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Study of Microbiome in Liver Transplantation

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Tianxiao Jiang

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Erstes Gutachten:	Prof. Dr. Joachim Andrassy
Zweites Gutachten:	Priv. Doz. Dr. Christian Schulz
Drittes Gutachten:	Priv. Doz. Dr. Eberhard Lurz
Viertes Gutachten:	Prof. Dr. Gerald Denk

Dekan: Prof. Dr. med. Thomas Gudermann

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Abstract

Introduction: Biliary complications are major contributors to morbidity after liver transplantation (LT). Recent evidence suggests that the biliary tract harbors a resident microbiome, yet its clinical relevance in LT remains unclear. This study aimed to investigate the biliary microbiome at the time of transplantation and explore its relationship to post-transplant outcomes.

Methods: In this prospective observational study, 31 LT cases were enrolled. Paired donor bile fluid and biliary mucosa samples were collected intraoperatively. Active bacterial communities were profiled using RNA-based 16S rRNA sequencing. Microbiome composition, diversity, and associations with clinical parameters were statistically analyzed.

Results: The biliary tract exhibited a distinct, non-sterile microbiome. Microbiome composition remained largely stable intraoperatively. Lower microbial richness in donor bile was linked to ITBL, while higher diversity was observed in cholangitis. Opportunistic and biofilm-forming genera were enriched in cases with biliary leaks or strictures. Donor antibiotic exposure reduced microbial diversity, whereas corticosteroid treatment was associated with increased richness.

Discussion: This study represents the first RNA-based analysis to characterize the biliary microbiome in LT. The findings reveal that microbial profiles present at transplantation are associated with subsequent biliary complications, suggesting their potential as early biomarkers. Donor treatment regimens influence microbiome structure, highlighting opportunities for targeted interventions. These insights expand current understanding of microbiota-host interactions in transplantation and may inform future strategies to mitigate post-LT complications.

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Abbreviation	Full Form
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ASV	Amplicon Sequence Variant
BMI	Body mass index
cDNA	Complementary DNA
CRP	C-reactive protein
DCD	Donors after circulatory death
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ERCP	Endoscopic retrograde cholangiopancreatography
ESLD	End-stage liver disease
FDR	False Discovery Rate
FMT	Fecal microbiota transplants
GFR	Glomerular filtration rate
GSK	Backtable bile
GSR	Reperfusion bile
HTK	Histidine-tryptophan-ketoglutarate

IBD	Inflammatory bowel disease
IRI	Ischemia-reperfusion injury
ITBL	Ischemic-type biliary lesions
IVC	Inferior vena cava
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
LPS	Lipopolysaccharides
LT	Liver transplantation
MaAsLin2	Multivariate Association with Linear Models
MDR	Multi-drug resistant
MELD	Model for End-Stage Liver Disease
MP	Machine perfusion
MRCP	Magnetic Resonance Cholangiopancreatography
MSK	Backtable biliary mucosa
MSR	Reperfusion biliary mucosa
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-anastomotic strictures
NGS	Next-generation sequencing
OTU	Operational Taxonomic Unit
PBC	Primary biliary cholangitis
PCoA	Principal Coordinates Analysis

PCR Polymerase chain reaction

PERMANOVA Permutational Multivariate Analysis of Variance

PSC Primary sclerosing cholangitis

PTBD Percutaneous transhepatic biliary drainage

RDP Ribosomal Database Project

RIN RNA Integrity Number

RNA Ribonucleic acid

RNase Ribonuclease

ROS Reactive oxygen species

SCS Static cold storage

UW University of Wisconsin

WBC White blood cells

1. Introduction

1.1 Overview of Liver Transplantation and Challenges

Liver transplantation (LT) remains the definitive therapeutic intervention for individuals diagnosed with end-stage liver disease (ESLD) or acute liver failure [1, 2], representing one of the most significant advances in modern medicine [3]. Since its introduction, LT has continuously evolved, with improvements in surgical techniques [4], immunosuppressive therapies [5], and organ preservation methods [2], all contributing to better survival rates and quality of life for patients [5]. However, despite these advancements, LT is accompanied by substantial risks, including acute and chronic complications that can significantly impact patient outcomes and graft longevity [6, 7]. Among these, biliary complications are notably frequent and challenging, representing a critical factor in both short- and long-term graft success [8, 9].

1.1.1 Surgical Overview of Liver Transplantation

LT involves replacing the patient's diseased liver with a healthy donor organ through a complex, multi-step surgery (Figure 1). The process begins with recipient hepatectomy, where the patient's liver is removed by clamping primary blood vessels, such as the hepatic artery, portal vein, and inferior vena cava (IVC) [5], to establish vascular control and minimize blood loss [10]. Liver graft preservation during transport is predominantly using static cold storage (SCS), which has relied on cooling the organ in solutions such as histidine-tryptophan-ketoglutarate (HTK) or University of Wisconsin (UW) to reduce cellular metabolism [11]. However, an increasing number of centers, including the Department of General, Visceral, Vascular, and Transplant Surgery at LMU Klinikum, now employ machine perfusion as an advanced preservation technique once the liver arrives at the recipient hospital. This method circulates

oxygenated, nutrient-rich perfusate through the donor liver at either normothermic or hypothermic conditions, significantly enhancing cell viability and minimizing ischemic injury, particularly beneficial for high-risk or marginal grafts [11-13]. The broader implementation of MP is currently limited by logistical and economic constraints. Future directions in the field are exploring the initiation of perfusion at the time of organ procurement to eliminate cold ischemia entirely. This so-called “ischemia-free” transplantation paradigm maintains continuous oxygenated perfusion from donor explant through implantation, an approach that early clinical studies have shown to be feasible and associated with reduced injury [14, 15].

Concurrently, the donor liver undergoes back-table preparation to optimize graft quality before implantation. After preparation, the donor liver is implanted, beginning with the vena cava anastomosis and later anastomosis of the portal vein and hepatic artery are created to allow initial reperfusion, re-establishing blood flow to the graft. Finally, the biliary anastomosis is performed at the end of the procedure, either as a choledocho-choledochostomy (duct-to-duct anastomosis) or as a choledocho-jejunostomy (Roux-en-Y hepaticojejunostomy) [16], thereby restoring biliary continuity. Reperfusion is a critical phase that introduces a risk of ischemia-reperfusion injury (IRI) [17], particularly affecting the biliary epithelium [17, 18]. Given its susceptibility to ischemic damage, biliary health remains central to transplant success, as biliary complications are among the leading causes of graft dysfunction and post-transplant morbidity[19].

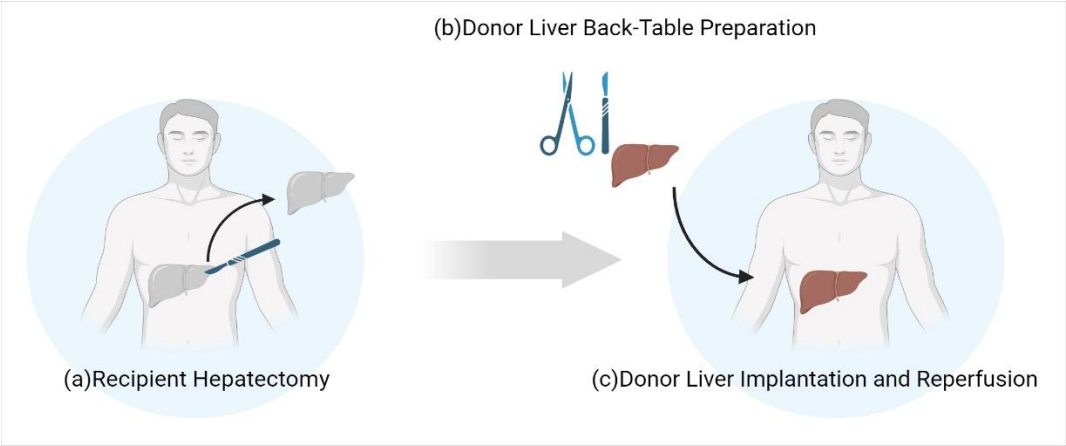

Figure 1 Key Steps in Liver Transplantation: (a) recipient hepatectomy, where the diseased liver is surgically removed; (b) donor liver back-table preparation, during which the donor liver is preserved and prepared for implantation; and (c) donor liver implantation and reperfusion, where the donor liver is implanted into the recipient and blood flow is restored.

1.1.2 Key Challenges Impacting Liver Transplantation Outcomes

LT remains an critical intervention for patients with ESLD, yet it is often associated with complex, multifaceted challenges that hinder both immediate and long-term success. Central among these complications is IRI, an inherent consequence of LT. IRI results from the temporary interruption of blood flow during transplantation, followed by sudden reoxygenation upon reperfusion. This process leads to the generation of reactive oxygen species (ROS) and triggers pro-inflammatory cytokine release, leading to oxidative stress and cellular injury [20]. Due to its sole arterial supply, the biliary epithelium is highly susceptible to ischemic injury [21]. Severe IRI frequently manifests in bile duct necrosis and ischemic-type biliary lesions (ITBL), with long-term repercussions on graft functionality and overall survival[22].

Efforts to mitigate IRI have led to the implementation of advanced graft

preservation strategies, including hypothermic and normothermic machine perfusion[11, 12]. Despite these advancements, IRI continues to pose a fundamental challenge in LT, as ischemic stress is associated with greater susceptibility to biliary complications and compromised graft survival [23]. Continuous improvements in organ preservation are therefore pivotal in addressing this limitation.

Biliary complications, notably bile duct strictures, biliary leaks, and ITBL, remain a prevalent and intricate challenge post-transplantation [24]. These issues disrupt bile flow, leading to adverse conditions such as cholangitis, recurrent infections, and, in extreme cases, graft failure. ITBL poses a particular threat as it frequently arises from ischemic injury, resulting in non-anastomotic strictures that impair bile flow even with adequate hepatic artery perfusion [25]. Management of these complications is complex, often necessitating repeated endoscopic interventions, such as endoscopic retrograde cholangiopancreatography (ERCP), to address strictures and leaks. In severe or recurrent cases, surgical revision or re-transplantation may be unavoidable, increasing patient morbidity and healthcare costs [26].

Another critical challenge is the elevated susceptibility to infections, a result of immunosuppressive therapies required to prevent graft rejection. Immunosuppression inherently increases the patient's vulnerability to bacterial, viral, and fungal pathogens. Post-transplant procedures, particularly ERCP, introduce risks by facilitating pathogen entry into the biliary tract, which can intensify inflammation and lead to recurrent strictures, further compromising biliary health [27]. Persistent infections also increase the likelihood of multi-drug resistant (MDR) organism colonization, possibly leading to recurrent episodes of cholangitis and escalating chronic inflammation within the graft, exacerbating graft injury [28].

Management of these infections, especially those caused by MDR pathogens, is challenging and often necessitates prolonged antibiotic use, contributing to both resistance development and potential drug toxicity [29, 30]. Preventive measures such as rigorous antibiotic management protocols, are crucial to minimize the risk for chronic infections and MDR colonization. Additionally, further research into the biliary microbiome's role in transplant health may provide insights into improving biliary outcomes and reducing complications [29].

1.2 Biliary Complications in Liver Transplantation

Biliary complications are among the most common and significant challenges faced in LT, affecting up to 10-30% of recipients [6-9]. These complications include biliary strictures, cholangitis, ITBL, and bile leaks, each of which may compromise graft viability and affect postoperative well-being. Biliary complications remain a major leading causes of graft failure and the need for repeat LT. Therefore, understanding their prevalence, etiology, and management is crucial for improving long-term outcomes.

1.2.1 Prevalence and Clinical Impact of Biliary Complications

In an effort to reduce the incidence of biliary complications in LT, maintaining key principles of surgical anastomoses is of utmost importance, including reducing tension at the anastomosis, ensuring regular suture intervals, maintaining adequate perfusion, and avoiding damage to the biliary epithelium [25]. Although Roux-en-Y hepaticojejunostomy is capable of maintaining optimal perfusion and reducing anastomotic tension, the duct-to-duct approach is typically preferred due to its closer resemblance to natural anatomy, avoidance of bowel manipulation, shorter operation time, and especially easier endoscopic access in case of biliary complications [31].

Post-transplant biliary complications are most frequently manifested as bile leaks and biliary strictures. While bile leakage most commonly occurs at the site of anastomosis, it may also arise from the liver parenchyma, particularly in split LT, or due to inadvertent injury to the biliary tract during graft preparation, both of which should be avoided to reduce postoperative morbidity [21, 32, 33]. Biliary anastomotic leakage most frequently presents within the first month following LT [34, 35]. Recent studies have suggested that local microbiota can significantly influence anastomotic healing in gastrointestinal surgeries [36-38], and similar mechanisms might play a role in LT. Bacterial proteolytic enzymes may compromise tissue repair through degradation of nascent collagen fibers, as has been observed with *Enterococcus* spp. [37]. Contrary to prior assumptions, the biliary system harbors a diverse but balanced microbial community, even under physiological conditions [39, 40]. Surgical trauma, antimicrobial therapy, and underlying pathological conditions may disrupt the balance between protective and pathogenic bacteria, influencing biliary healing and potentially contributing to leakage and stricture formation [37, 38].

Biliary anastomotic strictures represent a relatively common postoperative complication, occurring in 5% to 10% of LT recipients, with most cases developing within the first year [41, 42]. Mild postoperative edema at the anastomotic site is commonly observed and generally transient. However, persistent inflammation at the site can lead to fibrotic scar tissue formation, ultimately resulting in anastomotic strictures, cholestasis, and cholangitis [43, 44]. The pathogenesis of anastomotic strictures is multifactorial, often involving inadequate mucosa-to-mucosa anastomosis, local ischemia, and fibrotic healing [35]. Diffuse ischemic injury and biliary leakage are recognized risk factors that predispose anastomotic strictures [45]. Endoscopic therapy, including stent placement and dilation, is generally effective and achieves high

short-term success rate. However, long-term outcomes are inconsistent, with some patients requiring repeated interventions due to stricture recurrence or incomplete resolution [46, 47].

Non-anastomotic strictures (NAS) are complex complications that frequently arise from prolonged preservation-related ischemic intervals, IRI, immune-mediated injury, or infections [48]. Unlike anastomotic strictures, NAS can impact broader segments of the biliary tree, making repeated interventions or even re-transplantation necessary [26]. Hepatic artery thrombosis has long been recognized as a major etiological factor in the development of NAS since the early days of LT, and the development of these strictures is often linked to an ischemic or immune-mediated injury mechanism [49, 50].

In cases where the arterial blood supply by the hepatic artery remains intact, NAS can develop into ITBL. ITBL involves injury affecting the native, non-anastomotic segments of the biliary tract, which leads to biliary sludge, intraductal cast formation, filling defects, ductal dilation and segmental stenosis [51]. These complications significantly increase patient morbidity, as graft failure due to ITBL often necessitates re-transplantation or may ultimately result in patient death. [26]. The incidence of NAS after LT is reported to be up to 19%, while ITBL rates range between 3% and 16% [52]. Recognized contributors to NAS pathogenesis encompass macrovascular factors such as the thrombosis of hepatic artery, microvascular factors such as extended ischemia duration, graft preservation methods, DCD, or donor vasopressor administration, and immune-mediated factors such as ABO incompatibility, rejection episodes, autoimmune diseases, CMV infection, and chemokine polymorphisms [53]. Injury to the biliary epithelial lining and disruption of the peribiliary microvascular plexus are also linked to the formation of these strictures [54].

The local microbiota has been suggested as an additional contributor to biliary complications after LT. Bile duct infection represent a common cause of post-LT complications, and recent studies have implicated enteric bacteria in the development of cholangitis, leading to reduced prognosis in LT recipients [28].

Cholangitis, characterized by bile duct infection, is another common complication in LT patients and often results from biliary obstruction. Prompt use of antibiotics and drainage procedures is essential to effectively manage this condition and prevent further complications such as sepsis or graft failure. Recurrent episodes of cholangitis can impair patient quality of life and contribute to chronic graft dysfunction, underscoring the importance of proper treatment[55].

1.2.2 Current Management Strategies

The management of biliary complications in LT is multifaceted, involving endoscopic, medical, and surgical interventions. ERCP is the first-line treatment for anastomotic strictures, allowing direct visualization and intervention [56]. Techniques such as balloon dilation and stent placement are often employed to relieve obstructions and maintain bile flow. However, treating non-anastomotic strictures with ERCP can be challenging due to their diffuse nature, necessitating multiple interventions and specialized expertise [57, 58].

Percutaneous transhepatic biliary drainage (PTBD) is an option when ERCP is not feasible, such as in patients with altered anatomy or complex strictures. PTBD provides an alternative route for bile drainage and can be combined with ERCP for better outcomes [59].

Antibiotic therapy remains an essential component of managing biliary infections such as cholangitis. Prophylactic antibiotics are often given prior to biliary interventions to reduce infection risks due to possible pathogen

colonization. However, the rising prevalence of MDR organisms complicates the management of these infections, necessitating broad-spectrum antibiotics or tailored treatments based on microbial sensitivities [60, 61].

In cases where endoscopic or percutaneous approaches are unsuccessful, surgical options are limited. Surgical revision is typically only feasible in select cases, such as converting a duct-to-duct anastomosis to a choledocho-jejunostomy in the presence of persistent bile leakage or isolated stenosis [62]. In most other scenarios, surgical re-intervention is not viable, leaving liver re-transplantation as the only remaining option. Re-transplantation is generally reserved for patients with severe, recurrent biliary complications leading to chronic graft failure. However, limited organ availability and increased morbidity make re-transplantation a challenging option[42].

Recent advances in machine perfusion techniques, such as normothermic and hypothermic machine perfusion, have shown promise in reducing the incidence of biliary complications by improving graft preservation and minimizing ischemic injury [63, 64]. Machine perfusion provides continuous oxygenated perfusion of the liver graft during storage, preserving bile duct viability and reducing the risk of ITBL, particularly in high-risk grafts [12].

1.2.3 Multifactorial Causes of Biliary Complications

Biliary complications in LT are influenced by multiple factors, including IRI, immune responses, microbial influences, surgical techniques, donor and also recipient related factors [65, 66].

As previously discussed, the bile ducts are particularly susceptible to IRI, which makes them vulnerable to necrosis and stricture formation [66]. Strategies to mitigate IRI, such as machine perfusion, have been developed to maintain continuous oxygenation and preserve bile duct integrity. In parallel, ongoing

research is investigating pharmacological interventions and donor management strategies to further reduce IRI and improve graft outcomes [67-69]..

Donor-related variables, including age, comorbidities, and cause of death (e.g., donation following brain death vs. DCD), significantly increase the risk for biliary complications [67]. Grafts from older donors or those with steatosis are more vulnerable to ischemic injury. Liver retrieved from DCD, which undergo longer warm ischemia periods, have an inherently higher risk for ITBL. Management of these high-risk grafts involves careful donor selection, reduced cold ischemia times, and the use of enhanced preservation techniques like hypothermic oxygenated perfusion to mitigate risks [42, 70].

Recipient-specific variables, including underlying liver diseases, anatomical variations, and co-existing conditions, can also influence biliary complication risks. Patients with primary sclerosing cholangitis (PSC) are particularly prone to recurrent biliary issues due to the chronic inflammatory and fibrotic nature of their disease, which often results in bile duct stenosis. In such cases, a choledochojejunostomy may be required instead of standard duct-to-duct anastomosis [71]. While imaging modalities such as magnetic resonance cholangiopancreatography (MRCP) can aid in preoperative assessment, they seldom alter the surgical approach. Instead, perioperative management plays a more critical role in minimizing biliary complications in high-risk recipients [72, 73].

The immune response is a major contributor to the pathogenesis of biliary complications. Acute cellular rejection can lead to bile duct damage if not promptly addressed, while chronic rejection results in progressive bile duct loss, leading to fibrosis and strictures. Immunosuppressive therapy is critical for preventing rejection, but striking the right balance is essential to minimize the

risk of both rejection and infections. Advances in immunosuppressive regimens, such as biologics and precision dosing strategies, aim to optimize outcomes by managing immune responses effectively [74].

The biliary microbiome, traditionally the biliary tract was believed to be sterile – at least in the healthy, is now increasingly recognized for its role in biliary complications following LT. Dysbiosis, characterized by an imbalance in the microbial population, can exacerbate biliary injury, particularly in patients with biliary stents or repeated endoscopic interventions. MDR organisms pose additional challenges, complicating treatment and increasing graft failure risk. Recent studies have highlighted the concept of a "biliary pathobiome," suggesting that shifts in microbial communities may contribute to or protect against biliary complications[23, 75-77]. Advances in next-generation sequencing (NGS) have enabled detailed profiling of the biliary microbiota [78], offering valuable understanding of microbiome-associated risks and guiding personalized treatments.

1.3 Role of the Microbiome in Health and Disease

The human microbiome is now understood to be more than a collection of commensal microbes, it represents an essential symbiotic partner, influencing immune system function, nutrient metabolism, and even host behavior [79]. The gut microbiome, in particular, is central to immune health, supporting the differentiation of immune cells and producing metabolites that modulate both local and systemic immune responses [80]. The diversity of microbial species across body sites like the gut, skin, and biliary tract is crucial for maintaining local homeostasis and therefore host health, with each site harboring unique bacterial compositions adapted to its specific environment [81]. The liver, through the gut-liver axis, interacts closely with the microbiome. It receives a

constant influx of microbial products through portal circulation, which affects immune regulation, liver health, and response to liver injury. This continuous cross-talk plays a role in maintaining immune tolerance and preventing autoimmune responses against beneficial microbial residents. Furthermore, as shown by recent research, disturbances in the gut microbiome can impact liver function and immune responses, illustrating the gut-liver axis's significance in health and disease [82, 83].

1.3.1 Microbiome-Immune Interactions and Dysbiosis

The human microbiome, comprising a complex network of bacteria, fungi, viruses, closely regulates immune functions, shaping both innate and adaptive responses. A balanced microbiome promotes immune tolerance through the generation of beneficial compounds such as short-chain fatty acids (SCFAs) and secondary bile acids [84], which support intestinal barrier function and prevent excessive inflammation. The anti-inflammatory properties of SCFAs play a critical role in supporting epithelial integrity and promote regulatory T-cell (Treg) differentiation, reducing the risk of inflammatory diseases and autoimmunity [85].

When microbial diversity is disrupted, a condition known as dysbiosis, the immune system can become dysregulated, often triggering chronic inflammatory responses. Pathogenic bacteria can overpopulate, releasing inflammatory molecules like lipopolysaccharides (LPS), which activate immune pathways and increase cytokine production [86]. This process is implicated in systemic diseases underscoring the far-reaching impact of dysbiosis [87]. Elevated levels of circulating LPS, for instance, can induce low-grade inflammation, which has been linked to metabolic disorders and liver diseases, highlighting the microbiome's central role in immune regulation[88].

1.3.2 The Gut-Liver Axis

The gut-liver axis refers to the intricate and reciprocal relationship between the gastrointestinal system and the liver [89], allowing them to interact via the portal vein and bile acid pathways (Figure 2). Through this axis, the liver processes metabolites from gut bacteria, including SCFAs, bile acids, and bacterial by-products like LPS. These microbial metabolites are vital for immune modulation within the liver, with bile acids serving as one of the most impactful signaling molecules in this interaction [90].

Bile acids, derived from hepatic cholesterol, are conjugated and then secreted into the small intestine and continuing into the colon [91]. Gut bacteria deconjugate and convert these primary bile salts into acids and subsequently secondary bile acids via specific microbial enzymes [92]. These secondary bile acids not only aid digestion but despite local microbiome-host interaction in the intestine, they also signal within the liver, influencing processes such as cholesterol metabolism and immune tolerance. Liver disease can alter this balance: reduced bile acid flow to the intestines, as seen in cholestatic conditions, results in fewer secondary bile acids, often linked to a reduction in beneficial bacteria that perform key metabolic conversions [93]. Furthermore, bile acid composition changes post-transplant in liver patients, where an increase in secondary iso- and oxo-bile acids signals the reestablishment of beneficial bacteria like Firmicutes while reducing potentially harmful strains [92], including Proteobacteria. This reconstitution supports a healthy microbiome and acts as a protective factor against infections by opportunistic pathogens like *Clostridium difficile*, which secondary bile acids help suppress [94].

In healthy individuals, the gut's defenses, including the epithelial barrier, mucus, and antimicrobial peptides, prevent the translocation of bacteria or their components, such as LPS, into the liver [95]. However, conditions that disrupt

these barriers, such as dysbiosis and inflammation, can increase bacterial translocation. This escalation in microbial influx activates immune responses in the liver, leading to chronic inflammation, fibrosis and, in severe cases, cirrhosis. Liver diseases like PSC and primary biliary cholangitis (PBC) are closely associated with dysbiosis, with studies showing that microbial imbalance can fuel inflammatory liver conditions [96]. In PSC, approximately 75% of cases exhibit significant gut dysbiosis, further indicating the microbiome's role in the gut-liver axis and the development of liver diseases [97, 98].

Emerging research highlights that interventions targeting the gut microbiome may be beneficial for managing gut-liver axis disruptions. For example, increasing microbial diversity with probiotics, prebiotics, or fecal microbiota transplants (FMT) has shown improvements in bile acid metabolism and reducing inflammation [99]. These interventions help maintain bile acid balance, protect gut integrity, and decrease systemic LPS levels, which together support liver health and reduce the risk of inflammation.

