Analysis of risk factors via microbiome from living donor and recipient in living donor liver transplantation

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ABSTRACT

Background

Acute rejection (AR) after liver transplantation (LT) significantly increases the risk of graft failure and mortality. Despite the importance of the gut-liver axis, there was limited research on the link between acute rejection and the gut microbiome in liver transplantation. This study investigates the predictive value of donor and recipient microbiomes for AR in living donor liver transplantation (LDLT).

Methods

This study was a prospective study based on stool samples collected from donors before surgery and recipients pre- and post-LDLT, including twenty-four paired stool samples. Based on biopsy-proven acute rejection (BPAR), the identification and abundance of gut microbes, and prediction of gene families were analyzed using full-length 16s rRNA gene sequencing.

Results

Gut microbiome changes before and after LT were associated with a decrease in alpha diversity and an increase in specific taxa like *Clostridium innocuum* and *Streptococcus salivarius* post-transplant. BPAR occurred in 5 out of 24 patients (20.8%) at a median of 46 days (ranging from 7 to 194 days) after LT in our cohort. Preoperative microbiome analysis showed specific taxa associated with AR risk, including *Massilioclostridium* at a level of ≥ 0.0049% in donors and *Veillonellaceae* at a level of ≥ 0.0051% in recipients. Post-transplant analyses revealed that certain microbiota, such as *Enterococcus faecium*, were more abundant in recipients without AR and correlated with a lower incidence of AR. The interaction between the gut and liver was examined through functional pathway prediction with gut-derived compounds such as PWY-6906 (including the O-linked N-acetylglucosamine pathway) and TEICHOICACID-PWY (including the IL-

12 secretion from macrophages pathway).

Conclusion

The study highlights the potential of microbiome profiling in predicting AR risks in LDLT patients and suggests that tailored immunosuppressive strategies could be developed for high risk groups. Additionally, it is expected that certain gut microbiome post LT can create an environment that reduces the incidence of rejection. These findings underscore the importance of personalized treatment for recipients in liver transplantation.

Key words: Microbiome, Liver transplantation, Acute rejection, personalized treatment, *Massilioclostridium, Veillonellaceae, Enterococcus faecium*

1. Introduction

1.1. Acute rejection

Acute rejection (AR) post-liver transplantation (LT) is an immune response against a foreign organ. Data from A2ALL and SRTR indicate that the incidence of acute rejection ranges from 15.6% to 26.9%, significantly raising the risk of graft failure and mortality, with hazard ratios (HR) of 6.79 and 8.81, respectively (Levitsky et al. 2017). This study identified the following risk factors for acute rejection: non-related donor, older recipient age, Black and Asian race, hepatitis C virus, primary sclerosing cholangitis, and primary biliary cholangitis.

A recent review (Choudhary et al. 2017) lists additional risk factors for AR, including autoimmune liver disease etiology prior to LT, cytomegalovirus infection, non-compliance or low levels of immunosuppression, positive lymphocyte cross-match, older recipient age, ethnic origin, male donor to female recipient, higher donor age, increased cold ischemia time, and living versus deceased donor liver transplantation.

Despite these findings, there is limited research on the link between acute rejection and the microbiome in liver transplantation.

1.2. Microbiome

The human gastrointestinal tract hosts a complex community of microorganisms known as the gut microbiome. Due to its anatomical proximity, the liver has a bidirectional relationship with the intestine and its microbiota, known as the 'gut-liver axis,' characterized by circular causality (Wirth et al. 2023). The liver acts as a primary defense against antigens and toxins from the gut. End-stage liver disease and liver transplantation are often associated with changes in the gut microbiome, influenced by antibiotic therapy, surgical interventions, anatomical alterations from surgery, biliary complications, and immunosuppressive treatments (Kriss et al. 2019). Studies have shown that liver transplantation can improve gut microbiota diversity in patients with end-stage liver diseases by increasing beneficial bacteria and reducing pathogenic gram-negative bacteria (Bajaj et al. 2018), even under immunosuppressive therapy (Ling et al. 2016). The gut-liver axis describes an interaction between the liver and intestine via multiple communication pathways such as portal vein and biliary tract. The liver plays a critical role in neutralizing harmful substances like bacterial toxins produced by the gut microbiota (Giannelli et al. 2014).

1.3. Relation between liver transplantation and Microbiome

The influence of the gut microbiome on liver transplantation can be viewed in three phases: pre-transplant, peri-transplant, and post-transplant. Before surgery, high portal pressure can compromise intestinal wall integrity, reduce the conversion to secondary fecal bile acids, and increase the risk of small intestinal bacterial overgrowth. Gut dysbiosis, favoring pathogenic bacteria, is also a concern. During the peri-transplant period, immunosuppressive agents raise the infection risk. Additionally, hypotension and reperfusion injury weaknes the immunity system, and medication and ischemia may lead to loss of gut mucosal barrier. Post-surgery, following factors influence the gut microbiome: increased microbial diversity, a reduction in pathogenic taxa, decreased endotoxemia, increased secondary fecal bile acids, elevated urinary TMA-N-oxide levels, and decreased short-chain fatty acids (Duong, Bajaj 2021).

Recent reviews have examined the gut microbiome as one of the prognostic factors after LT, with evidence linking the microbiome to infections, obesity, diabetes mellitus, cardiovascular diseases, acute kidney injury, and cancer following LT. However, there is limited study to ascetain its relationship with acute rejection after LT (Doycheva, Leise, Watt 2016).

1.4. Living donor liver transplantation

LT is the definitive treatment for end-stage liver disease and hepatocellular carcinoma. Despite its treatment outcomes, in some countries, only few numbers of patients can receive LT due to a shortage of deceased donors. In such cases, living donor liver transplantation (LDLT) is considered a viable solution. LDLT involves unique considerations, including donor safety (Lee et al. 2017) and the genetic similarities between the donor and the recipient. In general, the donor's microbiome is expected to maintain a different, healthier liver-gut axis compared to the recipient’s. However, research on how the donor's microbiome affects the recipient is scarce.

1.5. The aim of study

This study aimed to determine whether the microbiomes of donors and recipients in LDLT can predict acute rejection. The research focused on three main questions:

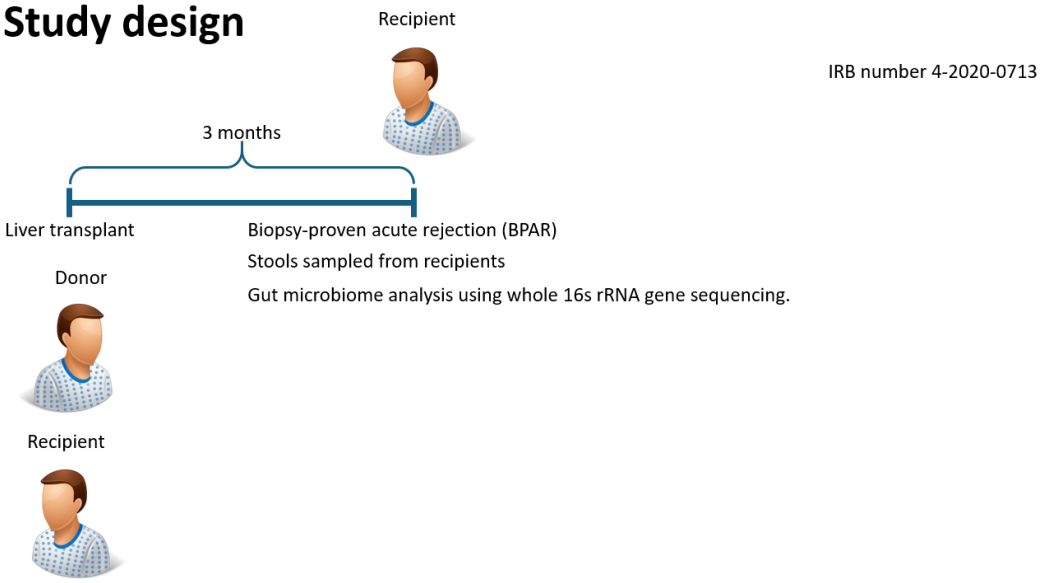
1) Can the preoperative donor microbiome predict acute rejection after LDLT?
2) Can the preoperative recipient microbiome predict acute rejection after LDLT?
3) Can a specific microbiome in recipients after LDLT reduce the incidence of rejection?

2. Methods

2.1. Study design

This study was a prospective, matched study. Stool samples were collected from donors before surgery to investigate whether the preoperative microbiome of donors influences recipient outcomes after liver transplantation. Similarly, stool samples were collected from recipients before surgery to examine whether their preoperative microbiome affects post-transplant outcomes. Additionally, to understand the microbiome changes post-liver transplantation, stool samples were collected from recipients three months after surgery. The study design and flow are illustrated in Figure 1..

Figure 1. Study design.



2.2. Patients

From July 2021 to August 2023, informed consents were obtained from individuals who underwent LDLT at Severance Hospital, Seoul, Korea. Stool samples were collected from 24 participants: once from donors before surgery; total two times from recipients before surgery and three-months after surgery.

2.3. Institutional Review Board

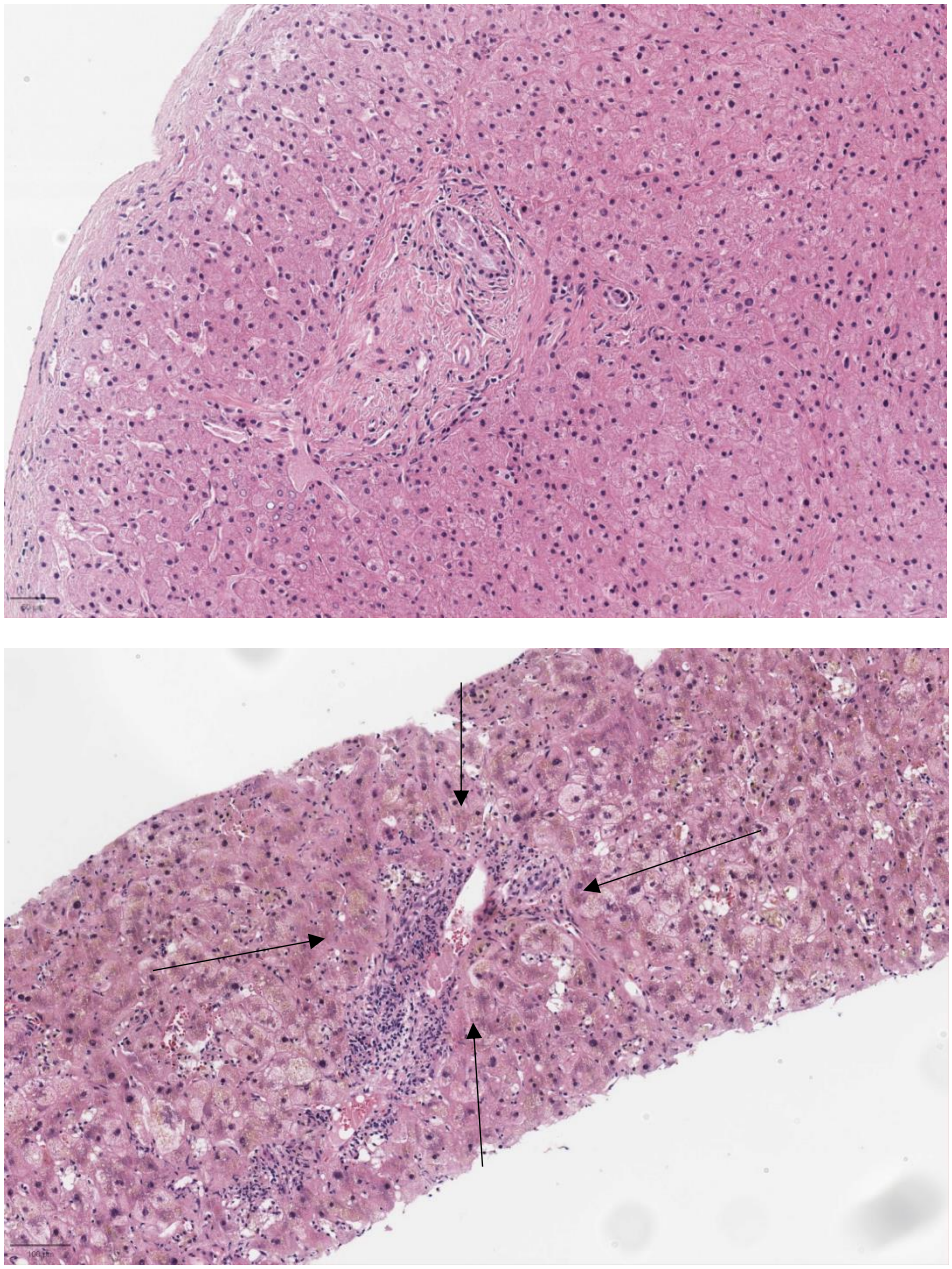
The study was conducted following the Declaration of Helsinki to minimize potential risks to donors and recipients. The institutional review board (IRB) of Yonsei University Health System reviewed the study design. Participants provided informed consent, were informed that their participation was voluntary, and could withdraw at any time. Samples and information on post-transplant rejection were collected from participants (IRB number 4-2020-0713)

2.4. Biopsy proven acute rejection

According to standard post-liver transplantation treatment guidelines, acute rejection is suspected if levels of AST, ALT, total bilirubin, and gamma-GT rise, prompting a percutaneous or transjugular liver biopsy. A pathologist diagnoses biopsy proven acute rejection (BPAR) based on the Banff Working Group on liver allograft pathology (Demetris et al. 2016). Differences in the rejection activity index are observed in slides of healthy donors and recipients with acute rejection, based on the extent of portal inflammation, bile duct inflammation and damage, and venous endothelial inflammation (Figure 2).

Figure 2. Healthy liver donor (up) and acute cellular rejection (down).

In contrast to healthy liver donors, acute rejection in recipients after liver transplantation was characterized by the infiltration of lymphocytes, primarily T cells, in the portal tracts. (Indicated by black arrows)



2.5. DNA Extraction and Quantification

DNA extraction from stool samples of donors and recipients before liver transplantation, and recipients three months after transplantation, was performed using the DNeasyPowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was quantified using Quant-IT PicoGreen (Invitrogen).

