease (MELD score) was not affected by FMT. Furthermore, a comprehensive survey of the effects of FMT on cirrhosis itself and portal hypertension-related derangements is still lacking.

Until now, it has not been confirmed in which part of the alimentary tract do microbiota affect the development and aggravation of liver cirrhosis and portal hypertension the most. In cirrhosis, the bile acid metabolism and bacteria translocation occur primarily in terminal ileum. A recent study showed that in cirrhotic rats, dysbiosis in terminal ileum resulted in immune dysregulation. While decontamination with antibiotics reversed the process. Although the potential role of FMT in cirrhosis has drawn attention, the optimal source of microbiota in the alimentary tract is still unclarified.

This study therefore aimed to find out the optimal way of gut microbial transplantation accompanied by mechanistic survey from the perspective of basic research. We tested the effects of microbiota transplants from different part of intestine, including feces or gut materials. Furthermore, the impacts of microbiota transplantations on portal hypertension-related parameters, especially of the splanchnic and collateral systems, were studied.
Material and Methods

Animal model: BDL and sham

Male Sprague-Dawley rats weighing 240-270 g at the time of surgery were used for experiments. The rats were housed in plastic cages and allowed free access to food and water. All rats were fasted for 12 hours before the operation. Rats with secondary biliary cirrhosis were induced with common bile duct ligation (BDL) [13]. Under Zoletil 50 (Tiletamine + Zolezepam) anesthesia (0.8 mg/kg, intramuscularly), the common bile duct was doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second above the entrance of the pancreatic duct, followed by section of the common bile duct between the ligatures. The rats were allowed to recover. A high yield of secondary biliary cirrhosis was noted 4 weeks after BDL. To avoid the coagulation defects, BDL rats received weekly vitamin K injection (50 μg/kg intramuscularly). The sham group only received laparotomy and wound closure without ligating the bile duct.

This study has been authorized by Taipei Veterans General Hospital Animal Committee with experimental approval (IACUC 2018-222). Animal work had taken place in Taipei Veterans General Hospital. After hemodynamic measurements, the rats were euthanized during blood sample collection, under anesthesia status. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals, 8th edition, 2011" published by the National Research Council, the United States.

Study Design

Rats received either BDL to induce liver cirrhosis or sham operation as surgical control. To reduce the preexisting gut microbiota and to facilitate the repopulation of
donor microbiota, antibiotics were administered on the 3rd, 4th and 5th days after operations. Sham or BDL rats were then randomly divided into three groups: 1. Control (vehicle), 2. Fecal material transplantation (FMT), 3. Gut material transplantation (GMT). Transplantations were performed for 5 consecutive days since the 7th day. Experiments were performed on the 28th day. Parallel groups received sham or BDL operation without antibiotics and transplantation were employed to exclude the effects of antibiotics.

To evaluate the therapeutic effect of FMT, BDL rats were randomly divided into three groups: 1. No treatment (naïve), 2. Control (vehicle), 3. Fecal material transplantation (FMT). In this series, antibiotics were given on the 17th to 19th days after operation. Transplantation were performed since 21st day. Experiments were performed on the 35th day.

**Microbiota transplantation**

*Preparation of donor gut and feces material*

The gut material was collected from donor rats at the time when they were just sent from the animal breeding center without any treatment. After anesthesia, the gut material of terminal ileum and fecal pallets were collected respectively. The collected material was placed in transfer buffer (pre-reduced sterile phosphate buffered saline containing 0.05 % cysteine HCl, 2 mL/g) on ice. The material was then immediately homogenized and centrifuged at 800 g for 2 minutes. After that, the supernatant was collected, mixed with glycerol to make the concentration of glycerol equal to 10%, and stored at -80 °C until being used [14].

*The application of microbiota transplantation*
Before the transplantation, the recipient rats received antibiotics including imipenem (50 mg/kg/day), vancomycin (50 mg/kg/day) and esomeprazole (30 mg/kg/day) once daily on the 3rd, 4th, and 5th days after operations by oral gavage to reduce the preexisting gut microbiota and to facilitate the repopulation of donor microbiota.

Forty-eight hours after the last dose of antibiotics treatment, recipient rats were oral-gavaged with fecal supernatant (2.4 ml/kg, equivalent to 0.43 g/kg of fecal or gut material) for 5 consecutive days [15]. During the treatments, body weight (BW), food/water intake and any adverse response were monitored daily.

**Measurement of systemic and portal hemodynamics**

The right carotid artery was cannulated with a PE-50 catheter that was connected to a pressure transducer. Continuous recordings of mean arterial pressure and heart rate were performed on a multi-channel recorder (MP45, Biopac Systems Inc., Goleta, CA, U.S.A.). The external zero reference was placed at the level of the mid-portion of the rat. To measure portal pressure, the abdomen was then opened with a mid-line incision, and the mesenteric vein was cannulated with a 18G catheter connected to the transducer [16].

Superior mesenteric artery (SMA) was identified at its aortic origin and a 5-mm segment was gently dissected free from surrounding tissues. Then a pulsed-Doppler flow transducer (TS420, Transonic system Inc., Ithaca, NY, U.S.A.) was placed to measure the SMA flow [16]. Hepatic inflow via the portal vein was also measured by placing a flow probe around the portal vein as proximal to the liver as possible.

Cardiac output was measured by thermodilution, as previously described. Briefly, a thermistor was placed in the aortic arch just distal to the aortic valve and the thermal indicator (100 μL of normal saline) was injected into the right atrium through a PE-50 catheter. The aortic thermistor was connected to a cardiac output computer Cardiomax III (Columbus Instruments International Co., OH, U.S.A.). Five thermodilution curves were obtained for each cardiac output measurement. The final
value was obtained from the arithmetic mean of the computer results. Cardiac index (ml/min/100g BW) was calculated as cardiac output per 100g BW. Systemic vascular resistance (mmHg/ml/min/100g BW) was calculated by dividing mean arterial pressure by cardiac index. SMA resistance (mmHg/ml/min/100g BW) was calculated by (mean arterial pressure-portal pressure)/SMA flow per 100 g BW. Hepatic vascular resistance (mmHg/ml/min/100 g BW) was calculated by portal pressure/hepatic inflow (portal part) per 100 g BW.

**Hepatic fibrosis determination with Sirius red staining**

Liver paraffin section was stained with Sirius red staining kit (Polysciences Inc., Warrington, PA, U.S.A.). Image J was used to measure the percentage of Sirius red-stained area. Briefly, grayscale image was used, then the stained red collagen was isolated using thresholding function. After that, the thresholded area was measured and shown as the percentage of thresholded area in the whole liver section.

**Immunofluorescent study for the mesenteric vascular density**

Mesenteric angiogenesis was quantified by CD31-labelled microvascular networks in rat mesenteric connective tissue windows. From each rat, at least two mesenteric windows (wedge-shaped regions of connective tissue bordered by the intestinal wall and the ileal blood vessel pairs) were dissected free, washed in PBS, dried on gelatin slides, and fixed in 100% MeOH (-20 °C for 30 min). Slides were then incubated overnight at 4 °C with the primary antibody mouse anti-rat CD31-biotin (AbD Serotec, Oxford, UK). Then secondary antibody (CY2-conjugated streptavidin; Jackson ImmunoResearch, West Grove, PA, U.S.A.) was applied for 1 hour at room
temperature. The image within the whole mesenteric window was thresholded by Image J software (available for download from the National Institutes of Health (http://rsb.info.nih.gov/ij)). The mesenteric vascular density was shown as the percentage of thresholded area within the whole window.