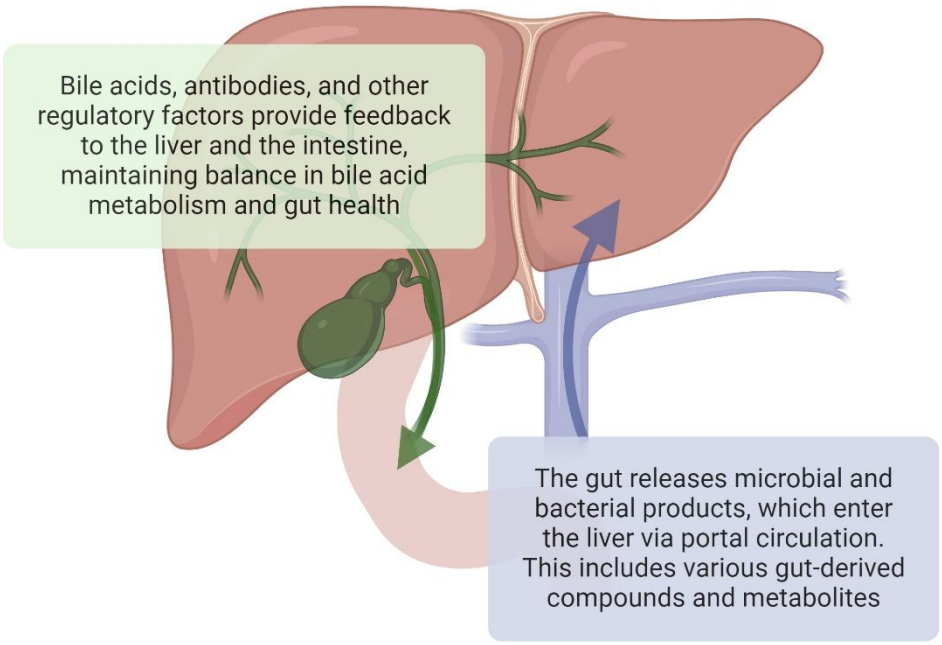

Figure 2 Illustration of the gut-liver axis

1.3.3 Biliary Microbiome and Its Role in Liver Health

The biliary tract contains a specialized microbiome that plays an essential role in liver and biliary health, influencing immune responses, bile acid metabolism, and overall hepatic function. Even in healthy individuals, the biliary tract is not sterile, it hosts a microbial community that includes Proteobacteria and *Enterococcus* spp., which are adapted to the bile's antimicrobial properties [100, 101]. This community is crucial in maintaining a balanced microenvironment that prevents pathogenic invasion. However, in diseases such as PSC, ESLD, or following interventions like LT and endoscopic procedures, significant shifts in this microbiome, often toward pathogenic bacterial populations, can disrupt the delicate balance of the biliary ecosystem [75].

Under normal conditions, bile acids act as both metabolic regulators and antimicrobials, shaping the biliary microbiome by restricting pathogenic bacteria

while supporting a controlled environment for bile-tolerant bacteria, such as Firmicutes and Proteobacteria [102]. These bacteria interact closely with bile acid metabolism, modulating primary and secondary bile acid synthesis. However, dysbiosis in the biliary tract disrupts this balance. In conditions like PSC, there is an observed increase in potentially pathogenic bacteria, such as *Klebsiella* and *E. coli*, which contribute to an inflammatory milieu that can promote fibrosis and other chronic biliary complications [27, 103, 104]. Reduced bile acid secretion, often a consequence of biliary diseases, further facilitates colonization by these harmful bacteria, increasing risks of inflammation and infection.

Dysbiosis in the biliary tract is closely linked to various liver and biliary diseases. In PSC, for instance, the biliary microbiome often undergoes shifts that lead to an enrichment of pro-inflammatory bacterial species [75]. This shift contributes to a carcinogenic microenvironment within the biliary ducts, with elevated levels of Proteobacteria and a concurrent decrease in beneficial bile-metabolizing bacteria. Such microbial imbalances are associated with ongoing inflammation, fibrosis, and disease progression. In patients with non-anastomotic strictures (NAS), which are common post-transplant complications, the reduction of beneficial bacteria such as *Enterococcus* spp. suggests that these microorganisms are crucial in maintaining a stable biliary microenvironment, potentially protecting against disease exacerbation [58, 77, 105, 106].

Medical interventions, such as biliary stenting, ERCP, and recurrent antibiotic therapy, significantly alter the biliary microbiome by introducing intestinal bacteria or favoring the growth of MDR organisms [57]. Patients undergoing ERCP, for instance, often display high rates of colonization by *Enterococcus* and *E. coli*, increasing their susceptibility to post-procedural infections like cholangitis [57]. Prolonged stenting procedures encourage the colonization of

biofilm-forming bacteria, including *Streptococcus* and *Fusobacterium*, which can adhere to the stent surfaces and persist despite antibiotic treatments [23]. These microbial changes are particularly concerning for immunocompromised patients, such as LT recipients, where MDR infections can lead to severe complications and impact graft survival [107, 108].

While data on the biliary microbiome are limited, modern microbial analysis techniques, such as NGS, have shed light on the microbial diversity within bile ducts. These studies reveal distinct microbial profiles across different biliary conditions, underscoring the significance of profiling specific bacteria in understanding disease mechanisms. In one study involving 208 biliary samples, *Enterococcus faecalis* and *Escherichia coli* were found to be predominant in various biliary complications, highlighting a possible role for these bacteria in the pathogenesis of conditions like NAS and anastomotic strictures [109]. Differences in microbial diversity and abundance between healthy individuals and those with biliary diseases support the need for a detailed microbial profile to discern specific bacterial roles in biliary health and disease progression. Further research into the biliary microbiome may provide innovative therapeutic approaches to address biliary complications. Possible interventions include probiotics or targeted antibiotics aimed at restoring microbial balance without disrupting beneficial species [110]. Such microbiome-modulating therapies could help in managing recurrent infections and reducing the incidence of bile duct complications, particularly in high-risk patients like LT recipients [89, 111]. By identifying microbial profiles associated with adverse outcomes, clinicians can develop strategies to prevent infections and improve transplant success, potentially leading to personalized therapies based on individual microbiome compositions [89].

1.4 Biliary Microbiome in Liver Transplantation

1.4.1 Biliary Microbiome Variability in Donor-Recipient Contexts

The biliary microbiome, though less extensively studied than the gut microbiome, is gaining recognition as an influential factor in LT research [89, 108, 111]. This microbial ecosystem, residing in the bile ducts, encompasses species specifically adapted to the bile-rich environment and resistant to its antimicrobial effects [112]. Transplantation entails the integration of donor and recipient microbiomes, a process that may affect the consistency and interactions within the biliary microbial niche. Variability in the biliary microbiome between donors and recipients has been observed, with each transplant scenario representing a unique microbial composition influenced by donor health, age, prior antibiotic treatments, and the recipient's own microbial environment [77, 113].

Studies to date have highlighted that the recipient's pre-existing microbiome can interact with the transplanted biliary microbiome, with complex, and often unpredictable, outcomes [113]. For instance, disruptions in the recipient's immune response due to immunosuppressive therapy can lead to an imbalance, or dysbiosis, favoring pathogenic species [114]. This environment may compromise graft viability by increasing susceptibility to infections and inflammatory responses within the bile ducts [115]. However, a substantial knowledge gap persists regarding the precise mechanisms by which how donor and recipient microbial profiles influence post-transplant outcomes and how these interactions can be modulated to promote long term graft survival.

1.4.2 Donor-Specific Factors in Biliary Microbiome Transfer

The biliary microbiome in donors is affected by a range of variables, such as age, health status, and prior medical treatments, though evidence on how these

factors specifically affect microbial transfer during transplantation remains limited. Some studies suggest that aging and underlying health conditions can alter microbial diversity in various parts of the body, potentially including the biliary tract [113]. However, direct data on how donor age shapes the biliary microbiome and whether this microbiome is transferred intact to the recipient is not yet well-established.

Similarly, donor exposure to antibiotics could play a role in modifying microbial communities within the biliary tract, although this effect has been primarily studied in the gut rather than in bile [116]. Antibiotics, while often necessary, can reduce microbial diversity and promote the persistence of antibiotic-resistant strains [117]. Studies on other microbial environments indicate that antibiotic-resistant species, such as *Escherichia* coli and *Klebsiella* pneumoniae, may proliferate when beneficial microbes are depleted [118]. It is plausible that these dynamics might extend to the biliary microbiome, with implications for post-transplant outcomes if resistant strains are present in the donor's bile. However, research directly linking donor antibiotic exposure to biliary microbiome transfer in LT remains scarce.

1.4.3 Recipient Factors and the Impact of Immunosuppression

The recipient's unique physiological and microbial environment is critical in determining the successful integration of the transplanted biliary microbiome. Pre-existing liver diseases, such as cirrhosis, PSC, and non-alcoholic fatty liver disease (NAFLD), frequently lead to microbial imbalances, or dysbiosis, within the gut-liver axis even before transplantation [119-121]. This dysbiosis can create a challenging post-transplant environment, predisposing the recipient to complications such as infections and inflammatory responses that jeopardize graft stability [111, 122]. Research indicates that certain pro-inflammatory bacterial species, often present in recipients with pre-existing liver dysbiosis,

may persist after transplantation, exacerbating immune reactions and elevating the risk of graft dysfunction [118].

The requirement for immunosuppressive therapy post-transplant adds another layer of complexity. Immunosuppressants are essential to prevent graft rejection, but they can also weaken the recipient's natural regulatory control over microbial populations, increasing the likelihood of microbial imbalance. This immunosuppression creates a favorable environment for potentially pathogenic bacteria to proliferate. For example, *Enterococcus faecalis* and *Clostridium difficile*, both resilient under conditions of reduced immune surveillance, are more likely to thrive in immunosuppressed environments [123]. Their growth raises the risk of conditions such as cholangitis, which can lead to graft failure if not managed carefully [54]. Immunosuppressive therapy is also associated with reduced microbial diversity in both the biliary and gut microbiomes, a factor frequently linked with increased susceptibility to MDR organisms [27, 30].

The effect of MDR strains is especially concerning, as bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* tend to dominate under antibiotic pressure, leading to infections that are complex and difficult to treat with conventional antibiotics [29]. These infections, fueled by reduced diversity and an environment favoring resistance, can undermine graft health and complicate recovery. Moreover, the persistence of MDR organisms in the biliary tract raises the risk of recurrent infections and inflammation, which are challenging to control in immunosuppressed patients [124].

Additionally, prophylactic and therapeutic antibiotic administration, routinely used in the post-transplant period, can significantly alter microbial dynamics. While necessary to prevent immediate post-operative infections, these antibiotics often deplete beneficial bacteria, creating opportunities for MDR

species to proliferate [125]. Research shows that prolonged antibiotic exposure in LT recipients can disrupt microbial resilience, making it difficult to re-establish a balanced biliary microbiome [125]. This interaction between a recipient's underlying dysbiosis, immunosuppressive therapy, and antibiotics underscores the complexity of managing microbial health post-transplant. These combined factors suggest the need for innovative strategies, such as microbiome-targeted therapies or adjusted immunosuppressive protocols, to maintain a stable biliary microbiome and reduce the likelihood of post-transplant complications [126, 127].

Experimental findings from gastrointestinal studies further support the idea that certain bacteria, including *Pseudomonas aeruginosa*, *Enterococcus* species, *Candida* species, and *Serratia marcescens*, may play significant roles in tissue healing and immune modulation [128-131]. These bacteria produce enzymes that interfere with collagen and extracellular matrix stability, potentially delaying healing by promoting local degradation. By activating the body's plasminogen system, which contributes to tissue remodeling, these bacteria may disrupt the delicate balance required for proper healing [131, 132]. When microbial stability is compromised, this balance can shift, leading to increased risks of biliary strictures or anastomotic leaks due to inadequate tissue repair mechanisms. While these effects are documented in gastrointestinal studies, they suggest similar risks within the biliary tract in LT recipients [89].

1.4.4 Research Gaps

Current investigations into the biliary microbiome in the context of LT remain scarce, with the majority of studies focusing on the gut microbiome rather than bile-specific microbial communities [89, 105, 111, 113, 127]. While it is known that microbial dysbiosis in the gut influences immune responses and may contribute to post-transplant complications, the specific contributions of the

biliary microbiome remain underexplored. There is a need to understand how distinct microbial communities within the biliary tract might influence transplant outcomes, particularly given the bile ducts' direct involvement in complications such as cholangitis, ITBLs, and bile duct strictures.

Some preliminary studies have pointed to variations in the biliary microbial composition between LT recipients with and without post-transplant complications, suggesting that certain microbial profiles may be associated with biliary health [23, 77]. However, the data remain scarce and are often derived from small cohort studies, making it difficult to draw general conclusions. Moreover, donor-related factors such as age, medical history, and antibiotic exposure are likely to influence the biliary microbiome, detailed investigations into how these variables shape microbial transfer and affect transplant outcomes are lacking. Addressing these critical knowledge gaps through systematic, large-scale investigations is essential to elucidate the role of the biliary microbiome in LT and to establish predictive microbial signatures that may inform risk stratification and targeted interventions.

1.5 Research Hypothesis and Objectives

1.5.1 Research Hypothesis

This study hypothesizes that the biliary microbiome plays a significant role in determining post-transplant outcomes, with certain microbial profiles at transplantation potentially increasing the risk of biliary complications, including ITBL, anastomotic strictures, leaks, and cholangitis. It is further anticipated that donor clinical factors, such as immunosuppressive and antibiotic treatments are also expected to influence these microbial communities, impacting overall outcomes.

1.5.2 Study Objectives

The study aims to:

1. Characterize the biliary microbiome composition in bile and mucosa at the time of LT, assessing its stability and niche-specific differences.
2. Identify microbial signatures associated with post-transplant biliary complications, including ITBL, anastomotic strictures, biliary leaks, and cholangitis.
3. Evaluate the impact of donor factors (e.g., age, ICU stay, antibiotic/corticosteroid exposure) and graft parameters (e.g., cold and warm ischemia time, machine perfusion) on biliary microbiome diversity and its predictive value for post-transplant outcomes.

2. Materials and Methods

2.1 Materials

The study materials include reagents, equipment, consumables, commercial kits, and software tools. A complete list of product details and suppliers is provided in Appendix A (Table 4-8).

2.2 Methods

2.2.1 Study Design

The here presented work is part of a prospective observational clinical study conducted at the Klinikum der Universität München, a high-volume LT center with expertise in microbiome research. The study aims to investigate the biliary microbiome in LT patients and its potential impact on post-transplant complications. Patients will be followed throughout the transplantation process and post-transplant period to assess microbiome-related changes. The study spans 36 months, including patient recruitment, sample collection, microbiome sequencing, data analysis, and final interpretation. The study was approved by the Institutional Review Board, and all participants provided written informed consent in accordance with the General Data Protection Regulation (GDPR) [133].

2.2.2 Patient Recruitment

LT recipients at the Klinikum der Universität München were enrolled following informed consent. The study population consists of patients undergoing LT, with comprehensive clinical and laboratory data recorded to facilitate post-transplant microbiome analysis.

Inclusion criteria required patients to be at least 18 years old and scheduled for

LT. The study mandated the availability of preoperative clinical and laboratory data and required donor biliary samples to be collected at predefined time points.

Exclusion criteria encompassed patients younger than 18 years, those unable to provide informed consent. Patients undergoing multi-organ transplantation were also excluded. Furthermore, cases where donor and recipient biliary samples were insufficient or unavailable were not included.

Clinical data, including demographic parameters, indication for LT, MELD (Model for End-Stage Liver Disease) score, immunosuppressive therapy, and post-transplant complications, were systematically recorded. Additionally, laboratory parameters such as liver function markers (ALT, AST, bilirubin, ALP), inflammatory markers (CRP, WBC count), renal function indicators (creatinine, GFR), and metabolic parameters (glucose, albumin) were extracted from the hospital database.

To ensure comprehensive longitudinal data collection, patient follow-up visits were scheduled at predefined intervals at 3, 6, 12, 18, 24, and 36 months following LT. These follow-ups included assessments of liver function recovery, antibiotic usage, and the occurrence of post-transplant complications such as biliary strictures, infections, and rejection episodes. Emergency interventions such as ERCP, PTCD, and surgical revisions were documented, along with corresponding clinical outcomes.

2.2.3 Sample Collection

The present work specifically focuses on the intraoperatively obtained donor samples, selected according to pre-established criteria including sample integrity, completeness of clinical metadata, and availability of sequencing data, in accordance with the scientific hypothesis.

Biliary samples were collected intraoperatively at two predefined time points: during backtable preparation and following reperfusion. At the backtable phase, prior to implantation, bile and biliary mucosa samples were obtained from the donor liver in a sterile surgical environment. Using sterile syringes, bile was aspirated directly from the donor bile duct before any flushing or modification of the bile duct anatomy. Biliary mucosa samples were obtained using sterile surgical forceps, ensuring minimal disruption to the surrounding tissue. The collected bile and mucosal specimens were immediately transferred into sterile, pre-labeled tubes and stored on ice until further processing.

Following vascular anastomosis and the re-establishment of hepatic circulation in the recipient, additional bile and biliary mucosa samples were collected at the reperfusion phase. Bile was aspirated from the common bile duct before the completion of biliary reconstruction, ensuring that the sample was representative of early post-reperfusion biliary contents. Mucosal biopsies were again obtained using sterile instruments. All samples were promptly placed into pre-labeled sterile collection tubes and stored on ice before being transported to the laboratory for preservation.

To monitor for potential contamination during sample asservation, negative controls were systematically included. These comprised sterile swabs exposed to the operating room environment and sterile saline rinses of sampling instruments, collected in parallel with clinical specimens. All control samples were processed and analyzed alongside bile and mucosal specimens to detect possible environmental or procedural contamination. To maintain sample integrity, bile samples were immediately aliquoted, snap-frozen, and then stored at -80°. Mucosal biopsies were preserved in RNAlater for subsequent nucleic acid extraction. The time of collection, storage conditions, and relevant perioperative parameters, including donor ischemia time and recipient clinical

details, were meticulously recorded in a standardized database.

All handling procedures were conducted adhered to standardized biosafety and aseptic protocols to minimize contamination and degradation of samples.

2.2.4 RNA-Based Microbiome Profiling and Primer Region Selection

Microbiome profiling in this study is based on total RNA extraction to selectively capture transcriptionally active bacteria in low-biomass compartments such as bile and biliary mucosa. This approach excludes residual or extracellular DNA from non-viable organisms and enhances biological relevance by targeting expressed ribosomal RNA. As demonstrated by Vasapolli et al., RNA-based 16S rRNA sequencing allows reliable profiling of viable bacterial communities across mucosal surfaces, with increased ecological resolution in clinical specimens[134].

Complementary DNA synthesized from total RNA is used as a template for of 16S rRNA gene amplification. In this study, the V1–V2 hypervariable regions are targeted using established primers (27F/338R). This choice is supported by prior observations indicating that V3–V4 primers may cause co-amplification of human mitochondrial DNA in tissue-based samples, thereby compromising bacterial sequence specificity and taxonomic accuracy. The V1–V2 region provides adequate phylogenetic resolution while avoiding such host background interference [135].

2.2.5 Sample Preparation and Mechanical Disruption

Sample processing was initiated immediately after defrosting to prevent nucleic acid degradation. Bile samples were subjected to centrifugation (6000 × g, 5 min) to remove debris before further processing. Mucosal biopsies were minced using sterile scissors and forceps under aseptic conditions.

To ensure effective lysis of bacterial cells, mechanical disruption was performed using bead beating. For tissue samples, mechanical disruption was performed using the TissueLyser LT at 50 Hz for 2 minutes in 700 µL RLT buffer with β-Mercaptoethanol. Bile samples were lysed in (1.4 mm ceramic, 0.1 mm silica, and 4 mm glass beads) through bead-beating.

After centrifugation (15000 × g, 10 min, 4 °C), the resulting supernatant was carefully transferred into a sterile, nuclease-free microcentrifuge tube, ensuring the pellet remained undisturbed to prevent contamination.

2.2.6 RNA Isolation from Bile and Tissue Samples

RNA extraction from bile and biliary mucosa was performed with the QIAGEN RNeasy Mini Kit, applying modified protocols suited for liquid and tissue materials [136].

To facilitate RNA binding, The lysate was combined with 100% ethanol in a 1:1 volume ratio and vortexed before transferring onto RNeasy Mini spin columns (max 650 µL per run). The membrane was then washed with 350 µL of Buffer RW1, followed by two rinses using 500 µL of Buffer RPE. Each washing step was accompanied by centrifugation (10000 × g, 30 seconds) to eliminate salts and other impurities. To completely remove residual ethanol prior to elution, a final centrifugation (10000 × g, 2 minutes) was carried out.

To maximize RNA yield, two elution steps were performed using 52 µL of RNase-free water per elution. Each step included a short incubation at room temperature followed by centrifugation (10,000 × g, 1.5 minutes, 4 °C). In the case of biliary mucosa samples, the elution volume was adjusted to 41 µL per elution step, leading to a final RNA yield of approximately 80 µL. The purified RNA is processed for TURBO DNase treatment to remove any residual DNA contamination.

2.2.7 TURBO DNase Treatment for Genomic DNA Removal

To remove residual genomic DNA contamination, RNA samples isolated from bile and biliary mucosa biopsies were subsequently treated with TURBO DNase. DNase digestion was carried out using the TURBO DNA-free™ Kit (Ambion) [137]. A volume of 80 µL purified RNA was transferred into a sterile, RNase-free tube kept on ice. Subsequently, RNase-free water, TURBO DNase and its buffer, RiboGuard RNase Inhibitor were added to achieve a final volume of 100 µL. The mixture was then incubated at 37 °C for 20 minutes to ensure complete digestion of DNA.

The reaction was terminated by adding TURBO DNase Inactivation Reagent, with a minimum of 2 µL and ideally up to 10 µL, followed by incubation for 3 minutes. The samples were then centrifuged and the supernatant which containing the DNase-treated RNA, was transferred to a new RNase-free tube, ensuring that the milky inactivation reagent was not disturbed or carried over.

To further purify and concentrate the RNA, samples were subjected to ethanol precipitation following the RNA precipitation protocol. This step ensured the removal of any residual inactivation reagent and optimized the RNA yield and integrity for downstream applications.

2.2.8 RNA Precipitation and Quality Control

Following TURBO DNase treatment, RNA samples were precipitated to concentrate and purify the RNA, ensuring optimal integrity for downstream applications. To precipitate RNA, sodium acetate, glycogen, and three volumes of pre-chilled 100% ethanol were added. The mixture was briefly vortexed and then incubated at -80°C for a minimum of 1–2 hours, preferably overnight, to enhance RNA recovery.

The samples underwent centrifugation (15000 × g, 30 min, 4 °C), after which the upper phase was gently aspirated with a 1000 µL pipette. To further purify the RNA and remove salts, 1 mL of ice-cold 70% ethanol was added to the pellet, followed by gentle vortexing and centrifugation. The supernatant was removed in two steps: first by discarding 900 µL with a larger pipette and then by removing the remaining 100 µL. A final brief centrifugation step (10 sec at 4°C) was performed to remove residual ethanol.

Air-drying of the RNA pellet was performed, with drying time minimized to prevent over-drying, which could affect RNA solubility. The dried pellet was resuspended, and in cases where cDNA (complementary DNA) synthesis was immediately performed, 1 µL of random hexamers and dNTPs were added.

A Nanodrop spectrophotometer (Thermo Fisher) was used to assess RNA quality, ensuring minimal protein and salt contamination. RNA integrity was further assessed using an Agilent Bioanalyzer, where an RNA Integrity Number (RIN) $\geqslant$7 was considered acceptable for downstream applications. Samples with sufficient quality were used for cDNA synthesis.

2.2.9 cDNA Synthesis by Reverse Transcription PCR

After RNA precipitation and quality assessment, complementary DNA (cDNA) was generated from purified RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) [138]. All reagents, tubes, and pipettes used in this step were treated to be RNase-free, and work surfaces were decontaminated with 70% ethanol and RNase Zap to prevent RNA degradation. The entire process was conducted on ice whenever possible, and a PCR cabinet was used with a 15-minute UV light treatment prior to handling samples to minimize contamination.

For each reaction, purified 8µL RNA was placed into a PCR tube kept on ice. To this, 1 µL random hexamer primers and 1 µL dNTP mix were added. The reaction was incubated in a thermocycler to denature RNA secondary structures (65 °C, 5 minutes), followed by immediate cooling on ice for at least 1 minute to prevent re-hybridization.

A master mix was prepared by combining RT buffer, $MgCl_2$, DTT, RNaseOUT , and SuperScript III Reverse Transcriptase. The reaction was incubated at 25 °C for 10 minutes to facilitate primer annealing, followed by 50 °C for 50 minutes to synthesize cDNA. Enzyme inactivation was achieved by heating the reaction to 85 °C for 5 minutes.

To remove residual RNA, each reaction received 1 µL of RNase H and was incubated at 37 °C for 20 minutes. The resulting cDNA was either utilized directly for downstream processes, such as 16S rRNA gene amplification.

2.2.10 16S rRNA Gene Amplification by PCR with GoTaq

PCR was performed using GoTaq Polymerase (Promega) with the primer pair 27F (10 pmol/µL) and 338R (10 pmol/µL). Due to the high sensitivity of 16S PCR to contamination, all procedures were carried out in a PCR cabinet, which was pre-treated with UV light for 15 minutes prior to use. To further prevent contamination, filtered pipette tips were used, and gloves were worn throughout the process. Every step was performed on ice to maintain the stability of reaction components.

Each PCR reaction had a final volume of 20 µL, containing 4.0 µL of 5× GoTaq Buffer, 2.0 µL of dNTPs (2.5 mM each, 10 mM total), 0.5 µL of 27F primer, 0.5 µL of 338R primer, 0.2 µL of GoTaq Polymerase, and a variable volume of template DNA, depending on its concentration, typically within the range of 100 pg to 100 ng. The remaining volume was adjusted with nuclease-free water.

Lower template DNA amounts were preferred for sequencing purposes to minimize amplification bias.

PCR conditions included an initial denaturation (95 °C, 3 min), 29 cycles of denaturation (95 °C, 1 min), annealing (56 °C, 40 s), and extension (72 °C, 40 s), followed by a final extension (72 °C, 5 min) and a hold at 4 °C.