2.6. Library construction and Sequencing

Sequencing libraries were prepared according to the PacBio amplicon Template Preparation and Sequencing protocols to amplify the 27F and 1492R regions. Input gDNA (2ng) was PCR amplified with 10× LA PCR Buffer II (Mg2+ free), 2.5mM of dNTP mix, 2.5mM MgCl2, 500nM each of the F/R PCR primer, and 5U of TaKaRa LA Taq (Takara, Kusatsu, Japan). PCR cycling conditions were 5 min at 94°C for heat activation, followed by 25 cycles of 30 sec at 94°C, 30 sec at 53°C, and 90 sec at 72°C, with a final extension of 5 min at 72°C. The primer pair with asymmetric barcoded adapters for amplification was: 27F-F: 5'-AGRGTTYGATYMTGGCTCAG -3', 1492-R: 5'- RGYTACCTTGTTACGACTT -3'. The PCR product was purified with SMRTbell cleanup beads, quantified using Quant-IT PicoGreen (Invitrogen), and qualified using TapeStation D5000 Screen Tape (Agilent Technologies, Waldbronn, Germany). For PacBio Sequel IIe sequencing, 500ng of pooled amplicon DNA was used for library preparation. A 10uL library was prepared using the PacBio SMRTbell prep kit 3.0. SMRTbell templates were annealed with the Sequel II Bind Kit 3.1 and Int Ctrl 3.1. The Sequel II Sequencing Kit 2.0 and SMRT cells 8M Tray were used for sequencing. SMRT cells (Pacific Biosciences) using 10hr movies were captured for each SMRT cell using the PacBio Sequel IIe (Pacific Biosciences) sequencing platform by Macrogen (Seoul, Korea). The subsequent steps followed the PacBio Sample Net-Shared Protocol, available at https://www.pacb.com/

2.7. Analysis of gut environment based upon 16s rRNA sequencing

Bacterial identities, abundances, and predicted functional pathways were analyzed using Quantitative Insights Into Microbial Ecology 2 (Qiime1.9) and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2 v2.3.0 beta (Douglas et al. 2020).

2.8. Statistical analyses

During data preprocessing, missing values were imputed using proximity matrix predictions from random forest (random Forest R package). For alpha diversity, 16s rRNA sequence reads were rarefied to standardize sequencing depth (GUniFrac R package). Alpha diversity in each group was measured by observed amplicon sequence variants (ASVs; vegan R package), Chao1 (vegan R package) for richness, and Shannon H (diversity(index = "shannon") of vegan R package), Simpson (diversity(index = “simpson”) of vegan R package), Inverse Simpson (diversity(index = "invsimpson") of vegan R package), evenness (Shannon / Log(observed ASVs)), and Fisher’s alpha (fisher.alpha of vegan R package) for evenness. Beta diversity analysis normalized sequence reads to relative abundance, visualized by non-metric dimensional scaling (NMDS) based on Bray-Curtis dissimilarity (metaMDS(distance = "bray") of vegan R package), and tested by analysis of similarity (ANOSIM) (anosim(distance = "bray", permutations = 9999) of vegan R package). Significant bacterial taxa in each group were used to generate circular cladograms with GraPhlAn, identifying signature bacterial lineages associated with clinical outcomes (Asnicar et al. 2015). Significant functional pathways inferred by PICRUSt2 were visualized with column charts of median fold changes. All variables were statistically tested by Student’s t-test (t.test(var.equal = TRUE) of stats R package), Welch’s t-test (t.test(var.equal = FALSE) of stats R package), Mann-Whitney U test (wilcox.test(exact=FALSE, correct=TRUE) of stats R package) for continuous data, and Chi-squared test (stats R package) for categorical data. For paired samples, the argument paired = TRUE was included. Classification and regression tree analysis (CART) used the rpart and rpart.plot R packages. Data analysis and visualization were conducted in R with a 5% significance level (R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Published online 2022).

3. Results

3.1. Baseline characteristics in liver transplant recipients

This study examined the baseline characteristics and clinical outcomes of patients undergoing LDLT, with a particular focus on the differences between those who experienced BPAR and those who did not. BPAR occurred in 5 out of 24 patients (20.8%) at a median of 46 days (ranging from 7 to 194 days) after LT in our cohort. The mean age of patients in the no BPAR group was 58.5 ± 7.7 years, while in the BPAR group, it was 58.8 ± 4.1 years, showing no significant difference (P = 1.000). The gender distribution also did not significantly differ, with males comprising 68.4% (n= 13) of the no BPAR group and 40.0% (n=2) of the BPAR group (P = 0.326). A notable finding was the significant difference in body mass index (BMI) between the groups. Patients in the no BPAR group had a mean BMI of 26.31 ± 3.7 kg/m^2, compared to 19.9 ± 3.5 kg/m^2 in the BPAR group, with a P value of 0.001, indicating a significant lower BMI in the BPAR group. The causes of liver transplantation, including chronic hepatitis B, alcoholic liver disease, non-B non-C, hepatocellular carcinoma, were not significantly different between the groups (P = 0.635). The MELD scores were similar between the no BPAR group (11.8 ± 6.9) and the BPAR group (11.0 ± 3.2) with a P value of 0.617. The distribution of CTP scores (A/B/C) also showed no significant differences (P = 0.477). Hypertension was present in 31.6% (n=6) of the no BPAR group and none in the BPAR group (P = 0.280). Diabetes mellitus prevalence was similar, with 31.6% (n=6) in the no BPAR group and 40.0% (n=2) in the BPAR group (P = 1.000). The mean age of donors was 38.3 ± 15.1 years in the no BPAR group and 41.0 ± 17.0 years in the BPAR group (P = 0.783). The donor gender distribution (male) was 63.2% (n=12) in the no BPAR group and 80.0% (n=4) in the BPAR group (P = 0.631). Donor BMI showed no significant difference between the groups (P = 0.120). Operative time averaged 555.2 ± 94.1 minutes for the no BPAR group and 532.0 ± 55.9 minutes for the BPAR group (P = 0.783). Warm ischemic time and cold ischemic time were also similar between groups (P = 0.836 and P = 0.731, respectively). Intraoperative transfusion of RBC packs did not differ significantly, with means of 4.6 ± 3.6 in the no BPAR group and 3.8 ± 3.7 in the BPAR group (P = 0.629). Postoperative ICU stay averaged 3.4 ± 1.6 days for the no BPAR group and 2.6 ± 0.9 days for the BPAR group (P = 0.297). The length of hospital stay post-surgery was 19.3 ± 5.5 days for the no BPAR group and 27.4 ± 23.3 days for the BPAR group (P = 0.945). The follow-up duration was shorter in the BPAR group (336.8 ± 95.4 days) compared to the no BPAR group (473.2 ± 215.4 days), nearing statistical significance (P = 0.063). The estimated GRWR was 1.10 ± 0.17 in the no BPAR group and 1.23 ± 0.24 in the BPAR group (P = 0.783). The measured GRWR was 1.19 ± 0.24 in the no BPAR group and 1.23 ± 0.22 in the BPAR group (P = 0.297).

There was no significant difference in the use of high doses of tacrolimus between the groups (P = 0.568), nor in the prevalence of high intrapatient variability (P = 0.549). However, positive

lymphocyte cross-matching was significantly higher in the BPAR group (40.0%, n=2) compared to the no BPAR group (0%, $P = 0.036$). The prevalence of ABO incompatibility was 36.9% (n=7) in the no BPAR group and 20.0% (n=1) in the BPAR group, showing no significant difference ($P = 0.631$). Detailed information is provided in Table 1

Table 1. Baseline characteristics in liver transplant recipients

Baseline characteristics	No BPAR (n=19)	Yes BPAR (n=5)	*P* value
Age, years	58.5 ± 7.7	58.8 ± 4.1	1.000
Gender, male	13 (68.4)	2 (40.0)	0.326
BMI	26.31 ± 3.7	19.9 ± 3.5	0.001
Causes of LT HBV Alcoholic NBNC HCC	5 (26.3) 8 (42.1) 3 (15.8) 8 (42.1)	0 (0) 2 (40.0) 1 (20.0) 2 (40.0)	0.635
MELD score	11.8 ± 6.9	11.0 ± 3.2	0.617
CTP score, A/B/C	12 (63.2)/ 6 (31.6) / 1 (5.3)	2 (40.0) / 3 (60.0) /0 (0)	0.477
Hypertension	6 (31.6)	0 (0)	0.280
Diabetes	6 (31.6)	2 (40.0)	1.000
Donor Age	38.3 ± 15.1	41.0 ± 17.0	0.783
Donor Gender, male	12 (63.2)	4 (80.0)	0.631
Donor BMI	24.5 ± 3.0	24.5 ± 2.9	0.120
Operation time, min	555.2 ± 94.1	532.0 ± 55.9	0.783
Warm ischemic time, mins	45.5 ± 13.0	46.4 ± 19.2	0.836
Cold ischemic time, mins	118.0 ± 25.8	121.2 ± 40.6	0.731
Intraoperative transfusion of RBC, packs	4.6± 3.6	3.8 ± 3.7	0.629
Postop ICU stay, days	3.4 ± 1.6	2.6 ± 0.9	0.297
Post hospital day, days	19.3 ± 5.5	27.4 ± 23.3	0.945
Follow up duration, days	473.2 ± 215.4	336.8 ± 95.4	0.063

GRWR, estimated	1.10 ± 0.17	1.23 ± 0.24	0.783
GRWR, measured	1.19 ± 0.24	1.23 ± 0.22	0.297
High dose of tacrolimus	4 (21.1)	2 (40.0)	0.568
High IPV	5 (26.3)	2 (40.0)	0.549
Lymphocyte cross matcha, positive	0 (0)	2 (40.0)	0.036
ABO incompatible	7 (36.9)	1 (20.0)	0.631

Values are expressed as n (%) or mean ± standard deviations.

Abbreviations: BMI, body mass index; LT, liver transplantation; HBV, hepatitis B virus; NBNC, non-hepatitis B virus and non-hepatitis C virus; HCC, hepatocellular carcinoma; MELD, Model for End-Stage Liver Disease; RBC, red blood cells; GRWR, graft to recipient weight

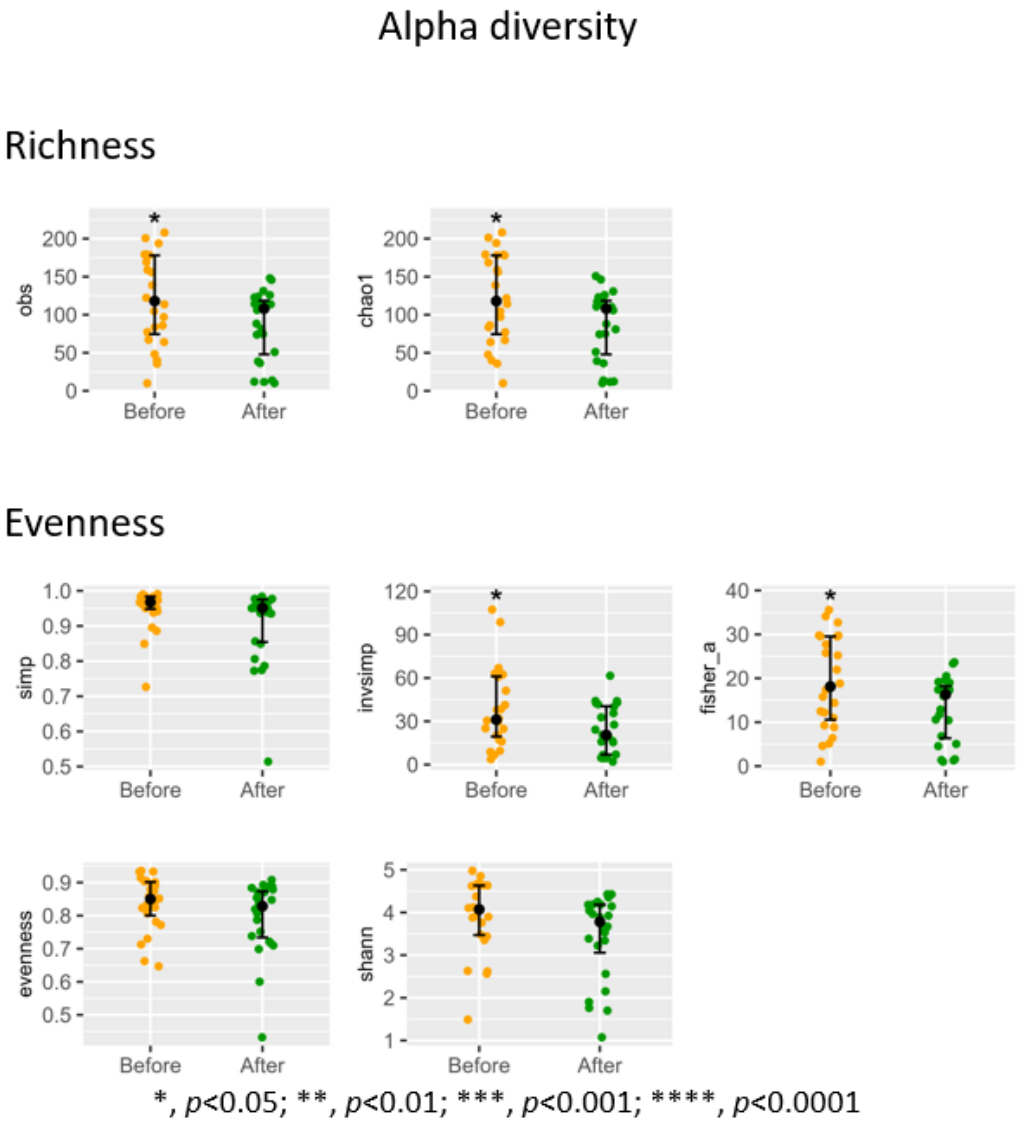
3.2. Gut microbial changes in recipients between before and after LT

3.2.1. alpha diversity and beta diversity

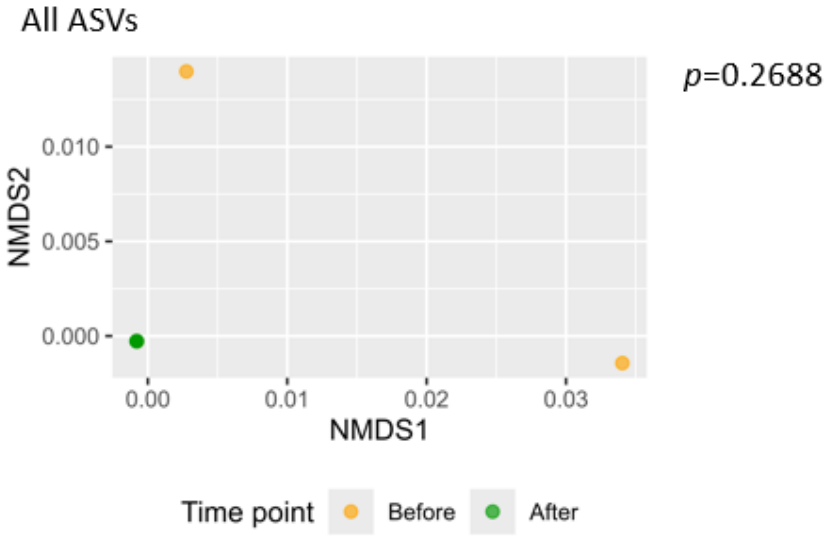
There are changes in the alpha diversity of the microbiome before and after liver transplantation, with decreases in richness and evenness. Observations and Chao alpha diversity indices show that richness decreases after liver transplantation compared to before ($P < 0.05$). In terms of evenness, indices such as invsimpson and fisher_alpha indicate an increase in alpha diversity ($P < 0.05$). However, indices like simpson, shannon, and evenness did not show statistical differences. For all ASVs, beta diversity showed no difference before and after surgery ($P = 0.268$; Figure 3).

