



Original Article



The Impact of Liver Graft Preservation Method on Longitudinal Gut Microbiome Changes Following Liver Transplant: A Proof-of-concept Study

Gail A.M. Cresci^{1,2,3*}, Qiang Liu^{4,5#}, Naseer Sangwan^{3,6}, Darren Liu³, David Grove², David Shapiro², Khaled Ali^{4,5}, Beatrice Cazzaniga^{4,5}, Luca Del Prete^{4,5}, Charles Miller^{4,5}, Koji Hashimoto^{4,5} and Cristiano Quintini⁷

¹Department of Gastroenterology, Hepatology and Nutrition, Digestive Disease Institute, Cleveland, OH, USA; ²Department of Inflammation and Immunity, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA; ³Cleveland Clinic Lerner College of Medicine, Cleveland Clinic, Cleveland, OH, USA; ⁴Department of General Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH, USA; ⁵Transplant Center, Cleveland Clinic, Cleveland, OH, USA; ⁶Cardiovascular and Metabolic Sciences/Shared Laboratory Resources, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA; ⁷Department of General Surgery, Digestive Disease Institute, Cleveland Clinic, Abu Dhabi, United Arab Emirates

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Abstract

Background and Aims: End-stage liver disease is associated with disruptions in gut microbiota composition and function, which may facilitate gut-to-liver bacterial translocation, impacting liver graft integrity and clinical outcomes following liver transplantation. This study aimed to assess the impact of two liver graft preservation methods on fecal microbiota and changes in fecal and breath organic acids following liver transplantation. **Methods:** This single-center, non-randomized prospective pilot study enrolled liver transplant patients whose grafts were preserved using either static cold storage or ex situ normothermic machine perfusion (NMP). Fresh stool and breath samples were collected immediately before surgery and at postoperative months 3, 6, and 12. Stool microbiota was profiled via 16S rRNA gene sequencing, stool short-chain fatty acids were measured using gas chromatography/mass spectrometry, and breath volatile organic compounds (VOCs) were analyzed with selected-ion flow-tube mass spectrometry. **Results:** Both cohorts experienced a loss of microbiota diversity and dominance by single taxa. The NMP cohort demonstrated enrichment of several beneficial gut taxa, while the static cold storage cohort showed depletion of such taxa. Various gut bacteria were found to correlate with stool short-chain fatty acids (e.g., lactic acid, butyric acid) and several VOCs. **Conclusions:** Fecal microbiota alterations associated with end-stage liver disease do not fully normalize to a healthy control profile following liver transplantation. However, notable differences in microbiota composition and function were observed between liver graft preservation methods. Future research with larger randomized cohorts is needed to explore whether the NMP-

associated shift in gut microbiota impacts clinical outcomes and if breath VOCs could serve as biomarkers of the clinical trajectory in liver transplant patients.

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Introduction

Orthotopic liver transplantation (OLT) is a lifesaving treatment for end-stage liver disease (ESLD). However, due to the ongoing disparity between the supply and demand for suitable donor livers, alternative therapeutic strategies have been explored to improve the quality of marginal livers.¹ Compared to static cold storage (SCS), the current standard of care, ex situ normothermic machine perfusion (NMP) is a method to enhance organ preservation and assess and improve marginal graft viability.^{1,2} In contrast to SCS, where the liver graft undergoes cold ischemia, ex situ normothermic machine perfusion maintains the liver graft in a near-physiological state with adequate oxygen and nutrient supply, thereby normalizing metabolism.³ These benefits can improve liver graft viability during preservation, resulting in less ischemia-reperfusion injury.³

Interest has grown regarding the role of the gut microbiome in health and disease. The gut microbiome is a complex ecosystem composed of trillions of microbes, including bacteria, fungi, protozoa, archaea, and phages. It is an important source of beneficial metabolites and plays a key role in maintaining gut barrier integrity and modulating immune function and inflammation.⁴ There exists a mutualistic, bidirectional relationship between the gut microbiome and its host, particularly within the immune system. The gut microbiome influences the development and function of the immune system, while the host immune system helps shape the composition

Keywords: Ex situ normothermic liver perfusion; Static cold storage; Short-chain fatty acids; Microbiota; Volatile organic acids; Breath metabolites.

#Contributed equally to this work.

*Correspondence to: Gail A. M. Cresci, Department of Inflammation and Immunity, Cleveland Clinic, 9500 Euclid Avenue, NB20, Cleveland, OH 44195, USA. ORCID: <https://orcid.org/0000-0002-5717-7099>. Tel: +1-216-445-0874, E-mail: crescig@ccf.org.

and diversity of the microbiome. The gut microbiome directly interacts with the liver via the portal vein, which carries gut-derived products to the liver. In turn, the liver communicates with the gut microbiome by feedback through bile acids and bioactive mediators released from the liver to the intestine.⁵ This bidirectional relationship is termed gut-liver axis.

The gut microbiome is involved in the pathophysiology of many chronic diseases, including liver disorders.⁴ Gut dysbiosis, an alteration in gut microbial composition and function, has been identified in patients with ESLD and is associated with poor outcomes during the pre-transplant period.^{6,7} Gut dysbiosis can affect medication metabolism, alter the production of beneficial microbially-derived metabolites, and impair the gut barrier, promoting the translocation of pathogens and pathogen-associated molecular patterns to the liver.⁵ Studies have investigated gut microbial changes following liver transplantation and have shown that the gut microbiome⁷⁻⁹ and its related functionality⁷ change from pre- to post-liver transplant. However, little is known regarding the impact of liver preservation methods on gut microbiome and metabolome changes following liver transplant. NMP has been reported to favorably affect liver graft physiology and metabolism. Given the crosstalk between the liver and the gut, including the gut microbiome,⁵ we hypothesized that improving liver graft physiology and metabolism through NMP would have a favorable impact on the gut microbiome compared to SCS in patients with ESLD receiving a liver transplant. To test this hypothesis, we assessed longitudinal changes in the gut microbiome and metabolome in patients undergoing liver transplantation utilizing SCS or NMP liver graft preservation methods.