**In situ perfusion preparation**

**SMA perfusion**

The *in situ* perfusion technique was modified from the *in vitro* SMA perfusion [17]. The abdomen was opened and an 18-gauge Teflon cannula served as the inlet was inserted in SMA. Another 16-gauge Teflon cannula as the outlet was inserted in the proximal end of the superior mesenteric vein (SMV). A ligature was tied over the proximal site of the insertion site to exclude the liver and collateral from perfusion. The animal was then transferred into the upper compartment of a warm chamber (37±0.5 °C). The temperature around the perfusion area was continuously monitored with a thermometer placed inside the mesentery and maintained at approximately 37±0.5 °C with a thermostatic pad and temperature-controlled infrared lamp. An open circuit perfusion was started with Krebs solution (composition in mM: NaCl, 118; KCl, 4.7; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; CaCl$_2$, 2.5; NaHCO$_3$, 25; dextrose, 11.0; pH, 7.4; 37±0.5 °C) via the mesenteric cannula by a roller pump (model 505S; Watson-Marlow Limited, Falmouth, Cornwall, UK). The perfusate was equilibrated with carbogen gas (95% O$_2$-5% CO$_2$) by a silastic membrane lung. The SMV cannula was opened to allow a free and complete washout of the blood. Pneumothorax was created by opening slits through the diaphragm to increase resistance in pulmonary arteries and prevent the perfusate from entering left heart chambers. The SMA was then perfused with
oxygenated (95% O\textsubscript{2}-5% CO\textsubscript{2}) Kreb solution containing 3% wt/vol albumin (factor V bovine serum albumin; Sigma, St. Louis, MO, U.S.A.). The effluent of the perfused tissue was collected in a reservoir placed at the lower compartment of the warm chamber and was not recirculated. To continuously monitor and record the pressure of this territory, a Spectramed DTX transducer attached to the Gould model RS 3400 recorder (Gould Inc., Cupertino, CA) was connected to a side arm placed just proximal to the perfusion cannula, with the zero placed at the level of right atrium. Because the temperature and pressure of the system stabilized within 10 minutes, all the experiments were performed 15 minutes after starting perfusion at a constant flow rate of 15 ml/min. The perfusion flow rate was kept constant throughout the whole experiment, so the changes in perfusion pressure reflected the changes in splanchnic vascular resistance. Only one concentration-response curve was performed in each preparation. In each individual preparation, after testing experimental agents, the contracting capability of the splanchnic vasculature was challenged with a 125-mM potassium chloride solution at the end of experiments.

Liver perfusion

The in situ perfusion system was performed as previously described with some modification [16]. Briefly, both jugular veins were cannulated with 16-gauge Teflon cannulas to ensure an adequate outflow without any resistance even at the highest flow rates. Heparin (200 U/100 g body weight) was injected through one of the cannulas. The abdomen was then opened and a 16-gauge Teflon cannula was inserted in the portal vein. The hepatic artery was ligated. To exclude the collaterals from perfusion, the second loose ligature around the distal portal vein was tied. An open circuit perfusion was then started with oxygenated Krebs solution perfusion in warm chamber.
Both the jugular vein cannulas were simultaneously opened to allow a complete washout of the blood. Pneumothorax was created by opening slits through the diaphragm. All the experiments were performed 15 minutes after starting perfusion at a constant rate of 40 ml/min.

**Portosystemic collateral system perfusion**

The *in situ* perfusion system was performed as previously described [18]. Briefly, both jugular veins were cannulated with 16-gauge Teflon cannulas. The abdomen was then opened and an 18-gauge Teflon cannula was inserted in the distal SMV and fixed with cyanoacrylate glue. To exclude the liver from perfusion, the second loose ligature around the portal vein was tied. All the experiments were performed 15 minutes after starting perfusion at a constant rate of 12 ml/min.

**Portal-systemic shunts analysis: microsphere distribution method**

Portal-systemic shunts was determined using the technique described by Chojkier and Groszmann and substituting color for radioactive microspheres; 30,000 of 15-μm yellow microspheres (Dye Track; Triton Technology, San Diego, CA, U.S.A.) was slowly injected into the spleen [19]. The rats were euthanized, and the livers and lungs were dissected and placed into new polypropylene centrifuge tubes. The number of microspheres in each tissue was determined following the protocol provided by the manufacturer. In brief, 3,000 blue microspheres (Dye Track) were added to each tube as an internal control. Tissue was digested overnight with 1 M KOH at 60 °C and thoroughly sonicated. After centrifugation, the supernatant was removed, and the pellets were washed once with 10 % Triton X-100 and twice with acidified ethanol. At the end of the process, a minimum pellets containing the
microspheres were allowed to dry overnight. The microspheres were diluted with 200 μl of acidified Cellosolve acetate (Spectrum Chemicals, Gardens, CA, U.S.A.). The absorbance of the solution was read at 448-nm (yellow) and 670-nm wavelengths (blue) by a spectrophotometer (Shimadzu, Columbia, MD, U.S.A.), and the number of microspheres was calculated by comparison with standards. Spillover between wavelengths was corrected with the matrix inversion technique. Portosystemic shunts were calculated as lung microspheres/(liver microspheres plus lung microspheres). Assuming a worst-case scenario in which two-thirds of the microspheres remain trapped in the spleen, this technique can detect a minimum shunt of 3.5 %. Studies using color microspheres have been shown to provide results similar to those using radioactive microspheres [20].

**Western analysis**

Tissue was immediately frozen in liquid nitrogen and stored at -80°C until required. The protein extracts were made by pulverization in grinder with liquid nitrogen, then a ratio of 1 ml of lysis buffer (phosphate-buffered solution containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) was added. 0.05% protease inhibitor cocktail solution (Roche Diagnostics GmbH, Penzberg, Germany) was applied for each 100 mg powdered sample. An aliquot of 20-40 μg protein from each sample that dissolved in sample buffer (63 mmol/l of Tris-HCL, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.005% bromphenol blue) and 10 μg positive control were separated on denaturing SDS-10% polyacrylamide gels by electrophoresis (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Prestained proteins markers (SDS-PAGE Standards, Bio-Rad) were used for molecular weight determinations. Proteins were
then transferred to a polyvinylidene difluoride membrane (Immum-Blot™ PVDF Membrane, Bio-Rad) by a semi-dry electroblotting system (Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell, Bio-Rad) for 1.5 h at 4 ℃. To block non-specific binding, membranes were blocked for 30 min with 3% non-fat dry milk in TBS-T, pH 7.4 (25 mmol/l Tris base-137 mmol/l NaCl-2.7 mmol/l KCl-1% Tween 20). Blots were incubated with the primary antibody (table 1). Then the blots were incubated for 60 min with the secondary antibody and washed. The specific proteins were detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Merk Millipore Co., Billerica, MA, U.S.A.) and scanned with a computer-assisted video densitometer and digitalized system (BioSpectrum® 600 Imaging System, Ultra-Violet Products Ltd., Upland, CA, U.S.A.). Then the signal intensity (integral volume) of the appropriate band was analyzed.

**Fecal sample collection, DNA extraction and 16S rRNA gene sequencing**

16S rRNA is made up of conserved and hypervariable regions. The former are not significantly different across microbial strains, whereas the later are genus or species-specific, and they differ in accordance to phylogenetic difference. Therefore, 16S rDNA are frequently used to identify species and are important for microbial phylogeny and taxonomic identification.