Figure 3. Alpha diversity and beta diversity regarding gut microbial changes in recipients between before and after LT.

Observations and Chao alpha diversity indices reveal a significant decrease in richness following liver transplantation compared to pre-transplant levels ($P < 0.05$). Furthermore, measures of evenness, including invsimpson and fisher_alpha indices, demonstrate a notable increase in alpha diversity. ($P < 0.05$)



Beta diversity



3.2.2. Significant Taxa

When examining the significant taxa in recipients before and after liver transplantation (LT), it was observed that at the phylum level, Bacteroidota, Campylobacterota, Fusobacteriota, and Thermodesulfobacteriota were abundant before surgery but decreased after surgery ($P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively)

At the class level, Bacteroidia, Betaproteobacteria, Desulfovibrionia, Epsilonproteobacteria, and Fusobacteriia were abundant before liver transplantation but decreased after the transplantation ($P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively).

Using this approach, significant differences at the order, family, genus, and species levels before and after liver transplantation can be identified. This yields a taxonomy of significant bacterial lineages. These are represented by the effect size as the median fold-change (mFC). Significant bacterial lineages that are abundant before liver transplantation include:

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[22]Odoribacteraceae|[81]Parabacteroides distasonis

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[22]Odoribacteraceae|[24]Rikenellaceae|[28]Alistipes|[50]A. onderdonkii

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[23]Prevotellaceae|[45]Prevotella|[84]P. copri

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[23]Prevotellaceae|[45]Prevotella|[85]P. stercorea

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|[44]Phocaeicola|[82]P. plebeius

[1]Bacteroidota|5Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|[44]Phocaeicola|[83]P. vulgatus

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|Genus|[52]Bacteroides ovatus

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|Genus|[53]Bacteroides stercoris

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|Genus|[54]Bacteroides thetaiotaomicron

[1]Bacteroidota|[5]Bacteroidia|[10]Bacteroidales|[16]Bacteroidaceae|Genus|[55]Bacteroides uniformis

[26]Turicibacteraceae|[47]Turicibacter|[91]T. sanguinis

[30]Catenibacterium|[61]C. mitsuokai

[40]Longibaculum|[75]L. muris

[36]Faecalibacillus|[70]F. intestinalis

[33]Dialister|[65]D. succinatiphilus

[43]Megasphaera|[80]M. micronuciformis

[89]Streptococcus vaginalis

[87]Streptococcus oralis

[86]Streptococcus intermedius

[74]Latilactobacillus curvatus

[35]Evtepia|[69]E. gabavorous

[39]Gemmiger|[72]G. formicilis

[73]Intestinimonas timonensis

[20]Eubacteriales Family XIII Incertae Sedis|[49]Zhenpiania|[94]Z. hominis

[20]Eubacteriales Family XIII Incertae Sedis|[42]Massilioclostridium|[77]M. coli

[62]Clostridium disporicum

[41]Marseillibacter|[76]M. massiliensis

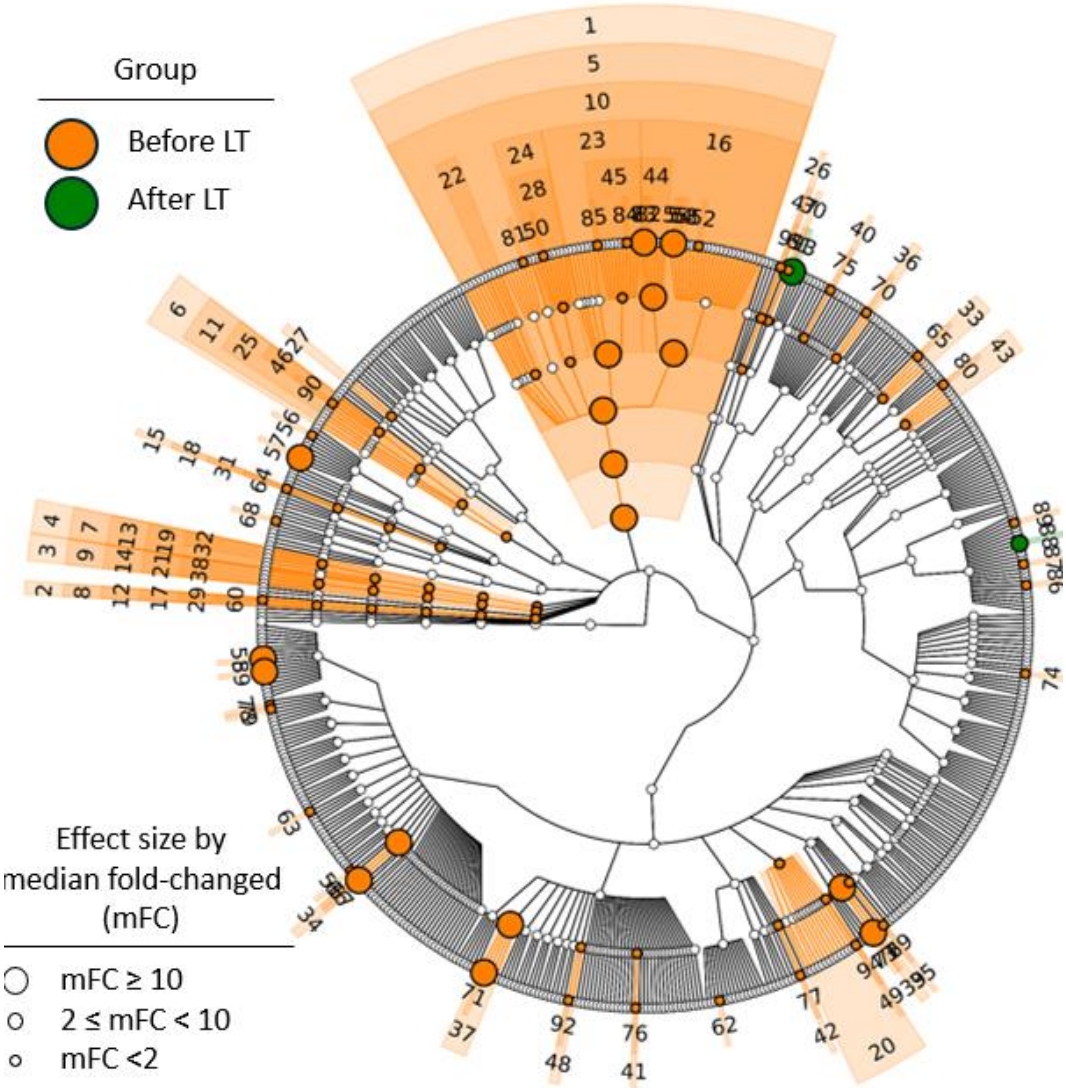
Significant bacterial taxa more abundant after LT include [93][Clostridium] innocuum and [88]Streptococcus salivarius in Figure 4.

There was a significant difference in beta diversity before and after liver transplantation, using the significantly clustered method. (P=0.0001; Figure 5)

Figure 4. Significant bacterial lineages regarding gut microbial changes in recipients between before and after LT.

This analysis produces a taxonomy of notable bacterial lineages, depicted by effect size in terms of median fold-change (mFC). The bacterial lineages significantly abundant before and after liver transplantation are listed below.

LT, liver transplantation.



Significant bacterial taxa more abundant before LT

[48]Vescimonas|[92]Vescimonas coprocola
[37]Faecalibacterium|[71]F. duncaniae
[34]Dorea|[66]D. longicatena
[34]Dorea|[67]D.phocaeensis
[51]Anaerobutyricum hallii
[63]Coprococcus catus
[78]Mediterraneibacter faecis
[79]Mediterraneibacter glycyrrhizinilyticus
[58]Blautia intestinalis
[59]Blautia obeum
[2]Campylobacterota|[8]Epsilonproteobacteria|[12]Campylobacterales|[17]Campylobacteraceae|[29]Campylobacter|[60]C. concisus
[3]Fusobacteriota|[9]Fusobacteriia|[14]Fusobacteriales|[21]Fusobacteriaceae|[38]Fusobacterium
[4]Thermodesulfobacteriota|[7]Desulfovibrionia|[13]Desulfovibrionales|[19]Desulfovibrionaceae|[32]Desulfovibrio
[68]Eggerthella guodeyinii
[15]Mycobacteriales|[18]Corynebacteriaceae|[31]Corynebacterium|[64]C. dentalis
[56]Bifidobacterium bifidum
[57]Bifidobacterium pseudocatenulatum
[6]Betaproteobacteria|[11]Burkholderiales|[25]Sutterellaceae|[46]Sutterella|[90]S. wadsworthensis
[27]Aggregatibacter

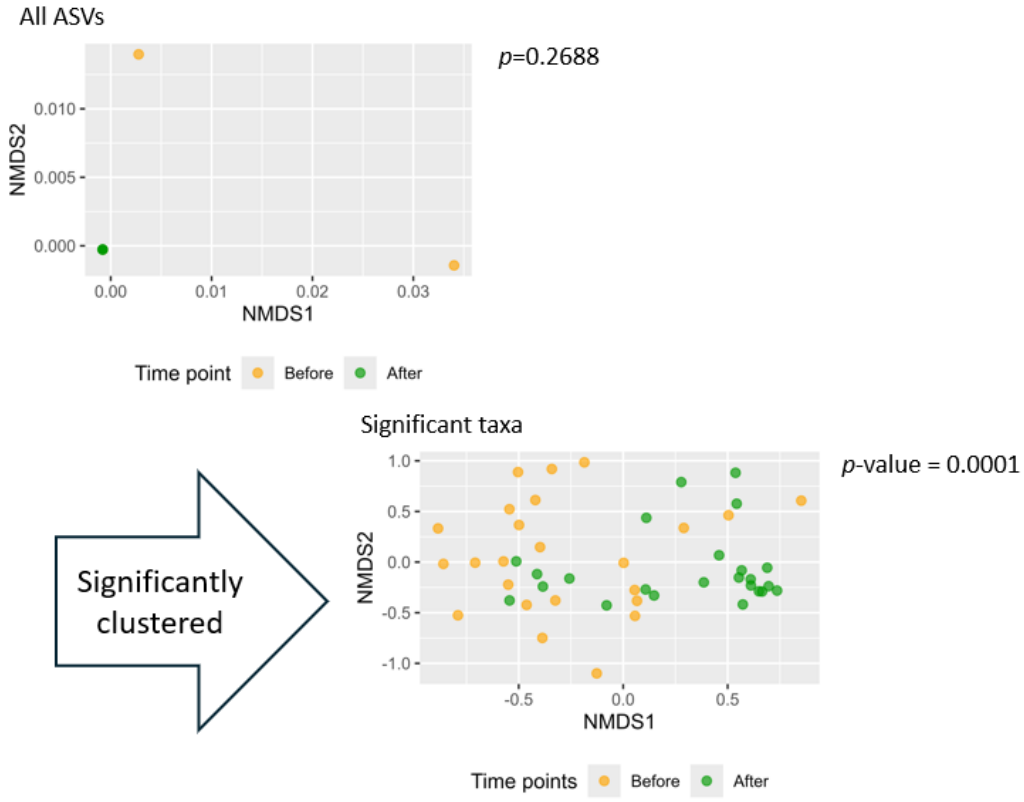
Significant bacterial taxa more abundant after LT

[93][Clostridium] innocuum
[88]Streptococcus salivarius

Figure 5. Beta diversity using significant clustered methods regarding gut microbial changes in recipients between before and after LT.

Unlike the analysis of all ASVs, there was a significant difference in beta diversity before and after liver transplantation, using the significantly clustered method.

ASVs, amplicon sequence variants; LT, liver transplantation.



3.2.3. Significant functional pathways *in silico*

Using significant functional pathways in *silico*, the pathways triggered before and after liver transplantation are as follows. Before liver transplantation, many pathways are observed: P42-PWY, PWY-5971, COLANSYN-PWY, PWYG-321, PWY-5989, PWY-6703, PWY-6282, PWY-7664, THISYN-PWY, NAGLIPASYN-PWY, BIOTIN-BIOSYNTHESIS-PWY, PWY-1269, PWY-6519, PWY-6467, PWY-6969, PWY-7323, PWY-5347, PWY-7456, TCA PWY-6147, HOMOSER-METSYN-PWY, MET-SAM-PWY, PWY-6895, PWY-5659, PWY-6891, FASYN-INITIAL-PWY, P108-PWY, PWY-7371, PWY-6263 and PWY-6572. Functional pathways more abundant after LT include PWY-7220, PWY-7222, LACTOSECAT-PWY, and P341-PWY. (Figure 6 and Table 2)

Figure 6. Significant functional in *silico* regarding gut microbial changes in recipients between before and after LT.

By analyzing significant functional pathways in silico, we identified the pathways activated before and after liver transplantation. Prior to liver transplantation, pathways such as PWY-6572, PWY-6263, and PWY-7371 were prevalent. Post-transplantation, pathways including LACTOSECAT-PWY, P341-PWY, PWY-7222 and PWY-7220 were notably triggered**.**

LT, liver transplantation.

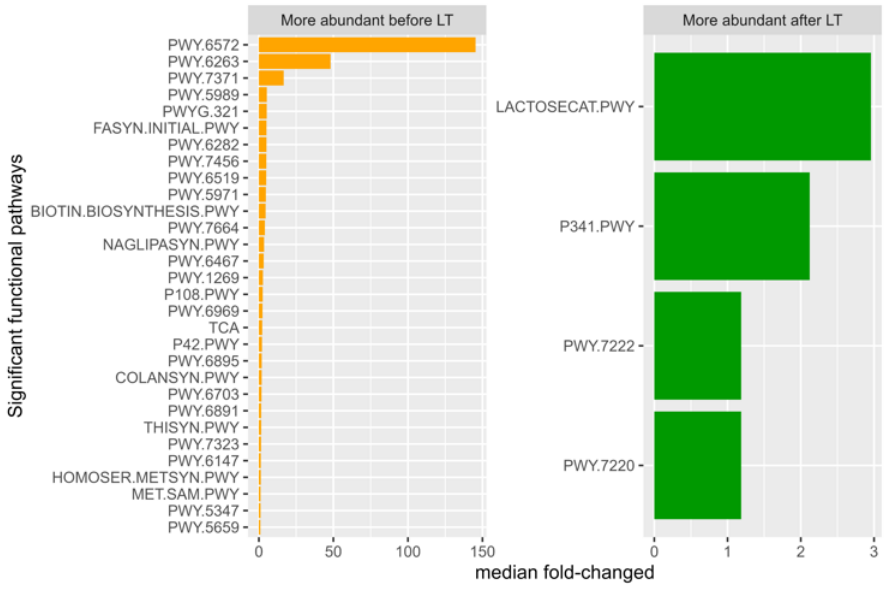


Table 2. Significant functional pathways and description in silico regarding gut microbial changes in recipients between before and after LT.