Methods

Study participants

All research was conducted in accordance with the Declarations of Helsinki and Istanbul, and all procedures were approved by the Cleveland Clinic Institutional Review Board. Written consent was obtained from all subjects. Patients were recruited from two non-randomized, prospective, longitudinal clinical studies conducted between 2018 and 2020 at the Cleveland Clinic (Cleveland, OH). A total of 41 OLT patients (>18 years of age) receiving conventionally accepted livers were included. Thirty-one patients received standard-of-care treatment with SCS from donor to recipient (SCS: IRB# 18-119 – “Biorepository of liver tissue and blood sample after liver transplantation”), and ten patients received liver grafts that were exposed to ex situ machine perfusion for liver graft preservation (NMP: IRB# 15-549 – “A Phase 1 Pilot Study to Assess Safety and Feasibility of Normothermic Machine Preservation in Human Liver Transplantation”). Details regarding the inclusion/exclusion criteria and treatment for NMP can be found in Liu *et al*.² Briefly, all livers in both groups underwent standard liver procurement with cold flush using organ preservation solution at the donor hospital. The livers were then transported to our institution on cold storage in an ice box. Livers in the SCS group underwent back-table preparation with a cold flush repeated at our institution, after which they were transplanted into the recipient following hepatectomy. Livers in the NMP group also underwent back-table preparation with cold flush, after which they were moved into NMP. The perfusate for NMP consisted of packed red blood cells, fresh frozen plasma, albumin, and some medications (e.g., heparin).² The liver grafts during NMP were continuously infused through both the portal vein and the hepatic artery with oxygenated warm perfusate to maintain physiological metabolism at body temperature. After NMP, the liver grafts were flushed with cold saline and organ

preservation solution to thoroughly remove the sanguineous perfusate, followed by the standard transplant procedure.

Fresh fecal samples were obtained from patients immediately before liver transplant and at three, six, and twelve months post-transplant via rectal swab or clean catch before entering the commode. Samples were collected, kept on ice for <24 h, and stored at –80°C until analysis. Breath samples were obtained at the same study time points and analyzed within 4 h. Fecal and breath samples were also obtained from an age-matched cohort of healthy controls without chronic diseases and compared with the baseline/pre-transplant time point.

Patient data collection

Demographic data (age, sex, race, ethnicity), organ preservation technique, type of organ donation, MELD-Na, malnutrition diagnosis, liver cancer diagnosis, length of hospital stay after transplant, and presence of transplant rejection were collected retrospectively from the patient’s electronic medical record. Liver function laboratory studies and medication data were collected at each study visit. Liver function studies included total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and gamma-glutamyl transferase. Medication data included proton pump inhibitors (PPIs)/histamine-2 (H2) antagonists, corticosteroids, immunosuppressive therapies, and antibiotic use. As diet impacts the gut microbiome, a self-reported seven-day food frequency questionnaire was obtained at each time point, focusing on the subject’s typical weekly consumption of food groupings (dairy, meat/fish, vegetables, fruits, fats, sweets, and fiber-containing foods).

16S rRNA gene amplicon sequencing and bioinformatics

Fecal sample DNA extraction, 16S rRNA gene amplicon sequencing, and bioinformatics analyses were performed using methods described earlier.⁸⁻¹² Briefly, raw 16S amplicon sequences and metadata (metabolomics) were demultiplexed using the `split_libraries_fastq.py` script implemented in QIIME2.^{9,10} The demultiplexed fastq file was split into sample-specific fastq files using the `split_sequence_file_on_sample_ids.py` script from QIIME2. Individual fastq files without non-biological nucleotides were processed using the Divisive Amplicon Denoising Algorithm (hereinafter referred to as DADA) pipeline.^{9,11} The output of the DADA2 pipeline (a feature table of amplicon sequence variants, or ASV table) was processed for alpha diversity (Simpson Diversity Index) and beta diversity (Bray-Curtis dissimilarity) analysis using the `phyloseq`¹² and `microbiomeSeq` (<http://www.github.com/umerijaz/microbiomeSeq>) packages in R.

Metabolomics analysis

The fecal sample analysis for short-chain fatty acid (SCFA) was performed using modified methods previously described.^{13,14} Briefly, fecal sample supernatant aliquots were mixed with 50 µL 2-Butanol/Pyridine (3:2) containing six heavy-labeled internal standards. The carboxylic acids were then derivatized with Isobutyryl chloroformate. After derivatization, the sample was mixed with hexane, and the hexane layer was removed for gas chromatography-mass spectrometry (MS) analysis. The quantification of SCFAs—acetic acid, butyric acid, isovaleric acid, lactic acid, propionic acid, and succinic acid—was performed using isotope dilution gas chromatography-GC/MS. Sample results are reported as the wet weight of fecal material. Metabolomics (metadata) were analyzed alongside 16S rRNA data as described above.

Exhaled breath collection and analysis

The selected-ion flow tube (SIFT)-MS technology and instrument used in this study have been previously described and validated.^{15,16} Breath collection and analysis were performed as previously described.¹⁷ Before breath sample collection, each participant performed a mouth rinse to minimize contamination from volatile organic compounds (VOCs) produced in the mouth. Exhaled gas was collected by having the subject inhale to total lung capacity, followed by exhaling into a Mylar collection bag at a constant flow. The Mylar collection bag was then capped and stored in an incubator at 37°C until analysis. Analysis was performed within four hours of sample collection, and bags were flushed with nitrogen between patients. Exhaled breath samples were analyzed using SIFT-MS with a VOICE200 SIFT-MS machine (Syft Technologies Ltd, Christchurch, New Zealand). The samples were analyzed in two modes. The first mode was the full mass scan mode, where the instrument analyzed the sample by looking at the mass-to-charge ratio versus product ion count. The charged precursor ions used were hydronium ion, oxygen, and nitrosonium ion, and the mass range analyzed was 15 amu to 200 amu. The second mode was the selected ion mode, where the product concentration was measured, and the rate constant was used to calculate the concentration of the VOC in the sample. VOC concentrations are represented in parts per billion.

Statistical analysis

Descriptive statistics were calculated for all patient clinical variables. Patients were stratified by liver preservation, SCS, and NMP. The Wilcoxon Rank-Sum test was used to compare the medians of continuous variables. Chi-squared and Fisher's exact tests were used to compare categorical variables. Statistical analyses were performed in R (version 4.1.2). Differences in mean values of fecal SCFAs and breath metabolites among cohorts were evaluated by one-way analysis of variance and Student's t-tests, with means compared using Tukey's multiple comparisons in GraphPad Prism (version 9.4). Significance was determined as $p < 0.05$.