Fecal samples were collected and fecal DNA was extracted according to the previous literature [21]. In brief, sample (250 mg) was suspended in 250 μl of guanidine thiocyanate, 0.1M Tris (pH 7.5), 40 μl of 10% N-lauroyl sarcosine, and 500 μl 5% N-lauroyl sarcosine. Mechanical disruption with beads were applied for DNA extraction. 2 μl of a 10-mg/ml solution of RNAase was added for RNA removal. Alcohol precipitation was performed for nucleic acids recovery. Standard PCR was
performed with 0.75 units of Taq polymerase (Roche) and 20 pmol/μL of the forward and reverse primers were run in a Mastercycler gradient (Eppendorf). Amplicons were pooled in equal concentration. The pooled amplicons (2 nM) was subjected to sequencing via Illumina MiSeq technology. Sequences were analyzed with QIIME 1.8.021 using an in-house script. Raw sequences of low quality were filtered out with a minimum acceptable Phred score of 20. Similar sequences were clustered into Operational Taxonomic Units (OTUs) or taxa by UCLUST algorithm based on 97% of similarity. Representative sequences of each OTU were aligned using PyNAST against Greengenes template alignment (gg_13_8). The basic local alignment search tool (BLAST) was performed to assign taxonomy for each OUT and the combination of two microbial databases (Greengenes and PATRIC).

**Statistical analyses**

The data other than sequence analyses were expressed as mean ± S.E.M. Statistical analyses was performed using repeated t-test (for in situ perfusion study) or one-way ANOVA as appropriate. LSD was used for the post-hoc test. SPSS version 21 software for Windows (SPSS Inc., Chicago, IL, U.S.A.) was used for analyses. Results is considered statistically significant at two-tailed P-values of less than 0.05.

For sequence analysis, pairwise comparisons were performed using OTU tables generated from each sample. Samples that contained fewer reads than the rarefaction depth were removed for the alpha and beta diversity analyses. Richness provided by alpha diversity was computed with Simpson and Shannon index. Sample clustering was performed using PCA methods based on UniFrac metrics. The Shapiro-Wilk test was used to check normality of the data. UniFrac weighted distance was analyzed by
pairwise comparisons between the study groups with the non-parametric test Kruskal-Wallis one-way analysis of variance. LSD was used for the post-hoc test.
Results

Microbiota transplantation ameliorated portal hypertension

Rats received BDL to induce liver cirrhosis and portal hypertension. After antibiotics treatment, sham-operated (S) or BDL (B) rats randomly received vehicle as control (C), feces (F, FMT), or material in gut/terminal ileum (G, GMT). To exclude the possible effects of antibiotics, parallel groups without any antibiotics and treatment were employed (N, naïve). The results were evaluated 28 days after operations (figure 1A).

Typical hemodynamic changes of portal hypertension were induced successfully in BDL group. Compared to SC group, BC group had significantly higher portal pressure (figure 1B, SC v.s. BC: 9.3±0.3 v.s. 15.7±1.6 (mmHg), \( P < .001 \)). Portal hypertension is the net results of interaction of hepatic, splanchnic and collateral system. Hyperdynamic change in systemic circulation is one of the characteristics of liver cirrhosis and affects splanchnic system. The following experiments therefore determined the phenotype change in the portal hypertension-related systems (figure 1C). In systemic circulation, cardiac index increased and systemic resistance decreased in BDL rats (SC v.s. BC, cardiac index: 26.8±1.1 v.s. 37.7 ± 4.1 (ml/min/100 g), \( P = .007 \); systemic resistance: 5.0±0.4 v.s. 3.7 ± 0.5 (mmHg/ml/min/100 g), \( P = .019 \)). Taken together, the results suggested that the BDL rats developed significant portal hypertension and hyperdynamic circulation.

It is worth noting that both FMT or GMT significantly reduced portal pressure (figure 1B, BC v.s. BF v.s. BG: 15.7±1.6 v.s. 11.2±1.2 v.s. 12.3±1.6 (mmHg), BC v.s. BF, \( P = .010 \); BC v.s. BG, \( P = .046 \)). On the other hand, FMT or GMT did not affect body weight, cardiac index or systemic resistance in cirrhotic rats (figure 1C).
Figure 1D shows plasma biochemistry data. Both plasma AST and total bilirubin levels increased significantly in BDL groups (SC v.s. BC: AST: 87±9 v.s. 667±43 (U/L), \(P < .001\); ALT: 51±5 v.s. 129±9 (U/L), \(P = .103\); total bilirubin: 0.03±0.00 v.s. 8.94±0.49 (mg/dl), \(P < .001\)). TNFα level also increased in cirrhotic rats (SC v.s. BC: 4.2±0.4 v.s. 25.7±4.5 (pg/ml), \(P = .001\)). There was no significant difference in AST, ALT, total bilirubin or TNFα level among BC, BF and BG groups. Antibiotics treatment did not affect the hemodynamic factors or plasma biochemistry results in sham or BDL groups.

**Microbiota transplantation did not influence the hepatic vasoconstriction and fibrosis**

In hepatic system, hepatic vascular contractility and fibrosis are main components affecting portal hypertension. Figure 2A reveals that microbiota transplantation decreased hepatic blood flow from portal part, significantly (BC v.s. BF v.s. BG, 8.2±1.8 v.s. 4.9±0.8 v.s. 4.2±0.4 (ml/min/100 g), BC v.s. BF, \(P = .068\); BC v.s. BG, \(P = .026\)). However, hepatic resistance was not affected (BC v.s. BF v.s. BG, 2.7±0.8 v.s. 2.9±0.8 v.s. 3.1±0.4 (mmHg/ml/min/100 g), BC v.s. BF, \(P = .783\); BC v.s. BG, \(P = .615\)). Liver perfusion experiment was further performed to determine the hepatic vascular responsiveness to vasoconstrictors, showing that there was no significant difference in hepatic vascular pressure change between BC and BF groups (figure 2B). Liver fibrosis severity was also evaluated by Sirius red-stained area. To minimize the bias, the staining area was determined in whole frame of the liver section (figure 2C). Consistent with the finding of hepatic resistance, liver fibrosis severity was higher in cirrhotic rats, which was not affected by microbiota transplantation (SC v.s. BC v.s. BF v.s. BG v.s. BN, 0.8±0.2 v.s. 20.3±0.8 v.s.)
18.2±3.0 v.s. 20.8±0.5 v.s. 23.4±0.7 (%), SC v.s. BC, \( P < .001 \); BC v.s. BF, \( P = .325 \); BC v.s. BG, \( P = .787 \); BC v.s. BN, \( P = .125 \). On the other hand, 3 days of antibiotics treatment in this study did not affect hepatic flow or liver fibrosis.

Liver fibrogenesis-related proteins expression levels were evaluated between SC and BC groups (supplementary figure 1). The protein expression of \( \alpha \)-SMA, TIMP and TGF\( \beta \) significantly upregulated in BDL group while comparing to sham group (\( \alpha \)-SMA: 0.1±0.0 v.s. 0.8±0.1, \( P < .001 \); TIMP: 0.4±0.0 v.s. 0.6±0.1, \( P = .033 \); TGF\( \beta \): 0.4±0.0 v.s. 0.6±0.0, \( P = .006 \). On the other hand, phospho-eNOS significantly downregulated (1.1±0.1 v.s. 0.7±0.1, \( P = .013 \). The protein expressions in BDL group were compatible with liver cirrhosis. The protein expressions were then evaluated among BDL groups (figure 3 and supplementary figure 2). Consistent with the results of Sirius red staining, they were not affected by microbiota transplantation. FXR-related signal transduction protein expressions, including FGF 15, SHP, phospho-eNOS, GCH, Cystathionase and phospho-moesin were not significantly different among BDL groups, either. Taken together, microbiota transplantation did not significantly influence the liver in cirrhotic rats.