Functional pathways more abundant in either pre-LT or post- LT group. LT, liver transplantation

a) Functional pathways more abundant in before LT

Pathway	Description
P42-PWY	incomplete reductive TCA cycle
PWY-5971	palmitate biosynthesis II (bacteria and plants)
COLANSYN-PWY	colanic acid building blocks biosynthesis
PWYG-321	mycolate biosynthesis
PWY-5989	stearate biosynthesis II (bacteria and plants)
PWY-6703	preQ0 biosynthesis
PWY-6282	palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate)
PWY-7664	oleate biosynthesis IV (anaerobic)
THISYN-PWY	superpathway of thiamin diphosphate biosynthesis I
NAGLIPASYN-PWY	lipid IVA biosynthesis
BIOTIN-BIOSYNTHESIS-PWY	biotin biosynthesis I
PWY-1269	CMP-3-deoxy-D-manno-octulosonate biosynthesis I
PWY-6519	8-amino-7-oxononanoate biosynthesis I
PWY-6467	Kdo transfer to lipid IVA III (Chlamydia)
PWY-6969	TCA cycle V (2-oxoglutarate:ferredoxin oxidoreductase)
PWY-7323	superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis

PWY-5347	superpathway of L-methionine biosynthesis (transsulfuration)
PWY-7456	mannan degradation
TCA	TCA cycle I (prokaryotic)
PWY-6147	6-hydroxymethyl-dihydropterin diphosphate biosynthesis I
HOMOSER-METSYN-PWY	L-methionine biosynthesis I
MET-SAM-PWY	superpathway of S-adenosyl-L-methionine biosynthesis
PWY-6895	superpathway of thiamin diphosphate biosynthesis II
PWY-5659	GDP-mannose biosynthesis
PWY-6891	thiazole biosynthesis II (Bacillus)
FASYN-INITIAL-PWY	superpathway of fatty acid biosynthesis initiation (E. coli)
P108-PWY	pyruvate fermentation to propanoate I
PWY-7371	1,4-dihydroxy-6-naphthoate biosynthesis II
PWY-6263	superpathway of menaquinol-8 biosynthesis II
PWY-6572	chondroitin sulfate degradation I (bacterial)

b) Functional pathways more abundant in after LT

Pathway	**Description**
PWY-7220	adenosine deoxyribonucleotides de novo biosynthesis II
PWY-7222	guanosine deoxyribonucleotides de novo biosynthesis II
LACTOSECAT-PWY	lactose and galactose degradation I
P341-PWY	glycolysis V (Pyrococcus)

3.3. The post-LT clinical outcomes linked with donors' gut microbiome

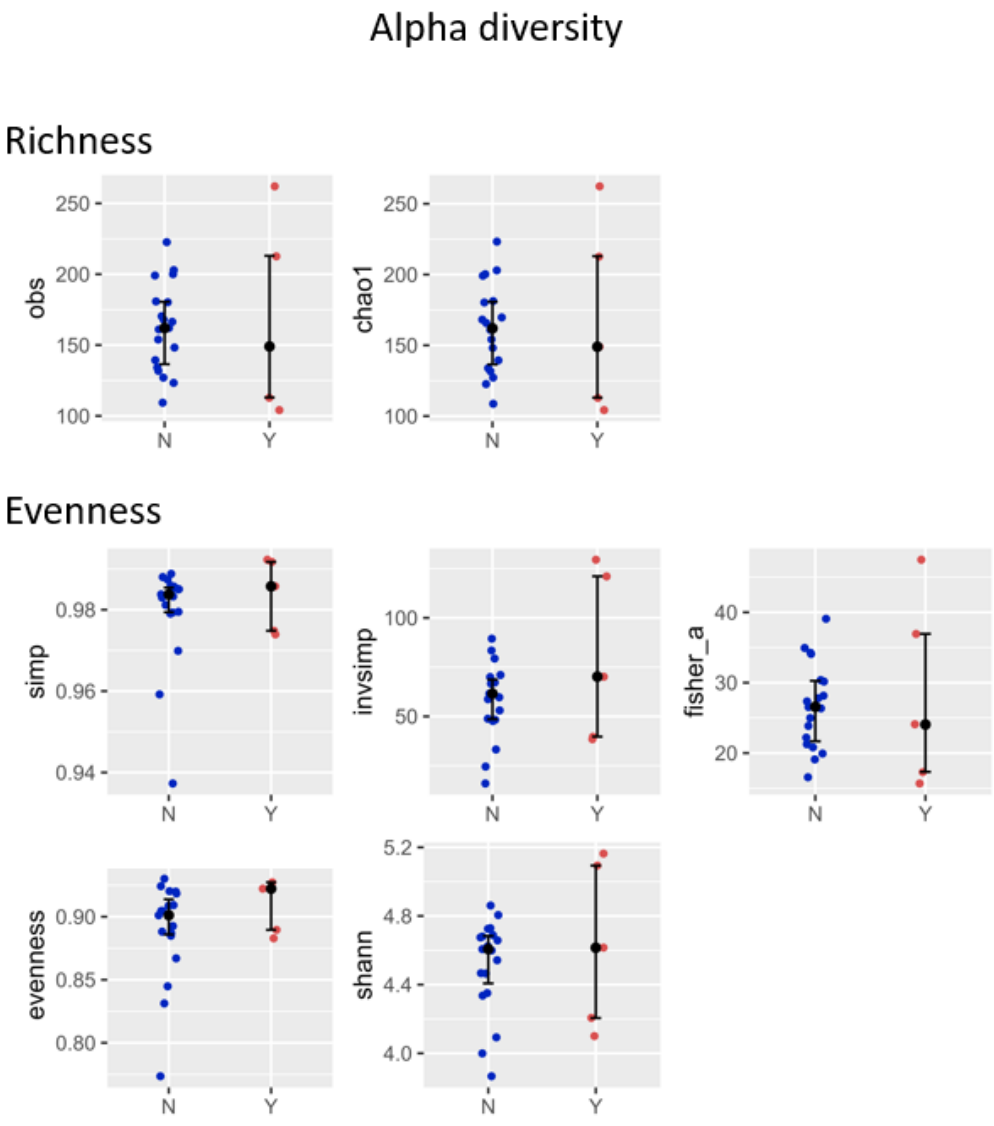
3.3.1. Alpha diversity and beta diversity

Regarding the pre-LT donor microbiome, when comparing the diversity between the BPAR group and the non-BPAR group, there was no statistically significant difference in alpha diversity and beta diversity between the BPAR group and the non-BPAR group. (Figure 7)

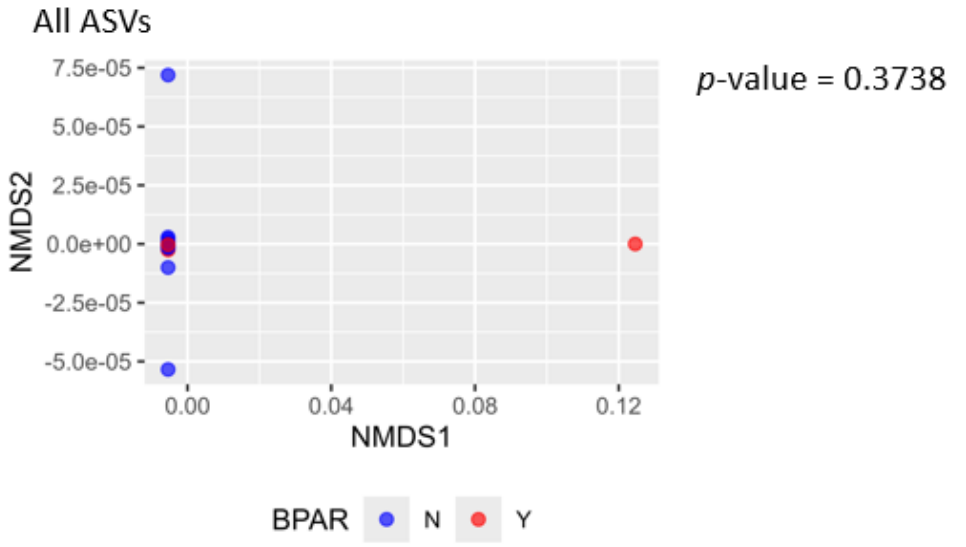
Figure 7. Alpha diversity and beta diversity regarding the post-LT clinical outcomes linked with donor's gut microbiome before LT.

In examining the pre-LT donor microbiome, no statistically significant differences in alpha and beta diversity were found between the BPAR group and the non-BPAR group.

BPAR, biopsy proven acute rejection ; LT, liver transplantation.



Beta diversity



*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.3.2. Significant taxa

In the group where BPAR did not occur after liver transplantation, the donor's microbiome before surgery had a higher abundance of the following significant bacterial taxa (Figure 8):

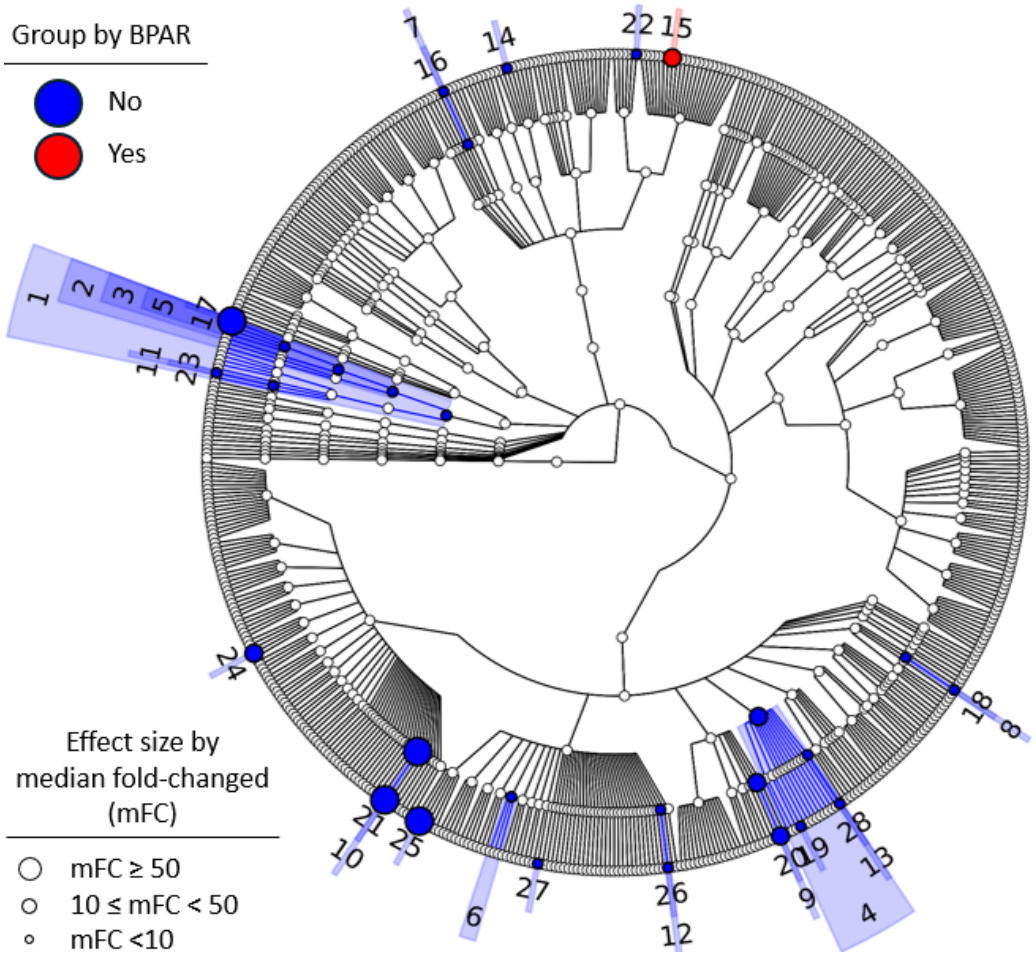
[1]Coriobacteriia|[2]Coriobacteriales|[3]Coriobacteriaceae|[5]Collinsella|[17]C. Aerofaciens,
[7]Barnesiella|[16]B. intestinihominis , [14]Alistipes onderdonkii , [22]Phocaeicola coprocola,
[8]Guopingia|[18]G. tenuis , [4]Eubacteriales Family XIII.
Incertae.Sedis|[13]Zhenpiania|[28]Zhenpiania.hominis , [4]Eubacteriales Family XIII.
Incertae.Sedis|Genus|[19]Lentihominibacter.hominis, [4]Eubacteriales Family XIII.
Incertae.Sedis|[9]Massilioclostridium|[20]M. coli, [12]Ruthenibacterium|[26]R. lactatiformans,
[27]Vescimonas fastidiosa, [6]Anaerotruncus , [25]Ruminococcus bromii,
[10]Oliverpabstia|[21]Oliverpabstia., [24]Roseburia inulinivorans , and
[1]Coriobacteriia|Order|Family|[11]Raoultibacter|[23]R. timonensis.

On the other hand, in the BPAR group, Bacteroides ovatus was more abundant in the donor's microbiome before LT. The significantly clustered method shows that there is a significant difference in beta diversity of gut microbiome after LDLT between the BPAR group and the non-BPAR group. (P=0.0004, Figure 9)

Figure 8. Significant bacterial lineages regarding the post-LT clinical outcome linked with donor's gut microbiome before LT.

In the cohort without BPAR after liver transplantation, the donor's pre-operative microbiome showed a greater presence of several key bacterial taxa, such as C. aerofaciens, B. intestinihominis, Zhenpiania.hominis, Lentihominibacter.hominis, and M. coli. Conversely, in the BPAR group, Bacteroides ovatus was more prevalent in the donor's microbiome prior to LT.

BPAR, biopsy proven acute rejection.; LT, liver transplantation.