The microbial variance was analyzed using ANOVA among sample categories while measuring the alpha diversity index with the `plot_anova_diversity` function in the `microbiomeSeq` package. Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed on all principal coordinates obtained during canonical correspondence analysis using the ordination function of the `microbiomeSeq` package. Differential abundance analysis was performed using the random-forest algorithm, implemented in the `DAtest` package (<https://github.com/Russel88/DAtest/wiki/usage#typical-workflow>). Briefly, differentially abundant methods were compared using false discovery rate, area under the receiver operating curve, empirical power, and false positive rate. Based on `DAtest`'s benchmarking, we selected LDA Effect Size and ANOVA as the methods of choice for performing differential abundance analysis. We assessed statistical significance ($p < 0.05$) throughout and, when necessary, adjusted p -values for multiple comparisons according to the Benjamini and Hochberg method to control the false discovery rate.¹² Linear regression (parametric test) and Wilcoxon (non-parametric) tests were performed on genera and ASV abundances against metadata variables using the base functions in R (version 4.1.2; R Core Team, 2021).¹⁵

Results

Patient characteristics

This study included a total of 41 patients who underwent

OLT. The median interquartile range (IQR) age at transplant was 55 years (47, 62) with 24 males (59%) and 17 females (41%). The majority of patients were White (38/41, 93%) (Table 1). Of the 41 patients, 10 (24%) underwent liver transplantation utilizing the NMP preservation technique. The most common type of organ donation was donation after brain death in 23 (57%), followed by donation after circulatory death in 11 (28%), and living donor liver transplantation in six (15%). The median (IQR) chemical MELD-Na score was 18 (13, 25). The median (IQR) length of stay after OLT was 10 days (8, 13). Eleven (28%) patients experienced acute cellular rejection within 12 months after their transplant.

Liver function analysis

At pre-transplant, patients in the SCS cohort had higher AST ($p = 0.035$), but there were no other differences between liver function indices between groups (Table 2). By the three-month post-transplant follow-up visit, those in the SCS cohort had higher levels of ALT ($p = 0.048$) and AST ($p < 0.06$). There were no statistically significant differences in liver function tests between liver preservation cohorts at six and twelve months post-transplant.

Diet and medication usage

There were no differences in dietary patterns between the two cohorts for the number of servings consumed over seven days from assessed food groups (data not shown). Both cohorts consumed foods from dairy, meat/fish, and starch groups daily, and sweet and fatty foods at least four times per week. Fiber-containing foods were rarely consumed. At pre-transplant, there was a higher usage of PPI/H2-blockers in the NMP cohort compared to the SCS cohort (100% vs 67% of patients; $p = 0.043$), but no differences occurred in the prevalence of other medication use (antibiotics, corticosteroids, and immunotherapy) between the two cohorts at any of the time points (Table 3).

Liver preservation method versus etiology of liver failure on microbiome diversity

16S rRNA gene sequencing was performed on samples collected at each time point to characterize temporal patterns of microbial community structure, richness, evenness, and diversity. Microbial diversity within each subject (alpha diversity – Simpson index) was quantified at each time point for each liver preservation method cohort (Fig. 1A). At pre-transplant, alpha diversity was higher in healthy controls compared to both liver preservation method cohorts (Fig. 1A). Alpha diversity did not differ between liver preservation cohorts at pre-transplant but was significantly different at three, six, and twelve months, with the largest differences shown at six months.

Principal components analysis (PCoA; Bray-Curtis) was performed to visualize the dissimilarities between subjects' gut microbiota at each time point (beta diversity) based on the type of liver preservation method (Fig. 1B). According to PC1 and PC2 analysis, the microbial communities were significantly separated from each other at each time point, with the greatest separation occurring at six and twelve months post-transplant.

Impact of liver preservation method on the abundance of individual bacterial taxa

Permutation testing of 16S data indicated that patients' overall core microbiota differed between the cohorts at each time point and changed over time within each preservation cohort, as shown in the heatmaps in Figure 2. To identify long-

Table 1. Patient demographics

Characteristic	N	Overall N = 41 ^a	SCS N = 31 ^a	NMP N = 10 ^a	p-value ^b
Age	41	55 (47, 62)	55 (48, 63)	54 (46, 62)	0.7
Gender	41				>0.9
Male		24 (59%)	18 (58%)	6 (60%)	
Female		17 (41%)	13 (42%)	4 (40%)	
Ethnicity	41				0.14
White		38 (93%)	30 (97%)	8 (80%)	
Black or African American		3 (7.3%)	1 (3.2%)	2 (20%)	
Type of liver donation	40				0.018
DBD		23 (57%)	19 (63%)	4 (40%)	
DCD		11 (28%)	5 (17%)	6 (60%)	
LDLT		6 (15%)	6 (20%)	0 (0%)	
MELD-Na	41	18 (13, 25)	17 (12, 22)	23 (18, 27)	0.13
Malnutrition	41				0.6
None		14 (34%)	10 (32%)	4 (40%)	
Mild		11 (27%)	7 (23%)	4 (40%)	
Moderate		9 (22%)	8 (26%)	1 (10%)	
Severe		7 (17%)	6 (19%)	1 (10%)	
Length of stay (Days)	39	10 (8, 13)	11 (9, 14)	10 (8, 10)	0.2
Rejection	40	11 (28%)	7 (23%)	4 (40%)	0.4

^aMedian (IQR); n (%), ^bWilcoxon rank-sum test; Fisher's exact test. DBD, donation after brain death; DCD, donation after circulatory death; LDLT, live-donor liver transplantation; SCS, static cold storage; NMP, normothermic machine perfusion; MELD, End-Stage Liver Disease.

term effects of the liver preservation method on bacterial taxa following liver transplant, mean relative abundances of genus-like phylogenetic groups pairwise comparisons were made between pre-transplant and 12-month time points (Fig. 3). There were a similar number of variations in the toptaxa noted in the SCS and NMP cohorts (9 and 10, respectively). Noteworthy are the taxa that exhibited remarkable changes between time points. The *Anaerostipes* genus decreased in both cohorts. The *Bacteroides* genus increased in the SCS cohort. And both *Lelliota* and *Faecalibacterium* genera decreased in the SCS cohort. The NMP cohort had several genera increase in abundance, including *Ruminococcus*, *Blautia*, *Lactobacillus*, *Oscillospira*, *Coprococcus*, *Tyzzereella*, *Oscillibacter*, and *Faecalitalea*. These data show changes in several key taxa comprising a healthy gut microbiome, which is important because these taxa can generate key biologically important metabolites (e.g., SCFAs). There were also changes in several taxa associated with inflammation or infection following liver transplant.