To confirm successful amplification, gel electrophoresis was performed. A 2% agarose gel was preferred for optimal resolution, but a 1% agarose gel could also be used depending on the downstream application. DNA bands corresponding to the expected ~330 bp amplicon were visualized under UV light. To ensure reaction specificity and detect contamination, both negative and positive controls were included in each PCR run. The negative control verified the absence of contamination in reagents or handling. The positive control used bacterial DNA to confirm successful amplification and reagent functionality.

Following electrophoresis, samples were used for sequencing. Proper handling procedures were maintained throughout to ensure the integrity of the amplicons.

2.2.11 Library Preparation and Illumina MiSeq Sequencing

After reverse transcription, the V1–V2 hypervariable region of the bacterial 16S rRNA gene was amplified. Amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter) to remove residual primers, nucleotides, and non-specific products. Fragment integrity (~330 bp) was confirmed using the Agilent 2100 Bioanalyzer, and cDNA concentration was quantified with a Qubit fluorometer (Thermo Fisher Scientific) for accurate normalization..

Sequencing was performed by the Helmholtz Centre for Infection Research (Braunschweig, Germany) using an Illumina MiSeq platform with a 2 × 300 bp paired-end strategy. Cluster density was adjusted to yield ~15,000 forward and reverse reads per sample. A PhiX control library was included as an internal

control. Demultiplexing and FASTQ generation were carried out using MiSeq Reporter software.

2.2.12 Quality Control and Sequence Filtering

Initial quality control was conducted using DADA2 in R, an amplicon denoising algorithm that corrects sequencing errors while preserving biological diversity. Sequences were filtered to remove reads with ambiguous bases, sequencing artifacts, and low-quality scores, retaining only reads with a Phred score $\geqslant$20. Additionally, reads shorter than 200 bp were discarded to ensure complete coverage of the V1-V2 region.

Host DNA and other non-target sequences were filtered out using bbduk.sh (BBTools). Three sequential filtering steps were performed to remove large ribosomal subunit sequences, small ribosomal subunit sequences, and human genomic reads, ensuring only microbial DNA was retained for downstream analysis. Paired-end reads were then merged using FLASH, which aligned overlapping forward and reverse reads to reconstruct high-fidelity full-length V1-V2 amplicons.

To further enhance sequence quality, chimera detection and removal were conducted using vsearch in reference-based mode, with sequences compared against the SILVA 16S rRNA reference database. Chimeric sequences, likely generated during PCR amplification, were excluded from further analysis to ensure the reliability of taxonomic classification.

2.2.13 OTU Clustering and Representative Sequence Selection

Following sequence quality filtering, taxonomic classification was performed using both ASV (Amplicon Sequence Variant) inference and traditional Operational Taxonomic Unit (OTU) clustering. ASVs were inferred using DADA2, clustering reads at 100% sequence identity, allowing for high-

resolution discrimination of closely related bacterial taxa. Additionally, sequences were clustered into OTUs at a 97% similarity threshold using vsearch, enabling comparisons with previous studies that employed traditional OTU-based classification.

Representative sequences from each OTU were aligned against the SILVA 16S rRNA reference database, and taxonomic assignments were performed using the Ribosomal Database Project (RDP) Classifier. To further refine taxonomic annotations, a manual curation step was implemented for the 1,000 most abundant phylotypes, cross-referencing annotations against the NCBI taxonomy database. Only sequences corresponding to well-characterized type strains were retained, while environmental sequences and ambiguous taxonomic assignments were excluded to enhance classification accuracy.

To standardize sequencing depth across samples and minimize potential biases introduced by uneven read distribution, rarefaction-based normalization was applied to the OTU table. This ensured comparability across datasets and allowed for robust microbial diversity analysis. The final OTU tables were used for ecological analyses, including alpha diversity metrics (Shannon and Simpson indices) and beta diversity comparisons (Bray-Curtis dissimilarity), as well as taxonomic composition visualization and correlation with clinical metadata.

2.2.14 Statistical and Computational Analysis

Following OTU clustering and taxonomic classification, processed sequencing data were prepared for comprehensive statistical analysis. Normalization procedures were applied to ensure comparability across samples. The final OTU tables were used as the basis for microbial diversity analysis, functional annotation, differential abundance testing, and correlation with clinical

metadata.

Alpha diversity was assessed using Chao1, Shannon, and Simpson indices, which were calculated for each sample to evaluate microbial richness and evenness. Statistical comparisons of alpha diversity between groups were conducted using the Mann–Whitney U test for non-normally distributed data and Welch's t-test for normally distributed data. One-way ANOVA was used for multi-group comparisons.

Beta diversity was evaluated using UniFrac distance metrics to assess microbial compositional differences between groups. These distances were visualized through Principal Coordinates Analysis (PCoA), and PERMANOVA (Permutational Multivariate Analysis of Variance) was applied to test for significant differences in microbial community structure between sample categories.

Differential abundance analysis was conducted using multiple methods to detect taxa that differ significantly across groups. DESeq2 and edgeR were used for differential abundance testing, applying log2 fold-change estimates and Benjamini-Hochberg false discovery rate (FDR) correction to adjust for multiple comparisons. The LEfSe (Linear Discriminant Analysis Effect Size) method was also employed to identify microbial taxa that significantly differed across groups based on their effect size.

To investigate associations between microbial composition and clinical parameters, Spearman's rank correlation was used to analyze the relationship between microbial taxa and continuous variables such as MELD score, donor age, and cold ischemia time. MaAsLin2 (Multivariate Association with Linear Models) was employed to account for confounding factors and identify robust microbial associations with clinical metadata. The effects of antibiotic exposure

on microbial diversity were assessed by stratifying patients based on antibiotic regimens and evaluating microbial shifts using Wilcoxon rank-sum tests and PERMANOVA.

Analyses were performed in R (v4.2.0) and Python (v3.9). QIIME2 was used for microbiome data processing, and phyloseq in R facilitated diversity analysis. Data visualization was carried out using ggplot2 (R) and Seaborn (Python), with GraphPad Prism (GraphPad Software) used for additional statistical comparisons and graphical representations. Results from this computational pipeline provided key insights into the dynamics of the biliary microbiome in LT and its potential impact on graft function and post-transplant outcomes.

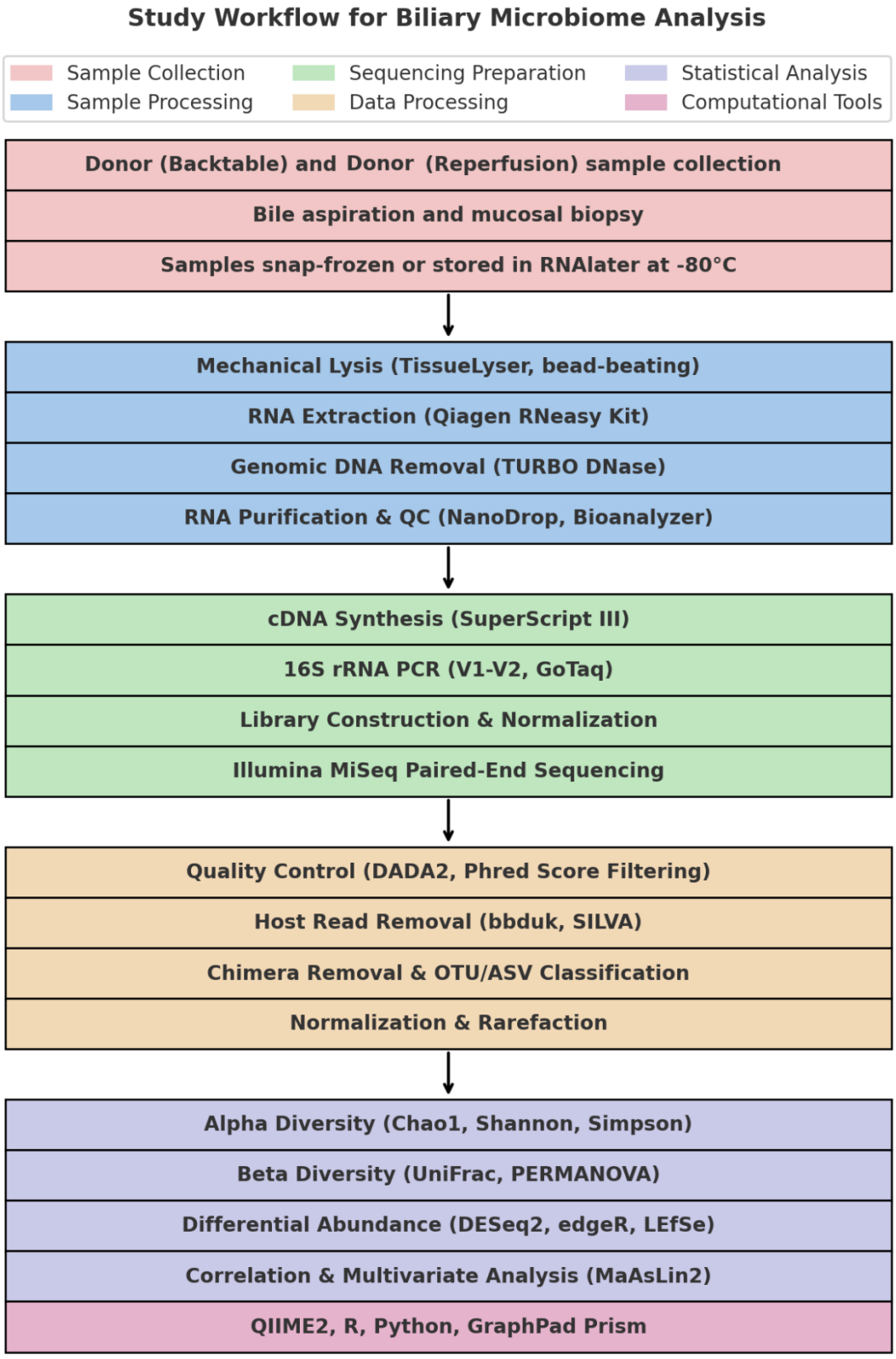

Figure 3 Biliary Microbiome Analysis: Experimental Workflow Overview

3. Results

3.1 Patients and Sample Characteristics

3.1.1 Sample Characteristics

Table 1 Sample Characteristics

Total Number of Samples	49
Backtable Phase Samples	30
Bile Fluid Samples	26
Biliary Mucosa Samples	4
Reperfusion Phase Samples	19
Bile Fluid Samples	14
Biliary Mucosa Samples	5

A total of 49 samples were collected from 31 transplanted liver grafts, comprising 40 bile fluid samples and 9 biliary mucosa samples. Based on the surgical phase of collection, 30 samples were obtained during the back-table phase prior to implantation, while 19 were collected following graft reperfusion in the recipient. Specifically, bile fluid accounted for 26 samples in the back-table phase and 14 in the reperfusion phase, whereas biliary mucosa samples were distributed as 4 during the back-table phase and 5 after reperfusion. Notably, all samples were derived exclusively from donor grafts.

These 49 samples represent a defined subset of the total sample collection carried out over the entire SMILE study period. Over the course of the entire study period, a total of 584 samples were obtained intraoperatively, including both donor and recipient specimens.

3.1.2 Donor Characteristics

Table 2 Donor Characteristics

Number of Donors	31
Median Age (Range)	44 (12–84)
Sex (Male:Female)	15:16
Median BMI (Range)	25.5 (17.9–39.6)
Median ICU Stay (Range, days)	3 (1–14)
Cause of Death (Ischemia by different causes: Cerebral hemorrhage)	52%:48%
At least one Comorbidity	87%
Received Corticosteroids	77%
Received Desmopressin	35%
Received Penicillin-Based Antibiotics	61%
Received Vancomycin/Linezolid	16%

Donor clinical characteristics are summarized in Table 2. The median donor age was 44 years (range: 12–84), with 48% being male. The median body mass index (BMI) was 25.5 (range: 17.9–39.6), and the median ICU stay prior to organ procurement was 3 days (range: 1–14). The primary causes of donor death were evenly distributed between ischemia of various origins (52%) and cerebral hemorrhage (48%). The sex distribution was balanced, with a slight predominance of female donors (52%). Notably, 87% of donors had at least one

pre-existing comorbidity. During their ICU stay, 77% receiving corticosteroids, and desmopressin was administered in 35% of cases. Antibiotic therapy was common, with most donors receiving one to two different agents. 61% were treated with a penicillin-based antibiotic, while 16% received broad-spectrum agents such as vancomycin or linezolid.

3.1.3 Recipient Characteristics

Table 3 Recipient Characteristics

Number of Recipients	31
Median Age (Range)	52 (6–69)
Sex (Male:Female)	17:14
Median Postoperative ICU Stay (Range, days)	28 (4–80)
Machine Perfusion (Yes:No)	23:8
Recipients with Biliary Complications	11 (35%)
Anastomotic Biliary Stricture	7 (22.6%)
Biliary Leak	5 (16.1%)
Cholangitis	3 (9.7%)
Ischemic-Type Biliary Lesion (ITBL)	2 (6.5%)

In total, 31 LT recipients were included in the study, with complete clinical data available (Table 3). Recipients had a median age of 52 years (range 6–69) and 55% were male. The median postoperative ICU stay for recipients was 28 days (range 4–80), reflecting the critical condition and intensive care needs of this cohort. Machine perfusion of the liver graft was utilized in 74% of cases (23 out

of 31), whereas the remaining 26% underwent standard SCS only. Biliary complications developed in 11 of 31 recipients (35%). The most common complication was anastomotic biliary stricture, occurring in 7 patients. Biliary leaks were noted in 5 patients, while cholangitis and ITBL each occurred in 2 patients. Some recipients experienced more than one complication.

3.2 Microbial Composition Across Liver Transplantation

3.2.1 Microbial Composition

Figure 4 presents the microbial composition at the phylum level across the four sample groups: backtable bile, reperfusion bile, biliary mucosa before reperfusion, and biliary mucosa after reperfusion. The figure indicates that Proteobacteria is the most dominant phylum in backtable bile (88.4%) and reperfusion bile (79.1%), which is consistent with the large gray section in the corresponding stacked bars. In contrast, in biliary mucosa before reperfusion (47.6%) and biliary mucosa after reperfusion (45.3%), the relative abundance of Proteobacteria is lower, with an increase in Actinobacteria (25.3% before reperfusion, 27.0% after reperfusion) and Firmicutes (20.6% before reperfusion, 23.8% after reperfusion). Bacteroidetes shows moderate representation across all groups, with its highest relative abundance in biliary mucosa before reperfusion (6.0%).

Figure 5 represents the microbial composition at the genus level across the same groups. The genus-level figure shows that *Bradyrhizobium* represents 59.4% of the total microbial community in backtable bile and 57.2% in reperfusion bile, which is reflected in the large orange section of the stacked bars. In contrast, in biliary mucosa before reperfusion, the composition is more diverse, with *Brevundimonas* (7.4%), *Acinetobacter* (3.9%), *Pseudomonas* (3.2%), and *Sphingobacterium* (3.0%) showing increased representation

compared to bile. In biliary mucosa after reperfusion, these genera remain present with similar relative abundances, with *Brevundimonas* at 8.1% and *Acinetobacter* at 3.8%, indicating a stable distribution in mucosal-associated bacteria.

The genus *Exiguobacterium*, which was identified in differential abundance analysis as significantly reduced in mucosa compared to bile, is reflected in the stacked bar plot with 2.1% relative abundance in backtable bile and 1.6% in reperfusion bile, while it is almost undetectable in biliary mucosa before reperfusion (0.1%) and after reperfusion (0.0%). Similarly, *Haematobacter*, another significantly different genus, follows the same trend, with 6.4% in backtable bile and 4.9% in reperfusion bile, but is nearly absent in biliary mucosa.

The overall microbial composition at both phylum and genus levels suggests that biliary mucosa harbors a more diverse microbial community compared to bile, with a greater representation of Actinobacteria and Firmicutes, while bile is dominated by Proteobacteria. The genus-level distribution shows a more even microbial community in biliary mucosa, whereas bile samples are primarily composed of a single dominant genus. The relative abundances from the table match the stacked bar plots, confirming consistency between the numerical data and the visual representation.

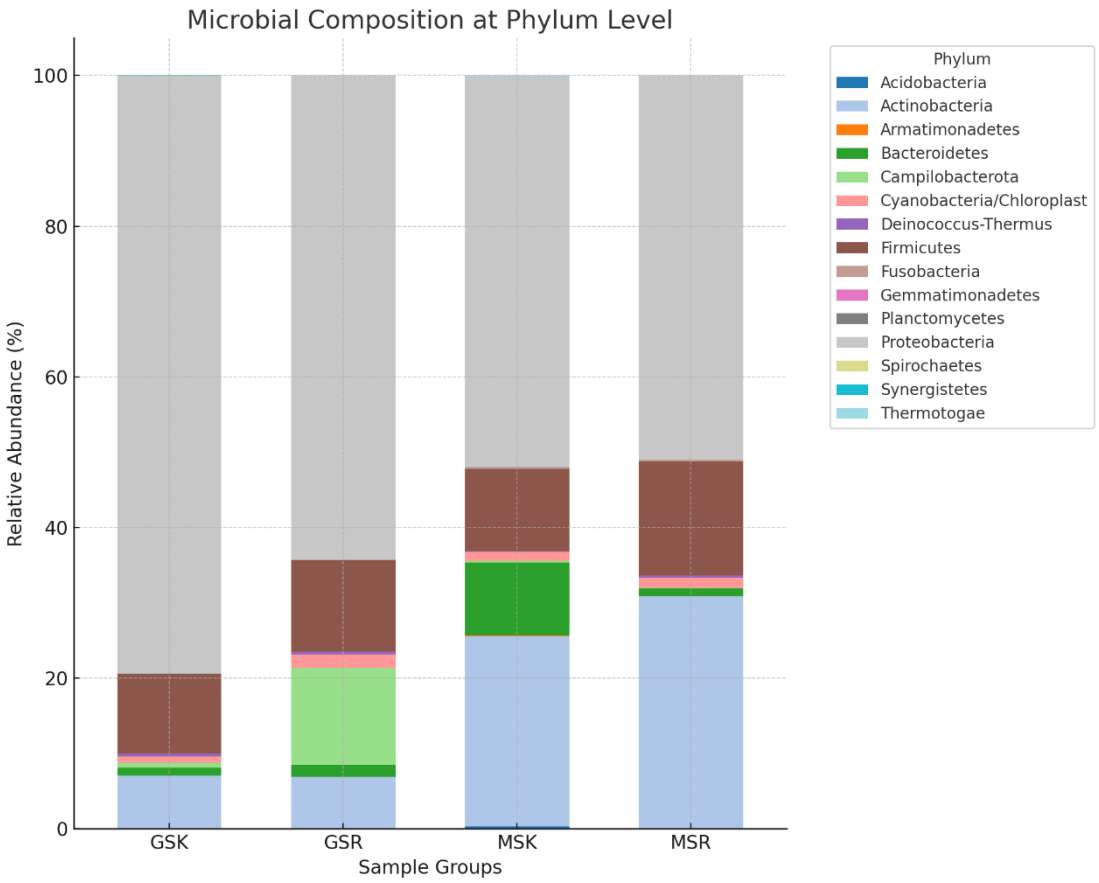

Figure 4 Microbial composition at phylum level: backtable bile (GSK), reperfusion bile (GSR), backtable biliary mucosa (MSK), and reperfusion mucosa (MSR).

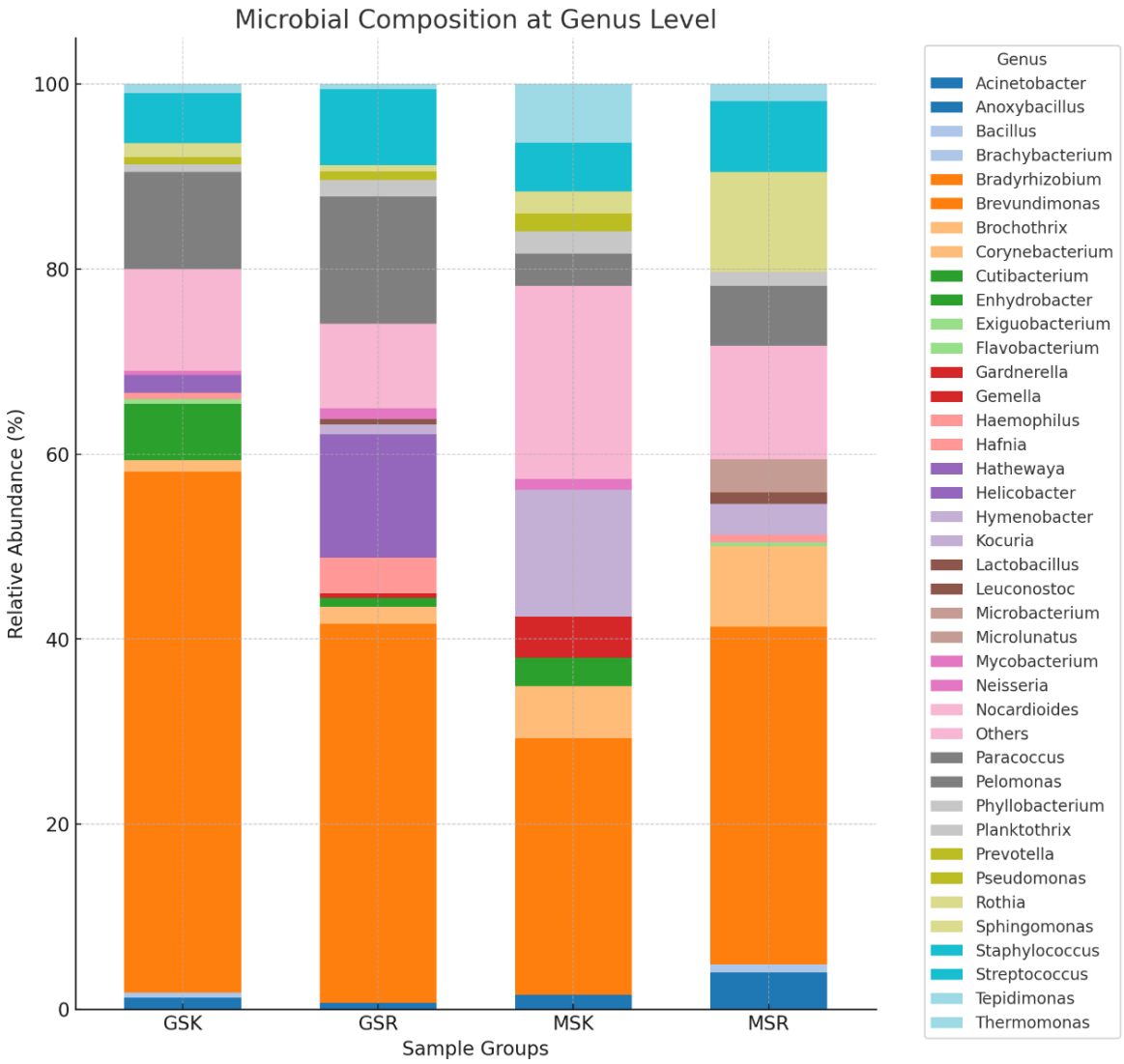

Figure 5 Microbial composition at genus level: backtable bile (GSK), reperfusion bile (GSR), backtable biliary mucosa (MSK), and reperfusion mucosa (MSR).

3.2.2 Differential Abundance Analysis

The differential abundance analysis using DESeq2 and LEfSe identified minimal statistically significant findings, with only *Hyphomicrobium* being significantly depleted in biliary mucosa compared to backtable bile (log2FC = -25.07, FDR = 0.067 in DESeq2). No other bacterial taxa met the statistical thresholds (FDR < 0.1) in these analyses. However, edgeR identified multiple

genera with significant differences across the four comparisons (Figure 6 and 7).

In the comparison between backtable bile and reperfusion bile (Figure 6 A), several genera showed significant shifts in abundance following reperfusion. Genera such as *Haematobacter* and *Exiguobacterium* were significantly more abundant in pre-reperfusion bile, *Brachybacterium* and *Chelatococcus* were significantly enriched in post-reperfusion bile. In the biliary mucosa before and after reperfusion comparison (Figure 6 B), reperfused mucosa showed increased abundance of several genera including *Microlunatus*, *Sphingomonas*, and *Thermomonas*, *Gardnerella* were significantly more abundant in pre-reperfusion mucosa.

In the comparison of biliary mucosa and bile before reperfusion (Figure 7 A), mucosal samples exhibited significantly higher levels of genera such as *Cumulibaater* and *Rubellimicrobium*, while *Exiguobacterium* and *Leuconostoc* were more abundant in bile. Post-reperfusion comparison (Figure 7 B) showed genera such as *Thermomonas* and *Dermabacter* significantly enriched in mucosa, while genera like *Helicobacter* and *Gemella* were more abundant in bile.