Significant bacterial taxa more abundant in BPAR-No

[1]Coriobacteriia|[2]Coriobacteriales|[3]Coriobacteriaceae|[5]Collinsella|[17]C. Aerofaciens
[7]Barnesiella|[16]B. intestinihominis
[14]Alistipes onderdonkii
[22]Phocaeicola coprocola
[8]Guopingia|[18]G. tenuis
[4]Eubacteriales Family XIII. Incertae.Sedis|[13]Zhenpiania|[28]Zhenpiania.hominis
[4]Eubacteriales Family XIII. Incertae.Sedis|Genus|[19]Lentihominibacter.hominis
[4]Eubacteriales Family XIII. Incertae.Sedis|[9]Massilioclostridium|[20]M. coli
[12]Ruthenibacterium|[26]R. lactatiformans
[27]Vescimonas fastidiosa
[6]Anaerotruncus
[25]Ruminococcus bromii
[10]Oliverpabstia|[21]Oliverpabstia.intestinalis
[24]Roseburia inulinivorans
[1]Coriobacteriia|Order|Family|[11]Raoultibacter|[23]R. timonensis

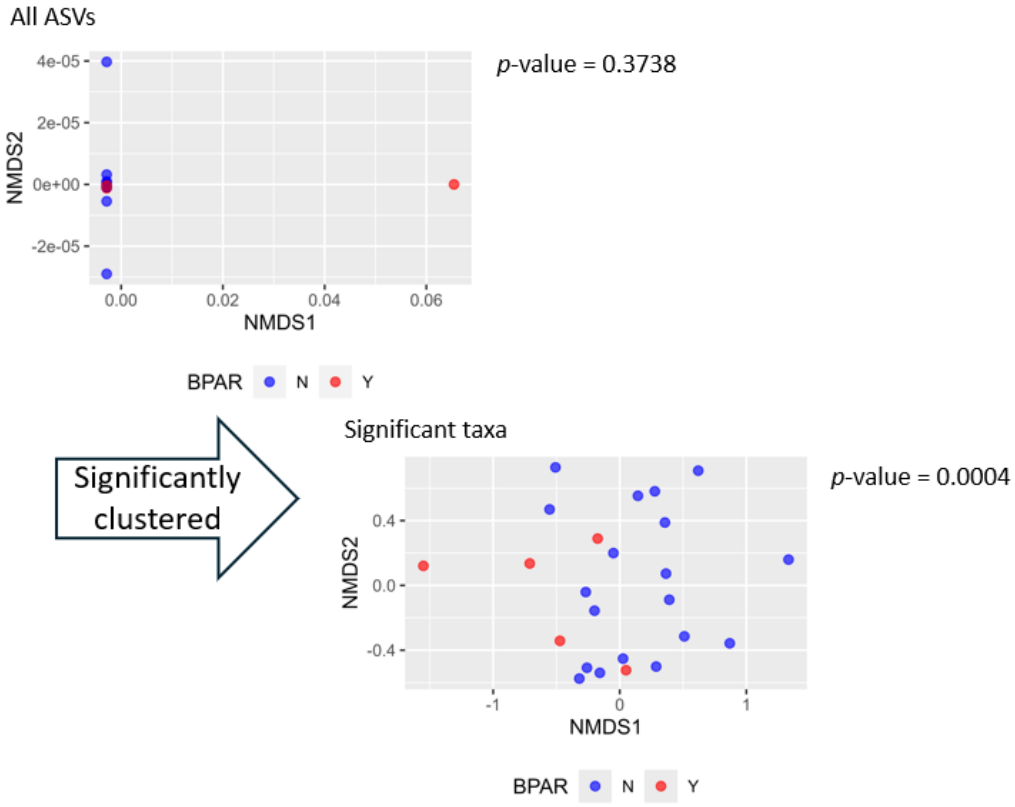
Significant bacterial taxa more abundant in BPAR-Yes

[15]Bacteroides ovatus

Figure 9. Beta diversity using significant clustered methods regarding the post-LT clinical outcome linked with donor's gut microbiome before LT.

When compared to the analysis of all ASVs, the significantly clustered method revealed a significant difference in beta diversity before and after liver transplantation.

ASVs, amplicon sequence variants; LT, liver transplantation.



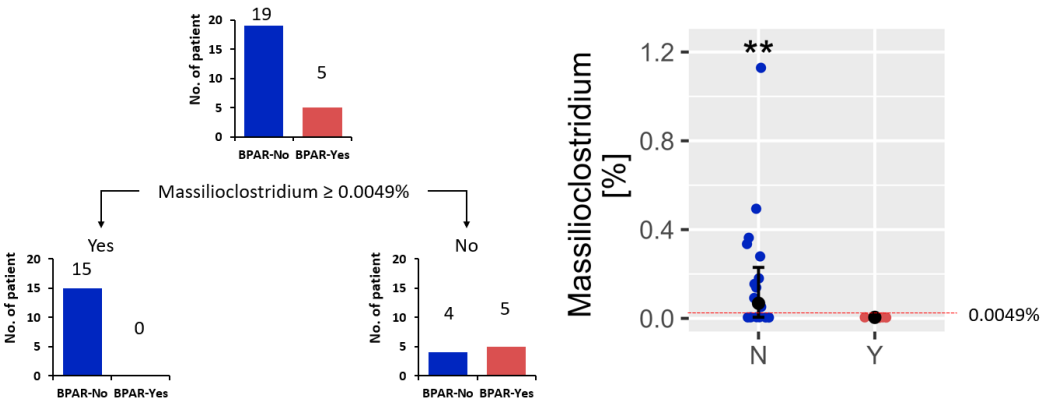
3.3.3. Decision tree using significant bacterial lineages

According to the decision tree using significant bacterial lineages, if the donor's microbiome has Massilioclostridium at a level of ≥ 0.0049%, there were no cases of BPAR, whereas if the level was below 0.0049%, all 5 of the 5 BPAR cases were successfully predicted. Thus, if the preoperative donor microbiome has Massilioclostridium at a level of < 0.0049%, the risk of BPAR increases. (P < 0.01; Figure 10)

Figure 10. Decision tree using significant bacterial lineages regarding the post-LT clinical outcome linked with donors' gut microbiome before LT.

Based on the decision tree analysis of significant bacterial lineages, no cases of BPAR were observed when the donor's microbiome contained Massilioclostridium at levels ≥ 0.0049%. In contrast, when levels were below 0.0049%, all 5 BPAR cases were accurately predicted.

BPAR, biopsy proven acute rejection.; LT, liver transplantation.



*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001

3.3.4. Significant functional pathways *in silico*

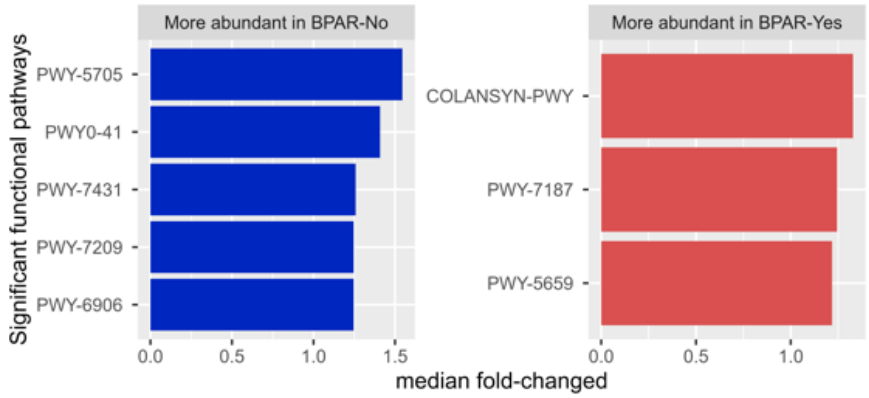
Functional pathways more abundant in the group where BPAR did not occur include PWY-5705 (allantoin degradation to glyoxylate III), PWY0-41 (allantoin degradation IV (anaerobic)), PWY-7431 (aromatic biogenic amine degradation (bacteria)), PWY-6906 (chitin derivatives degradation), and PWY-7209 (superpathway of pyrimidine ribonucleosides degradation).

On the other hand, in the group where BPAR occurred, COLANSYN-PWY (colanic acid building blocks biosynthesis), PWY-7187 (pyrimidine deoxyribonucleotides de novo biosynthesis II), and PWY-5659 (GDP-mannose biosynthesis) were observed. (Figure 11)

Figure 11. Significant functional pathways and description in *silico* regarding the post-LT clinical outcome linked with donor' gut microbiome before LT.

In the group without BPAR, functional pathways such as PWY-5705, PWY0-41, PWY-7431, PWY-6906, and PWY-7209 were more abundant. Conversely, in the group experiencing BPAR, pathways including COLANSYN-PWY, PWY-7187, and PWY-5659 were more prominent.

BPAR, biopsy proven acute rejection. ; LT, liver transplantation.



Functional pathways more abundant in BPAR-No

pathway	description
PWY-5705	allantoin degradation to glyoxylate III
PWY0-41	allantoin degradation IV (anaerobic)
PWY-7431	aromatic biogenic amine degradation (bacteria)
PWY-6906	chitin derivatives degradation
PWY-7209	superpathway of pyrimidine ribonucleosides degradation

Functional pathways more abundant in BPAR-Yes

pathway	description
COLANSYN-PWY	colanic acid building blocks biosynthesis
PWY-7187	pyrimidine deoxyribonucleotides de novo biosynthesis II
PWY-5659	GDP-mannose biosynthesis

3.4. The post-LT clinical outcomes linked with recipients' gut microbiome before LT

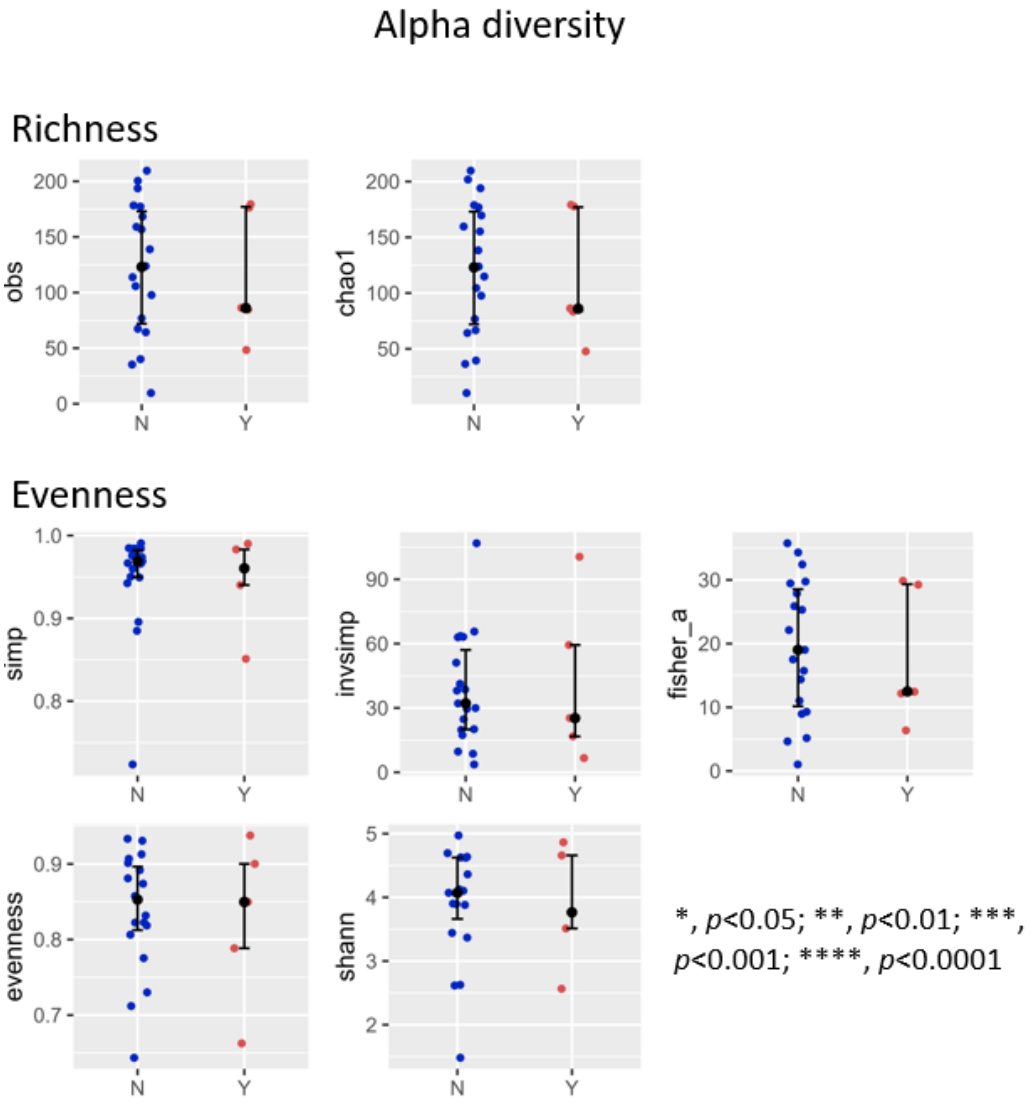
3.4.1. Alpha diversity and beta diversity

To determine whether the recipient's microbiome before liver transplantation affects post-surgery BPAR, we conducted alpha and beta diversity analyses using the same method as before. There was no statistically significant difference in alpha diversity and beta diversity between the BPAR group and the non-BPAR group. (Figure 12)

Figure 12. Alpha diversity and beta diversity regarding the post-LT clinical outcomes linked with recipients' gut microbiome before LT.

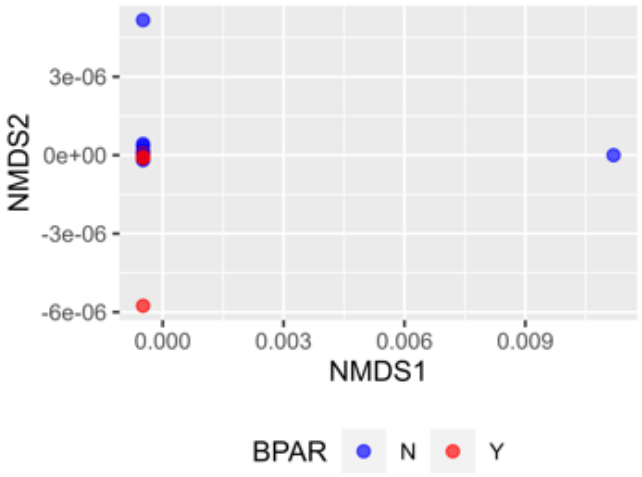
No statistically significant differences in alpha or beta diversity were found between the BPAR and non-BPAR groups.

BPAR, biopsy proven acute rejection; LT, liver transplantation.



Beta diversity

All ASVs



p-value = 0.5922

3.4.2. Significant taxa

In the group where BPAR was not observed, Veillonellaceae, Gemmiger, and Prevotella copri were more abundant in the recipient's microbiome before LT.

Regarding significant bacterial lineages, in the group where BPAR was not observed, Veillonellaceae, Gemmiger, and Prevotella copri were more abundant in the recipient's microbiome before LT.