**Microbiota transplantation attenuated overt vasodilatation and angiogenesis in splanchnic system**

The effects of microbiota transplantation on splanchnic system are shown in figure 4A. BDL rats had significantly higher SMA flow and lower SMA resistance as compared with the sham rats (SC v.s. BC, SMA flow: 5.1±0.4 v.s. 7.3±0.7 (ml/min/100 g), \( P = .005 \); SMA resistance: 25.1±2.3 v.s. 16.7±1.8 (mmHg/ml/min/100 g), \( P = .009 \). FMT had a trend toward reduction of SMA flow in cirrhotic rats while GMT exerted significant effect (BC v.s. BF v.s. BG, 7.3±0.7 v.s.
5.9±0.8 v.s. 5.6±0.7 (ml/min/100 g), BC v.s. BF, \( P = .074 \); BC v.s. BG, \( P = .030 \). Arginine vasopressin (AVP)-induced SMA vasoconstriction was significantly enhanced by FMT (figure 4B, \( P = .015 \)), suggesting that microbiota transplantation reversed splanchnic overt vasodilatation in cirrhosis. Splanchnic angiogenesis was determined by vascular density in whole mesenteric window (figure 4C) to avoid selection bias. The mesenteric vascular density increased significantly in BDL rats (SC v.s. BC, 0.22±0.10 v.s. 1.59±0.35 (%), \( P < .001 \)), which was reduced by both fecal and gut material transplantation (BC v.s. BF v.s. BG v.s. BN: 1.59±0.35 v.s. 0.68±0.19 v.s. 0.79±0.13 v.s. 1.73±0.36 (%); BC v.s. BF, \( P = .004 \); BC v.s. BG, \( P = .011 \); BC v.s. BN, \( P = .627 \)). Antibiotics treatment in this study exerted no observable effects on splanchnic blood flow or mesenteric angiogenesis.

Mesenteric angiogenic proteins expression levels were determined between SC and BC groups (supplementary figure 3). The results showed that BDL group had significant upregulation of phospho-eNOS, iNOS, VEGF, phsopho-VEGFR2, COX1, HIF1\( \alpha \), PDGF and Akt (phospho-eNOS: 0.5±0.1 v.s. 1.3±0.2, \( P = .012 \); iNOS: 0.3±0.0 v.s. 0.6±0.1, \( P = .008 \); VEGF: 0.1±0.0 v.s. 0.7±0.1, \( P = .001 \); phospho-VEGFR2: 0.3±0.0 v.s. 0.6±0.1, \( P = .004 \); COX1: 0.6±0.1 v.s. 0.9±0.1, \( P = .001 \); HIF1\( \alpha \): 0.2±0.0 v.s. 0.7±0.1, \( P < .001 \); PDGF: 0.3±0.0 v.s. 0.7±0.1, \( P < .001 \); Akt: 0.5±0.1 v.s. 0.9±0.0, \( P < .001 \)). These findings were compatible with molecular change in splanchnic system in cirrhosis that overt vasodilatation and mesenteric angiogenesis resulted in hyperdynamic change. The protein expressions were then evaluated among BDL groups (figure 5 and supplementary figure 4), showing that eNOS phosphorylation was downregulated in both microbiota transplantation groups. And FMT had more significant effect compared to GMT (BC v.s. BF v.s. BG: 1.6±0.2 v.s. 0.6±0.2 v.s. 1.1±0.2; BC v.s. BF, \( P < .001 \); BC v.s. BG: \( P = .048 \); BF v.s. BG: \( P =
In brief, microbiota transplantation effectively ameliorated splanchnic hyperdynamic circulation in liver cirrhosis, which are at least partly, though inhibition of overt vasodilatation, pathological angiogenesis and downregulated eNOS activation.

**Microbiota transplantation ameliorated portosystemic collateral shunting degree**

The portosystemic collateral shunting degree was evaluated by splenorenal shunt (SRS) flow, the most prominent intra-abdominal shunting vessel in rodents, and microsphere method (Figure 6A). The results showed that SRS flow increased significantly in BDL group compared to sham group (SC v.s. BC: 0.03±0.00 v.s. 0.37±0.05 (ml/min/100 g), \(P < .001\)), which was reduced significantly by both microbiota transplantations (BC v.s. BF v.s. BG v.s. BN: 0.37±0.05 v.s. 0.22±0.08 v.s. 0.22±0.06 v.s. 0.49±0.08 (ml/min/100 g); BC v.s. BF, \(P = .045\); BC v.s. BG, \(P = .040\); BC v.s. BN, \(P = .086\)). The shunting degree evaluated by microsphere method showed consistent result (figure 6B): shunting severity increased in BC group and was reversed in BF group (SC v.s. BC v.s. BF: 10.7±3.1 v.s. 74.1±5.0 v.s. 56.8±8.2 (%), SC v.s. BC: \(P < .001\); BC v.s. BF: \(P = .047\)). Furthermore, SRS flow correlated well with the microsphere data (figure 6C, \(P < .001, r^2=0.757\)). The collateral vascular contractility was evaluated by portosystemic collateral perfusion (figure 6D). The result revealed that microbiota transplantation significantly enhanced vascular contractility of collateral vessels (BC v.s. BF, \(P = .024\)). Taken together, microbiota transplantation ameliorated portosystemic shunting and enhanced collateral vasoconstriction in cirrhosis.

**Therapeutic effects of microbiota transplantation**
To explore the therapeutic effects of microbiota transplantation, the rats received FMT or vehicle since the 21st day after BDL operation (figure 7A). There was no treatment in the BN group to exclude the effects of antibiotics. Consistent with previous findings, FMT since the stage of liver fibrosis still ameliorated portal pressure (figure 7B, BN v.s. BC v.s. BF: 16.7±0.4 v.s. 17.0±1.0 v.s. 14.4±0.5 (mmHg), BC v.s. BF, P = .017; BC v.s. BN, P = .753). The collateral vessel blood flow decreased at the same time (figure 7C, BN v.s. BC v.s. BF: 3.7±0.5 v.s. 4.0±0.6 v.s. 2.1±0.5 (ml/min/100 g), BC v.s. BF, P = .022; BC v.s. BN, P = .765). FMT had no effects on liver fibrosis (figure 7D, BN v.s. BC v.s. BF: 25.4±1.2 v.s. 25.2±0.3 v.s. 23.8±0.5 (%), BC v.s. BF, P = .161; BC v.s. BN, P = .833). Interestingly, FMT significantly attenuated mesenteric angiogenesis even at the stage of liver fibrosis (figure 8A, BN v.s. BC v.s. BF: 3.0±0.6 v.s. 3.1±0.6 v.s. 1.2±0.3 (%), BC v.s. BF, P = .018; BC v.s. BN, P = .848). It is worth noting that antibiotic treatment exerted no observable effects in this experiment.

The SMA morphology was further evaluated to determine the effects of FMT on splanchnic vessels (figure 8B). The cross section of SMA was stained with α-SMA to highlight the smooth muscle layer. The results showed that FMT increased wall thickness and wall area of SMA (BC v.s. BF, SMA wall thickness: 88.4±4.0 v.s. 103.1±4.0 (μm), P = .024; SMA wall area: 0.20±0.02 v.s. 0.24±0.01 (mm²), P = .043). In addition, phospho-myosin intensity over smooth muscle layer of SMA was determined by immunofluorescent study. Since myosin phosphorylation is the last and key step in the signal transduction of smooth muscle constriction, the intensity may reflect vasoconstriction. The result revealed that FMT group had increased phospho-myosin intensity (12.47±0.59 v.s. 14.37±0.52 (1/μm²), P = .032). This finding consisted with the findings of increasing splanchnic vasoconstriction and
downregulated phospho-eNOS in FMT group. Taken together, FMT significantly changed splanchnic vascular morphology. This might reverse vascular remodelling in cirrhotic rats and inhibit the following mesenteric angiogenesis.