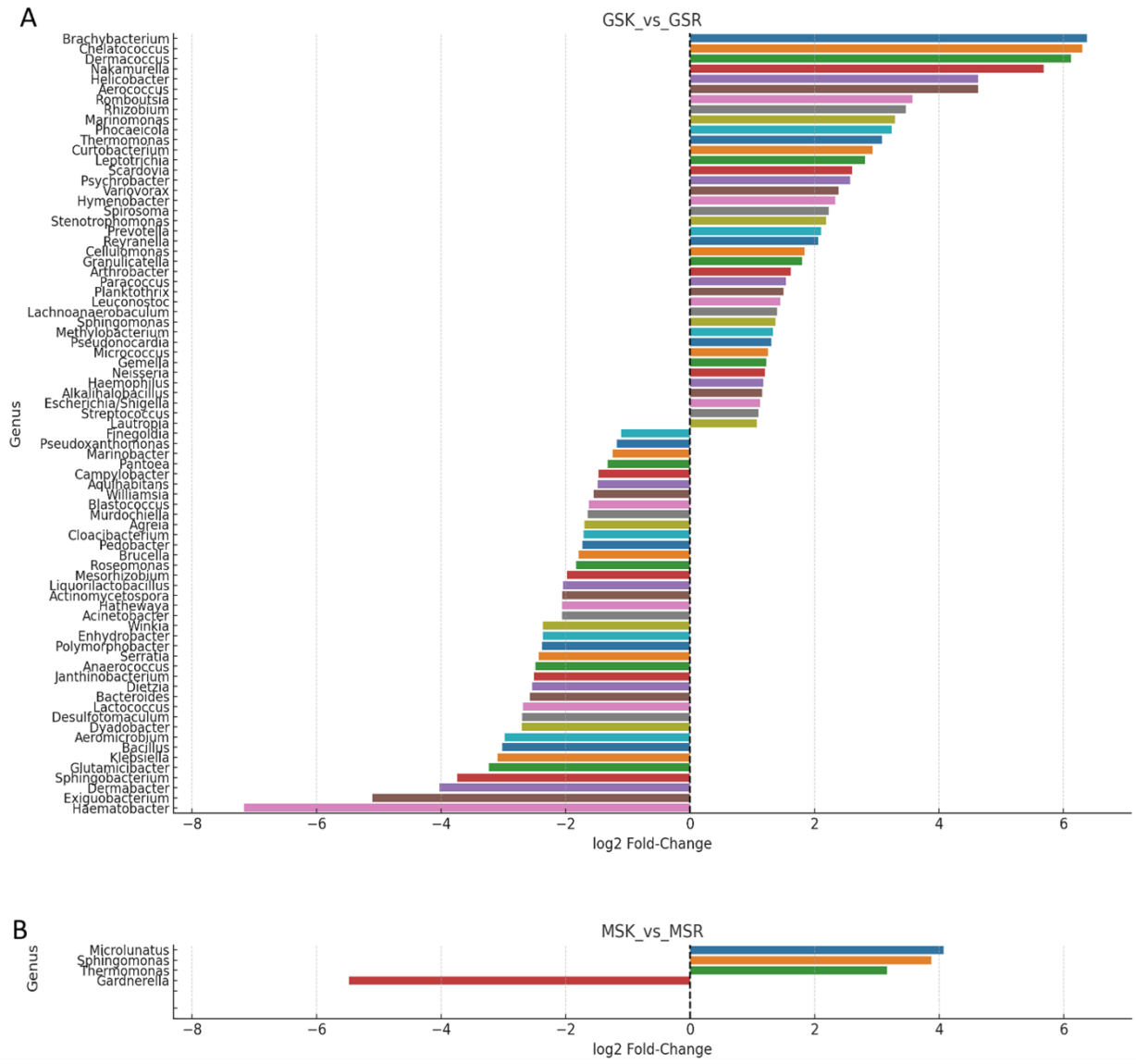

Figure 6 edgeR Differential Abundance: A, bile before and after reperfusion (GSK vs GSR). B, biliary mucosa before and after reperfusion (MSK vs MSR)

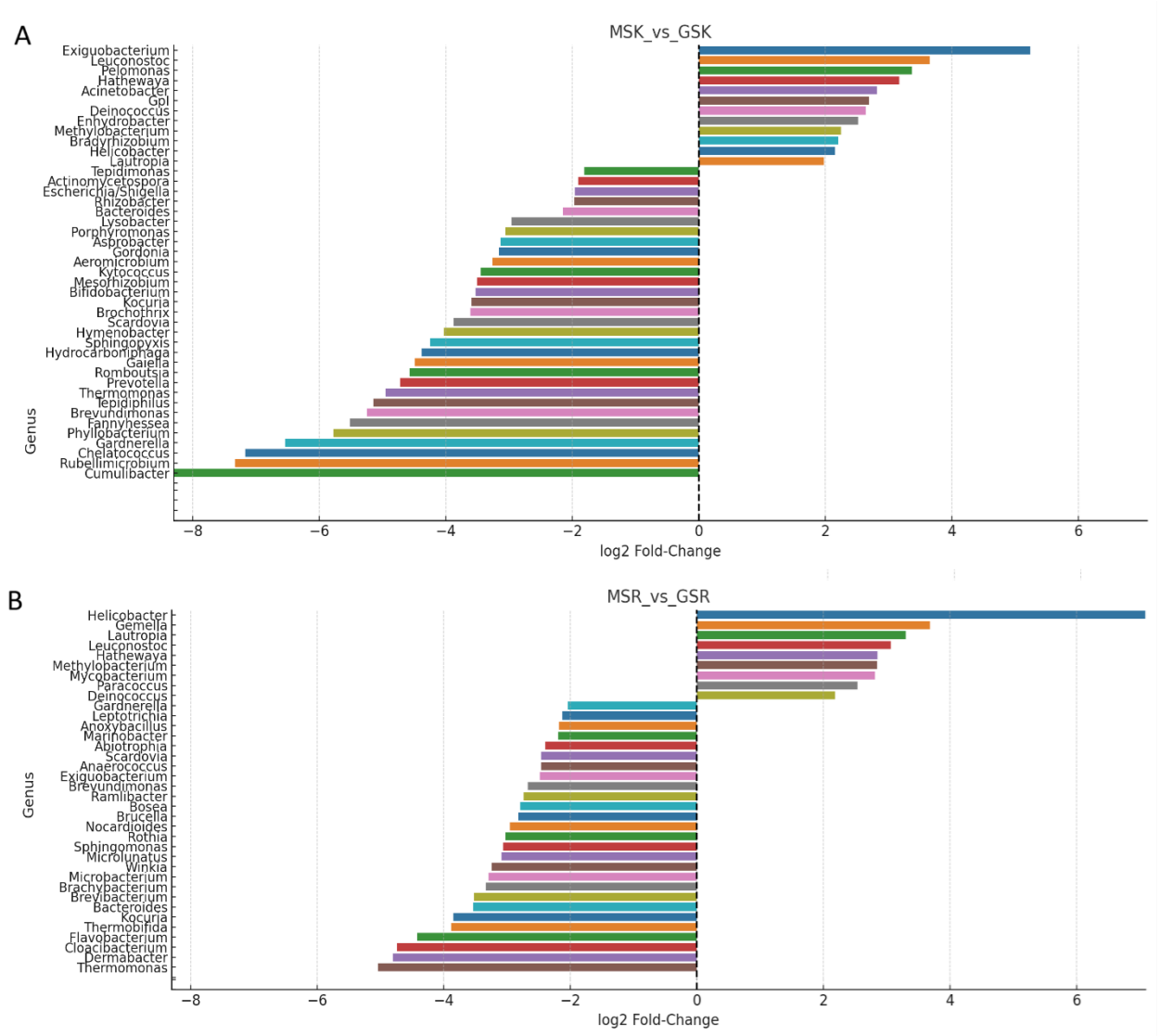

Figure 7 edgeR Differential Abundance: A, biliary mucosa and bile before reperfusion (MSK vs GSK). B, biliary mucosa and bile after reperfusion (MSR vs GSR)

3.2.3 Alpha Diversity

The alpha diversity analysis compared microbial diversity between donor bile before reperfusion and donor bile after reperfusion, biliary mucosa before reperfusion and biliary mucosa after reperfusion, as well as between donor bile

and biliary mucosa. The Shannon, Simpson, and Chao1 indices were used to assess microbial diversity (Figure 8).

The results shows difference in the Shannon index between donor bile and biliary mucosa before reperfusion (Figure 8 C, $p < 0.05$), suggesting a notable variation in microbial diversity between bile and biliary mucosa prior to reperfusion. However, no significant differences were observed in other comparisons, including donor bile before reperfusion versus donor bile after reperfusion, biliary mucosa before reperfusion versus biliary mucosa after reperfusion, and biliary mucosa after reperfusion versus donor bile after reperfusion across all diversity indices ($p > 0.05$).

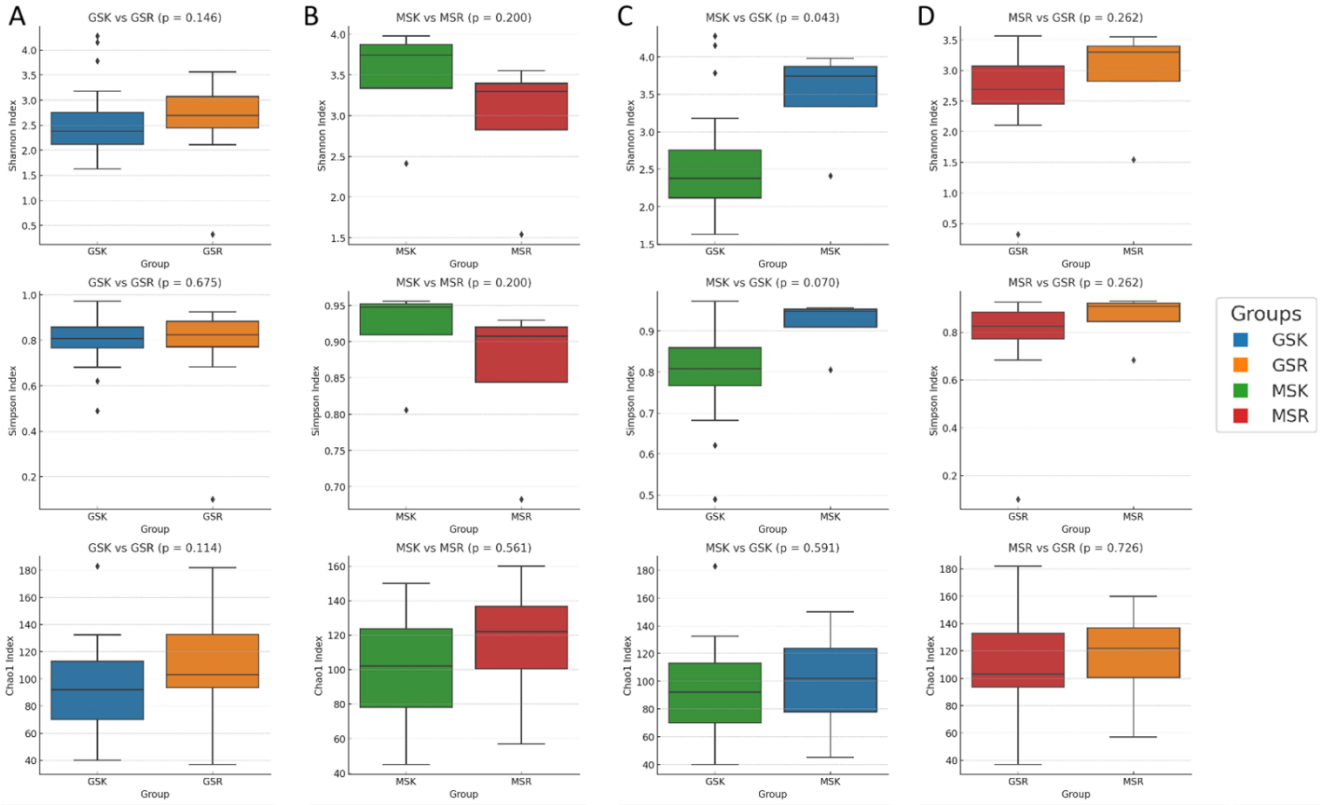

Figure 8 Alpha diversity comparisons between sample groups: A, backtable bile vs reperfusion bile (GSK vs GSR). B, backtable biliary mucosa vs reperfusion biliary mucosa (MSK vs MSR). C, backtable mucosa vs backtable bile (MSK vs

GSK). D, reperfusion mucosa vs reperfusion bile (MSR vs GSR).

3.2.4 Beta Diversity

The beta diversity analysis was conducted to evaluate microbial compositional differences across donor bile and biliary mucosa samples collected during LT at the back table and reperfusion stages. PCoA was used to visualize microbial clustering, and Pairwise PERMANOVA was applied to assess statistical differences between groups. The PCoA plot (Figure 9) showed a clear distinction between donor bile and biliary mucosa, indicating inherent differences in microbial composition between these anatomical sites. In contrast, microbial diversity remained stable between the back table and reperfusion stages within each tissue type. Confidence ellipses were included in the visualization to represent group dispersion.

Pairwise PERMANOVA results showed no significant differences between donor bile samples collected at the back table and reperfusion stages ($F = 0.9650$, $p = 0.4430$) or between biliary mucosa samples at these stages ($F = 0.5072$, $p = 0.7880$), indicating no detectable shifts in microbial composition during this period (Figure 6 B and 6 C). A significant difference was observed between biliary mucosa and bile during back table preparation ($F = 2.2399$, $p = 0.0460$), supporting distinct microbial compositions between these compartments, though this result was not significant after multiple testing correction (Figure 6 D). The comparison between biliary mucosa and bile at reperfusion showed no significant difference ($F = 1.1104$, $p = 0.2880$), with confidence ellipses suggesting some degree of separation (Figure 6 E).

These results indicate that while donor bile and biliary mucosa exhibit distinct microbial compositions, microbial diversity within each tissue type remains

stable across transplantation stages. The lack of significant short-term changes suggests that the transplantation process does not induce major shifts in microbial communities at these time points.

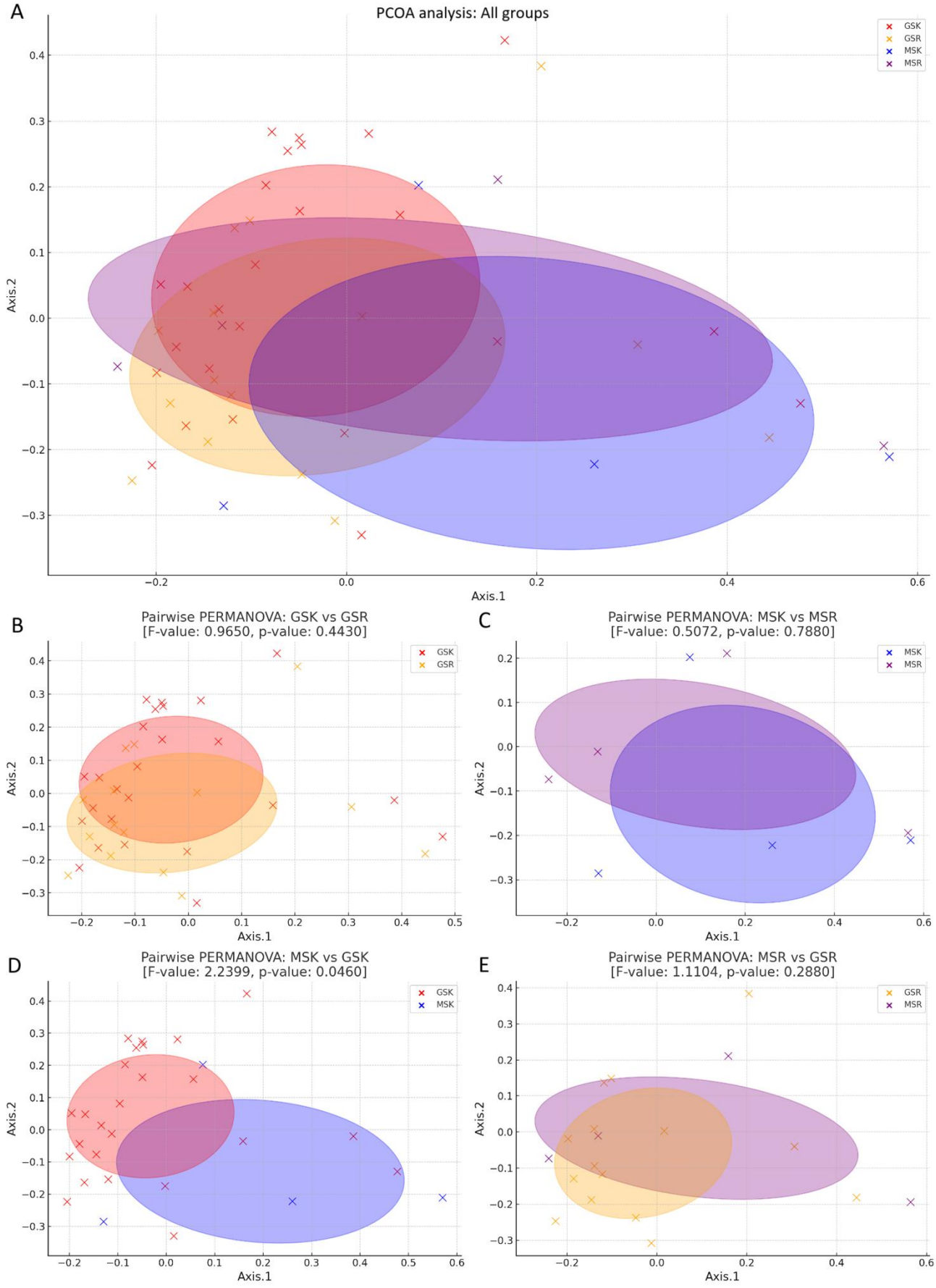
A
PCOA analysis: All groups
GSK
GSR
MSK
MSR
Axis.1
Axis.2
B
Pairwise PERMANOVA: GSK vs GSR
[F-value: 0.9650, p-value: 0.4430]
C
Pairwise PERMANOVA: MSK vs MSR
[F-value: 0.5072, p-value: 0.7880]
D
Pairwise PERMANOVA: MSK vs GSK
[F-value: 2.2399, p-value: 0.0460]
E
Pairwise PERMANOVA: MSR vs GSR
[F-value: 1.1104, p-value: 0.2880]

Figure 9 beta diversity and PCoA analysis: A, all groups: backtable bile (GSK), bile reperfusion (GSR), backtable biliary mucosa (MSK), reperfusion biliary mucosa (MSR). B, GSK vs GSR. C, MSK vs MSR. D, MSK vs GSK. E, MSR vs GSR.

3.2.5 Effect of Machine Perfusion on Biliary Microbiota

Alpha diversity metrics (Shannon, Simpson, Chao1) show no statistically significant differences between MP and No MP groups, suggesting that microbial richness and evenness remain unaffected by perfusion status (Figure 10 and 12). Similarly, beta diversity analysis using PCoA reveals no distinct clustering between MP and No MP groups, with PERMANOVA results confirming the absence of significant differences in overall microbial diversity (Figure 11 and 13). These findings indicate that machine perfusion does not substantially influence the diversity of the biliary microbiota.

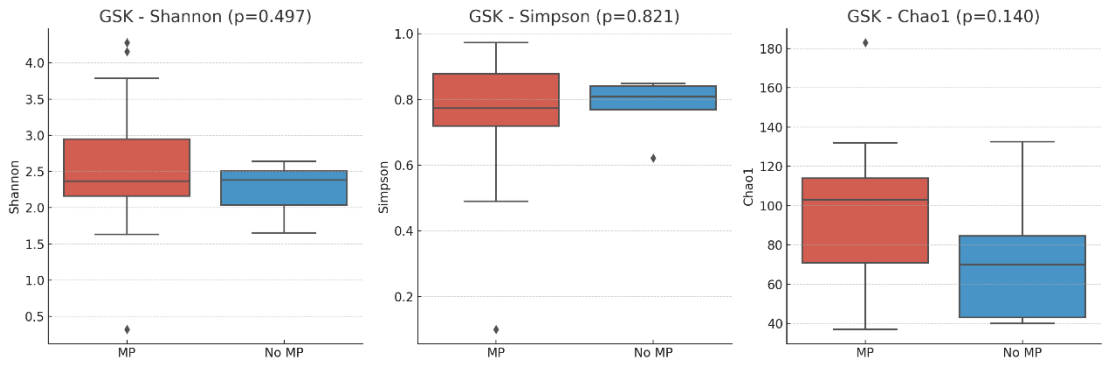

Figure 10 Alpha diversity of backtable bile (GSK) assessing the effect of machine perfusion (MP)

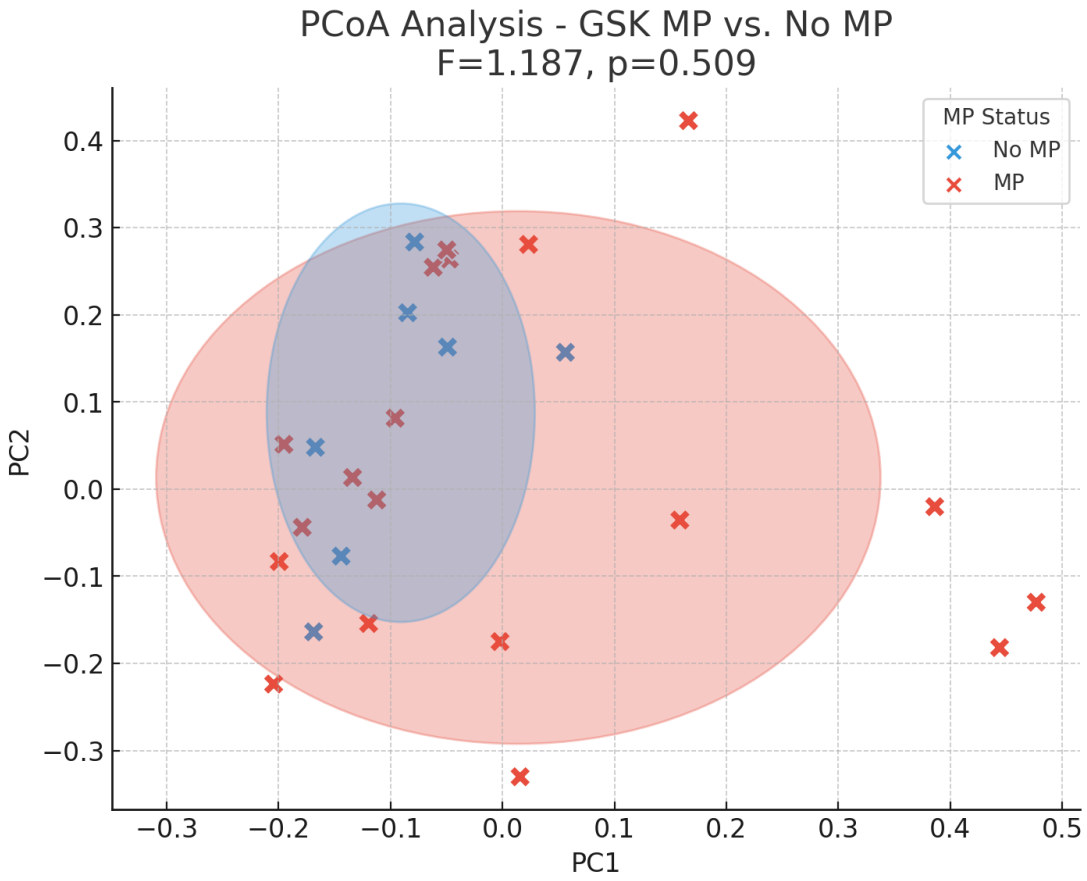

Figure 11 PCoA of beta diversity in backtable bile (GSK) assessing the effect of machine perfusion (MP)

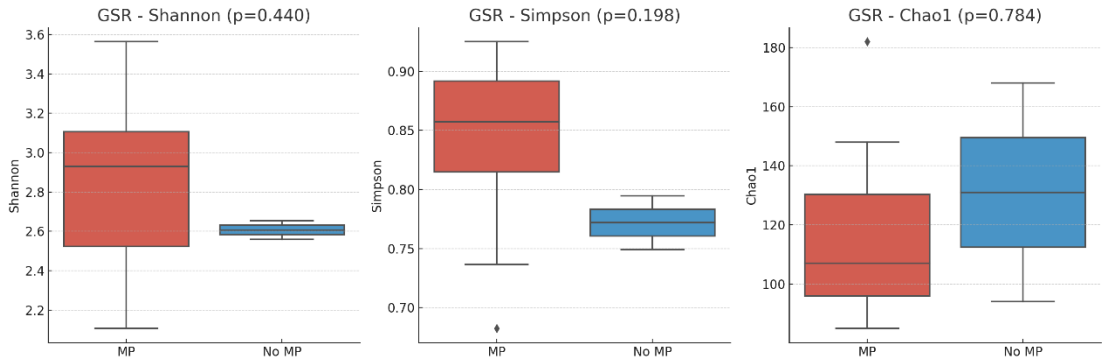

Figure 12 Alpha diversity of reperfusion bile (GSR) assessing the effect of machine perfusion (MP)

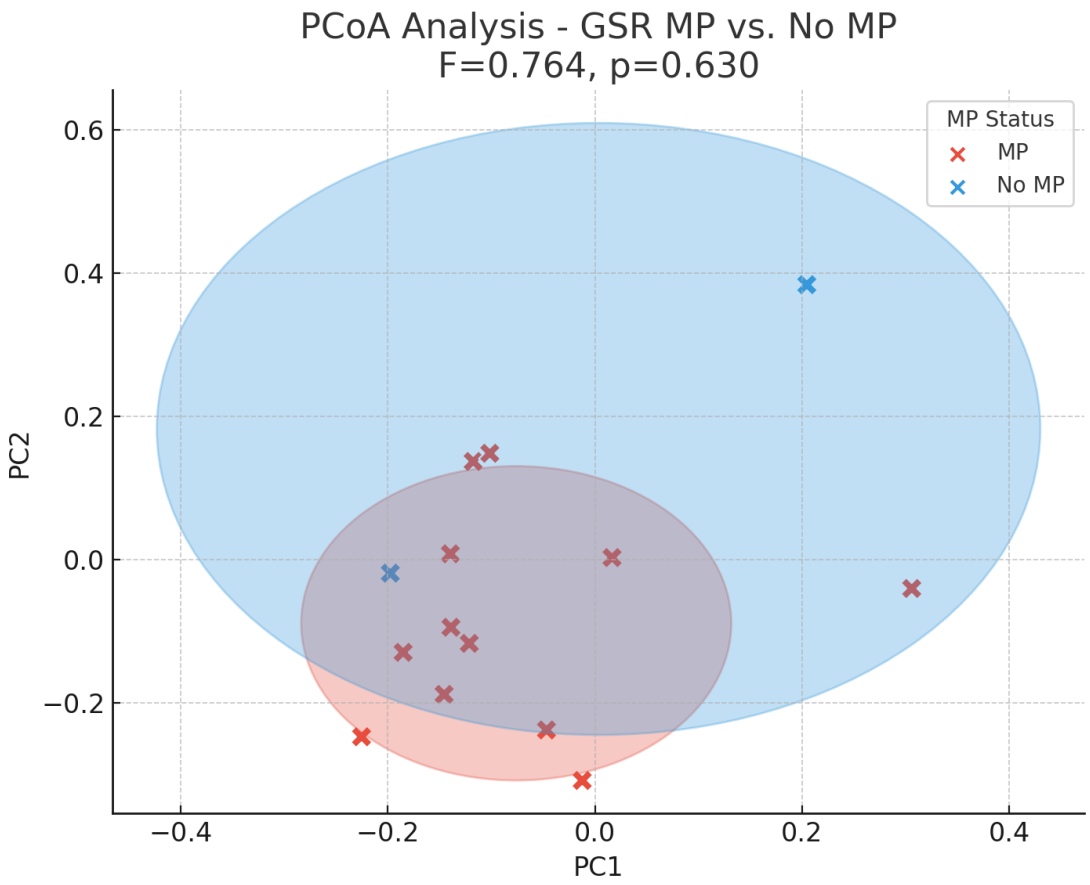

Figure 13 PCoA of beta diversity in reperfusion bile (GSR) assessing the impact of machine perfusion (MP)

3.3 Post-Transplant Outcomes and Biliary Complications

3.3.1 Alpha diversity

Alpha diversity (Shannon, Simpson, and Chao1 indices) was analyzed separately in bile fluid (Figure 14) and bile duct mucosa samples (Figure 15), comparing different biliary complications.

