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Functional Microbiomics in Liver Transplantation: Identifying Novel Targets for Improving Allograft Outcomes

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Abstract

Gut dysbiosis, defined as a maladaptive gut microbial imbalance, has been demonstrated in patients with end-stage liver disease, defined as a contributor to disease progression, and associated clinically with severity of disease and liver-related morbidity and mortality. Despite this well-recognized phenomena in patients with end-stage liver disease, the impact of gut dysbiosis and its rate of recovery following liver transplantation (LT) remains incompletely understood. The mechanisms by which alterations in the gut microbiota impact allograft metabolism and immunity, both directly and indirectly, are multifactorial and reflect the complexity of the gut-liver axis. Importantly, while research has largely focused on quantitative and qualitative changes in gut microbial composition, changes in microbial functionality (in the presence or absence of compositional changes) are of critical importance. Therefore, to translate functional microbiomics into clinical practice, one must understand not only the compositional but also the functional changes associated with gut dysbiosis and its resolution post-LT. In this review, we will summarize critical advances in functional microbiomics in LT recipients as they apply to immune-mediated allograft injury, posttransplant complications, and disease recurrence, while highlighting potential areas for microbial-based therapeutics in LT recipients.

INTRODUCTION

The human intestinal microbiome, composed of 10^{13} to 10^{14} individual microbes (or microbiota) and their genomes, is a central symbiotic participant in modulating host

metabolic and immune homeostasis along the gut-liver axis.¹⁻³ There is a bidirectional cross talk between the gut and the liver; intestinal dysbiosis (imbalance in intestinal microbiota) and increased bacterial translocation across the intestinal barrier are ubiquitous in patients with end-stage liver disease (ESLD) and have been associated with disease severity, complications including hepatic encephalopathy and hepatocellular carcinoma (HCC), and mortality.⁴⁻⁸ These maladaptive changes in the microbiome persist following liver transplantation (LT) though the impact of the severity and rate of recovery of dysbiosis on patient outcomes remains incompletely understood.

With the advent of advanced techniques to facilitate culture-independent characterization of microbiota and their products, such as large-scale metagenomics and metabolomics, our understanding of the degree and nature of gut dysbiosis in the clinical setting is advancing at a rapid pace. These tools are complemented by animal models, including germ-free and gnotobiotic models, which use the same techniques and allow further mechanistic investigation. As our technical expertise evolves, so too does our understanding of the functional consequences of dysbiosis in the LT recipient, highlighting potential avenues for microbial-based interventions to improve clinical outcomes. In this review, we will summarize critical advances in functional microbiomics in LT recipients as they apply to immune-mediated allograft injury, posttransplant complications, and disease recurrence, while highlighting potential areas for microbial-based therapeutics in LT recipients.

FORM VERSUS FUNCTION IN GUT DYSBIOSIS

Given its complexity, characterizing the composition of gut microbiota to correlate with clinical events can be challenging. Diversity measurements are used to characterize the complexity of microbial communities and provide an index for comparison and correlation. Alpha-diversity indices, such as the Shannon diversity index (SDI), are qualitative markers of the richness (number of bacterial species) and evenness (relative abundance of bacterial species) of gut microbiota in an individual ecosystem (eg, gut microbiota in a LT recipient).⁹ Higher SDI values indicate a larger number of bacterial taxa with a relatively even distribution of abundance, whereas low SDI values indicate a smaller number of bacterial taxa where a limited number are particularly dominant.¹⁰ Alpha-diversity indices such as SDI are used to characterize clinical subsets of patients (eg, patients with cirrhosis) and correlate them to a clinical event or outcome (eg, in-hospital mortality). In general, and specifically in liver disease, low-diversity dysbiosis (characterized by a low SDI) is associated with inflammation and disease progression.¹¹ For instance, low microbial alpha diversity is associated with hepatic inflammation in nonobese nonalcoholic fatty liver disease (NAFLD) patients,¹² with cirrhosis and subsequent severity of clinical decompensation,^{8,13} and with 30-day mortality in patients with cirrhosis.⁵

Beta-diversity indices, such as weighted and unweighted UniFrac, quantify compositional differences of microbial communities between independent samples (as opposed to within an individual ecosystem).¹⁴ Beta-diversity indices have been critical in advancing our understanding of the microbiome in the context of specific clinical conditions including liver disease. For instance, UniFrac principal component analysis has been essential to demonstrate differences in gut microbiota between healthy controls and patients with

cirrhosis as well as longitudinal changes in individual patients pre- and post-LT.^{13,15} However, they represent a qualitative description of microbial composition rather than an assessment of the functional changes in the microbiome that will be required for mechanistic understanding and targeted therapy.