**Microbiota transplantation improved dysbiosis in BDL rats**

The feces of SC, BC, BF, BG or N (naïve, original healthy fecal samples of the donor rats without any treatment) groups were analysed. The Simpson and Shannon alpha diversity index showed no significant difference among SC, BC, BF, BG or N groups (figure 9A). The beta diversity derived by principal component analysis with covariance matrix showed that liver cirrhosis induced remarkable change in microbiome while microbiota transplantation from both origins changed it (figure 9B). The MRPP on the pairwise UniFrac distance further confirmed the results (SC v.s. BC, A = 0.124, $P = .017$; BC v.s. BG, A = 0.089, $P = .016$; BC v.s. BF, A = 0.064, $P = .126$; SC v.s. N, A=0.189, $P = .002$).

Further analysis with linear discriminant analysis (LDA) effect size was performed (figure 9C). The results revealed that compared to SC group, BC group lacked Firmicutes. In addition, the Ruminococcus gauveauii and Tyzzerella, which belong to Lachnospiraceae family decreased. The Firmicutes, especially Lachnospiraceae family were not restored in BF or BG groups. Interestingly, Bifidobacterium increased in both BF and BG groups. On the other hand, based on Tax4fun from KEGG database, the relative abundance of VEGF signaling pathway reduced in both microbiota transplant groups (supplementary figure 5, BC v.s. BF, $P = .031$; BC v.s. BG, $P = .001$).
Discussion

The effects of microbiota transplantation on cirrhosis-induced portal hypertension has not been not clearly surveyed. Arab, et al. had reviewed the influences of MT on portal hypertension [22]. In the review, the authors noted that MT has potential benefits in primary sclerosing cholangitis, NAFLD, and alcohol-induced liver injury in mice. García-Lezana et al. showed that MT reverse portal hypertension in a rat model of NASH. However, there was no liver cirrhosis in the rats [23]. Following that, there were several comprehensive reviews pointed out the relationship between microbiota and portal hypertension [24]. Nevertheless, the impact of MT on cirrhosis-induced portal hypertension and the relevant hemodynamic derangements has not been clearly addressed. In this study, microbiota transplantation with fecal or gut material effectively attenuated portal hypertension and portosystemic collaterals. In addition, splanchnic hyperdynamic circulation, mesenteric pathological angiogenesis and eNOS phosphorylation were ameliorated. However, hepatic vascular resistance, severity of liver fibrosis and hepatic vascular constriction were not affected by microbiota transplantation. Taken together, microbiota transplantation from both feces or gut effectively control portal hypertension and extrahepatic derangements.

Liver cirrhosis induced by BDL significantly changed microbiome. Compared to the sham group, BDL rats significantly lacked Lachnospiraceae. It is worth noting that microbiota transplantation did not restore microbiome in cirrhotic rats towards that of the sham condition. Instead, the microbiota shifted to a new status. In both transplantation groups, Bifidobacterium increased significantly. These findings were consistent with several FMT studies in cirrhotic patients [9-10, 25]. Interestingly, microbiota transplantation from feces or gut exerted similar results in cirrhotic rats. In addition, there was no marked differences in the diversity of microbiota between the...
two groups at the end of experiments. To further clarify the relationship between microbiota and phenotype change, we analyzed the correlation between Lachnospiraceae or Bifidobacterium abundance and portal pressure-related derangements. Nevertheless, there were no significant correlations. This suggested that the beneficial effects of microbiota transplantation may be mainly derived from the whole composition of gut (terminal ileum) microbiota instead of single strain of bacteria. Furthermore, the “process of stool formation” from terminal ileum to anus did not add much as compared to the gut microbiota, in terms of their effects. This might also explain why microbiota transplantation from feces or gut exerted the similar beneficial effects.

Intrahepatic hemodynamics is determined by both structural and functional components. It has been well recognized that in liver cirrhosis, hepatic regeneration nodules and collagen fiber interfere with the portal blood inflow, and this is the structural component. On the other hand, the functional component is abnormal vasoconstriction of the intrahepatic vessels. The intrahepatic vascular tone is primarily regulated by NO via the eNOS pathway [26]. In this study, we found that BDL rats, as compared to sham rats, had significantly higher hepatic resistance. Liver fibrosis severity and related protein expressions, including α-SMA, TIMP and TGFβ also upregulated significantly in the BDL rats. On the other hand, eNOS phosphorylation downregulated significantly in BDL rats. Taken together, the structural and functional components alter in the hepatic system of BDL rats, which are compatible with the features of cirrhosis. However, the aforementioned changes could not be observed in BDL rats receiving microbiota transplantation. In addition, in situ liver perfusion showed that microbiota transplantation did not influence hepatic
vascular responsiveness to vasoconstrictors. We thus concluded that microbiota transplantation with this protocol exerted no impact on BDL-induced cirrhotic rats.

Pathological angiogenesis has been recently recognized as an important triggering and maintenance factor of portal hypertension and portosystemic collaterals. The portal blood flow is mainly supplied by splanchnic system. During cirrhosis progression, abnormal splanchnic angiogenesis further increases portal inflow [27]. It has been documented that VEGF, a potent proangiogenic factor, plays a pivotal role in the process. In splanchnic organs, VEGF and receptor overexpression have been identified in cirrhotic rats [28]. Inhibition of VEGF receptor significantly attenuated splanchnic vascularization and portosystemic collaterals in animal models [27]. NO, a vasodilator, has also been recognized as a potent proangiogenic factor [29]. Its role in splanchnic vasodilation and angiogenesis in portal hypertension has also been widely surveyed [30]. In this study, splanchnic blood flow and vasodilation decreased significantly in microbiota transplantation groups. In addition, eNOS phosphorylation was downregulated. In brief, microbiota attenuated angiogenesis and vasodilation in splanchnic system that resulted in reduction of portal pressure and was through at least partly, the inhibition of eNOS activation.

It is worth noting that portosystemic collaterals decreased in microbiota transplantation groups. In this study, shunting severity was evaluated by two methods. The blood flow of SRS, the most prominent collateral vessel in cirrhotic rats, was measured directly. The results showed that SRS flow decreased significantly and consistently in both transplantation groups. Shunting severity was further evaluated by microsphere method. By injecting the microsphere into the spleen and measuring the distribution of microsphere in lung and liver, the shunting severity could be
quantitated. Both methods correlated well and suggested that microbiota transplantation effectively attenuated portosystemic collaterals.

Not until recently, the clinical significance of shunting vessels in portal hypertension was strengthened [3]. In a recent study, the images of 908 cirrhotic patients were analyzed by Baveno VI-SPSS group. They found that the severity of collateral vessel, as defined by cross-sectional spontaneous portosystemic shunts area, predicted 1-year survival of cirrhotic patients. Interestingly, in the training cohort, the collateral shunting area only correlated with overt hepatic encephalopathy, but not ascites, variceal bleeding, spontaneous bacterial peritonitis or hepatorenal syndrome. Taken together, the improvement of shunting severity is pivotal in the treatment of hepatic encephalopathy. Consistently, two randomized control trial suggested that FMT attenuated hepatic encephalopathy [9-10, 25]. However, the MELD score was not improved in the treatment group. The results are in accordance with our finding that microbiota transplantation did not significantly influence the liver. Furthermore, since hepatic encephalopathy is mainly induced by poor liver function and portosystemic shunts, our data provides the evidence that the efficacy of FMT on hepatic encephalopathy may be mediated through the reduced portosystemic collaterals.