In bile samples, microbial richness is reduced in ITBL cases (Figure 14 A), where the Chao1 index was significantly lower ($P < 0.001$). The Shannon and Simpson indices showed no significant difference ($P = 0.293$ and $P = 0.795$,

respectively). Anastomotic stenosis and biliary leak did not show significant differences in any alpha diversity metric ($P > 0.2$ for all). However, a trend of increased Shannon diversity was seen in cholangitis cases ($P = 0.259$), while Chao1 richness was significantly higher in the cholangitis group (Figure 14 D, $P = 0.007$). When comparing groups based on the total number of complications (0, 1, or 2), no significant differences were observed across any diversity metric ($P > 0.9$).

In mucosal samples, no comparisons reached statistical significance. Biliary leak cases showed lower Shannon diversity ($P = 0.235$) and Chao1 richness ($P = 0.055$) compared to non-leak cases, though these differences were not significant. Anastomotic stenosis cases also showed slightly reduced diversity and richness, but without statistical significance ($P > 0.15$). Total complication count had no significant impact on microbial diversity either ($P > 0.3$).

Overall, the most significant finding was the markedly reduced richness (Chao1) in bile fluid of ITBL cases ($P < 0.001$), while Chao1 richness was significantly higher in cholangitis cases ($P = 0.007$). All other comparisons were non-significant.

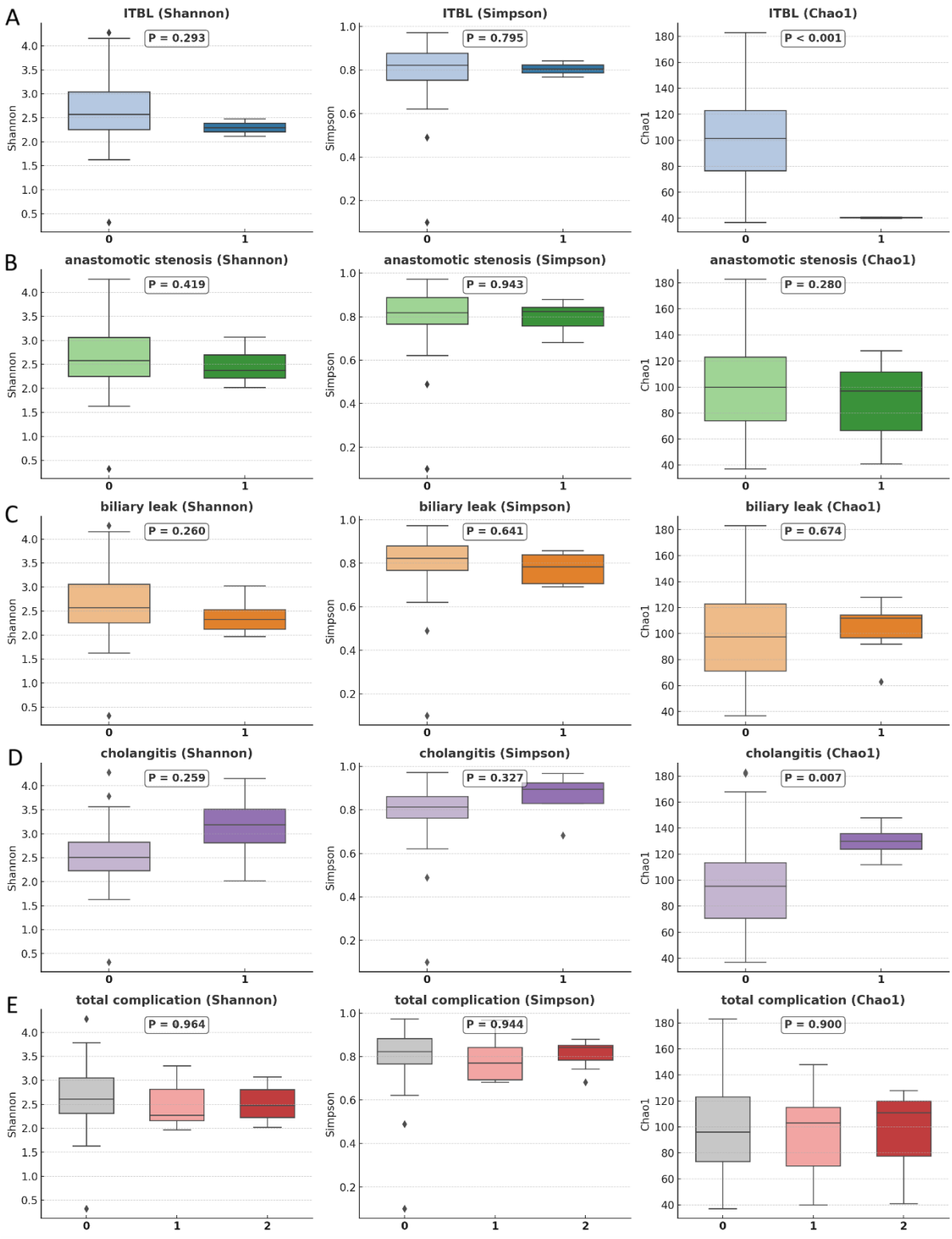

Figure 14 Alpha diversity of bile samples in cases with (1) and without (0) complications, including ITBL (A), anastomotic stenosis (B), biliary leak (C), cholangitis (D), and total complications (E)

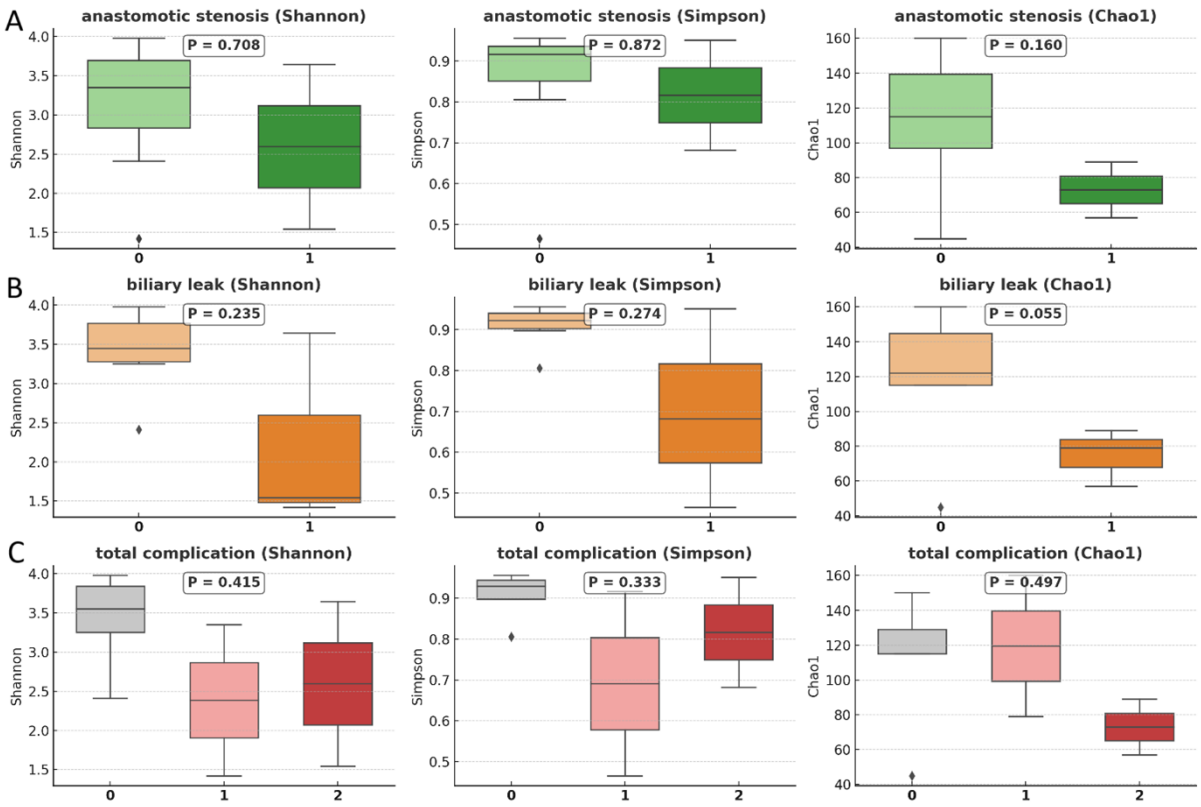

Figure 15 Alpha diversity of biliary mucosa samples in cases with (1) and without (0) complications, including anastomotic stenosis (A), biliary leak (B), and total complications (C)

3.3.2 Beta Diversity

Figure 16 and 17 present the results of the PERMANOVA analysis evaluating microbial beta diversity differences between groups stratified by the presence (1) or absence (0) of specific biliary complications. The PCoA plots illustrate microbial community clustering for bile and mucosa samples, with 95% confidence ellipses representing group-level variation. The corresponding PERMANOVA p-values indicate statistical comparisons between individuals with and without each complication.

None of the p-values reached statistical significance at the $p < 0.05$ threshold, indicating no significant differences in microbial beta diversity between affected and unaffected groups. In bile samples, the comparison between individuals

with cholangitis (1) and without cholangitis (0) exhibited the lowest p-value (p = 0.08), suggesting a potential trend, though not meeting significance criteria. Other comparisons, including ITBL (yes vs no), anastomotic stenosis (yes vs no), biliary leak (yes vs no), and total complication categories (2 vs 1, 2 vs 0, 1 vs 0), yielded p-values exceeding 0.10, further indicating no meaningful separation in microbial community composition between affected and unaffected groups.

Similarly, in mucosa samples, none of the comparisons demonstrated statistically significant differences, with p-values exceeding 0.10 across all categories. The observed microbial beta diversity does not appear to distinguish between individuals with and without biliary complications within the analyzed dataset.

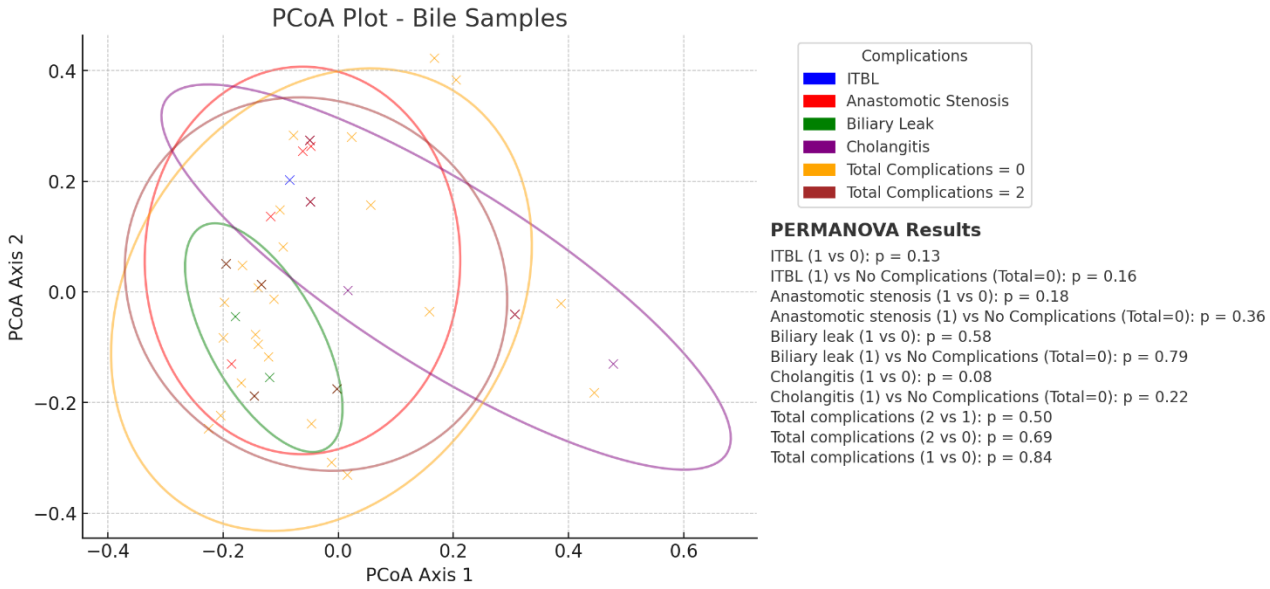

Figure 16 PERMANOVA analysis and PCoA of beta diversity of bile samples in cases with (1) and without (0) complications, including ITBL, anastomotic stenosis, biliary leak, cholangitis and total complications

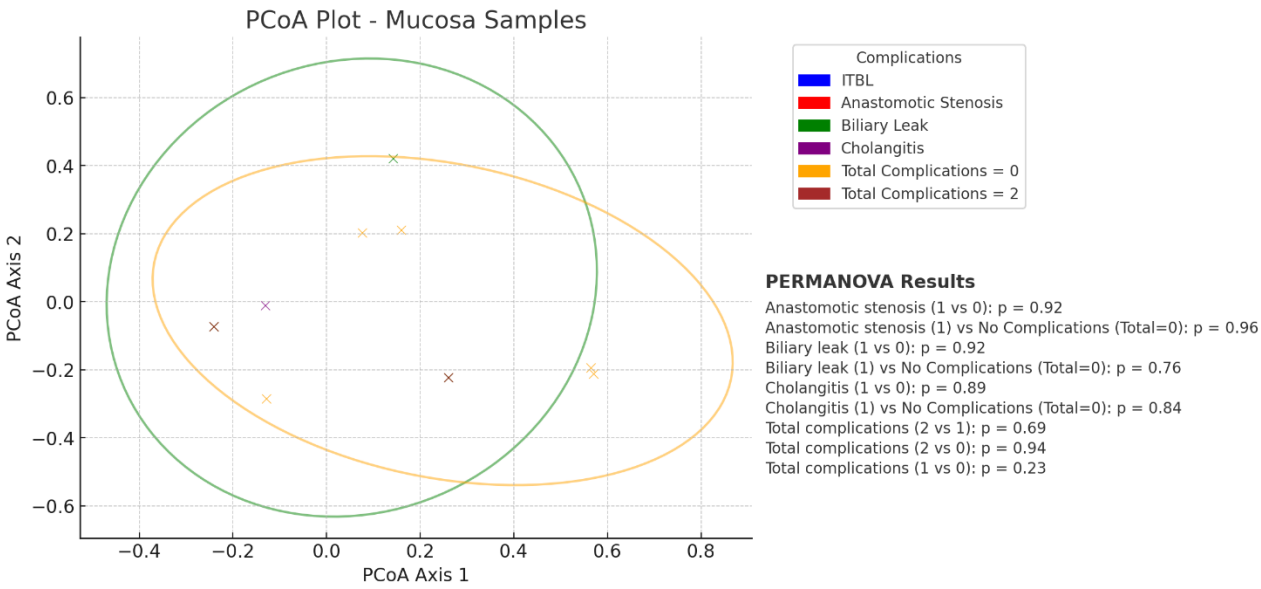

Figure 17 PERMANOVA analysis and PCoA of beta diversity of biliary mucosa samples in cases with (1) and without (0) complications, including anastomotic stenosis, biliary leak, cholangitis and total complications

3.3.3 Microbiota Abundance in Biliary Complications

The LDA Score plot (Figure 18) and the cladogram (Figure 19) identifies distinct bacterial taxa that differentiate patients based on postoperative outcomes. In patients with no complication, the microbiota included members of the order Campylobacterales (class Campylobacteria) and the families Burkholderiaceae and Acetobacteraceae, as well as the genus *Roseomonas*. Cholangitis samples exhibited the broadest range of taxa, with enriched genera including *Streptococcus*, *Haemophilus*, *Neisseria*, *Pseudoalteromonas*, *Brochothrix*, *Mesorhizobium*, *Aureimonas*, *Murdochiella*, *Asprobacter*, *Fusicatenibacter*, and *Rothia*. Several taxa were only classified at the family level, such as Yersiniaceae, Halomonadaceae, *Hyphomonadaceae*, and *Aurantimonadaceae*, while some were identified only at species level (e.g., "*elegans*", "*marmoris*", and "*parahominis*"). Biliary leak samples were characterized by enrichment of

the family *Chitinophagaceae* and the genus *Delftia*, whereas anastomotic stenosis was associated with the family *Cytophagaceae*, represented by the genus *Spirosoma*. ITBL-affected samples displayed a distinct microbial composition, including genera such as *Alkalihalobacillus*, *Gordonia* (*G. amarae*), *Janthinobacterium*, *Shinella*, *Rheinheimera*, *Paenirhodobacter*, *Rubellimicrobium*, and *Streptococcus* (*S. anginosus*), along with broader classifications such as the order Bacteroidales and phylum Acidobacteria.

The edgeR differential abundance analysis (Figure 20) further supports and the findings from the LDA-based taxonomic comparison. Among taxa enriched in cholangitis cases, several genera identified in the LDA analysis such as *Murdochiella*, *Aureimonas*, and *Neisseria* were also significantly elevated or differentially abundant in edgeR analysis, providing cross-validation of their association with this condition. In addition to the overlapping taxa, edgeR analysis identified several additional genera not detected in LDA but significantly associated with cholangitis, including *Shewanella* and *Aeromicrobium*. In the analysis of biliary leak and anastomotic stenosis, edgeR identified additional taxa that were not detected by LDA. For example, *Haematobacter* was enriched in biliary leak cases, while *Desulfotomaculum* exhibited increased abundance in anastomotic stenosis. Notably, *Brachybacterium* was enriched in both conditions.

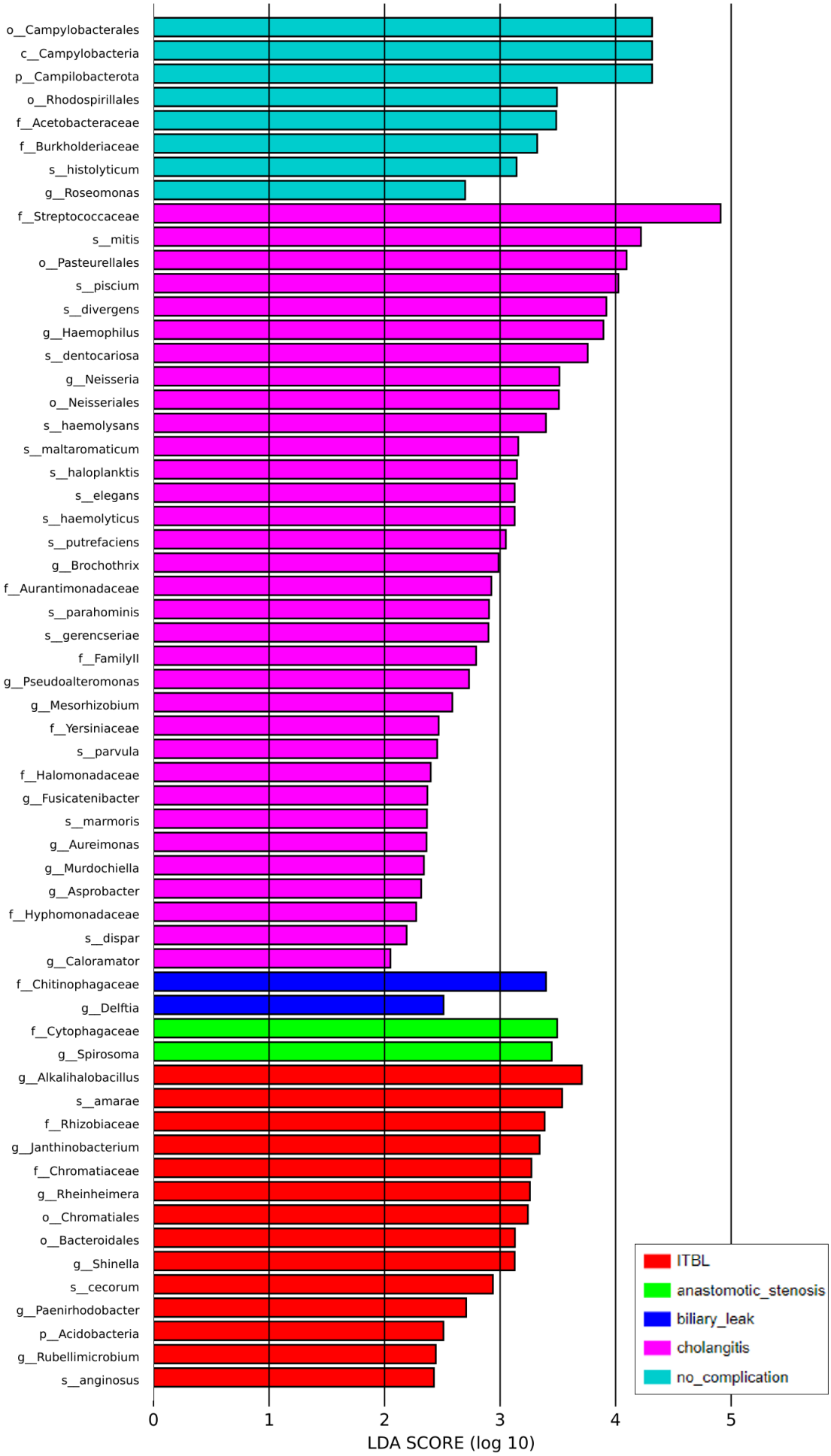
o__Campylobacterales
c__Campylobacteria
p__Campilobacterota
o__Rhodospirillales
f__Acetobacteraceae
f__Burkholderiaceae
s__histolyticum
g__Roseomonas
f__Streptococcaceae
s__mitis
o__Pasteurellales
s__piscium
s__divergens
g__Haemophilus
s__dentocariosa
g__Neisseria
o__Neisseriales
s__haemolysans
s__maltaromaticum
s__haloplanktis
s__elegans
s__haemolyticus
s__putrefaciens
g__Brochothrix
f__Aurantimonadaceae
s__parahominis
s__gerencseriae
f__FamilyII
g__Pseudoalteromonas
g__Mesorhizobium
f__Yersiniaceae
s__parvula
f__Halomonadaceae
g__Fusicatenibacter
s__marmoris
g__Aureimonas
g__Murdochiella
g__Asprobacter
f__Hyphomonadaceae
s__dispar
g__Caloramator
f__Chitinophagaceae
g__Delftia
f__Cytophagaceae
g__Spirosoma
g__Alkalihalobacillus
s__amarae
f__Rhizobiaceae
g__Janthinobacterium
f__Chromatiaceae
g__Rheinheimera
o__Chromatiales
o__Bacteroidales
g__Shinella
s__cecorum
g__Paenirhodobacter
p__Acidobacteria
g__Rubellimicrobium
s__anginosus
0
1
2
3
4
5
LDA SCORE (log 10)
ITBL
anastomotic_stenosis
biliary_leak
cholangitis
no_complication

Figure 18 LDA score plot identifying bacterial taxa associated with postoperative complications, including cholangitis, biliary leaks, anastomotic stenosis, ITBL and no complications

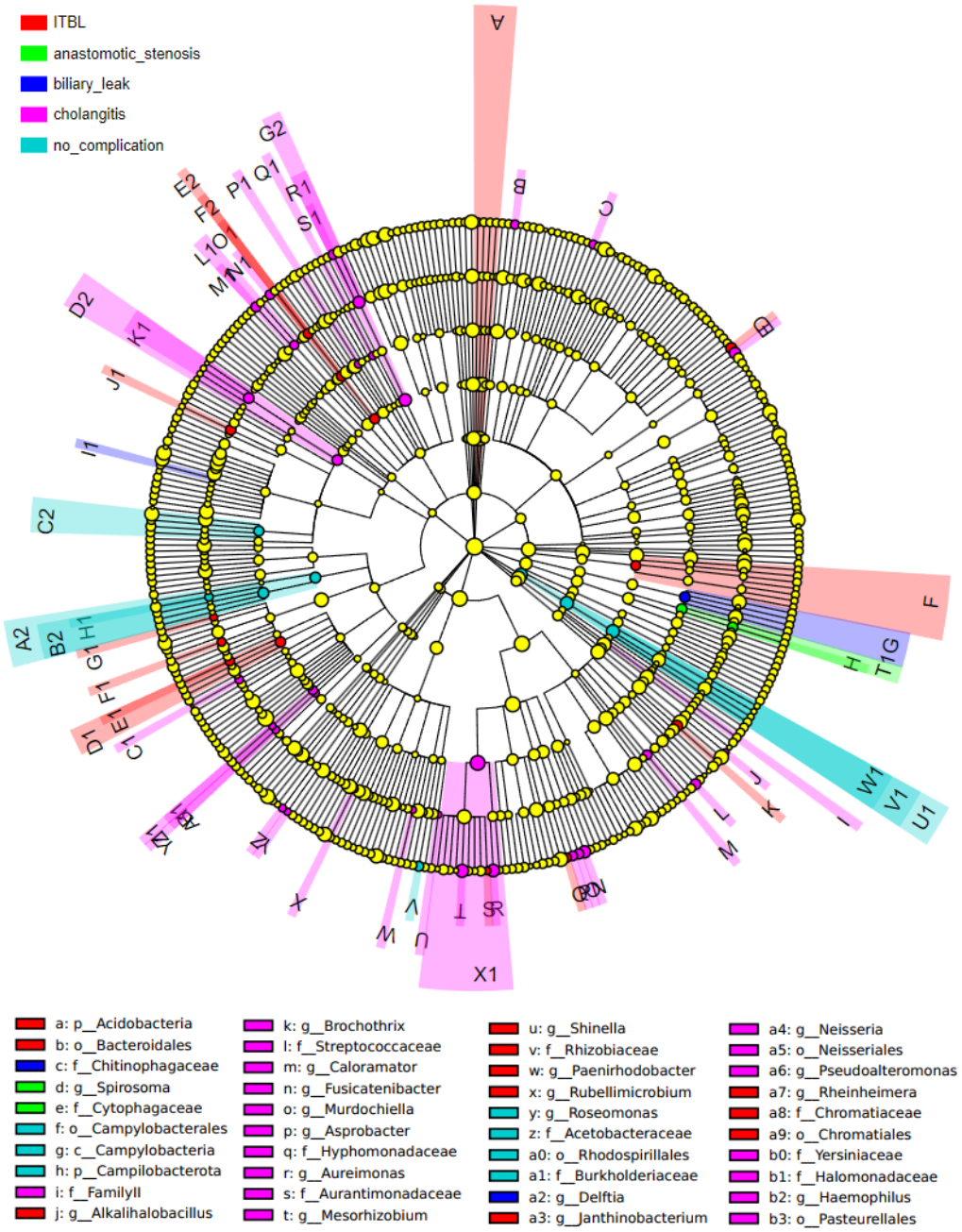

Figure 19 LEfSe cladogram highlighting bacterial taxa associated with ITBL, anastomotic stenosis, biliary leak, cholangitis, and no complication.