Correlation of gut microbiota and stool and breath organic acids

The gut microbiota can produce a variety of metabolites with a wide range of bioactivities. SCFAs are deemed beneficial metabolites with many biological roles, such as regulation of gut microbiota composition, immune function, gut barrier integrity, inflammation, and energy homeostasis.¹⁸ VOCs are released within the gut as metabolites, absorbed, and distributed among tissues and organs, ultimately reaching the lungs, where they can be released in exhaled breath.¹⁹ Formed and emitted as a result of normal and abnormal biological processes, some VOCs have been used as biomark-

ers of health conditions or pathologies,¹⁹ and several breath VOCs have been correlated with gut microbiota.²⁰ Since alterations in several bacterial taxa associated with a healthy gut microbiome and the production of beneficial metabolites were found following liver transplant, Spearman's rank correlation coefficient was employed to test for correlations between the mean relative abundances of genus-like phylogenetic groups and SCFA levels in stool and volatile organic acids in the breath. Samples at the six-month time point were analyzed due to increased alpha and beta diversity between cohorts at this time. Pooled samples showed multiple genera significantly correlated with several fecal SCFAs and breath metabolites (Fig. 4). Positive correlations were found between stool lactic acid and *Intestinibacter* and *Tyzzereella*, and between stool butyric acid and *Ruminococcus*. A negative correlation was observed between stool succinate acid and *Parabacteroides* genus. Breath hydrogen sulfide positively correlated with *Bacteroides* genus but negatively correlated with *Lactobacillus*, *Anaerotruncus*, *Sellimonas*, and *Ruminiclostridium* genera. *Coprobacillus* genus positively correlated with several breath metabolites, including triethylamine, trimethylamine, acetonitrile, ethane, acetaldehyde, 2-propanol, and 1-heptane. Acrylonitrile negatively correlated with several taxa (*Intestinibacter*, *Finegoldia*, *Prevotella*, *Parabacteroides*). Triethylamine positively correlated with *Roseburia* and *Coprobacillus* and negatively correlated with *Intestinibacter* and *Finegoldia*.

Correlation of factors that impact gut microbiome

Medications and nutritional status are known factors that impact the gut microbiome. Since liver transplant patients are often malnourished and receive multiple medications, we

Table 2. Liver function tests

Lab indices	N	Overall, N = 41 ^a	SCS, N = 31 ^a	NMP, N = 10 ^a	p-value ^b
Pre-transplant	41				
Bilirubin, total		3.1 (1.8, 5.4)	3.4 (1.6, 6.6)	2.7 (1.9, 4.0)	0.4
AST		52 (35, 91)	62 (44, 104)	38 (30, 47)	0.035
ALT		34 (24, 55)	38 (26, 54)	26 (23, 54)	0.3
ALP		159 (104, 244)	173 (108, 236)	144 (108, 328)	>0.9
GGT		-	-	-	
3 Mo. follow up	39				
Bilirubin, total		0.40 (0.30, 0.50)	0.40 (0.30, 0.50)	0.40 (0.30, 0.40)	0.2
AST		19 (14, 28)	23 (14, 31)	16 (12, 20)	0.060
ALT		17 (12, 24)	18 (16, 25)	12 (11, 15)	0.048
ALP		87 (70, 143)	87 (69, 148)	90 (71, 118)	0.8
GGT	38	45 (24, 83)	52 (32, 84)	24 (20, 61)	0.13
6 Mo. follow up	38				
Bilirubin, total		0.40 (0.30, 0.50)	0.50 (0.30, 0.60)	0.40 (0.30, 0.40)	0.10
AST		22 (16, 32)	22 (16, 31)	21 (14, 30)	0.5
ALT		21 (12, 33)	22 (13, 30)	17 (11, 33)	0.5
ALP		102 (76, 130)	99 (74, 136)	109 (91, 120)	0.9
GGT	37	36 (20, 65)	36 (20, 67)	34 (20, 52)	>0.9
12 Mo. follow up	37				
Bilirubin, total		0.50 (0.30, 0.70)	0.50 (0.40, 0.83)	0.30 (0.30, 0.40)	0.020
AST		21 (18, 33)	22 (18, 36)	20 (18, 24)	0.3
ALT		24 (15, 35)	24 (15, 33)	23 (13, 35)	0.6
ALP		99 (69, 153)	104 (70, 139)	85 (68, 288)	>0.9
GGT	32	36 (21, 85)	42 (25, 85)	24 (18, 50)	0.3

^aMedian (IQR); n (%). ^bWilcoxon rank-sum test. SCS, static cold storage; NMP, normothermic machine perfusion; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

tested for the impact of malnutrition diagnosis and the receipt of antibiotics and gastric acid-modulating medications on the relative abundance of genus-like phylogenetic cohorts. Pooling patient samples for both cohorts and all time points, we found that all these factors influenced select taxa abundance, particularly *Lelliottia* and *Bacteroides* genera (Fig. 5). Specifically, antibiotics and PPIs or H₂ antagonists were associated with a decreased relative abundance of *Lelliottia* and *Bacteroides* genera (Fig. 5A, B). A more severe malnutrition diagnosis (severe, moderate) was associated with a higher relative abundance of *Escherichia/Shigella* and *Enterococcus*, while *Lelliottia* and *Bacteroides* genera responded variably (Fig. 5C).

Discussion

Here, we show that patients with ESLD experience longitudinal changes in their fecal microbiota diversity, composition, and function during the perioperative liver transplant period. Importantly, we show for the first time that fecal microbiota responses post-liver transplant differ between the liver graft preservation methods utilized.

Normothermic machine perfusion optimizes liver graft availability and integrity, and it is safe and superior as a means to preserve marginal grafts compared to SCS.¹ The

unique ability to maintain the graft in a metabolically active state during the preservation period allows for the assessment of its viability.²¹ Despite these benefits, patients are still at risk for post-transplant complications such as graft rejection and infections, which require prophylactic treatment with immunosuppressants and antibiotics that can alter the gut microbiome. To ensure a successful transplant, liver graft integrity is carefully considered, and generally, all liver transplant patients follow the same treatment protocols. However, the role of the gut microbiome in the context of liver transplantation perioperative care seems to be overlooked as a potential confounding factor.

The clinical importance of understanding how the gut microbiome responds post-liver transplant lies in the rationale that persistent ESLD-associated gut dysbiosis could increase the likelihood of the liver graft being exposed to undesirable gut-derived byproducts through the gut-liver axis, which could compromise graft integrity and the patient's post-transplant course.