BDL is a widely accepted animal model simulating the pathophysiological changes of liver cirrhosis and portal hypertension [31]. However, there are some limitations of BDL model: BDL induces cirrhosis by bile stasis, which is not the most common form of liver injury in human practice. Furthermore, bile stasis itself may alter microbiota [32]. On the other hand, hepatotoxins such as thioacetamide (TAA) or carbon tetrachloride (CCl₄) induce liver cirrhosis through chronic hepatitis. However, these models still have limitations. For example, TAA can be delivered by
intraperitoneal injection or oral route. Nevertheless, intraperitoneal injection of TAA aggravates mesenteric angiogenesis and may interfere with the interpretation of the current findings on extrahepatic effects of the treatments. TAA delivered through oral gavage also inevitably impacts microbiota. On the other hand, the severity of portosystemic shunting in CCL₄ model is not prominent enough as compared to that of BDL [33]. Taken together, although the animal models of liver cirrhosis and portal hypertension have been extensively adopted and BDL is the one that fits the main theme of the current study the most, validation of the results in clinical study is absolutely necessary.

In conclusion, microbiota transplantations from both feces or gut effectively control portal hypertension, which is related to the amelioration of splanchnic hyperdynamic circulation, mesenteric angiogenesis and eNOS phosphorylation. The portosystemic shunting degree also reduced at the same time but the liver was unaffected. The clinical application of microbiota transplantation for the control of portosystemic collaterals-related complications deserves further investigation.
Data availability:

The RNA sequencing data is available at
https://drive.google.com/file/d/1AlMf3Kl2HjTmuYcsyigwpOLhDqFJTVTB/view?usp=sharing

The rest of data that support the findings of this study are available from the corresponding author, SJH, upon reasonable request.

Competing interests:

The authors report no conflict of interest.

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References

1. Garcia-Tsao, G. and Bosch, J. (2010) Management of varices and variceal hemorrhage in cirrhosis. N Engl J Med 362, 823-832.

2. Gracia-Sancho, J., Marrone, G. and Fernández-Iglesias, A. (2019) Hepatic microcirculation and mechanisms of portal hypertension. Nat Rev Gastroenterol Hepatol 16, 221-234.

3. Praktiknjo, M., Simón-Talero, M., Römer, J., Roccarina, D., Martínez, J., Lampichler, K., Baiges, A., Low, G., Llop, E., Maurer, M. H., Zipprich, A., Triolo, M., Maleux, G., Fialla, A. D., Dam, C., Vidal-González, J., Majumdar, A., Picón, C., Toth, D., Darnell, A., Abraldes, J. G., López, M., Jansen, C., Chang, J., Schierwagen, R., Uschner, F., Kukuk, G., Meyer, C., Thomas, D., Wolter, K., Strassburg, C. P., Laleman, W., La Mura, V., Ripoll, C., Berzigotti, A., Calleja, J. L., Tandon, P., Hernandez-Gea, V., Reiberger, T., Albillos, A., Tsochatzis, E. A., Krag, A., Genescà, J. and Trebicka, J.; Baveno VI-SPSS group of the Baveno Cooperation. (2020) Total area of spontaneous portosystemic shunts independently predicts hepatic encephalopathy and mortality in liver cirrhosis. J Hepatol 72, 1140-1150.

4. Wiest, R., Albillos, A., Trauner, M., Bajaj, J. S. and Jalan, R. (2017) Targeting the gut-liver axis in liver disease. J Hepatol 67, 1084-1103.

5. Acharya C, Bajaj JS. (2019) Altered Microbiome in Patients With Cirrhosis and Complications. Clin Gastroenterol Hepatol 17, 307-321.

6. Schwabl, P., Hambruch, E., Seeland, B. A., Hayden, H., Wagner, M., Garnys, L., Strobel, B., Schubert, T. L., Riedl, F., Mitteregger, D., Burnet, M., Starlinger, P., Oberhuber, G., Deuschle, U., Rohr-Udilova, N., Podesser, B. K., Peck-
Radosavljevic, M., Reiberger, T., Kremoser, C. and Trauner, M. (2017) The FXR agonist PX20606 ameliorates portal hypertension by targeting vascular remodelling and sinusoidal dysfunction. J Hepatol 66, 724-733.

7. Schirbel, A., Kessler, S., Rieder, F., West, G., Rebert, N., Asosingh, K., McDonald, C. and Fiocchi, C. (2013) Pro-angiogenic activity of TLRs and NLRs: a novel link between gut microbiota and intestinal angiogenesis. Gastroenterology 144, 613-623.e9.

8. Leffler, D. A. and Lamont, J. T. (2015) Clostridium difficile infection. N Engl J Med 372, 1539-48.

9. Bajaj, J. S., Kassam, Z., Fagan, A., Gavis, E. A., Liu, E., Cox, I. J., Kheradman, R., Heuman, D., Wang, J., Gurry, T., Williams, R., Sikaroodi, M., Fuchs, M., Alm, E., John, B., Thacker, L. R., Riva, A., Smith, M., Taylor-Robinson, S. D. and Gillevet, P. M. (2017) Fecal microbiota transplant from a rational stool donor improves hepatic encephalopathy: A randomized clinical trial. Hepatology 66, 1727-1738.

10. Bajaj, J. S., Salzman, N. H., Acharya, C., Sterling, R. K., White, M. B., Gavis, E. A., Fagan, A., Hayward, M., Holtz, M. L., Matherly, S., Lee, H., Osman, M., Siddiqui, M. S., Fuchs, M., Puri, P., Sikaroodi, M. and Gillevet, P. M. (2019) Fecal Microbial Transplant Capsules Are Safe in Hepatic Encephalopathy: A Phase 1, Randomized, Placebo-Controlled Trial. Hepatology 70, 1690-1703.

11. Bajaj, J. S. and Khoruts, A. (2020) Microbiota changes and intestinal microbiota transplantation in liver diseases and cirrhosis. J Hepatol 72, 1003-1027.

12. Muñoz, L., Borrero, M. J., Úbeda, M., Conde, E., Del Campo, R., Rodríguez-Serrano, M., Lario, M., Sánchez-Díaz, A. M., Pastor, O., Díaz, D., García-Bermejo, L., Monserrat, J., Álvarez-Mon, M. and Albillos, A. (2019) Intestinal
Immune Dysregulation Driven by Dysbiosis Promotes Barrier Disruption and Bacterial Translocation in Rats With Cirrhosis. Hepatology 70, 925-938.

13. Nevzorova, Y. A., Boyer-Diaz, Z., Cubero, F. J. and Gracia-Sancho, J. (2020) Animal models for liver disease - A practical approach for translational research. J Hepatol 73, 423-440.

14. Di Luccia, B., Crescenzo, R., Mazzoli, A., Cigliano, L., Venditti, P., Walser, J. C., Widmer, A., Baccigelupi, L., Ricca, E. and Iossa, S. (2015) Rescue of Fructose-Induced Metabolic Syndrome by Antibiotics or Faecal Transplantation in a Rat Model of Obesity. PLoS One 10, e0134893.

15. Durgan, D. J., Ganesh, B. P., Cope, J. L., Ajami, N. J., Phillips, S. C., Petrozino, J. F., Hollister, E. B. and Bryan, R. M. Jr. (2016) Role of the Gut Microbiome in Obstructive Sleep Apnea-Induced Hypertension. Hypertension 67, 469-74.

16. Hsu, S. J., Lee, F. Y., Wang, S. S., Hsin, I. F., Lin, T. Y., Huang, H. C., Chang, C. C., Chuang, C. L., Ho, H. L., Lin, H. C. and Lee, S. D. (2015) Caffeine ameliorates hemodynamic derangements and portosystemic collaterals in cirrhotic rats. Hepatology 61, 1672-84.