On the other hand, [1]Negativicutes|[2]Veillonellales|[3]Veillonellaceae ,[4]Gemmiger and [5]Prevotella|[6]Prevotella.copri were more abundant in the recipient's microbiome before LT. (Figure 13)

Using the significantly clustered method, there was a significant difference in beta diversity between the group where BPAR occurred and the group where it did not. (*P*=0.0023; Figure 14)

Figure 13. Significant bacterial lineages regarding the post-LT clinical outcome linked with recipients' gut microbiome before LT.

In the group without BPAR, the recipient's microbiome before LT had a higher abundance of Veillonellaceae, Gemmiger, and Prevotella copri.

BPAR, biopsy proven acute rejection.; LT, liver transplantation.

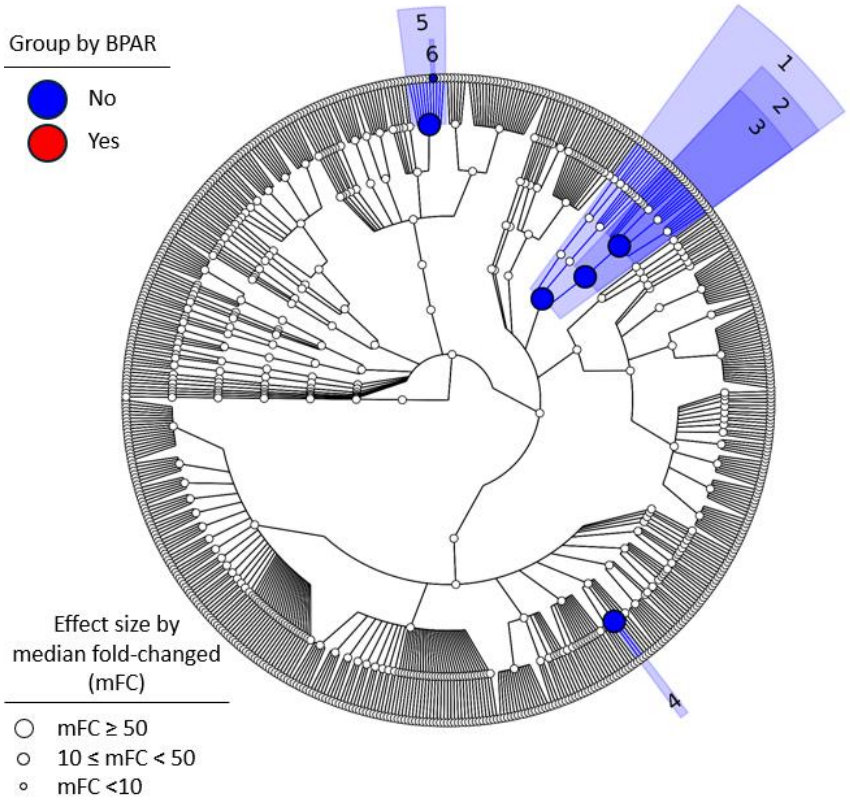
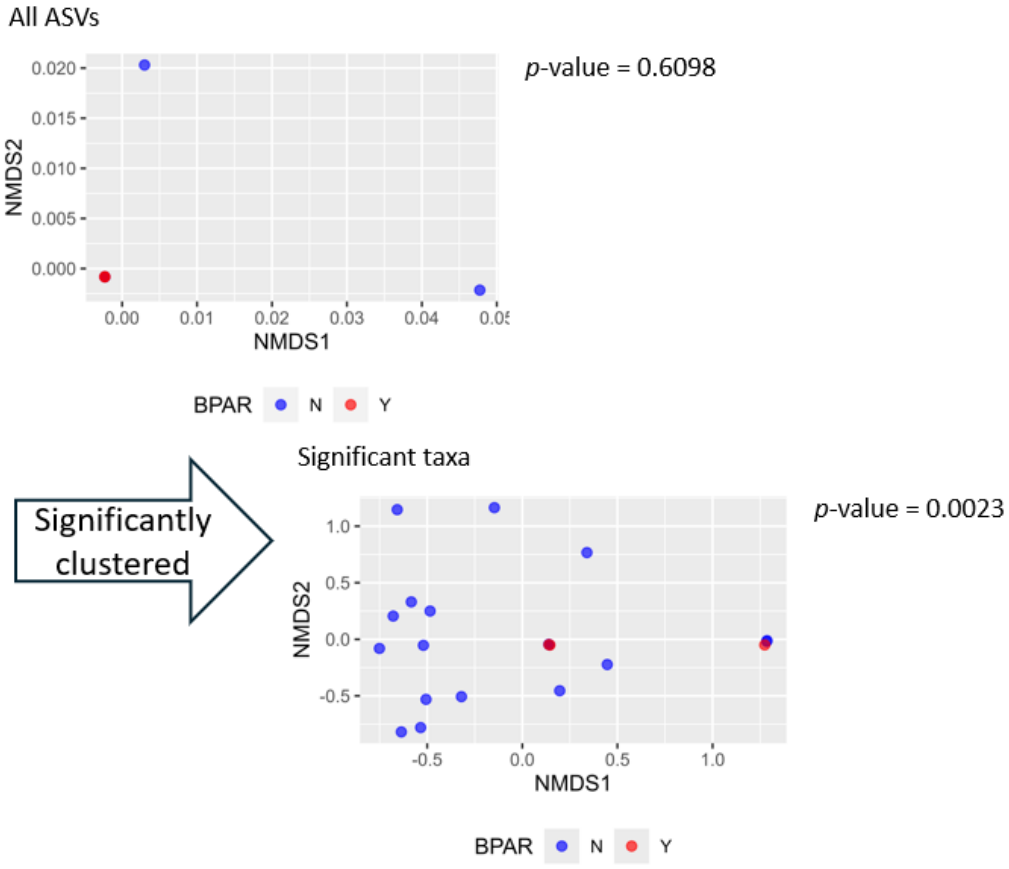


Figure 14. Beta diversity using significant clustered methods regarding the post-LT clinical outcome linked with recipients' gut microbiome before LT.

Compared to the analysis of all ASVs, the significantly clustered method demonstrated a marked difference in beta diversity before and after liver transplantation.

ASVs, amplicon sequence variants; LT, liver transplantation.



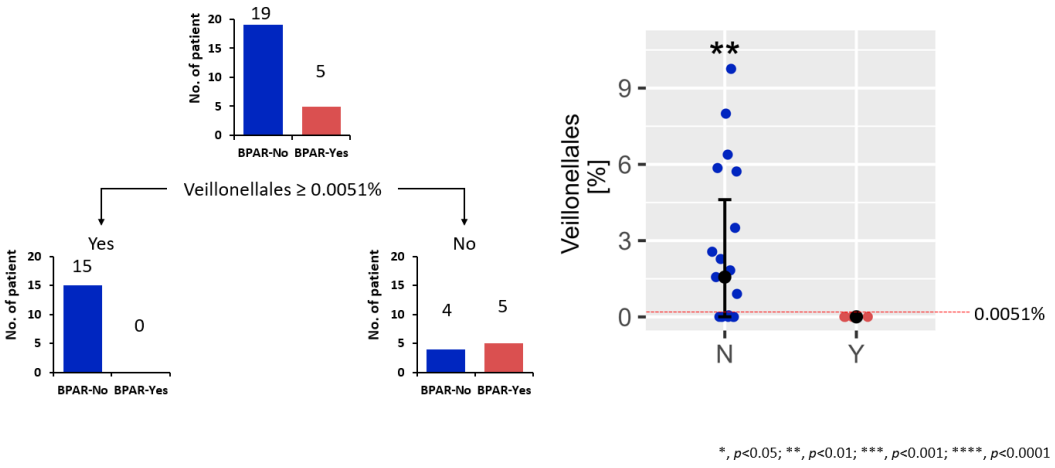
3.4.3. Decision tree using significant bacterial lineages

Based on the decision tree derived from significant bacterial lineages, if the pre-transplant recipient microbiome contains Veillonellales at a concentration of ≥ 0.0051%, no instances of BPAR were observed. Conversely, when the concentration was below 0.0051%, BPAR occurred in 5 out of 9 individuals, meaning all 5 BPAR cases were successfully predicted. Therefore, having Veillonellales at a level of < 0.0051% in the preoperative recipient microbiome is associated with increased risk of BPAR (P < 0.01; Figure 15).

Figure 15. Decision tree using significant bacterial lineages regarding the post-LT clinical outcome linked with recipients' gut microbiome before LT.

According to the decision tree analysis of significant bacterial lineages, no instances of BPAR were observed when the pre-transplant recipient microbiome had Veillonellales at concentrations of ≥ 0.0051%. However, when the concentration fell below 0.0051%, All of the 5 BPAR cases were successfully predicted.

BPAR, biopsy proven acute rejection.; LT, liver transplantation.

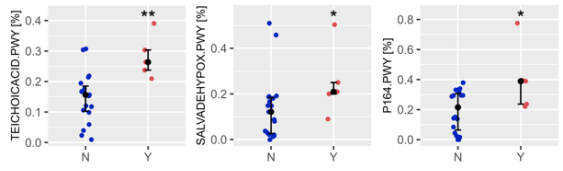


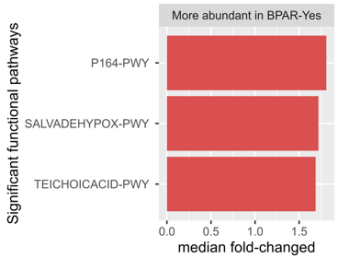
3.4.4. Significant functional pathways *in silico*

In the significant functional pathways in *silico*, no significant pathways were found in the group where BPAR did not occur. However, in the group where BPAR occurred, the pathways TEICHOICACID-PWY (teichoic acid (poly-glycerol) biosynthesis), P164-PWY (purine nucleobases degradation I (anaerobic)), and SALVADEHYPOX-PWY (adenosine nucleotides degradation II) were more abundant. (Figure 16)

Figure 16. Significant functional pathways and description in *silico* regarding the post-LT clinical outcome linked with recipients' gut microbiome before LT.

In the BPAR group, the pathways TEICHOICACID-PWY, P164-PWY, and SALVADEHYPOX-PWY were more prevalent. BPAR, biopsy proven acute rejection.; LT, liver transplantation.





Functional pathways more abundant in BPAR-No

Not observed

Functional pathways more abundant in BPAR-Yes

pathway	description
TEICHOICACID-PWY	teichoic acid (poly-glycerol) biosynthesis
P164-PWY	purine nucleobases degradation I (anaerobic)
SALVADEHYPOX-PWY	adenosine nucleotides degradation II

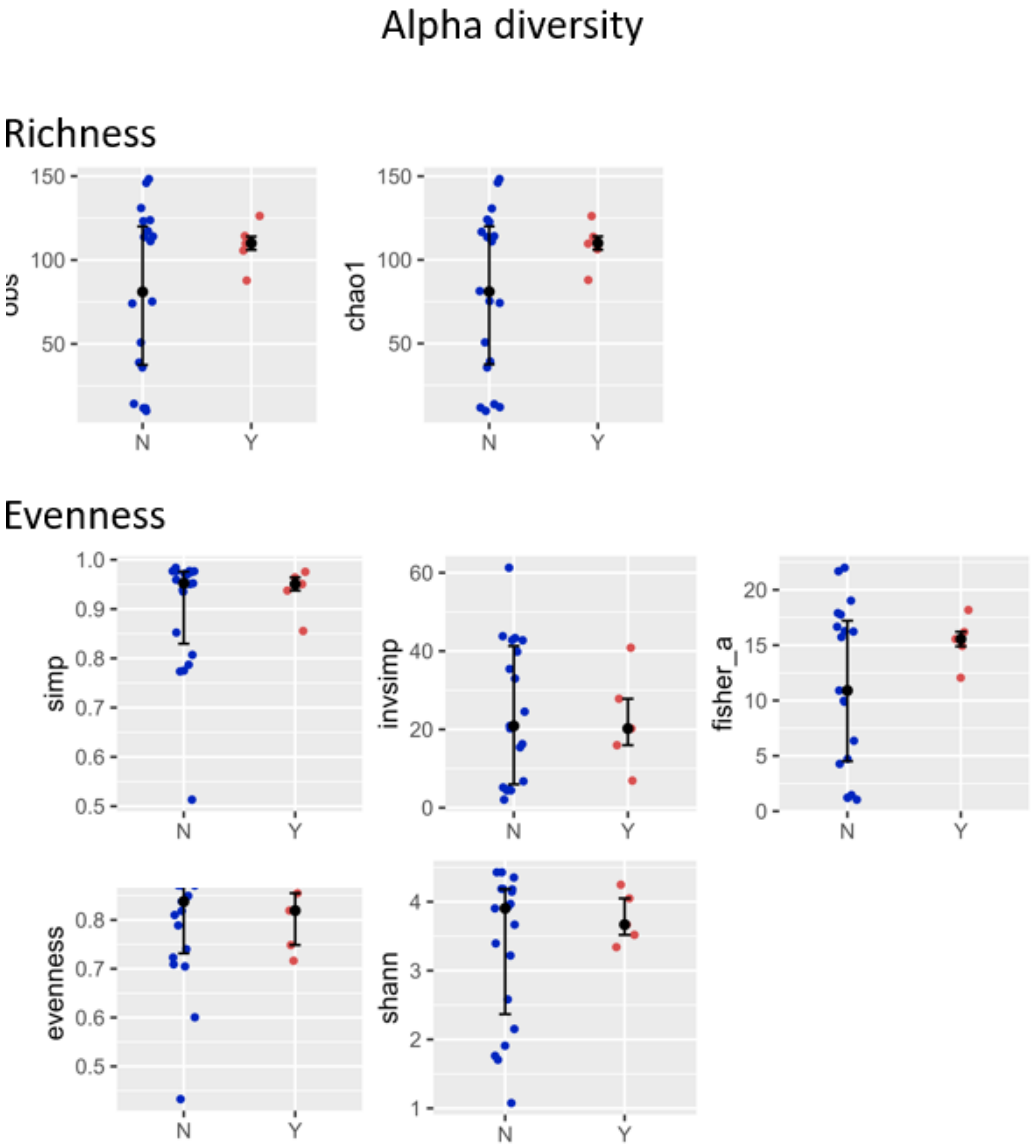
3.5. The post-LT clinical outcomes linked with recipients' gut microbiome after LT

3.5.1. Alpha diversity and beta diversity

To determine whether the recipient microbiome at 3 months post-liver transplantation influenced post-surgery BPAR, alpha and beta diversity analyses were conducted. There was no statistically significant difference in alpha diversity and beta diversity between the BPAR group and the non-BPAR group. (Figure 17)

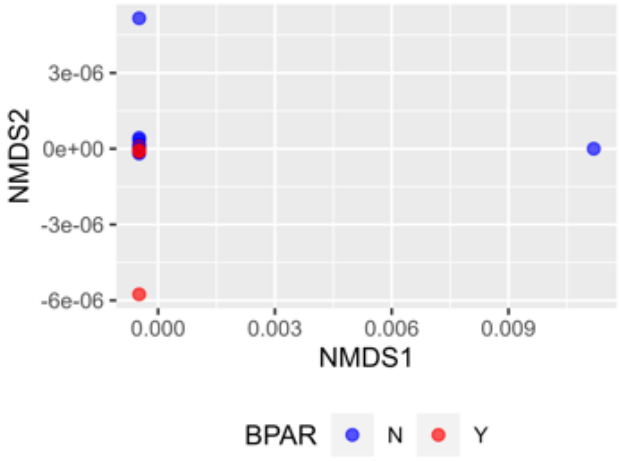
Figure 17. Alpha diversity and beta diversity regarding the post-LT clinical outcomes linked with recipients' gut microbiome after LT

No statistically significant differences in alpha or beta diversity were detected between the BPAR and non-BPAR groups in understanding the recipient's microbial community at 3 months post-LT. BPAR, biopsy proven acute rejection. ; LT, liver transplantation.