Alpha diversity is the most common indicator for assessing the health of the gut microbiota, and it is closely associated with the status of many diseases.²² Our pooled diversity data corroborates prior studies showing a decreased alpha diversity pre-transplant compared to healthy controls.⁷⁻⁹ However, here we show a variable alpha diversity response

Table 3. Medications

Medication type	N	Overall, N = 41 ^a	SCS, N = 31 ^a	NMP, N = 10 ^a	p-value ^b
Pre-transplant	40				
PPI/H2-blocker		30 (75%)	20 (67%)	10 (100%)	0.043
Antibiotics		28 (70%)	22 (73%)	6 (60%)	0.5
Steroids		4 (10%)	3 (10%)	1 (10%)	>0.9
Immunosuppression		1 (2.5%)	1 (3.3%)	0 (0%)	>0.9
3 Mo. follow up	39				
PPI/H2-blocker		27 (69%)	20 (69%)	7 (70%)	>0.9
Antibiotics		37 (95%)	28 (97%)	9 (90%)	0.5
Steroids		13 (33%)	9 (31%)	4 (40%)	0.7
Immunosuppression		39 (100%)	29 (100%)	10 (100%)	>0.9
6 Mo. follow up	38				
PPI/H2-blocker		23 (61%)	16 (57%)	7 (70%)	0.7
Antibiotics		37 (97%)	28 (100%)	9 (90%)	0.3
Steroids		11 (29%)	9 (32%)	2 (20%)	0.7
Immunosuppression		38 (100%)	28 (100%)	10 (100%)	>0.9
12 Mo. follow up	36				
PPI/H2-blocker		15 (42%)	11 (41%)	4 (44%)	>0.9
Antibiotics		35 (97%)	26 (96%)	9 (100%)	>0.9
Steroids		10 (28%)	6 (22%)	4 (44%)	0.2
Immunosuppression		34 (94%)	25 (93%)	9 (100%)	>0.9

^an (%), ^bFisher's exact test. SCS, static cold storage; NMP, normothermic machine perfusion; PPI, proton pump inhibitor.

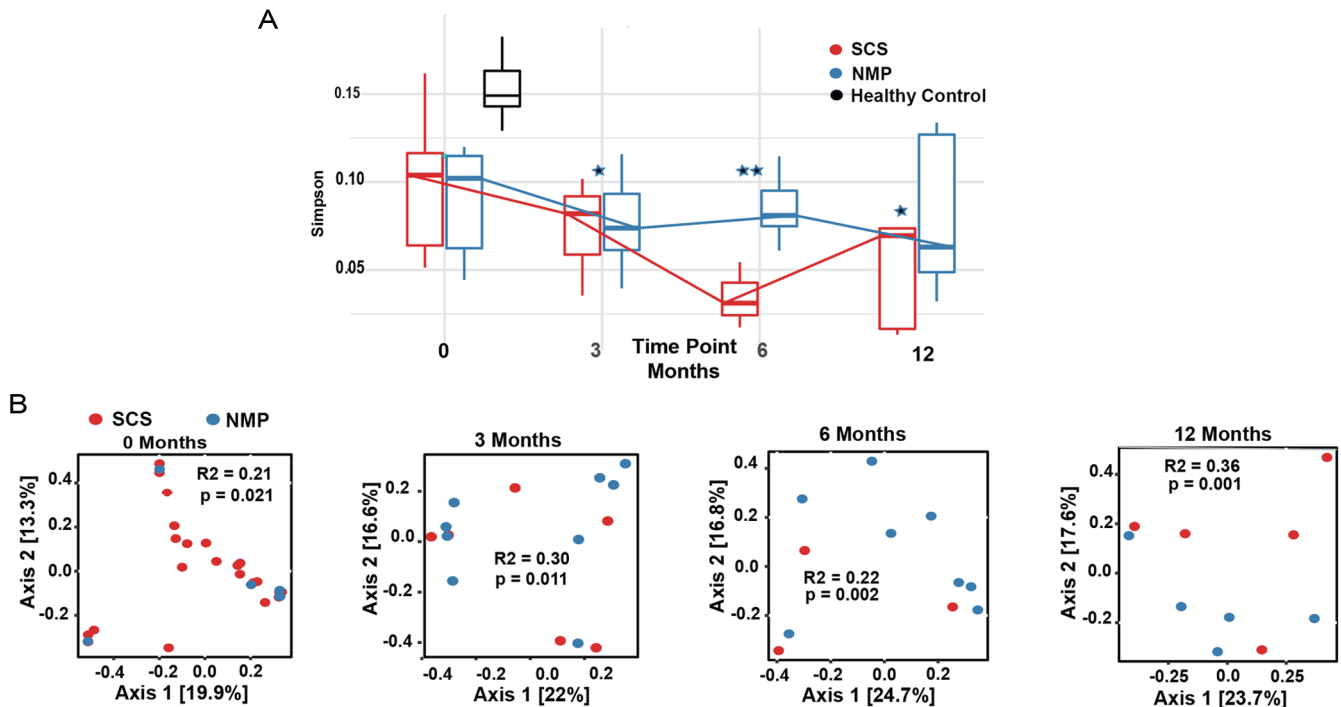


Fig. 1. Gut microbiota diversity. Alpha- and beta- diversity plots visualize the longitudinal changes in the microbial community structure of fecal samples in liver transplant recipients at pre-transplant, three, six, and twelve months post-transplant time points. (A) Box plots demonstrating alpha diversity (Shannon index) values. Box plots show the median, lower, and upper quartiles and are color-coded by SCS, NMP, and healthy control cohorts. (B) PCA depicting beta diversity patterns in SCS vs. NMP cohorts. *p < 0.05; **p < 0.01 (PERMANOVA with Benjamini-Hochberg FDR). PCA, Principal component analysis; SCS, static cold storage; NMP, normothermic machine perfusion; FDR, false discovery rate.