17. Tsai, M. H., Iwakiri, Y., Cadelina, G., Sessa, W. C. and Groszmann, R. J. (2003) Mesenteric vasoconstriction triggers nitric oxide overproduction in the superior mesenteric artery of portal hypertensive rats. Gastroenterology 125, 1452-61.

18. Mosca, P., Lee, F. Y., Kaumann, A. J. and Groszmann, R. J. (1992) Pharmacology of portal-systemic collaterals in portal hypertensive rats: role of endothelium. Am J Physiol 263, G544-50.

19. Chojkier, M. and Groszmann, R. J. (1981) Measurement of portal-systemic shunting in the rat by using gamma-labeled microspheres. Am J Physiol 240, G371-5.
20. Hodeige, D., de Pauw, M., Eechaute, W., Weyne, J. and Heyndrickx, G. R. (1999) On the validity of blood flow measurement using colored microspheres. Am J Physiol 276, H1150-8.

21. Godon, J. J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl Environ Microbiol 63, 2802-13.

22. Arab, J. P., Martin-Mateos, R. M. and Shah, V. H. (2018) Gut-liver axis, cirrhosis and portal hypertension: the chicken and the egg. Hepatol Int. 12, 24-33.

23. García-Lezana, T., Raurell, I., Bravo, M., Torres-Arauz, M., Salcedo, M. T., Santiago, A., Schoenenberger, A., Manichanh, C., Genescà, J., Martell, M. and Augustin, S. (2018) Restoration of a healthy intestinal microbiota normalizes portal hypertension in a rat model of nonalcoholic steatohepatitis. Hepatology 67, 1485-98.

24. Baffy, G. (2019) Potential mechanisms linking gut microbiota and portal hypertension. Liver Int 39, 598-609.

25. Bajaj, J. S., Fagan, A., Gavis, E. A., Kassam, Z., Sikaroodi, M. and Gillevet, P. M. (2019) Long-term Outcomes of Fecal Microbiota Transplantation in Patients With Cirrhosis. Gastroenterology 156, 1921-1923.e3.

26. Lee, F. Y., Colombato, L. A., Albillos, A. and Groszmann, R. J. (1993) Administration of N omega-nitro-L-arginine ameliorates portal-systemic shunting in portal-hypertensive rats. Gastroenterology 105, 1464-70.

27. Fernandez, M., Vizzutti, F., Garcia-Pagan, J. C., Rodes, J. and Bosch, J. (2004) Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. Gastroenterology 126:886-94.

28. Huang, H. C., Wang, S. S., Hsin, I. F., Chang, C. C., Lee, F. Y., Lin, H. C.,
Chuang, C. L., Lee, J. Y., Hsieh, H. G. and Lee, S. D. (2012) Cannabinoid receptor 2 agonist ameliorates mesenteric angiogenesis and portosystemic collaterals in cirrhotic rats. Hepatology 56, 248-58.

29. Papapetropoulos, A., García-Cardeña, G., Madri, J. A. and Sessa, W. C. (1997) Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest 100, 3131-9.

30. Iwakiri, Y. and Kim, M. Y. (2015) Nitric oxide in liver diseases. Trends Pharmacol Sci 36, 524-36.

31. Van Campenhout, S., Van Vlierberghe, H. and Devisscher, L. (2019) Common Bile Duct Ligation as Model for Secondary Biliary Cirrhosis. Methods Mol Biol 1981, 237-47.

32. Verbeke, L., Farre, R., Trebicka, J., Komuta, M., Roskams, T., Klein, S., Elst, IV, Windmolders, P., Vanuytsel, T., Nevens, F. and Laleman, W. (2014) Obeticholic acid, a farnesoid X receptor agonist, improves portal hypertension by two distinct pathways in cirrhotic rats. Hepatology 59, 2286-98.

33. Abraldes, J.G., Pasarín, M. and García-Pagán, J.C. (2006) Animal models of portal hypertension. World J Gastroenterol 12, 6577-84.
Table 1. List of antibodies for imunoblots

| Antibody      | Vendor       | Catalog     |
|---------------|--------------|-------------|
| Liver         |              |             |
| α-SMA         | Genetex      | GTX10034    |
| FGF15         | Abcam        | ab229630    |
| Cystathionase | Proteintech  | 12217-1-AP  |
| p-eNOS        | Genetex      | GTX129058   |
| eNOS          | Cell signaling | 32027s    |
| β-actin       | Genetex      | GTX629630   |
| p-moesin      | Cell signaling | 3141s    |
| moesin        | Cell signaling | 3146s    |
| GCH           | Abcam        | ab236387    |
| TGFβ          | Genetex      | GTX110630   |
| SHP           | Abcam        | ab186874    |
| TIMP          | Genetex      | GTX64360    |
| Mesentery     |              |             |
| p-eNOS        | Genetex      | GTX129058   |
| eNOS          | Cell signaling | 32027s    |
| iNOS          | Genetex      | GTX130246   |
| VEGF          | Merch Millipore | ABS82   |
| p-VEGFR2      | Genetex      | GTX50153    |
| VEGFR2        | Cell signaling | 9698s    |
| COX1          | Cell signaling | 4841s    |
| COX2          | Cell signaling | 12282s   |
| HIF-1α        | Genetex      | 127309      |
| β-actin       | Genetex      | GTX629630   |
| FGF15         | Abcam        | ab229630    |
| p-Akt         | Cell signaling | 9271S    |
| Akt           | Cell signaling | 4691L    |
| p-ErK         | Cell signaling | 4370S    |
| Erk           | Cell signaling | 9102S    |
| PDGF          | Millipore    | ABS82       |
Figure Legends

Figure 1. Effects of microbiota transplantation on systemic circulation. (A) Rats with sham (S) or bile duct ligation (B, BDL) surgery were randomly allocated into vehicle control (C), fecal material transplant (F) or gut material transplant (G) groups. The experiments were performed 28 days after operations when cirrhosis developed. (B) BC had higher portal pressure comparing to SC group. Microbiota transplantation significantly reversed portal hypertension in BF and BG groups as compared to BC group. n = 6, 7, 6, 6, 6, 7, 7 (C) In systemic circulation, cardiac index increased, and systemic resistance decreased in cirrhotic rats, which were not affected by microbiota transplantation. (D) Both plasma AST, total bilirubin and TNFα levels increased significantly in cirrhotic groups. There was no significant difference in AST, ALT, total bilirubin or TNFα level among BC, BF and BG groups. *P < .05, **P < .01, ***P < .001

Figure 2. The impacts of microbiota transplantation on the liver. (A) The hepatic resistance increased significantly in BC group compared to sham (SC) group. Microbiota transplantation did not affect liver hemodynamics. n = 6, 7, 6, 6, 6, 7, 7 (B) Liver perfusion surveyed hepatic vascular responsiveness to vasoconstrictors. There was no difference in hepatic vascular pressure change between the two groups. n = 6, 6 (C) Liver fibrosis severity evaluated by Sirius red stained area in whole section of the liver showed that liver fibrosis aggravated in BDL group. Microbiota transplantation did not affect the severity of liver fibrosis. Scale bars, left panels: 1 mm, right panels, 200 μm; n = 5, 6, 6, 6, 7; *P < .05, **P < .01, ***P < .001
**Figure 3** Liver fibrogenesis-related protein expression. There was no significant difference among groups (all \( p > 0.05 \)). \( n = 9, 9, 8 \)