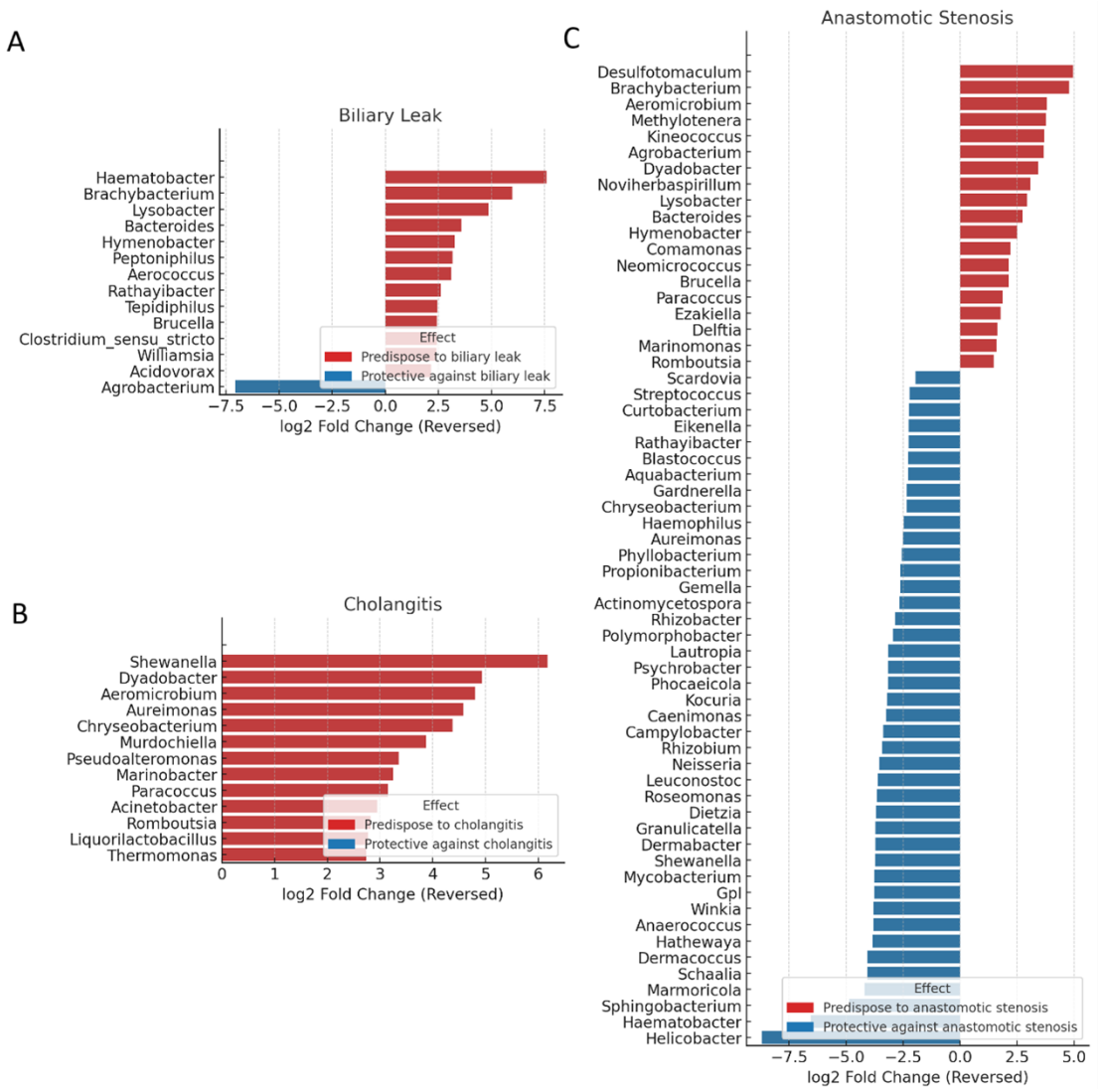

Figure 20 Differential abundance analysis of bacterial taxa associated with biliary leak (A), cholangitis (B), and anastomotic stenosis (C) using edgeR

3.3.4 Associations with MELD Score

To explore the potential influence of donor-derived biliary microbiota on post-transplant graft function, microbiome profiles collected from the donor bile duct at the time of LT were analyzed and correlated with the recipient's MELD score over 12 months following LT.

15 patients were included based on the availability of complete 12-month MELD score follow-up data. To evaluate post-transplant graft function, a MELD score of ⩽9 was defined as good graft function, while a score >9 was considered poor graft function (Figure 21). The 3-month MELD score was used to assess short-term recovery status (Figure 22), whereas the 12-month MELD score was used to evaluate long-term recovery (Figure 23).

Figure 22 shows the differential biliary microbial composition at the time of LT between patients with good and poor short-term recovery outcomes. The cladogram highlights distinct taxonomic groups enriched in each outcome group. In the poor short-term recovery group, higher relative abundances were observed for the bacterial family Peptococcaceae and Listeriaceae, the genus *Ezakiella*, the species *nigrificans* and *thermosphacta*, and the order Oceanospirillales. In contrast, the good short-term recovery group was characterized by enriched levels of the genus *Sphingorhabdus*, the species *aeria*, the family Prevotellaceae, and the order Pasteurellales. The corresponding LDA score plot confirms the discriminatory power of these taxa, with all listed genera showing LDA scores greater than 2 in absolute value.

Figure 23 displays the LEfSe results comparing donor bile microbiota associated with long-term recovery outcomes at 12 months post-transplantation. In the poor long-term recovery group, multiple genera were enriched, including *Rhizobium*, *Granulicatella*, *Lachnoanaerobaculum*, and *Schaalia*, as well as the species *rhizophila*, the family Nocardiaceae, and the order Sphingobacteriales. Conversely, only the family *Enterobacteriaceae* was found to be significantly enriched in the good long-term recovery group.

The relationship between donor-derived biliary microbiota at the time of LT and recipient MELD scores over time was further examined using Spearman correlation (Figure 24) and MaAsLin2 models (Figure 25). Among the taxa

identified by LEfSe, several genera associated with poor long-term recovery exhibited consistent positive correlations with MELD scores across timepoints in the MaAsLin2 or Spearman correlation analyses. Specifically, the genera *Rhizobium*, *Granulicatella*, and *Schaalia*, all enriched in the long-term poor recovery group, showed positive correlations with MELD scores at multiple postoperative timepoints. Similarly, the family Pasteurellaceae, also associated with poor long-term outcomes in LEfSe, demonstrated a slight positive correlation with MELD score. In contrast, none of the taxa enriched in the short-term recovery groups exhibited significant correlations with MELD scores over time. Several additional bacterial genera not identified in the LEfSe analysis showed statistically significant correlations with MELD scores over time. For instance, in the MaAsLin2 analysis (Figure 25), the well-characterized *Pseudomonas* showed a strong positive association with MELD score at 6 months post-LT, while *Flavobacterium* exhibited moderate positive correlations immediately after LT, as well as at 1 and 6 months after LT.

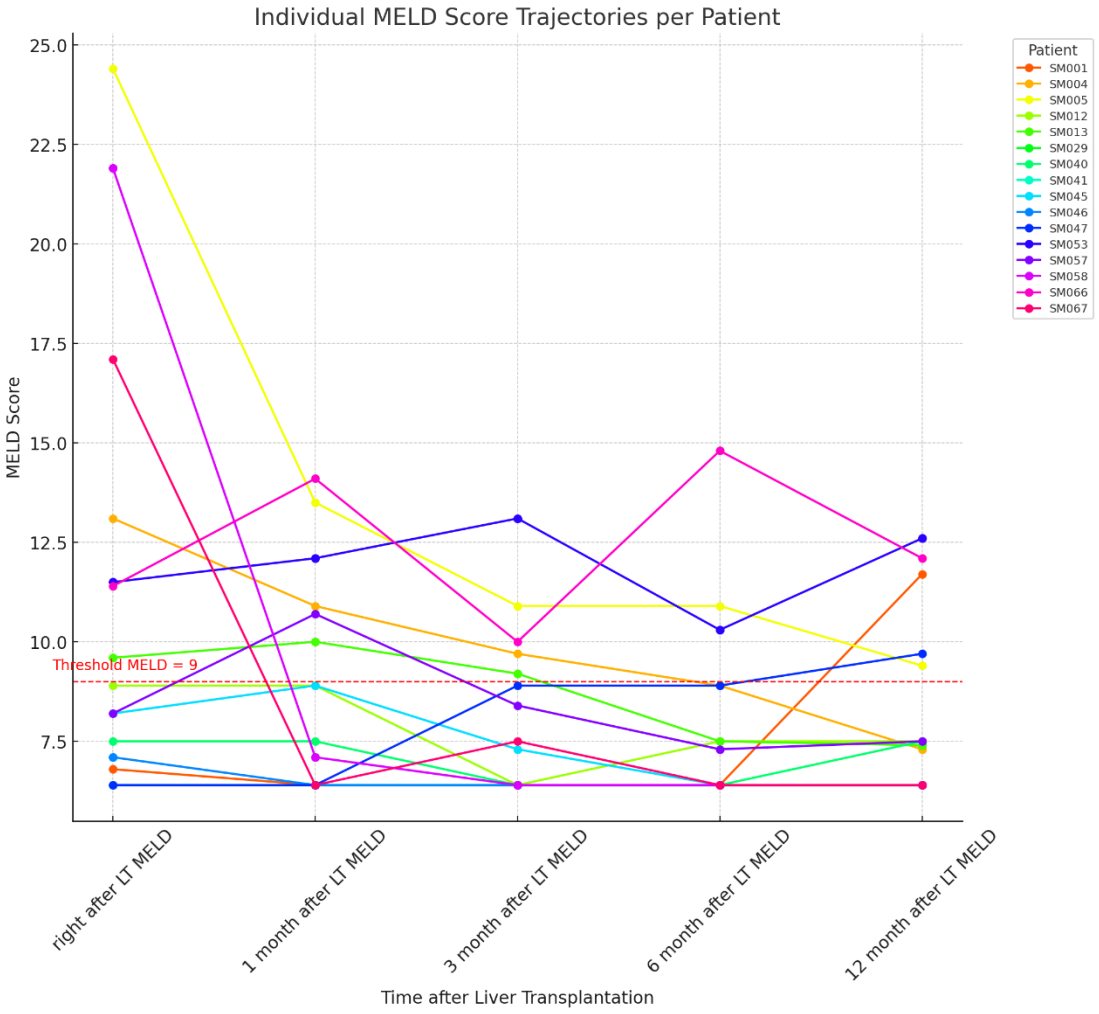

Figure 21 Individual MELD Score Over 12 Months Following LT

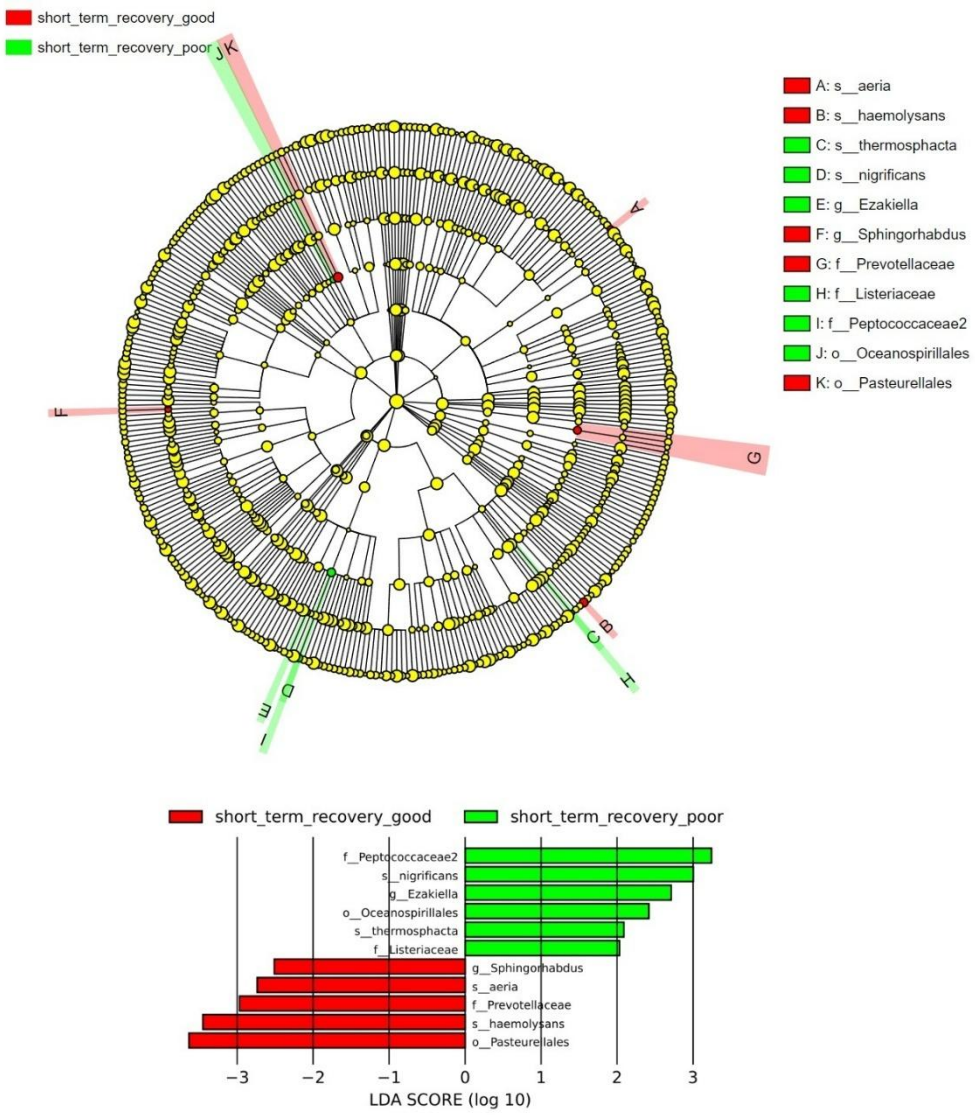

Figure 22 LEfSe-Based Comparison of Biliary Microbiota in Short-Term Recovery Outcomes

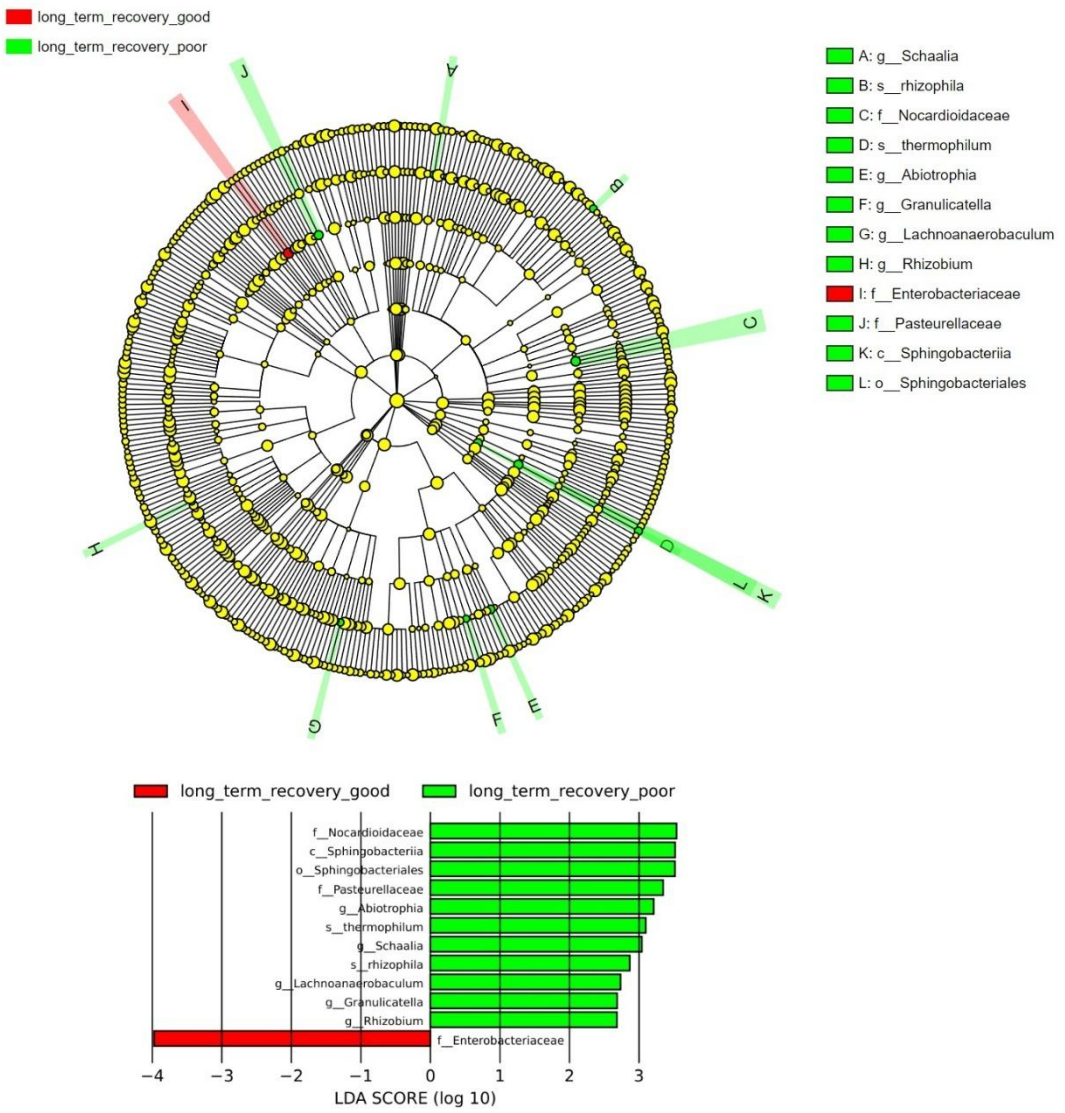

Figure 23 LEfSe-Based Comparison of Biliary Microbiota in Long-Term Recovery Outcomes

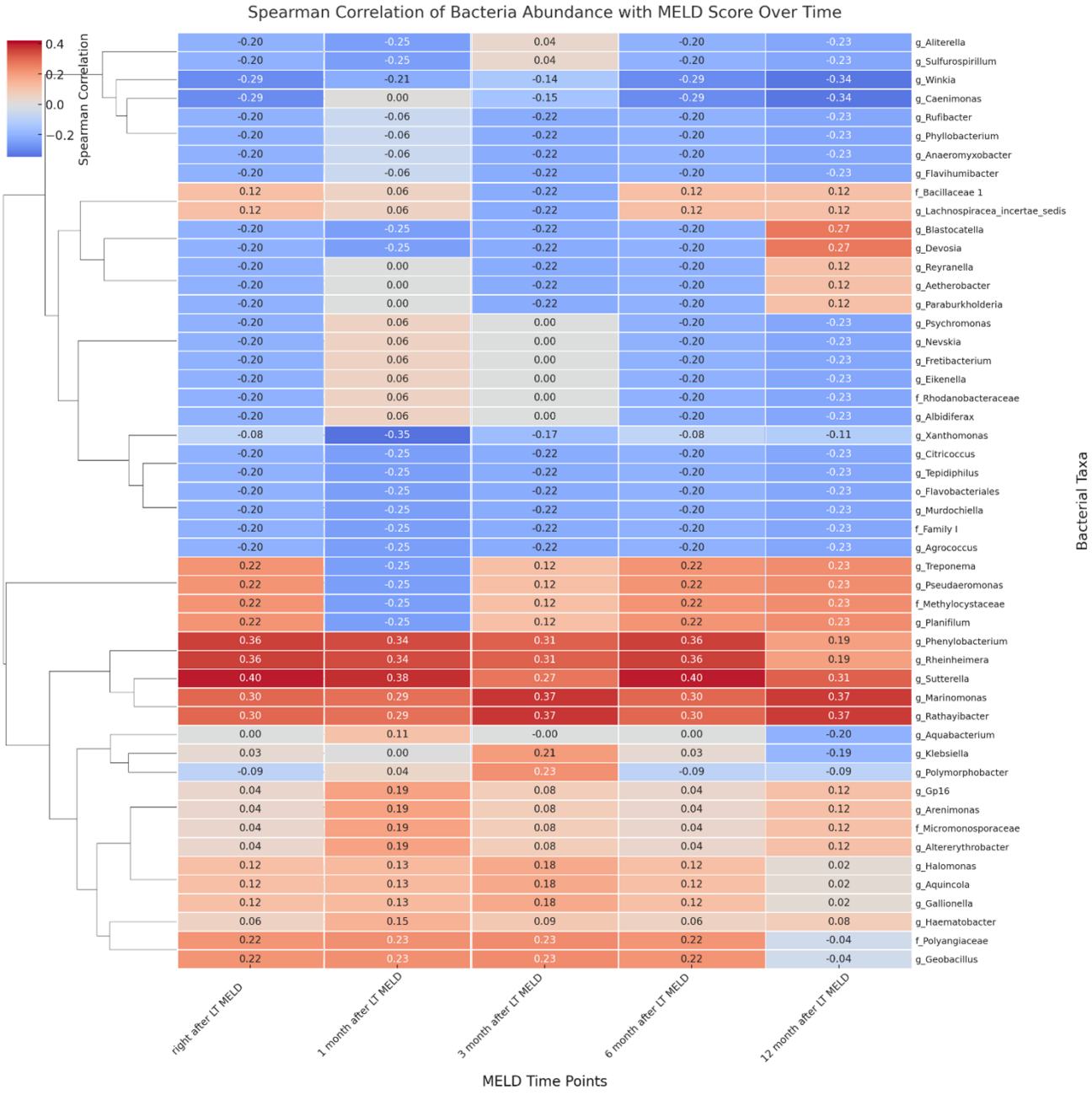

Figure 24 Spearman correlation heatmap of bacterial abundance with MELD score over time following liver transplantation

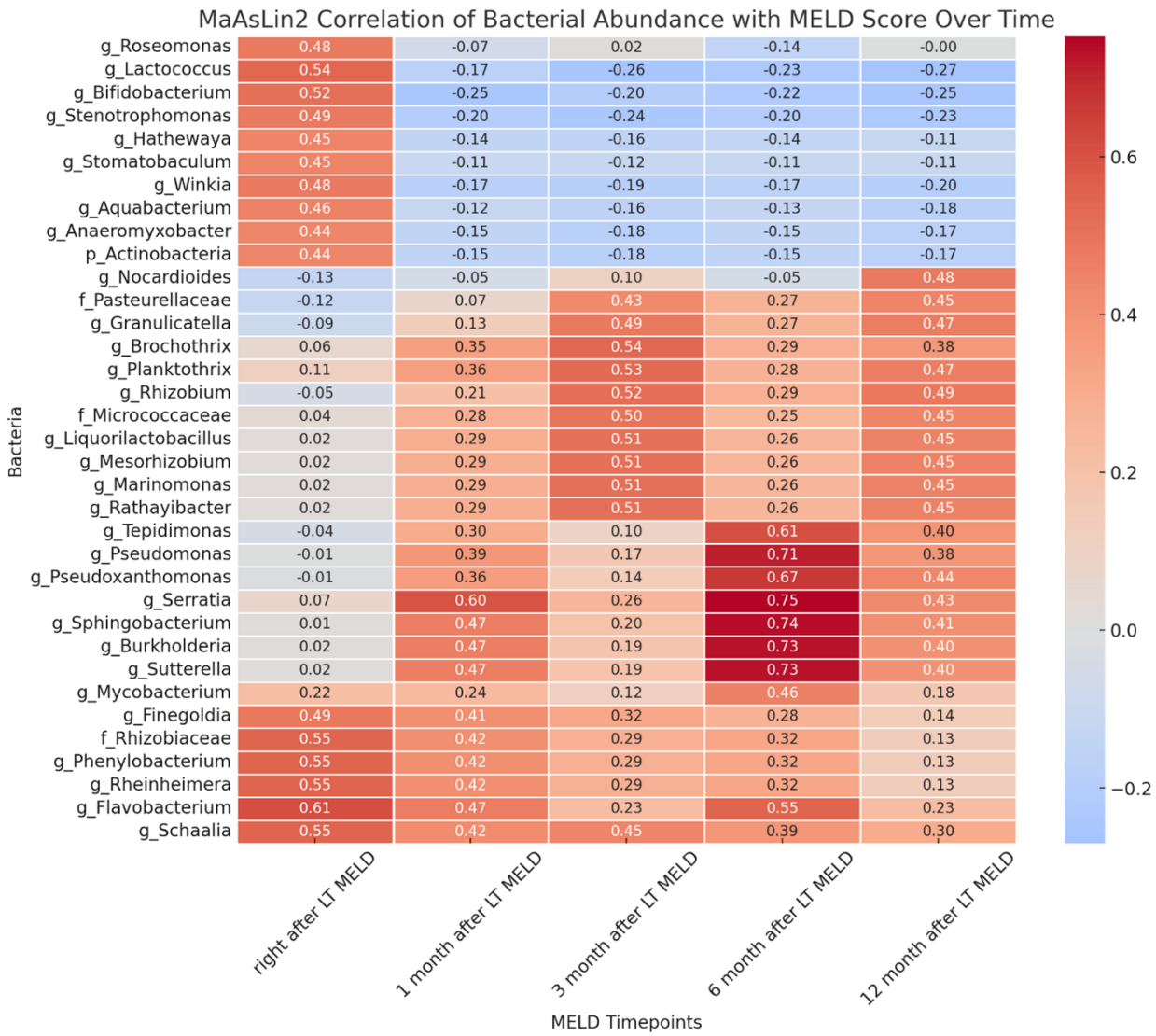

Figure 25 MaAsLin2 correlation heatmap of bacterial abundance with MELD score over time following liver transplantation

3.4 Impact of Donor Clinical Parameters on the Biliary Microbiome

3.4.1 Overview of Correlation

Spearman correlation heatmap (Figure 26) illustrates the associations between microbial taxa and donor clinical variables. Correlation strength is represented

by color, with red indicating positive and blue indicating negative associations, and white signifying weak or no correlation. Hierarchical clustering on the left groups microbial taxa with similar correlation patterns, helping to identify taxa that respond similarly to clinical conditions.