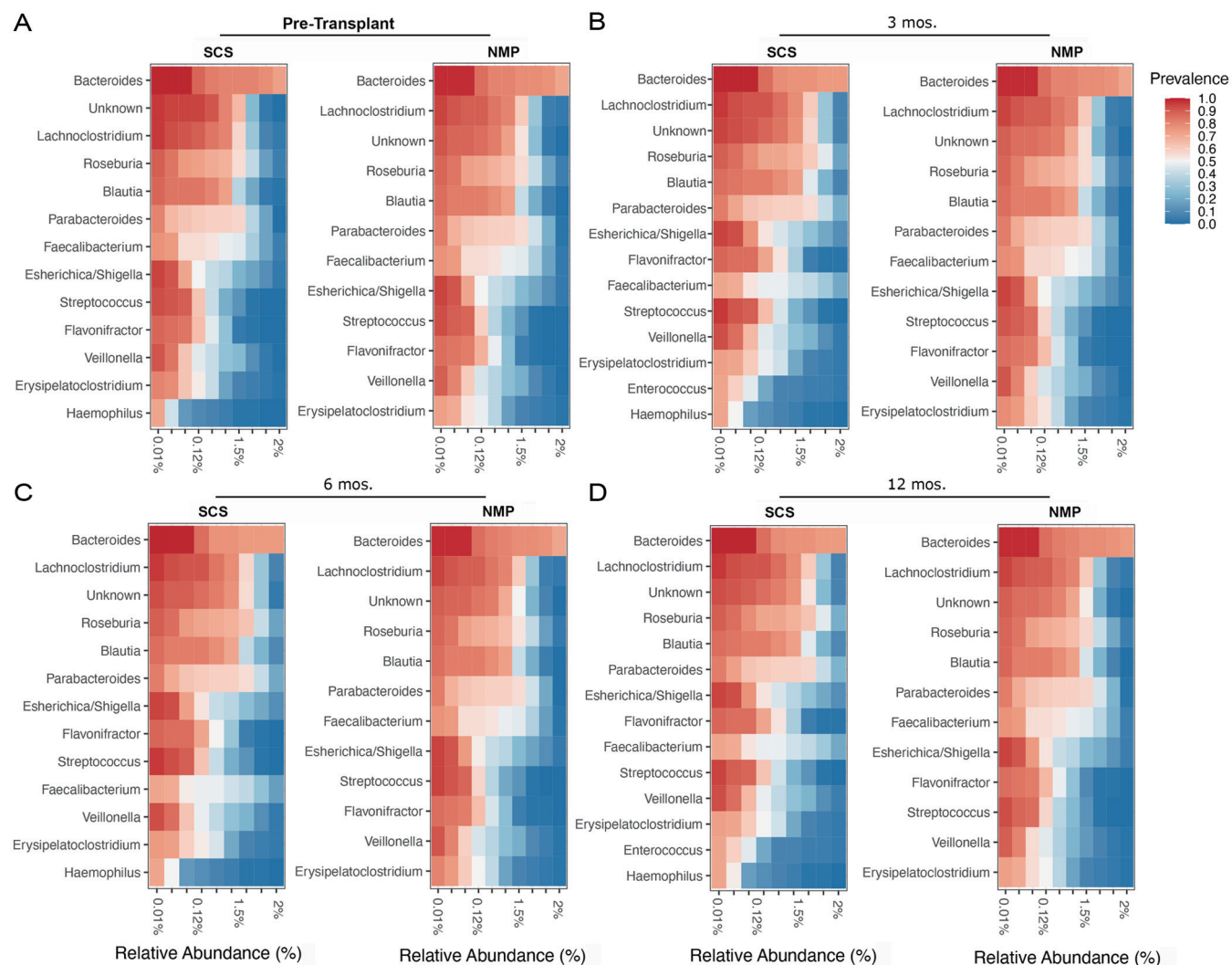


Fig. 2. Longitudinal core microbiome analysis. Longitudinal core microbiome analysis reveals significant taxonomic alterations in liver transplant recipients at (A) pre-transplant, and (B) three, (C) six, and (D) twelve months post-transplant. Heatmaps represent the longitudinal core microbiome of SCS and NMP cohorts at the genus level as a function of relative abundance. The x-axis represents detection thresholds (relative abundance), ranging from lower (left) to higher (right) values. Color shading indicates the prevalence of each bacterial genus among samples for each abundance threshold. SCS, static cold storage; NMP, normothermic machine perfusion.

post-transplant based on the liver preservation method utilized. Specifically, alpha diversity was lower at six months post-transplant in the SCS group, but by 12 months, it more closely resembled that of the NMP group. A higher diversity indicates a more stable gut microbiota, and these findings suggest that the gut microbiota of patients with SCS graft preservation was less rich and more dissimilar from that of patients receiving NMP at six months post-op, which could have implications for the function of their gut microbiota ecosystem and immunity.

Gut microbiota structure and function disturbances create vulnerability to infection by an overabundance of opportunistic enteric pathogens, which compete with commensals for space and nutrients. Analyzing characteristics and alterations in gut microbiota community structure at the genus level, we found changes in both beneficial and potentially harmful microbiota. Focusing on genera changes between pre- and 12 months post-transplant, several beneficial microbes decreased in the SCS cohort, including *Anaerostipes*,

Lactobacillus, and *Faecalibacterium*. These commensal gut microbes are associated with producing beneficial metabolites such as SCFA (e.g., butyrate).²³ Butyrate, known to support gut barrier integrity and the immune system,²⁴ is depleted with chronic alcohol exposure in animal and human studies.^{24,25} Targeting depleted butyrate levels with a butyrate prodrug (tributyryn) or a butyrate-targeting synbiotic protects the gut-liver axis by rescuing gut barrier integrity and liver injury induced by ethanol exposure in mouse models.^{24–26} Taken together, the depletion of beneficial bacteria and their metabolites suggests liver transplant patients may be at increased risk for compromised gut immune and intestinal barrier function perioperatively, which could impact the liver graft. Further investigation into restoring the gut microbiota and/or beneficial metabolites (e.g., SCFA) post-liver transplant is warranted.

Conversely, there was an increase in a few potentially harmful microbiota in the SCS cohort, including genera *Bacteroides* and *Lelliottia*. Although the number of taxa that un-

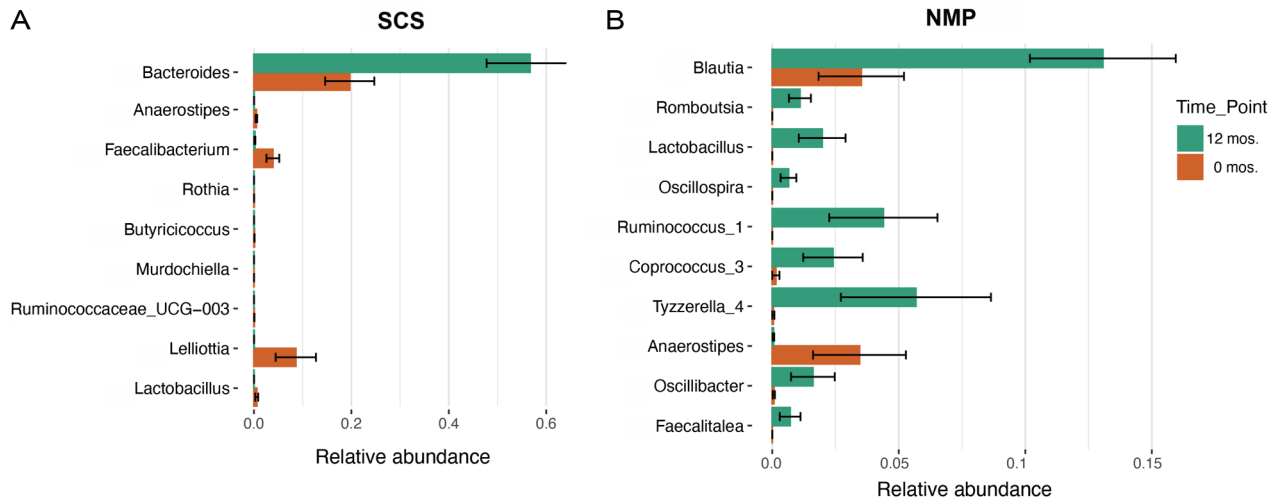


Fig. 3. Differential abundance of top taxa. Differential abundance analysis for (A) SCS and (B) NMP cohorts reveals statistically significant taxa between pre-transplant and 12-month time points. (White's nonparametric t-test with Benjamini-Hochberg FDR multiple test correction, adjusted $p \leq 0.05$). SCS, static cold storage; NMP, normothermic machine perfusion; FDR, false discovery rate.