**Figure 4.** The effects of microbiota transplantation on splanchnic system. (A) BC had significantly higher SMA flow and lower SMA resistance as compared to SC group. SMA flow was reversed in BG group as compared to BC group. \( n = 6, 7, 7, 6, 6, 6, 7, 7 \)
(B) The AVP (arginine vasopressin)-induced SMA territory vasoconstriction was significantly enhanced in cirrhotic rats receiving microbiota transplantation. \( n = 6, 6 \)
(C) Splanchnic angiogenesis was determined by vascular density in whole frame of mesenteric window to minimize selection bias. The mesenteric vascular density increased significantly in BC group, which was attenuated by microbiota transplantations (BF, BG). Scale bar: 1 mm; \( n = 6, 7, 7, 6, 6, 6, 7, 7 \); *\( P < .05 \), **\( P < .01 \), ***\( P < .001 \)

**Figure 5.** Mesenteric angiogenic protein expression. eNOS phosphorylation was downregulated in BF and BG groups. \( n = 8, 9, 8 \); *\( P < .05 \), **\( P < .01 \), ***\( P < .001 \)

**Figure 6.** The effects of microbiota transplantation on portosystemic collaterals. (A) The SRS flow increased significantly in BC group and reduced significantly in both BF and BG groups. \( n = 6, 7, 7, 6, 6, 7, 6 \)
(B) Microsphere method showed that the shunting severity increased in BC group and was reversed in BF group. \( n = 6, 6, 5 \)
(C) The SRS flow correlated with microsphere shunting degree well (\( p < 0.001, r^2 = 0.757 \)). (D) The collateral vascular contractility was evaluated by collateral perfusion, showing that
microbiota transplantation significantly enhanced vascular contractility of collateral vessels. \( n = 6, 6; \) *\( P < .05, **\( P < .01, ***\( P < .001 \)

**Figure 7. The therapeutic effects of microbiota transplantation.** (A) The rats received FMT or vehicle since the 21\(^{st}\) day after BDL operation when the liver fibrosis developed. There was no treatment in the BN group to exclude the effects of antibiotics. (B) Microbiota transplantation since the stage of liver fibrosis still ameliorated portal hypertension. \( n = 6, 7, 7 \) (C) The SRS flow decreased significantly in BF group. (D) There was no significant difference in liver fibrosis among three groups. Scale bars, left panels: 1 mm, right panels, 200 \( \mu \text{m}; \) *\( P < .05, **\( P < .01, ***\( P < .001

**Figure 8. The therapeutic effects of microbiota transplantation on splanchnic vasculature.** (A) Mesenteric angiogenesis decreased significantly in BF group even delivered since the stage of liver fibrosis. It is worth noting that antibiotics treatment exerted no observable effects in this experiment. \( n = 6, 7, 7 \); Scale bar: 1 mm. (B) The SMA morphology was further evaluated to determine the effects of microbiota transplantation on splanchnic vessels. The cross section of SMA was stained with \( \alpha \)-SMA to highlight the smooth muscle layer. The SMA wall thickness and area increased significantly in BF group. The intensity of phospho-myosin over smooth muscle layer may reflect vasoconstriction. The result revealed that BF group had increased phospho-myosin intensity. Scale bar: 100 \( \mu \text{m}; \) \( n = 7, 7; \) *\( P < .05, **\( P < .01, ***\( P < .001

**Figure 9. Microbiota analyses.** (A) The Simpson and Shannon alpha diversity index showed no significant difference among all groups. \( n = 6, 6, 6, 6 \) (B) Principal component analysis with covariance matrix
showed that liver cirrhosis induced remarkable change in microbiome while microbiota transplantation from both origins modified it. (C) Linear discriminant analysis effect size (LEfSe). The results revealed that compared to SC group, BC group lacked Firmicutes. In addition, Ruminococcus gauveauii and Tyzzerella, which belong to Lachnospiraceae family decreased. The Firmicutes, especially Lachnospiraceae family was not restored in microbiota transplantation groups. Interestingly, Bifidobacterium increased in both BF and BG groups (n = 6 in all groups). *P < .05, **P < .01, ***P < .001
(A) Experimental setup:

- **Day 0**: Sham, Antibiotics, Sham-naive (SN), Sham-Control (SC), Sham-FMT (SF), Sham-GMT (SG), BDL-Control (BC), BDL-FMT (BF), BDL-GMT (BG), BDL-naive (BN).
- **Day 3-5**: Transplantation (once per day):
  1. Control (vehicle)
  2. Feces
  3. Gut
- **Day 7-11**: Sacrifice

(B) Portal Pressure

(C) Systemic parameters:

- **Body Weight**
- **Blood Pressure**
- **Heart Rate**
- **Cardiac Index**
- **Systemic Resistance**

(D) Biochemical parameters:

- **AST**
- **ALT**
- **t-Bilirubin**
- **TNFα**
Liver Western Blot

- **α-SMA**
  - Band location: BC, BF, BG
  - α-SMA (42)

- **TIMP**
  - Band location: BC, BF, BG
  - TIMP (30)

- **TGFβ**
  - Band location: BC, BF, BG
  - TGFβ (23)

- **FXR**
  - Band location: BC, BF, BG
  - FXR (42)

- **SHP**
  - Band location: BC, BF, BG
  - SHP (28)

- **phospho-eNOS**
  - Band location: BC, BF, BG
  - phospho-eNOS (75)

- **GCH**
  - Band location: BC, BF, BG
  - GCH (28)

- **Cystathionase**
  - Band location: BC, BF, BG
  - Cystathionase (43)

- **phospho-moesin**
  - Band location: BC, BF, BG
  - phospho-moesin (78)

- **FGF 15**
  - Band location: BC, BF, BG
  - FGF 15 (25)

- **β-actin**
  - Band location: BC, BF, BG
  - β-actin (42)

- **moesin**
  - Band location: BC, BF, BG
  - moesin (78)

- **ΔGCH**
  - Band location: BC, BF, BG
  - ΔGCH (28)
Mesentery Western Blot

**phospho-eNOS**

**VEGF**

**COX1**

**HIF1α**

**iNOS**

**phospho-VEGFR2**

**COX2**

**FGF15**

**phospho-Akt**

**Akt**

**phospho-ErK**

**PDGF**

| Band location | BC | BF | BG |
|---------------|----|----|----|
| p-eNOS (133)  |    |    |    |
| eNOS (140)    |    |    |    |
| iNOS (131)    |    |    |    |
| VEGF (57)     |    |    |    |
| p-VEGFR2 (152)|    |    |    |
| VEGFR2 (230)  |    |    |    |

| Band location | BC | BF | BG |
|---------------|----|----|----|
| COX1 (70)     |    |    |    |
| COX2 (65)     |    |    |    |
| HIF1α (130)   |    |    |    |
| β-actin (42)  |    |    |    |

| Band location | BC | BF | BG |
|---------------|----|----|----|
| FGF15 (25)    |    |    |    |
| PDGF (57)     |    |    |    |
| P-Akt (60)    |    |    |    |
| Akt (60)      |    |    |    |
| P-ErK (42, 44)|    |    |    |
| Erk (42, 44)  |    |    |    |
| β-actin (42)  |    |    |    |
(A) Timeline of experimental procedures:

- **BDL** (Day 0)
- **Antibiotics** and **Transplantation**
  - **Control**
  - **Feces**
- **BDL-naive (BN)**
- **BDL-Control (BC)**
- **BDL-FMT (BF)**
- **Day 17-19**
- **Day 21-25**
- **Sacrifice**

(B) **Body Weight**

(C) **Portal Pressure**

(D) **SRS Flow**

(E) **Liver Fibrosis**

**Sirius Red Staining**

- BN
- BC
- BF

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