As shown in Figure 27, multiple microbial taxa exhibited moderate to strong Spearman correlations with donor clinical variables. For example, donor BMI was positively correlated with order Rhizobiales (r = 0.56), *Spirosoma* (r = 0.53), order Solirubrobacterales (r = 0.43), and order Enterobacterales (r = 0.43), while negatively correlated with *Aerococcus* (r = –0.44). Liver ischemia times also demonstrated distinct associations. Specifically, longer cold ischemia time was associated with increased abundance of *Deinococcus* (r = 0.41) and decreased abundance of *Agrobacterium* (r = –0.40) and *Pseudoxanthomonas* (r = –0.43). Total ischemia time correlated positively with *Roseomonas* (r = 0.38) and negatively with *Agrobacterium* (r = –0.45) and family Rhodobacteraceae (r = –0.40).

Antibiotic exposures showed marked associations with microbial profiles. Penicillin-based antibiotic use was negatively correlated with *Neisseria* (r = –0.52), *Rothia* (r = –0.51), *Lautropia* (r = –0.44), and *Granulicatella* (r = –0.42). Vancomycin/linezolid exposure demonstrated strong positive correlations with *Murdochiella* (r = 0.50), order Mycobacteriales (r = 0.50), and family Blastocatellaceae (r = 0.50). Desmopressin administration was positively associated with order Solirubrobacterales (r = 0.41) and *Microlunatus* (r = 0.40), while corticosteroid use showed a negative correlation with *Novosphingobium* (r = –0.43).

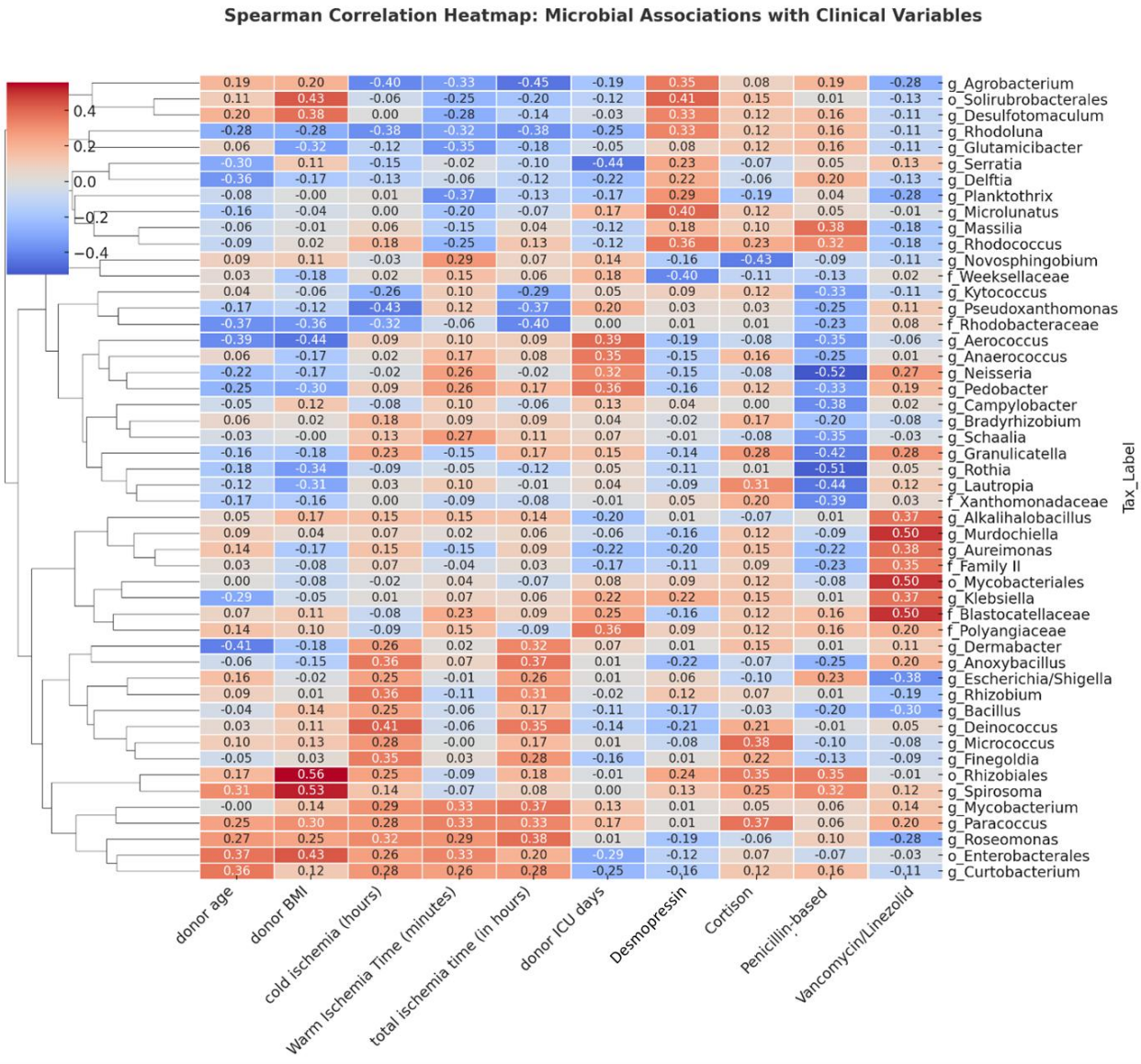

Figure 26 Spearman correlation heatmap of microbial associations with donor clinical variables in liver transplantation

3.4.2 Effects of Antibiotics and Cortisone on Bile Microbial Diversity and Composition

The bile microbiota of LT donors shows clear differences depending on exposure to antibiotics, cortisone, both, or neither, with notable variations in microbial diversity and taxonomic composition.

Alpha diversity analysis (Figure 27 A) indicates that microbial richness is lowest in the antibiotic-treated group and highest in the cortisone-treated group, as reflected in the Chao1 index. The untreated group and the combination treatment group exhibit intermediate richness values. The Simpson and Shannon indices show that microbial evenness follows the same pattern, with cortisone-treated donors displaying the most even microbial distribution, while antibiotics reduce both richness and evenness. The group receiving both treatments shows slightly higher richness than the antibiotic group but remains lower than the cortisone group. These results indicate that antibiotic exposure reduces microbial diversity, whereas cortisone administration is associated with a more diverse and evenly distributed bile microbiome.

Beta diversity analysis using Bray-Curtis and Jaccard distance metrics (Figure 27 B) does not show clear clustering of microbial compositions between treatment groups ($p > 0.05$). The ordination plots indicate that microbial community structure remains relatively stable despite differences in diversity. The cortisone-treated group exhibits the widest distribution, suggesting greater variability in microbial composition among individuals, while the antibiotic-treated group appears more tightly clustered. The untreated group retains a distinct microbial structure, with a broader distribution compared to the antibiotic group but with less variation than the cortisone-treated samples.

The LDA score plot (Figure 28) and cladogram (Figure 29) show distinct microbial compositions across treatment groups. In the antibiotic group, *Enhydrobacter*, *Schaalia*, *Flavobacterium*, and *Abiotrophia* are enriched. The cortisone-treated group shows increased abundance of *Haematobacter*, *Anaerococcus*, *Ruminococcus*, *Luteimonas*, *Winkia*, *Brevibacillus*, Gluconobacter, and *Paenibacillus*. The group receiving both antibiotic and cortisone treatment is characterized by enrichment of the species

salmoniphilum, which belongs to the genus *Carnobacterium*. The group without antibiotic or cortisone treatment displays increased abundance of *Planctomycetacia*, *Legionella*, *Blastocatella*, *Pseudonocardia*, and *Devosia*.

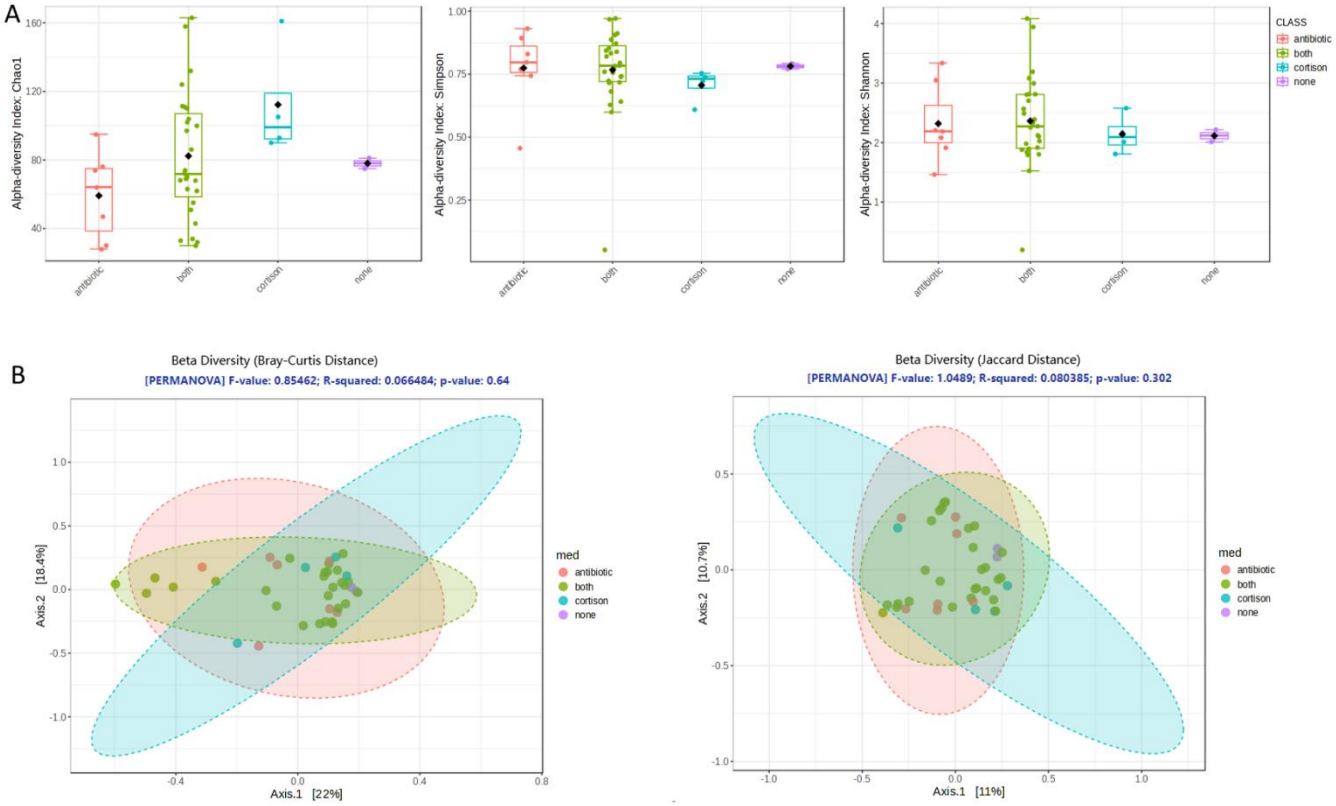

Figure 27 Alpha (A) and beta diversity (B) analysis of bile microbiota stratified by medication exposure (antibiotic, cortison, both, none).

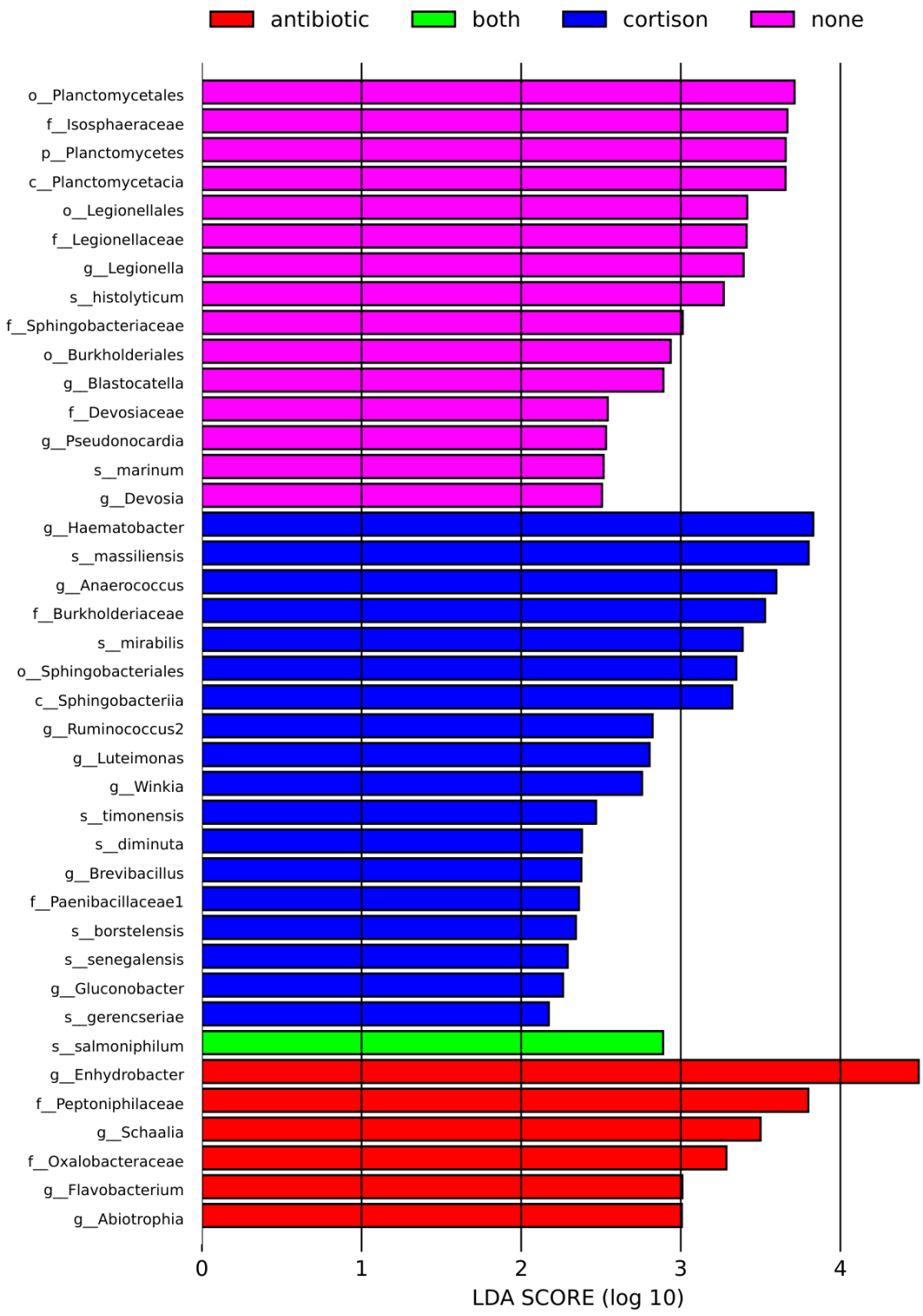

Figure 28 LDA score plot identifying differentially abundant bacterial taxa across medication groups (antibiotic, cortison, both, none)

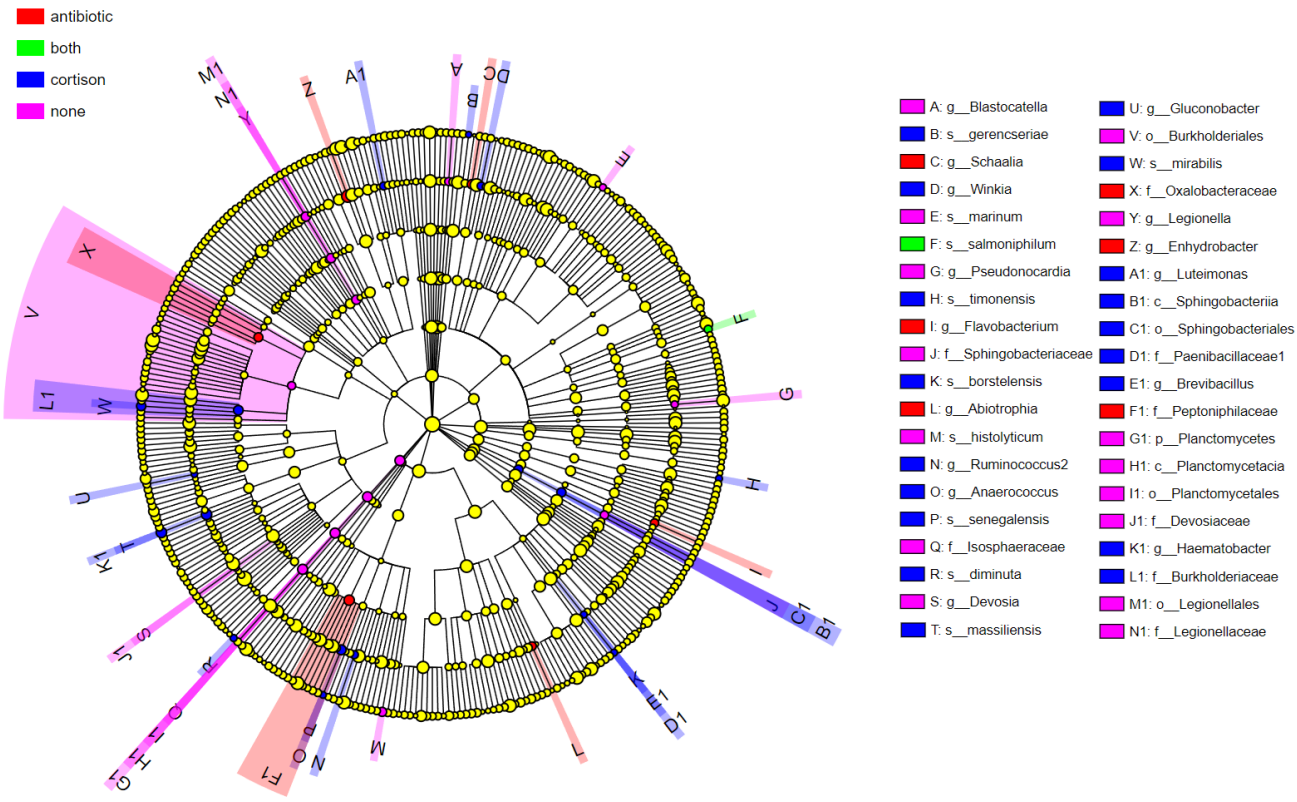

Figure 29 LEfSe cladogram identifying differentially abundant bacterial taxa across medication groups (antibiotic, cortison, both, none)

3.4.3 Cause of Death and Biliary Microbiota Diversity

The bile microbiota of LT donors does not show clear differences between donors who died from cerebral hemorrhage and those who died from ischemia by different causes. Beta diversity analysis shows overlapping microbial compositions between the two groups (Figure 30 A), suggesting that donor death type does not significantly impact overall community structure. Alpha diversity metrics, including Chao1, Shannon, and Simpson indices, do not show a clear distinction between the groups, with no substantial differences in

microbial richness or evenness (Figure 30 B). These findings indicate that the donor's cause of death does not strongly influence bile microbiota composition.

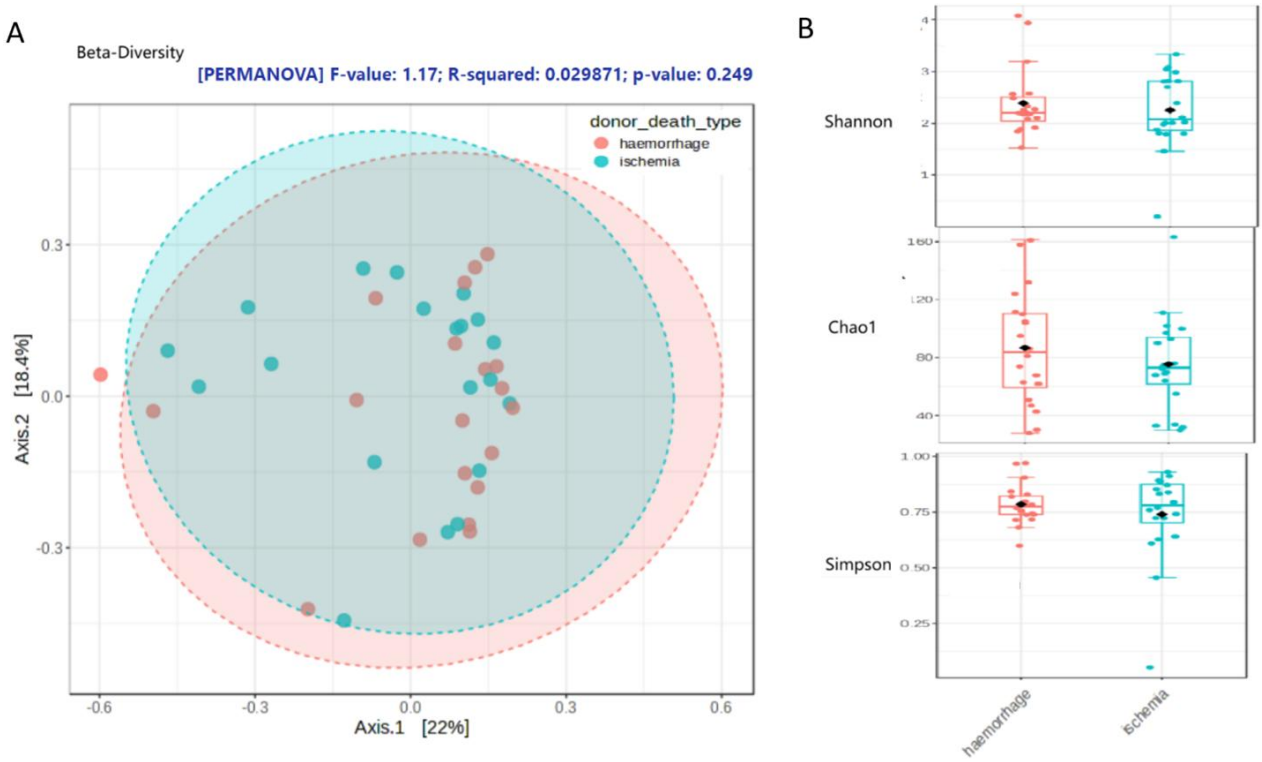

Figure 30 Alpha (B) and beta (A) diversity analysis of bile microbiota in liver transplant donors categorized by cause of death: cerebral hemorrhage and ischemia by different causes

4. Discussion

4.1 Interpretation of Key Findings

4.1.1 Biliary Microbiome Composition at Transplant

This study confirms the presence of a diverse biliary microbiome at the time of LT, challenging the traditional notion of biliary sterility [23, 54, 77, 113, 139]. Using RNA-based microbiome analysis, which selectively detects viable bacteria and therefore enhances result reliability, we analyzed bile fluid and biliary mucosa samples and identified distinct microbial communities between these compartments. Bile fluid was dominated by *Proteobacteria*, particularly during back-stage preparation, whereas biliary mucosa exhibited a more balanced distribution, with increased representation of *Actinobacteria* and *Firmicutes*. The relative abundance of *Proteobacteria* remained high in bile fluid even after reperfusion, though minor shifts in specific bacterial genera were observed.

Alpha diversity analysis revealed that the biliary mucosa exhibited significantly higher microbial diversity than bile prior to reperfusion, as indicated by a higher Shannon index ($p < 0.05$). This suggests that the mucosal surface initially harbors a richer and more evenly distributed microbial community. However, following reperfusion, microbial diversity did not differ significantly between bile and biliary mucosa. It is plausible that reperfusion injury and the associated inflammatory responses compromise the integrity of the cholangiocyte barrier, facilitating the translocation of mucosa-associated microbes into the bile and contributing to the convergence of alpha diversity between bile and biliary mucosa [110, 140].