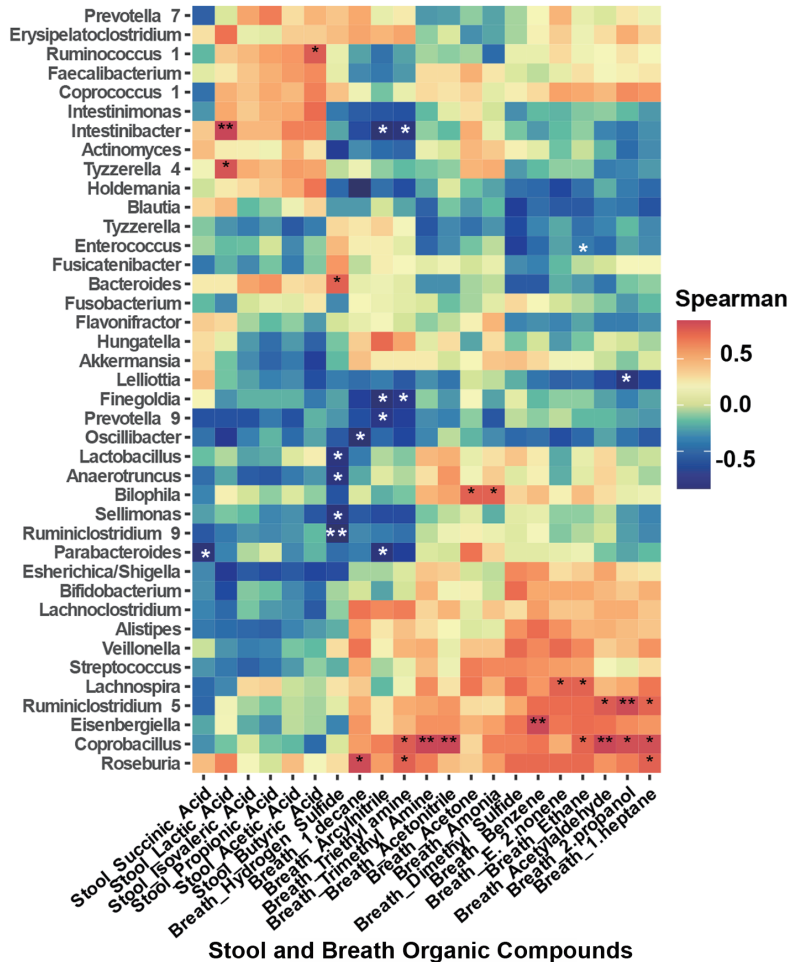


Fig. 4. Gut microbiota and fecal and breath organic compounds. All SCS and NMP samples across all time points for fecal and breath samples were pooled. A Spearman correlation heatmap displays the relationships between fecal and breath metabolites and fecal microbiota at the genus level. Red squares represent positive correlations. Blue squares represent negative correlations. White squares represent no correlation. * $p < 0.05$, ** $p < 0.01$. SCS, static cold storage; NMP, normothermic machine perfusion.