Beta diversity

All ASVs



p-value = 0.5922

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.5.2. Significant taxa

In the group where BPAR events occurred after liver transplantation, the following bacterial taxa were more frequently observed in the gut microbiome at 3 months post-surgery as followings:

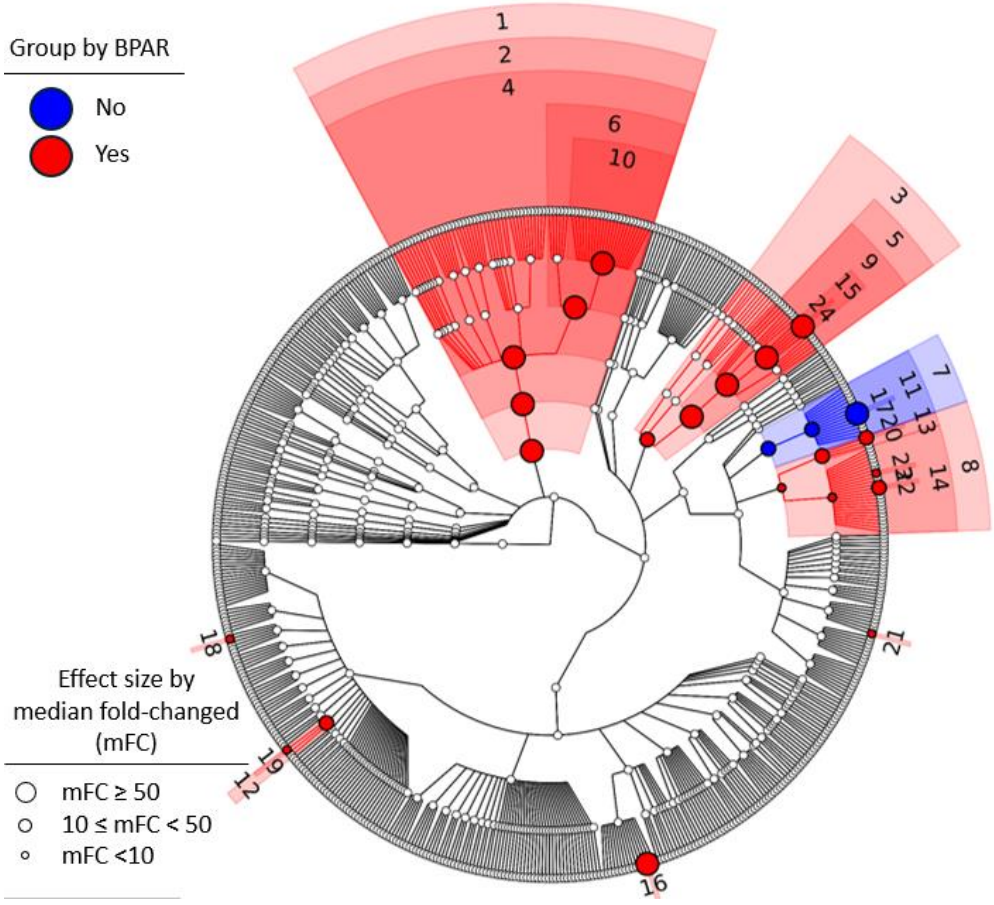
[1]Bacteroidota|[2]Bacteroidia|[4]Bacteroidales|[6]Bacteroidaceae|[10]Bacteroides, [3]Negativicutes|[5]Veillonellales|[9]Veillonellaceae|[15]Veillonella|[24]V. parvula, [8]Streptococcaceae|[13]Lactococcus|[20]L. lactis, [8]Streptococcaceae|[14]Streptococcus|[22]S. parasanguinis , [8]Streptococcaceae|[14]Streptococcus|[23]S. salivarius , [21]Limosilactobacillus albertensis, [16]Clostridium saudiense, [12]Lachnospira|[19]L. eligens, and [18]Lachnoclostridium pacaense. (Figure 18)

In groups where BPAR was not observed, E. faecium is more abundant in microbiome of recipients at 3 months post LT. Using the significantly clustered method, there was no a significant difference in beta diversity between the group where BPAR occurred and the group where it did not. (P= 0.128; Figure 19)

Figure 18. Significant bacterial lineages regarding the post-LT clinical outcome linked with recipients' gut microbiome after LT.

In groups without BPAR, E. faecium was more abundant in the recipients' microbiome three months post-LT. In contrast, in the BPAR group, Bacteroides, V. parvula, L. lactis, S. parasanguinis, S. salivarius, Limosilactobacillus albertensis, Clostridium saudiense, L. eligens, and Lachnoclostridium pacaense were abundant.

BPAR, biopsy proven acute rejection.; LT, liver transplantation.



Significant bacterial taxa more abundant in BPAR-No

[7]Enterococcaceae|[11]Enterococcus|[17]E. faecium

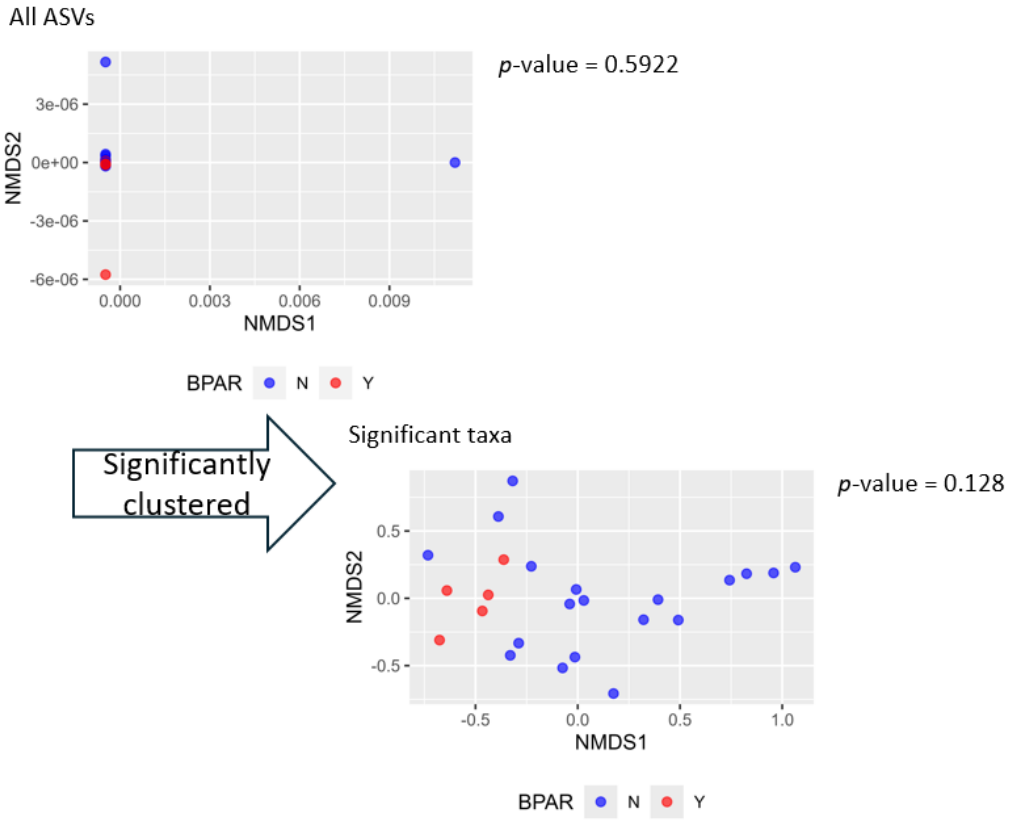
Significant bacterial taxa more abundant in BPAR-Yes

[1]Bacteroidota|[2]Bacteroidia|[4]Bacteroidales|[6]Bacteroidaceae|[10]Bacteroides
[3]Negativicutes|[5]Veillonellales|[9]Veillonellaceae|[15]Veillonella|[24]V. parvula
[8]Streptococcaceae|[13]Lactococcus|[20]L. lactis
[8]Streptococcaceae|[14]Streptococcus|[22]S. parasanguinis
[8]Streptococcaceae|[14]Streptococcus|[23]S. salivarius
[21]Limosilactobacillus albertensis
[16]Clostridium saudiense
[12]Lachnospira|[19]L. eligens
[18]Lachnoclostridium pacaense

Figure 19. Beta diversity using significant clustered methods regarding the post-LT clinical outcome linked with recipients' gut microbiome after LT.

The significantly clustered method did not reveal a notable difference in beta diversity between the group with BPAR and the group without BPAR; however, we observed a significant decrease in the statistical p-value and a noticeable trend.

ASVs, amplicon sequence variants; LT, liver transplantation.



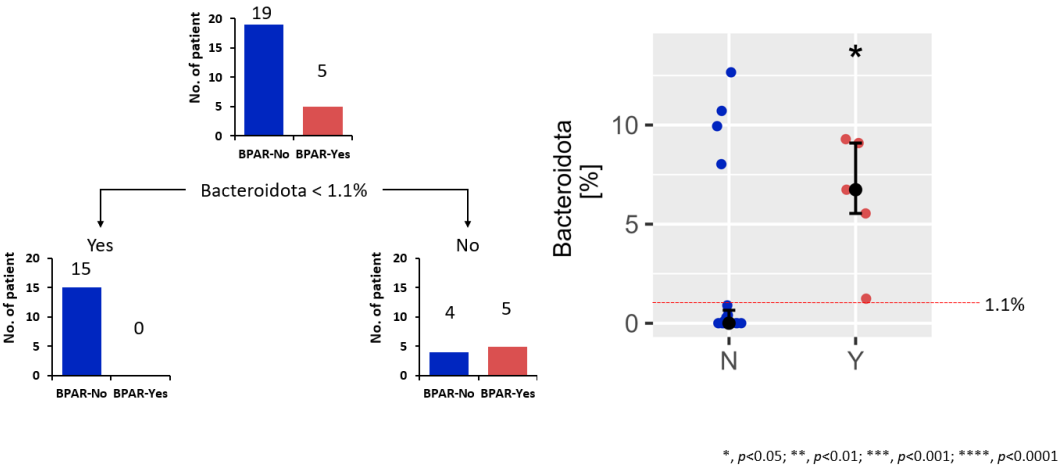
3.5.3. Decision tree using significant bacterial lineages

If the post-transplant recipient microbiome at 3 months contains Bacteroidot at a concentration of < 1.1%, no instances of BPAR were observed. Conversely, when the concentration was above 1.1%, PAR occurred in 5 out of 9 individuals, means all of the 5 BPAR cases were successfully predicted. ($P < 0.05$; Figure 20)

Figure 20. Decision tree using significant bacterial lineages regarding the post-LT clinical outcome linked with recipients' gut microbiome after LT.

Three months post-LT, BPAR was not seen if the recipient's microbiome contained less than 1.1% Bacteroidota. When the concentration was above 1.1%, BPAR occurred in 5 out of 9 individuals, meaning all 5 BPAR cases were successfully predicted.

BPAR, biopsy proven acute rejection; . LT, liver transplantation.



*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001

3.5.4. Significant functional pathways *in silico*

In the group where BPAR was not observed, the following significant functional pathways in *silico* were more abundant: PWY-5384 (sucrose degradation IV (sucrose phosphorylase), P161-PWY (acetylene degradation), DTDPRHAMSYN-PWY (dTDP-L-rhamnose biosynthesis I), PWY-621 (sucrose degradation III (sucrose invertase), PWY-6317 (galactose degradation I (Leloir pathway), PWY0-1586 (peptidoglycan maturation (meso-diaminopimelate containing), PWY0-1061 (superpathway of L-alanine biosynthesis), ARO-PWY (chorismate biosynthesis I), PWY-6163 (superpathway of aromatic amino acid biosynthesis), PWY-6151 (S-adenosyl-L-methionine cycle I), PWY4FS-8 (phosphatidylglycerol biosynthesis II (non-plastidic)), PWY4FS-7 (phosphatidylglycerol biosynthesis I (plastidic)), PWY-5097 (L-lysine biosynthesis VI), ANAGLYCOLYSIS-PWY (glycolysis III (from glucose)), PHOSLIPSYN-PWY (superpathway of phospholipid biosynthesis I (bacteria)), PWY0-1319 (CDP-diacylglycerol biosynthesis II) and PWY-5667 (CDP-diacylglycerol biosynthesis I). The detailed data were shown in Figure 21 and Table 3.

Figure 21. Significant functional pathways in *silico* regarding the post-LT clinical outcome linked with recipients' gut microbiome after LT.

In the non-BPAR group, the following significant functional pathways were more prevalent according to in silico analysis: PWY-5384, P161-PWY, DTDPRHAMSYN-PWY, and PWY-621. HEME-BIOSYNTHESIS-II and PWY-5861 were more abundant in the BPAR group.

BPAR, biopsy proven acute rejection. LT, liver transplantation.

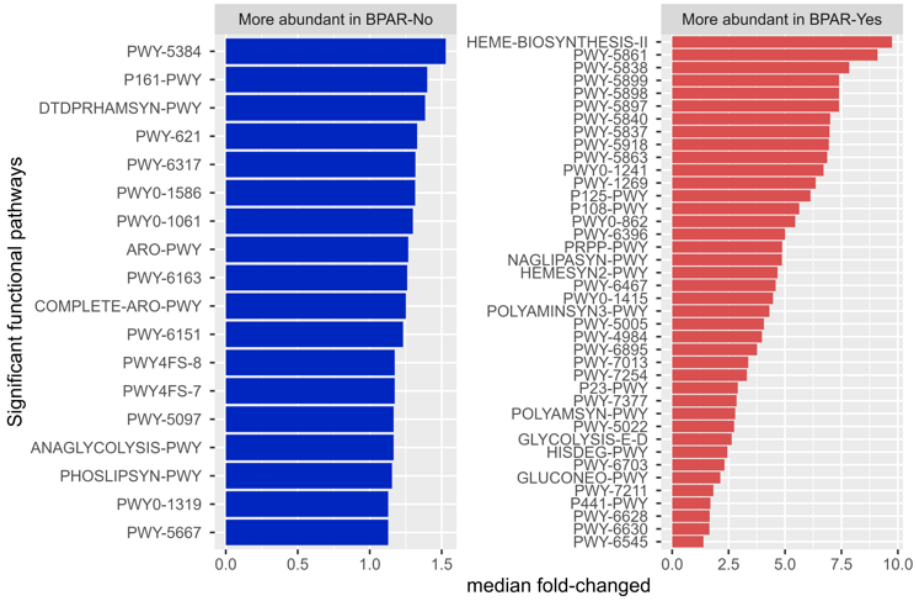


Table 3. Significant functional pathways and description in *silico* regarding the post-LT clinical outcome linked with recipients' gut microbiome after LT.