The distinct microbial communities observed between bile and mucosa suggest that different ecological pressures shape these microbiomes. Bile fluid is a dynamic and transient environment that favors the survival of bile-resistant bacteria, whereas biliary mucosa provides a relatively stable niche that supports a broader range of microbial taxa. The differences in microbial composition between these compartments are consistent with previous findings in non-transplant settings and highlight the potential role of the biliary microbiome in transplant-related complications [23, 77, 110, 141, 142].

4.1.2 Stability of the Biliary Microbiome During Transplantation

Contrary to concerns that IRI or surgical handling might induce immediate shifts in microbial composition [143, 144], our study demonstrates that the biliary microbiome remains largely stable throughout the transplantation process.

This analysis is only of interest because this study was based on RNA, which is generally less stable than DNA and therefore more likely to represent metabolically active and viable bacteria. In specialized microbial niches such as the biliary tract, this distinction is particularly important, as DNA-based methods may capture remnants from dead bacteria or environmental detritus. RNA-based profiling, by contrast, offers a more accurate snapshot of the living microbial community present during transplantation.

Beta diversity analysis using PCoA and PERMANOVA indicated no statistically significant differences between pre-reperfusion (backtable) and post-reperfusion bile or mucosal samples. These findings suggest that ischemic stress, temporary loss of bile flow, and surgical exposure do not induce major short-term disruptions in the beta diversity of biliary microbiota.

Differential abundance analysis revealed some microbial shifts in bile following reperfusion. *Haematobacter* and *Exiguobacterium* were among the most

significantly depleted genera post-reperfusion ($\log_2$ fold-change < –6), while genera such as *Dermacoccus*, *Nakamurella*, and *Helicobacter* exhibited increased abundance. These alterations may reflect early microbial responses to the reperfusion-induced changes in the biliary environment, including shifts in oxygen levels, bile composition, and immune activation. The increase in *Helicobacter* is of particular interest due to its potential association with biliary tract inflammation and epithelial injury [145]. In contrast, only a few genera in the mucosa showed significant changes, with *Gardnerella* markedly decreased and *Sphingomonas*, *Thermomonas*, and *Microlunatus* enriched after reperfusion, suggesting that the mucosal microbiota remains comparatively stable during early post-reperfusion phases. Despite these specific shifts, the overall microbial structure remained intact, as well as MP alters minimal changes in the bacterial community, suggesting that intraoperative alterations in the biliary microbiome are minimal, and major changes are more likely to be driven by long-term factors such as immune modulation, antibiotic exposure, and biliary interventions.

4.1.3 Association Between Microbiome and Post-Transplant Complications

A key finding of this study is that the composition of the biliary microbiome at the time of transplantation is associated with the development of specific biliary complications post-transplant.

Patients who developed ITBL exhibited significantly lower microbial richness (Chao1 index) in bile fluid compared to those who remained ITBL-free. ITBL cases were characterized by a depletion of beneficial commensal bacteria and an enrichment of environmental and opportunistic pathogens, including *Janthinobacterium*, *Shinella*, and family Rhizobiaceae. These taxa are known for their environmental resilience and potential involvement in inflammatory responses [146, 147], suggesting that a low-diversity bile microbiome at the

time of transplant may predispose patients to ITBL by impairing colonization resistance and allowing pathogenic bacteria to persist.

In contrast, patients who developed cholangitis exhibited higher microbial diversity in bile, with significantly increased Chao1 indices and a trend toward higher Shannon index compared to those without infections. These patients showed an enrichment of *Streptococcus*, *Pasteurellales*, *Neisseria*, *Haemophilus*, and *Pseudoalteromonas*, taxa commonly associated with opportunistic infections of the biliary tract. These facultative anaerobic bacteria such as *Streptococcus* are frequently associated with opportunistic infections of the biliary tract [148], suggesting that post-transplant biliary stasis, immunosuppression, or antibiotic-induced dysbiosis may create conditions that favor microbial overgrowth and infection. Other less common facultative anaerobes, such as *Pasteurellales* and *Haemophilus* spp., have been sporadically reported in case studies of biliary infections [110].

For patients who developed biliary leaks and anastomotic strictures, the microbial composition was characterized by an increased abundance of biofilm-forming bacteria, particularly family Chitinophagaceae, which is known for their ability to form resilient biofilms, which may impair biliary healing, promote chronic inflammation, and increase the risk of fibrosis [145, 149]. Although prior biliary interventions such as stenting can facilitate colonization by biofilm-forming bacteria, no such procedures were documented in donor records. The observed microbial signatures likely reflect an intrinsic biliary microbiota rather than procedure-induced colonization, potentially contributing to post-transplant complications.

4.1.4 Microbial Taxa with Similarities and Differences Across Conditions

A detailed comparison of microbial taxa across different conditions revealed several important trends. Certain bacterial genera, such as *Brevundimonas*, *Acinetobacter*, and *Pseudomonas*, were consistently present across all groups, suggesting that these taxa may constitute a core biliary microbiome that persists despite surgical and immunological changes. These bacteria likely exhibit resilience to bile flow and transplant-associated stress, maintaining their presence throughout the transplantation process. Distinct microbial signatures were observed in specific biliary complications, suggesting potential microbial contributions to their pathogenesis. In bile after reperfusion, *Helicobacter* was significantly enriched, reinforcing its association with bile duct colonization and chronic inflammation. The ability of *Helicobacter* to form biofilms and evade immune responses may contribute to the fibrotic processes underlying biliary strictures [150].

Pre-transplant donor management strategies were found to significantly influence the biliary microbiome composition. Donor livers exposed to antibiotics exhibited lower microbial diversity, with a depletion of commensal bacteria and an increased prevalence of antibiotic-resistant species. In contrast, corticosteroid exposure was associated with higher microbial diversity, potentially due to immunosuppressive effects that reduce host immune clearance of microbial populations. These findings suggest that the clinical management of donors prior to transplantation may have lasting effects on the recipient’s biliary microbiome and subsequent transplant outcomes.

4.1.5 Summary and Perspectives

This study confirms that the biliary microbiome is present at the time of LT and remains largely stable throughout the surgical process. While bile fluid is

predominantly composed of Proteobacteria, biliary mucosa harbors a more diverse microbial community. The distinction between these two compartments highlights the influence of local environmental conditions on microbial composition.

Although no major shifts in overall microbial diversity were observed between pre- and post-reperfusion samples, specific taxa exhibited significant changes, suggesting early microbial responses to transplantation. Importantly, the biliary microbiome at the time of transplant was associated with post-transplant complications, with lower microbial richness linked to ITBL and higher diversity observed in cholangitis cases. The presence of biofilm-forming bacteria in biliary leaks and anastomotic strictures further suggests a potential microbial role in post-transplant bile duct integrity.

These findings provide new insights into the biliary microbiome's relevance in LT. Future research should investigate its long-term impact on graft function and explore potential microbiome-targeted strategies to mitigate post-transplant complications.

4.2 Comparison with Existing Literature

4.2.1 The Biliary Microbiome: From Sterility to Clinical Relevance

Early beliefs held that the biliary tract was sterile under healthy conditions, but accumulating evidence has overturned this dogma. Multiple animal and human studies demonstrating that bile ducts do harbor a resident microbiome even in the absence of overt infection [39, 40, 100, 101]. However, research on the biliary microbiome in the context of LT remains limited, with the majority of existing studies focusing on the gut microbiome rather than bile-specific microbial communities [89, 105, 111, 113, 127].

Our findings extends current understanding by demonstrating the consistent presence of a complex biliary microbiome in donor liver bile at the time of transplantation. We observed a rich assortment of taxa in transplant bile samples, reinforcing reports that even “healthy” bile supports a microbial community. This recognition of a baseline biliary microbiome sets the stage for understanding its dynamics and impacts after LT.

A key focus of recent research has been the link between bile microbiota profiles and post-transplant biliary complications [77]. It reported that LT recipients with biliary symptoms had bile microbiomes heavily skewed toward Gram-negative opportunists [77]. Over 50% of the sequences in symptomatic patients’ bile belonged to Proteobacteria – with *Pseudomonas* (~23% relative abundance) among the top genera [77]. In contrast, asymptomatic control patients’ bile showed a more balanced community, enriched in Firmicutes such as *Enterococcus* and other commensal taxa. Our findings are in strong agreement with these patterns. In our cohort, *Pseudomonas* at the time of transplant showed a strong positive correlation with MELD scores over time, suggesting a potential association with poorer graft function. On the other hand, Lactococcus, a less virulent lactic acid bacterium, showed a weak negative correlation with MELD scores at 1, 3, 6, and 12 months post-LT. This congruence with previous study underscores a consistent theme: Proteobacteria-dominated bile communities are associated with post-LT biliary pathology [77], whereas a microbiome rich in benign, Gram-positive commensals correlates with stable outcomes.

4.2.2 Microbial Diversity and Its Implications for Transplant Outcomes

Aside from composition, the overall diversity of the bile microbiome has been examined as a factor in transplant outcomes. In our data, patients who had uncomplicated recoveries showed a trend toward higher alpha-diversity in their

bile at baseline compared to those who later developed complications, though this difference did not reach statistical significance. This observation mirrors the findings of previous study [77], who also found no significant difference in alpha-diversity between their symptom vs. control groups despite clear compositional differences. In other words, it may not be the sheer number of species in bile that influences outcomes, but rather the identity of key microbes. Both previous study and ours indicate that specific taxa (e.g. *Pseudomonas* or Proteobacteria) are better markers of risk or protection than broad diversity measures [77]. Our results therefore in agree with prior research in showing that bile microbiome composition differs markedly between patients with and without complications, even if the diversity indices do not differ significantly.

4.2.3 Prognostic and Modifiable Aspects of the Biliary Microbiome

Importantly, our study adds novel evidence regarding the timing of these microbiome differences. Whereas prior studies [77, 151] analyzed bile at the time complications were already manifest, we sampled bile at the time of transplant, before any post-operative biliary complication had occurred. We found that the presence of certain bacteria in this baseline sample was prognostically relevant, foreshadowing later complications. This prospective element of our study suggests that the microbiome differences are not merely a consequence of biliary injury; they precede and possibly contribute to the development of complications.

Research showed that an increase in Proteobacteria is often observed alongside biliary injuries after transplant, though it remained unclear whether this dysbiosis is a cause or an effect of the complication [23, 54, 77]. Our findings strengthen the argument for causality: detecting a Proteobacteria-heavy profile (for example, *Pseudomonas*-enriched bile) at transplantation could serve as an early warning sign of impending issues. Building upon

previous cross-sectional findings, our study demonstrates that a bile microbiome enriched in potentially pathogenic taxa may already be present at the time of transplantation and could serve as a predictor of subsequent biliary complications. This is a novel contribution to the field, suggesting a possible predictive biomarker role for the biliary microbiome.

Furthermore, it complements older studies based on culture, which found that any bacterial or fungal colonization of bile ("bacteriobilia" or "fungibilia") was associated with worse outcomes after post-LT biliary interventions [54]. We provide a modern, sequence-based confirmation and refinement of that concept, pinpointing which microbial communities are most deleterious. Notably, organisms like *Pseudomonas* that we and others have implicated are known for biofilm formation and resistance to bile's innate antimicrobial properties, potentially enabling persistent biliary colonization and mucosal damage [152]. Their early presence in the graft bile may thus actively drive the pathogenesis of complications, rather than simply overgrow after a complication has started.

Conversely, our data in agreement with previous research indicate that *Enterococcus* and similar commensals are abundant in uncomplicated cases, raising the possibility that they contribute to a more resilient bile environment that resists pathogenic takeover [77]. Interestingly, study shows that bile from patients with non-anastomotic strictures had lower proportions of *Enterococcus* compared to patients without complications [23], supporting the idea that a loss of commensal *Enterococci* is linked to worse outcomes.

Taken together, our study and prior research are largely in concordance: both identify a pathogenic vs. commensal shift in the bile microbiome as a key differentiator between complicated and uncomplicated post-transplant courses. Where we diverge is mainly in the timing and implications of these findings: by capturing microbiome signatures at transplant, we highlight their predictive

potential and strengthen the case for a causal role, which is a new insight building on the associations reported by earlier studies.

Another aspect of the biliary microbiome research is how stable or malleable this microbial community is in the post-transplant period. The existing literature suggests that the biliary microbiome is influenced by post-operative interventions but also exhibits resilience. For instance, a recent study analyzing bile samples from LT recipients with different types of biliary strictures reported that all samples contained a mix of common bile-associated phyla (e.g. Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, and Actinobacteria) regardless of complication type [23]. Notably, certain external factors were shown to selectively influence the biliary microbiome. For example, biliary stenting facilitates microbial ascent from the duodenum and promotes biofilm formation, resulting in substantial compositional shifts, including enrichment of biofilm-forming taxa such as *Streptococcus* and *Fusobacterium*. In contrast, antibiotic administration had minimal impact on overall microbial diversity in the same study, suggesting that the biliary microbiome may retain a degree of stability in the absence of direct biliary tract intervention. [23].

While our study was not primarily designed to longitudinally track bile microbiome changes post-LT, our findings do shed light on microbiome stability from a different angle. By identifying a resident microbiome at the moment of transplantation, we establish the baseline community that the organ carries into the recipient. This baseline likely serves as the foundation for post-operative microbial ecology. The fact that certain bacteria detected in the donor bile at transplant persisted long enough to be implicated in complications months later suggests that the graft's microbiome can engraft in the recipient and maintain itself over time (at least in the relative short term). In our cohort, standard perioperative antibiotic prophylaxis did not sterilize the bile – many of the

organisms present at transplant (including the potential pathogens) presumably survived or quickly re-populated after antibiotics, consistent with the resilience noted by previous research [23]. Thus, our data support the notion that the biliary microbiome, once established, is fairly stable in the immediate post-transplant period, unless perturbed by factors like instrumentation [23]. We also observed that donor characteristics had subtle effects on this baseline microbiome, for example, livers from donors with higher BMI tended to harbor distinct microbial communities, characterized by higher abundances of *Spirosoma* and Rhizobiales, at the time of transplant. This hints that the microbiome a recipient "inherits" with the new liver can vary with donor factors, though further research is needed to determine how long donor-derived microbial profiles persist and how they evolve under the influence of the recipient's body and medical treatments.

Broadly, our results fit with the emerging view that the biliary microbiome after transplant is shaped by both inoculum effects (what the donor liver brings) and post-operative factors (antibiotics, immunosuppression, biliary interventions) [23, 77, 151]. The existing literature is still sparse on longitudinal biliary microbiome data [23, 75-77], but the available evidence (including our study) suggests a scenario in which a core biliary community is present early on and can be modulated – though not wholly erased – by clinical interventions. Ultimately, maintaining a stable, balanced bile microbiome might be beneficial, as a stable community dominated by commensals could resist overgrowth by opportunistic invaders. This concept is indirectly supported by other observations, where *Enterococcus* was relatively depleted in patients with severe complications [23, 77]. Our work adds that preserving or even seeding a healthy bile microbiome at the time of transplant could be an appealing strategy to reduce complications down the line.

4.3 Methodological Strengths and Limitations

4.3.1 Strengths

This study is distinguished by several methodological strengths. First, the prospective study design with systematic sample collection at predefined surgical time points is a major advantage. By collecting specimens intraoperatively during back-table preparation and again after graft reperfusion, the research captured a rare temporal snapshot of the biliary microbiome from donor to recipient. This design minimizes recall bias and ensures that microbiome data precede clinical outcomes, strengthening the validity of any associations observed.

The carefully standardized sample collection protocols (sterile technique, immediate sample preservation on ice, snap-freezing of bile, and RNAlater storage for tissue) further bolster the study's reliability by maintaining sample integrity and minimizing contamination risks during the critical window of collection.

Another strength lies in the high-throughput sequencing techniques and bioinformatics pipeline employed. Targeted sequencing of the V1–V2 hypervariable regions of the 16S rRNA gene was conducted on the Illumina MiSeq platform (2×300 bp), enabling comprehensive and high-resolution profiling of the bacterial communities. Rigorous library preparation and downstream quality control (using DADA2 for denoising, chimera removal, and host DNA filtering) ensured that the resulting data were high-quality and free of technical artifacts. This approach enabled a comprehensive characterization of the biliary microbiome, capturing diverse taxa across samples.

Notably, this analysis was performed using RNA-based sequencing, which adds a layer of specificity by capturing transcripts from viable and metabolically

active bacteria. In contrast to DNA, which can persist after bacterial death or originate from environmental detritus, RNA is inherently less stable and more reflective of living microbial populations. This is particularly important in specialized and low-biomass environments such as the biliary tract, where RNA-based approaches improve the accuracy and clinical relevance of microbiome profiling.

Importantly, the study also integrated detailed clinical metadata with the sequencing data. Extensive donor and recipient information – such as donor age, cause of death, antibiotic usage, ischemia times, and recipient outcomes (e.g. occurrence of biliary complications, rejection episodes, etc.) – were systematically recorded and incorporated into the analysis. This integration allowed the researchers to perform robust correlation analyses, linking microbiome composition with clinical parameters. As a result, the study could identify meaningful patterns (for example, associations between certain microbial profiles and post-transplant complications or donor factors) that lend clinical context to the microbiome findings. In summary, the prospective, well-controlled design combined with advanced sequencing and rich metadata collection provided a strong foundation to generate insightful and clinically relevant results.

4.3.2 Limitations

Despite these strengths, several limitations of the methodology should be acknowledged. Foremost, the sample size was relatively small (31 donor-recipient pairs), which limits the statistical power to detect subtle microbial differences and may reduce the generalizability of the findings. With a modest cohort from a single transplant center, the study may not capture the full variability of biliary microbiomes seen in broader populations or different clinical settings. The limited number of cases also constrained subgroup analyses; for

instance, only a subset of transplants yielded biliary mucosa samples (9 tissue samples vs. 40 bile fluid samples were collected), which restricts the ability to draw definitive conclusions about microbiota differences between bile fluid and the biliary epithelium.

Additionally, because all samples were collected at one center under specific protocols, there is a possibility of selection bias or center-specific effects. Patients and grafts were subject to particular inclusion criteria and management practices, so results might reflect those specifics rather than a universal phenomenon. Moreover, the study lacks longitudinal microbiome data. All samples were obtained intraoperatively at the time of transplantation. Without post-operative longitudinal sampling, temporal changes in microbial community composition cannot be assessed, and potential associations between microbiome dynamics and the development of post-transplant complications remain unexplored.

Finally, the observational design of this study means that we can identify associations but cannot infer causality between microbiome features and clinical outcomes. Although the prospective sample collection establishes a temporal link (microbiome at transplant and outcomes thereafter), we did not intervene or experimentally manipulate the microbiome, nor did we obtain longitudinal microbiome samples during the post-transplant course. Thus, it remains unclear whether the microbial differences noted are a cause of subsequent biliary complications or merely correlates (or consequences) of other underlying factors. In future research, a combination of extended longitudinal monitoring and functional experiments (for example, in vitro cultures or animal models inoculated with bile microbes) would be needed to definitively establish causal roles and mechanistic pathways. Despite these limitations, our study provides a foundation by characterizing the biliary

microbiome at the critical peri-operative period using a RNA-based approach, and it highlights directions for more comprehensive and longitudinal investigation.

4.4 Clinical Implications

When placed in the context of the broader biliary microbiome research, our study's findings both corroborate prior knowledge and contribute new insights. We confirm fundamental points from earlier studies – notably, that the bile duct is not sterile and harbors a diverse microbiota, and that certain microbial signatures are associated with post-transplant complications [23, 77]. These areas of agreement strengthen the growing consensus that microbial factors are entwined with LT outcomes. At the same time, we expand on previous work by demonstrating that microbiome differences are detectable at the time of organ implantation, providing a temporal link between the donor organ's microbiome and the recipient's clinical course. This prospective element is a novel contribution that advances the field beyond the retrospective correlations reported by previous studies [23, 75-77].

In essence, our results suggest that the biliary microbiome could be used prognostically – an idea hinted at in prior literature but not established due to limited data. In fact, there has not been enough evidence to reliably predict transplant outcomes based on microbiome profiles. Our study helps fill this gap by showing that early bile microbiome profiling has potential predictive value, as we identified specific bacteria at transplant that correlated with complications that occurred weeks or months later. This finding opens the door to risk stratification: for example, a predominance of *Pseudomonas* in a donor bile sample might flag a patient for closer monitoring or preemptive measures, whereas a bile dominated by benign commensals could be reassuring.

Moreover, our work underscores the clinical relevance of biliary microbiome stability and diversity. We observed that a bile community skewed towards diverse, commensal-rich composition was associated with uneventful recoveries, supporting the broader concept that microbiome diversity and balance can be protective in critical illness settings. This aligns with the idea of "colonization resistance," where a diverse microbiota inhibits pathogenic overgrowth. Conversely, a perturbed, *Proteobacteria*-heavy community may lack these protective features and thus predispose to complications. In the literature, diversity itself has not consistently emerged as a discriminator in transplant cohorts [77], but community makeup clearly has – a nuance that our data reinforce.

Additionally, considering microbiome stability, our findings highlight that the donor organ's microbiota is an important piece of the puzzle. Rather than viewing post-transplant infections or cholangiopathies as arising solely from hospital-acquired bugs or immunosuppression effects, we show that the seeds of these issues might be present at transplantation. This perspective dovetails with recent studies showing that transplanted organs carry their microbiome and that these microbes can persist. It also suggests novel interventions: for instance, microbiome modulation at the time of transplant (such as targeted antimicrobial therapy or even probiotic/prebiotic treatments to shape the bile flora) could be explored to improve outcomes.

In summary, our study fits well into the existing body of research on the LT biliary microbiome by affirming known associations (microbial profiles linked to complications, bile not being sterile) and by adding new dimensions regarding timing and causality. We agree with prior works that opportunistic Gram-negatives like *Pseudomonas* are important players in post-LT biliary

complications [23], and that a commensal-rich microbiome may be conducive to graft health [23, 77].

We also observe, as others have, that the bile microbiome shows resilience in the face of antibiotics and tends to shift primarily when perturbed by interventions like stents [23]. Where we break new ground is in demonstrating microbiome-driven risk stratification at the point of transplant and highlighting the transplanted organ's microbiome as a foundational element of the post-transplant course. These contributions broaden the discussion from simply characterizing post-transplant dysbiosis to considering how we might proactively manage or leverage the biliary microbiome to improve LT outcomes.

Future studies can build on this by tracking how the bile microbiome evolves in larger cohorts over time and by testing whether modifying the bile microbial community can indeed prevent complications. Our findings, in concert with the reviewed literature, suggest that the stability, diversity, and composition of the biliary microbiome are integral to transplant success, and they invite a reimagining of post-transplant care that includes the microbial dimension as a key factor in graft health.

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6. Appendix A: List of Materials

Table 4 Reagents and Chemicals

Material	**Supplier**
Ethanol (Molecular Biology Grade)	Merck
Nuclease-free water	Ambion, Austin, TX, USA
GelRed Nucleic Acid Gel Stain	Biotium
Sodium Acetate (3 M, pH 5.5)	Sigma-Aldrich
Glycogen (Stock: 5 µg/µl)	Thermo Fisher Scientific
Buffer RLT + β-Mercaptoethanol	QIAGEN
Buffer RW1 and Buffer RPE	QIAGEN
RNAlater Stabilization Solution	QIAGEN
Acetic Acid (Glacial) 100%	Merck, Germany
Hydrochloric Acid (HCl) 2M	Titripur, Merck, Germany
Magnesium Chloride Hexahydrate (MgCl2.6H2O)	Fluka, Switzerland
Zinc Chloride (ZnCl2)	Merck, Germany
Phenol:Chloroform:Isoamyl Alcohol (25:24:1)	Sigma-Aldrich

TRIzol Reagent	Thermo Fisher Scientific

Table 5 Equipment

Equipment	**Supplier**
FastPrep-24 Homogenizer	MP Biomedicals, Santa Ana, CA, USA
TissueLyser II	QIAGEN
Cooling Centrifuge (15,000 rpm)	Eppendorf
Centrifuge 5424R	Eppendorf, Hamburg, Germany
NanoDrop One Spectrophotometer	Thermo Fisher Scientific
Qubit Fluorometer	Thermo Fisher Scientific
PCR Cabinet (UV Decontamination)	Thermo Fisher Scientific
Mastercycler X50	Eppendorf
Illumina MiSeq Sequencer	Illumina
Agarose Gel Electrophoresis System	Bio-Rad
High-performance computing clusters	Institutional Server
Bioanalyzer 2100	Agilent Technologies
Electroporator	Bio-Rad

Table 6 Consumables

Material	**Supplier**
Centrifuge tubes (15 ml, 50 ml)	TPP, Switzerland
Freezing tubes	Cryo.s, Greiner, Germany
Safe-Lock tubes (1.5 ml, 2 ml)	Eppendorf
PCR tubes (0.2 ml strips)	Thermo Fisher
Serological pipettes (5 ml, 10 ml, 25 ml)	Greiner Bio-One, Corning
96-well plates for PCR	Sarstedt, Germany
Filtered Pipette Tips	Eppendorf
Hydrophobic Pen	Agilent Technologies, USA

Table 7 Commercial Kits

Kit	**Supplier**
QIAamp DNA Microbiome Kit	QIAGEN, Hilden, Germany
RNeasy Mini Kit	QIAGEN
TURBO DNase	Thermo Fisher Scientific, Waltham, MA
GoTaq G2 Hot Start Green Master Mix	Promega, Madison, WI, USA
QIAseq 16S/ITS Region Panel	QIAGEN
Agencourt AMPure XP Beads	Beckman Coulter, Brea, CA, USA

SuperScript III First-Strand Synthesis Kit	Invitrogen
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems

Table 8 Software

Software	Version
QIIME2	v2022.2
vsearch	v2.15
FLASH	v1.2.11
R with ggplot2, phyloseq, vegan, DESeq2, edgeR, LEfSe, MaAsLin2	v4.2.0
GraphPad Prism	v9.0
Python	V3.9
Microsoft Excel	Office 365

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Affidavit

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I hereby declare, that the submitted thesis entitled

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Munich, 11.0.2026

Place, Date

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