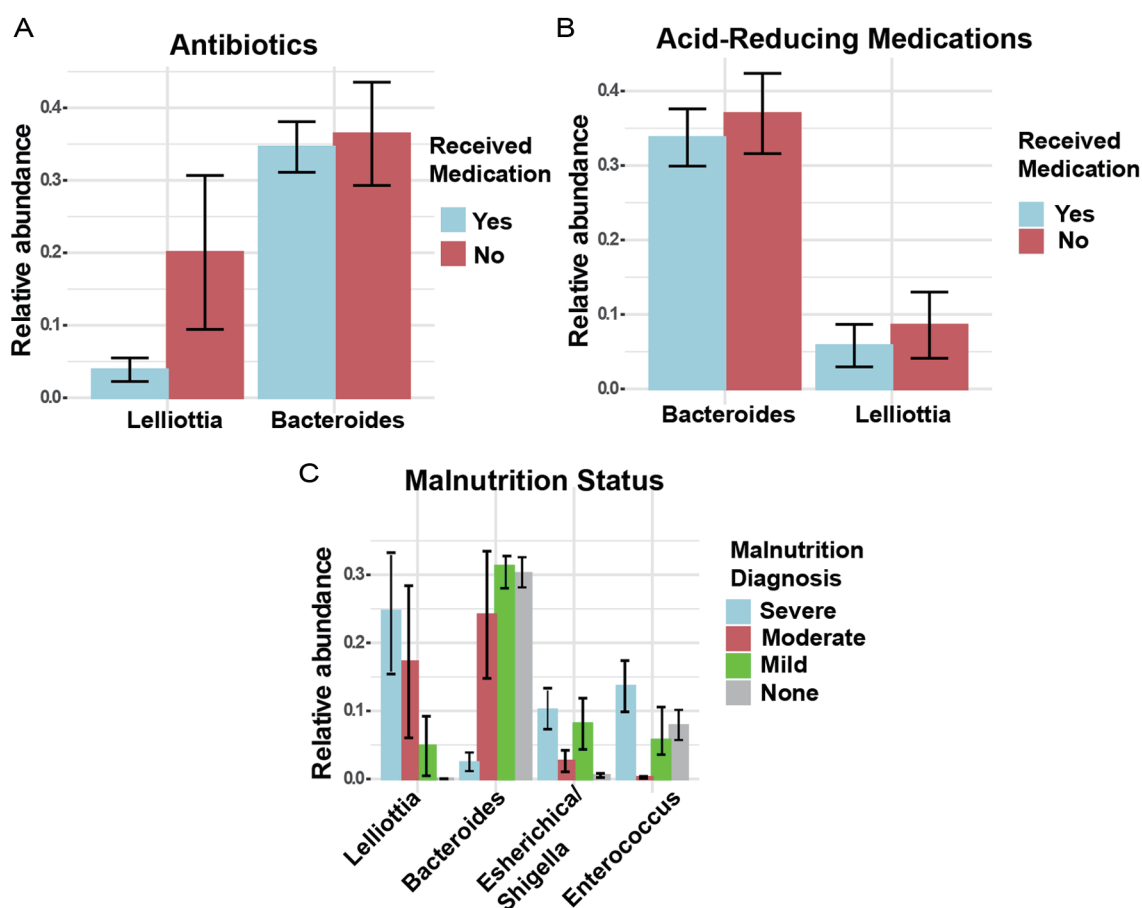


Fig. 5. Correlation of fecal microbiota with factors known to disturb gut microbiota. All patient samples across all time points were pooled. DAA of ASVs (genus-level) was performed: (A) Comparing patients receiving antibiotics (yes) versus those not receiving antibiotics (no); (B) Comparing patients receiving a PPI or histamine 2-antagonist (yes) versus those not receiving these medications (no); and (C) Assessing patients with varying levels of malnutrition (none, mild, moderate, or severe) at the pre-transplant time point. ASV tables were rarefied to the sample with the lowest number of sequences in each analysis. ASVs were assigned at the genus level, and genera with a relative abundance of more than 0.3% of the total were included in the DAA analysis. FDR estimates were calculated for multiple comparisons, with a significance threshold of $p < 0.05$. ASVs, amplicon sequence variants; DAA, differential abundance analysis; FDR, false discovery rate; PPI, proton pump inhibitor.

derwent major abundance changes was equivalent between the SCS and NMP cohorts, the NMP cohort demonstrated enrichment in beneficial bacteria such as *Blautia*, *Roboutsia*, *Lactobacillus*, and *Coprococcus*. This may be due to the NMP liver preservation method resulting in more favorable feedback of bile acids and bioactive mediators to the gut. Due to gut-liver crosstalk, an increase in potentially harmful microbes in the SCS cohort may contribute to the worsened liver function tests (ALT, AST) in the SCS group at the three-month time point. *Bacteroides* species and other gut commensal bacteria (e.g., *Enterococcus faecalis*) can be responsible for infections that have significant morbidity and mortality, such as intra-abdominal sepsis.^{27,28} Interestingly, our data also show that the use of medications that impact the gut microbiome, including antibiotics and acid-reducing agents, was related to the relative abundance of *Bacteroides* and *Lelliottia*. Moreover, malnutrition, sarcopenia, and frailty are prevalent complications in patients with ESLD and are associated with increased risk of morbidity and mortality.²⁹ We found that pre-transplant gut microbiota in more severely malnourished patients were enriched in *Escherichia/Shigella* and *Enterococcus*. Vancomycin-resistant enterococci colonization is common in liver transplant patients and associated with worse post-transplant outcomes.³⁰ Recently, Zhao *et al.*

identified *Enterococcus faecium* and *Escherichia coli* among the top four bacterial infections in post-liver transplant patients.³⁰ Based on the data shown here, it would be relevant to test whether these bacteria translocated from the gut.

Discriminatory metabolites from breath samples (VOCs) have been tested in many diseases as a non-invasive approach for disease detection,³¹ including liver diseases.³² Hanouneh *et al.* reported six VOCs (2-propanol, acetaldehyde, acetone, ethanol, pentane, and trimethylamine) were elevated in patients with liver disease compared to healthy controls.¹⁷ Fernandez del Rio *et al.* identified seven VOCs that were elevated in patients with cirrhosis compared to healthy controls, five of which significantly decreased post-transplant (limonene, methanol, 2-pentanone, 2-butanone, and carbon disulfide).³³ Here, we found multiple VOCs, including those previously identified with liver diseases, to correlate with several gut microbiota genera. For example, *Coprobacillus* genera positively correlated with breath triethylamine, trimethylamine, acetonitrile, ethane, acetaldehyde, 2-propanol, and 1-heptane. Recently, *Coprobacillus cateniformis*, a gram-positive and non-sporulating bacterium, caused bacteremia in an immunocompromised patient,³⁴ and its fecal levels decreased in patients with irritable bowel syndrome following fecal microbiota transplant, which correlated with

improved irritable bowel syndrome symptoms and fatigue.³⁵ Taken together, these findings warrant further investigation into whether breath VOCs could be used as a non-invasive biomarker for post-liver transplant microbiome disturbances and/or clinical course. To proceed, randomized studies with large patient cohorts, correcting for possible confounding factors and performing internal or external validation, are needed to confirm if VOCs can be reliably tested and quantified, used to detect changes in the perioperative period, identify even small changes or early signs of changes in liver graft integrity, or provide valuable insights into the diagnosis, prognosis, or treatment following liver transplant.

Conclusions

In summary, the current study is the first in the world to increase our knowledge of how the liver graft preservation method impacts fecal microbiota in the perioperative period of liver transplant patients. A highlight of this study, comparing SCS and NMP, is the 12-month follow-up to characterize how the gut microbiome responds longitudinally and correlates with fecal and breath organic compounds. A limitation of this study is that it is proof-of-concept with small group sizes of non-randomized cohorts, which limits statistical power. Additionally, many confounding variables limit the generalizability of these findings, including the lack of detailed information on diet, comorbidities, medications, and the fact that the majority of the study participants were White. Moreover, we were unable to categorize donor or recipient characteristics in the analysis due to limited case numbers, resulting in heterogeneity in donor and recipient distribution. However, our observation that fecal microbiota was impacted by the liver preservation method is novel and can help the transplantation community understand the benefits of NMP, which is increasingly used worldwide. Future research in larger randomized cohorts is warranted to determine whether the NMP-induced shift in gut microbiota composition and function impacts clinical outcomes and if breath VOCs could serve as biomarkers for the clinical course in liver transplant patients.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (GAMC, QL, CQ, CM, KH), acquisition of data (DS, KA, QL, BC, LDP, CQ), analysis and interpretation of data (NS, DL, GAMC, QL, QC), drafting of the manuscript (GAMC), critical revision of the manuscript for important intellectual content (QL, CQ), and study supervision (GAMC, QL, CQ). All authors have made significant contributions to this study and have approved the final manuscript.

Ethical statement

All research was conducted in accordance with the Declarations of Helsinki and Istanbul, and all procedures were approved by the Cleveland Clinic Institutional Review Board:

SCS: IRB# 18-119 – “Biorepository of liver tissue and blood sample after liver transplantation” and NMP: IRB# 15-549 – “A Phase 1 Pilot Study to Assess Safety and Feasibility of Normothermic Machine Preservation in Human Liver Transplantation”. Written consent was obtained from all subjects.

Data sharing statement

Data supporting the reported results can be requested by writing to the corresponding author.

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Cresci G.A.M. *et al*: Liver preservation method impact on the gut microbiome

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