Functional pathways more abundant in either the BPAR or non-BPAR group. BPAR, biopsy proven acute rejection.

a) Functional pathways more abundant in BPAR-No

Pathway	Description
PWY-5384	sucrose degradation IV (sucrose phosphorylase)
P161-PWY	acetylene degradation
DTDPRHAMSYN-PWY	dTDP-L-rhamnose biosynthesis I
PWY-621	sucrose degradation III (sucrose invertase)
PWY-6317	galactose degradation I (Leloir pathway)
PWY0-1586	peptidoglycan maturation (meso-diaminopimelate containing)
PWY0-1061	superpathway of L-alanine biosynthesis
ARO-PWY	chorismate biosynthesis I
PWY-6163	chorismate biosynthesis from 3-dehydroquinate
COMPLETE-ARO-PWY	superpathway of aromatic amino acid biosynthesis
PWY-6151	S-adenosyl-L-methionine cycle I
PWY4FS-8	phosphatidylglycerol biosynthesis II (non-plastidic)
PWY4FS-7	phosphatidylglycerol biosynthesis I (plastidic)
PWY-5097	L-lysine biosynthesis VI

ANAGLYCOLYSIS-PWY	glycolysis III (from glucose)
PHOSLIPSYN-PWY	superpathway of phospholipid biosynthesis I (bacteria)
PWY0-1319	CDP-diacylglycerol biosynthesis II
PWY-5667	CDP-diacylglycerol biosynthesis I

b) Functional pathways more abundant in BPAR-Yes

Pathway	**Description**
HEME-BIOSYNTHESIS-II	heme biosynthesis I (aerobic)
PWY-5861	superpathway of demethylmenaquinol-8 biosynthesis
PWY-5838	superpathway of menaquinol-8 biosynthesis I
PWY-5899	superpathway of menaquinol-13 biosynthesis
PWY-5898	superpathway of menaquinol-12 biosynthesis
PWY-5897	superpathway of menaquinol-11 biosynthesis
PWY-5840	superpathway of menaquinol-7 biosynthesis
PWY-5837	1,4-dihydroxy-2-naphthoate biosynthesis I
PWY-5918	superpathay of heme biosynthesis from glutamate
PWY-5863	superpathway of phylloquinol biosynthesis
PWY0-1241	ADP-L-glycero-β-D-manno-heptose biosynthesis
PWY-1269	CMP-3-deoxy-D-manno-octulosonate biosynthesis I
P125-PWY	superpathway of (R,R)-butanediol biosynthesis

P108-PWY	pyruvate fermentation to propanoate I
PWY0-862	(5Z)-dodec-5-enoate biosynthesis
PWY-6396	superpathway of 2,3-butanediol biosynthesis
PRPP-PWY	superpathway of histidine, purine, and pyrimidine biosynthesis
NAGLIPASYN-PWY	lipid IVA biosynthesis
HEMESYN2-PWY	heme biosynthesis II (anaerobic)
PWY-6467	Kdo transfer to lipid IVA III (Chlamydia)
PWY0-1415	superpathway of heme biosynthesis from uroporphyrinogen-III
POLYAMINSYN3-PWY	superpathway of polyamine biosynthesis II
PWY-5005	biotin biosynthesis II
PWY-4984	urea cycle
PWY-6895	superpathway of thiamin diphosphate biosynthesis II
PWY-7013	L-1,2-propanediol degradation
PWY-7254	TCA cycle VII (acetate-producers)
P23-PWY	reductive TCA cycle I
PWY-7377	cob(II)yrinate a,c-diamide biosynthesis I (early cobalt insertion)
POLYAMSYN-PWY	superpathway of polyamine biosynthesis I
PWY-5022	4-aminobutanoate degradation V
GLYCOLYSIS-E-D	superpathway of glycolysis and Entner-Doudoroff
HISDEG-PWY	L-histidine degradation I
PWY-6703	preQ0 biosynthesis

GLUCONEO-PWY	gluconeogenesis I
PWY-7211	superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis
P441-PWY	superpathway of N-acetylneuraminate degradation
PWY-6628	superpathway of L-phenylalanine biosynthesis
PWY-6630	superpathway of L-tyrosine biosynthesis
PWY-6545	pyrimidine deoxyribonucleotides de novo biosynthesis III

4. Discussions

This is the first paper predicting BPAR in LT recipients using abundant microbiota from both LT donors' and recipients' microbiomes pre-LT. Liver damage or disease consistently results in alterations to intestinal permeability and microbial composition via the gut-liver axis (Chassaing, Etienne-Mesmin, Gewirtz 2014). Conversely, enhanced liver graft function may aid in the restoration of intestinal microbiota (Ren et al. 2013), indicating that microbial profiling could serve as a potential biomarker for liver injury.

Despite the recognized importance of the gut-liver axis, research on the connection between acute rejection and the gut microbiome remains scarce. Studies in rats have demonstrated that hepatic rejection injury following LT can lead to early-phase changes in intestinal microbiota and subsequent intestinal barrier dysfunction, which may exacerbate hepatic rejection injury in the later stages (Ren et al. 2014). These authors emphasized that the high sensitivity of microbial alterations during AR after LT suggests that changes in the intestinal microbiota could predict early-phase AR and serve as a therapeutic target to mitigate rejection injury post-LT.

As far as we know, there is only one study that compares graft rejection and the gut microbiome in humans after liver transplantation. Kato et al. conducted a prospective study on the intestinal microbiota of 38 liver transplant recipients in Japan. They compared the Shannon diversity index of fecal samples collected from patients during episodes of AR with those from time-matched samples of patients who did not experience AR. For patients with AR, the Shannon diversity index was significantly lower in the post-transplant period compared to the pre-transplant period ($P < 0.01$, paired t-test). Additionally, the post-transplant samples from AR patients had a lower Shannon diversity index compared to time-matched samples from non-AR patients. In AR patients, the mean Shannon diversity index decreased before AR episodes, stayed low during the rejection period, and then increased after the rejection resolved (Kato et al. 2017). This study also revealed that there are changes in the microbiome before and after liver transplantation, which can influence the rejection response in living-donor liver transplantation. Compared to the Kato study, where AR was associated with an abundance of Bacteroides, Enterobacteriaceae, Streptococcaceae, and Bifidobacteriaceae, and non-AR was associated with Lactobacillaceae, Clostridiaceae, Ruminococcaceae, and Peptostreptococcaceae, our study found that BPAR was associated with an abundance of Bacteroidaceae, Veillonellales, Streptococcaceae, Clostridium saudiense, Lachnospira|19L. eligens, and Lachnoclostridium pacaense. Notably, post-transplantation, at 3 months, we found that in groups where BPAR was not observed, E. faecium was more abundant in the recipient's microbiome. Furthermore, if the recipient's microbiome had Bacteroidota levels below 1.1% at 3 months post-LT, the probability of BPAR decreased significantly ($P < 0.01$). Additionally,

a higher abundance of Enterococcaceae at 3 months post-surgery was associated with a lower incidence of BPAR.

Enterococcus faecium is currently being actively researched as a probiotic. Xiao et al. explored the genetic profile of strain B13 in E. faecium and confirmed its safety and efficacy. The strain exhibited strong probiotic characteristics in vitro, including high tolerance to acidic and bile salt conditions, and notable antioxidant activity. The absence of hemolytic and gelatinase activities, along with limited antibiotic resistance, indicated that its risks were manageable (Xiao et al. 2024). Zhang et al. also investigated the effects of dietary supplementation with E. faecium, finding that it significantly enhanced colonization in mature broilers and potentially boosted growth performance by altering the ileal microbiota (Zhang et al. 2024). In the future, after liver transplantation, Fecal microbiota transplantation or probiotics using E. faecium could create a gut environment that reduces the incidence of rejection. To achieve this, large-scale RCTs are needed.

The main strength of this paper is its ability to predict groups prone to post-surgery rejection based on the abundance of taxa in the donor's and recipient's microbiomes before surgery. Our research indicates that specific donor and recipient microbiomes before living-donor liver transplantation are associated with a higher risk of BPAR. In the group where BPAR was observed, Bacteroides ovatus was more abundant in the donor's microbiome before surgery. Conversely, if the preoperative donor microbiome contained Massilioclostridium at a level of $\geq 0.0049\%$, the risk of BPAR decreased significantly ($P < 0.01$). For recipients, in the group where BPAR was not observed, Veillonellaceae, Gemmiger, and Prevotella copri were more abundant in the preoperative microbiome. Additionally, if the preoperative recipient microbiome had Veillonellales at a level of $\geq 0.0051\%$, the risk of rejection decreased significantly ($P < 0.01$). These microbiomes can be used to identify high-risk groups for BPAR. Specifically, Massilioclostridium at a level of $\geq 0.0049\%$ in preoperative donors and Veillonellales at a level of $\geq 0.0051\%$ in preoperative recipients are significant predictor. For high-risk groups for rejection, using higher doses of immunosuppressants or stronger immunosuppressive agents can be an effective preventive measure (Ekberg et al. 2007, Group 1994). In fact, a study on rats after LT revealed that using optimal dosages of immunosuppressants stabilized the gut microbiota. The stable gut microbiota showed an increase in probiotics and a decrease in potential pathogenic endotoxin-producing bacteria (Jiang et al. 2018).

In significant functional pathways in *silico* regarding the recipients' gut microbiome before LT, TEICHOICACID-PWY is known to be related to the wall teichoic acid-dependent phagocytosis of intact cell walls of Lactiplantibacillus plantarum, which elicits IL-12 secretion from macrophage (Kojima et al. 2022) and this may be connected to the immune response and could be associated with hypoxanthine plasma levels being an effective, minimally invasive

biomarker for diagnosing metabolic dysfunction-related steatohepatitis. Regarding the donors' gut microbiome before LT, PWY-6906 is involved in the degradation of chitin derivatives. In this pathway, O-linked N-acetylglucosamine has a protective effect in preventing liver cirrhosis, suggesting that this pathway may play a role in protecting the liver. (Morino, Maegawa 2021)

The limitations of this study include single center data and a small sample size. Additionally, the timing of stool sample collection at 3 months post-liver transplantation does not necessarily coincide with the onset of rejection. Therefore, while we can predict outcomes for those without BPAR, the cause-and-effect relationship for those with BPAR remains unclear.

Nevertheless, our study is significant in that it has developed a model to predict BPAR after liver transplantation based on the preoperative microbiome of both the donor and the recipient.

5. Conclusion

Our study identified significant microbiome changes before and after liver transplantation that impact rejection responses in living-donor liver transplantation. We observed that the presence of Massilioclostridium at a level of ≥ 0.0049% in donor microbiomes and Veillonellaceae at a level of ≥ 0.0051% in recipient microbiomes were linked to a prediction of BPAR. Post-transplant, the abundance of E. faecium at 3 months in non-BPAR groups and lower levels of Bacteroidota were associated with a reduced probability of BPAR, while higher Enterococcaceae abundance correlated with a lower incidence of BPAR. Based on our findings, it may be possible in the future to identify high-risk groups through preoperative microbiome analysis and provide targeted management accordingly.

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Abstract in Korean

생체 간이식 전후의 기증자와 수혜자의 장내미생물의 변화양상을 통한 간이식 후 예후 인자 분석

배경

생체 간이식에서 공여자와 수혜자의 장내 미생물을 통한 위험 요인 분석. 간이식 후 급성 거부반응은 이식 실패와 사망 위험을 크게 증가시킨다. 장-간 축의 중요성에도 불구하고, 간이식에서 급성 거부반응과 장내 미생물군 간의 연관성에 대한 연구는 제한적이다. 본 연구는 생체 간이식에서 공여자와 수혜자의 장내 미생물이 급성거부반응을 예측하는 가치가 있는지 연구하였다.

방법

본 연구는 수술 전 공여자와 수술 전후 수혜자로부터 수집한 대변 샘플을 기반으로 한 전향적 매칭 연구하였다. 조직검사로 입증된 급성 거부반응에 따라 장내 미생물의 식별 및 풍부도, 유전자 군 예측을 전체 길이 16s rRNA 유전자 시퀀싱을 사용하여 분석했다.

결과

이식 전후의 장내 미생물 변화가 거부반응에 영향을 미치며, 이식 후 알파 다양성의 감소와 *Clostridium innocuum* 및 *Streptococcus salivarius*와 같은 특정 세균의 증가를 발견했다. 급성 거부반응은 우리 코호트에서 간이식 후 중간값 68.4일(범위 7~194일)에서 24명의 환자 중 5명 (20.8%)에서 발생하였다. 수술 전 장내 미생물 분석을 통해 거부반응 위험과 관련된 특정 세균을 확인했으며, 공여자의 경우 Massilioclostridium

이 0.0049% 이상, 수혜자의 경우 Veillonellaceae가 0.0051% 이상인 경우이다. 이식 후 분석에서는 *Enterococcus faecium*과 같은 특정 미생물이 급성거부반응이 없는 수혜자에서 더 풍부하게 나타났으며, 급성거부 발생률이 낮은 것과 상관관계가 있음을 발견했다. 장과 간의 상호작용은 PWY-6906(O-linked N-acetylglucosamine 경로를 포함) 및 TEICHOICACID-PWY(IL-12 분비 매크로파지 경로를 포함)와 같은 장내 유래 화합물을 포함한 기능 경로 예측을 통해 조사되었다.

결론

본 연구는 장내 미생물 프로파일링이 거부반응 위험을 예측할 수 있는 잠재력을 강조하며, 고위험군을 위한 맞춤형 면역억제 전략이 개발될 수 있음을 시사한다. 또한, 이식 후 수혜자의 특정 장내 미생물이 거부반응 발생률을 줄이는 환경을 조성할 것으로 기대된다. 이러한 결과는 간이식에서 공여자와 수혜자 모두를 위한 맞춤형 치료의 중요성과 근거를 강조할 것으로 보인다.

핵심되는 말 : 장내미생물, 간이식, 급성거부반응 개인 맞춤 치료, Massilioclostridium, Veillonellaceae, Enterococcus faecium