Metagenomics and (meta)metabolomics studies (describing changes in the functional potential of gut microbiota and microbial products, respectively) are now a focus to better understand functional consequences of dysbiosis.^{16,17} Advances in this area have led to a better understanding of the functional consequences of dysbiosis and led to recognition of clinically relevant changes that may have not otherwise been appreciated by more general compositional data approaches. Further integration of omics data with functional immune assays has also correlated immunophenotypes to specific bacterial taxa, further honing our ability to generate hypotheses on important immunomodulatory microbes for experimental follow-up and potential therapeutic targets in LT recipients.¹⁸ Ultimately, the impact of dysbiosis on allograft biology is due to both compositional and functional changes in the microbiome with both serving as potential targets for microbial-based therapies.

MEDIATORS OF GUT-LIVER CROSS TALK

Gut microbiota modulate systemic metabolic and immune functions via multiple mechanisms that may drive the impact of dysbiosis on subsequent allograft function.¹⁹ In addition to contributions from impaired mucosal immunity and portal hypertension, gut dysbiosis can result in a breakdown of the mucosal barrier, facilitating increased translocation of microbes and microbial products into the portal circulation.^{20,21} Commensal bacteria are essential for maintenance of the mucosal barrier, both directly via pathogen-associated molecular patterns (PAMPs) binding to pattern recognition receptors (PRRs; eg, Toll-like receptors) facilitating mucosal cell-mediated immunity²² and indirectly via production of short-chain fatty acids (SCFA), notably butyrate, which are essential for enterocyte health.²³ Additional barrier functions are altered in dysbiosis and associated with ESLD, notably reduced mucin production coinciding with loss of autochthonous *Akkermansia muciniphila*.²⁴

Once in the portal circulation, PAMPs (eg, lipopolysaccharide [LPS]) activate innate immune cells, including Kupffer cells, liver sinusoidal endothelial cells, and stellate cells in hepatic sinusoids, via PRR binding (eg, Toll-like receptor-4) and induce inflammation and fibrogenesis.^{25–28} Liver-related gut dysbiosis increases delivery of pro-inflammatory PAMPs to the liver and induces an inflammatory cytokine milieu, which in turn can shape adaptive immune function, for instance polarizing CD4⁺ T cells to a Th1 phenotype.^{29,30}

Microbial metabolites also play a critical role in the gut-liver axis.³¹ Dietary choline is converted into trimethylamine (TMA) by gut bacteria and subsequently converted to TMA-N-oxide (TMAO) after entering the portal circulation.³² While originally identified as an atherogenic microbial metabolite,³³ TMAO has now been associated with hepatic injury via increased triglyceride production and promotion of hepatic steatosis and elevated levels were associated with shorter transplant-free survival in patients with primary sclerosing cholangitis (PSC).^{34,35} Furthermore, bacterial conversion to TMA reduces conversion to

phosphatidylcholine, a metabolite that when deficient contributes to chronic liver disease in both nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis models.^{36,37}

In addition to their importance for maintaining intestinal barrier function, SCFA (butyrate, propionate, and acetate) are potent mediators of both hepatic immunity and metabolism. This occurs both by SCFA binding to G-protein coupled receptors and by transcriptional regulation via inhibition of histone deacetylases.³⁸ Both butyrate and propionate have been shown to reduce nuclear factor κ B gene expression via histone deacetylase inhibition, ameliorating macrophage activation in ischemic models of tissue injury.^{39–41} SCFA have also been shown to facilitate neutrophil recruitment, both directly via induction of neutrophil chemotaxis and indirectly via chemokine induction (eg, interleukin-8) in endothelial cells.^{38,41}

SCFA can also mediate systemic adaptive immune responses. Butyrate is known to impair maturation of dendritic cells, reduce costimulatory molecules, and impair cytokine production, collectively reducing subsequent T-cell activation and promoting induction of regulatory T cells (T_{REG}).³⁸ Reduced butyrate can in turn lead to decreased T_{REG}/Th17 ratio, which has been associated with liver allograft rejection.^{42,43} Saturated long-chain fatty acids are also produced via gut microbiota, notably *Lactobacillus*, and are essential to maintenance of intestinal barrier function. Long-chain fatty acids supplementation and *Lactobacillus* probiotic supplementation have both been shown to reduce mucosal and hepatic inflammation in a mouse model of alcohol-induced liver injury.⁴⁴

Gut microbiota metabolize ethanol into acetaldehyde and acetate via aldehyde dehydrogenase. Bacterial taxa rich in aldehyde dehydrogenase activity include Enterobacteriaceae, and abundance of this bacterial family can lead to accumulation of acetaldehyde, resulting in further breakdown of the mucosal barrier, increased inflammation, and adaptive immune activation.^{45,46}

Enterohepatic circulation of bile acids (BAs) also plays a critical role in the gut-liver axis. Gut microbiota convert primary BAs into secondary BAs via deconjugation, dehydrogenation, and dehydroxylation.⁴⁷ The balance of primary versus secondary BAs both in the intestinal lumen and portal circulation regulate metabolic functions, notably glucose and lipid metabolism, via binding to farnesoid X receptor (FXR) and Takeda G-protein coupled receptor-5 (TGR5).⁴⁸ Furthermore, both receptors are expressed ubiquitously on circulating monocytes/macrophages, and therefore dysbiosis-induced alterations in the circulating BA pool can be associated with tissue inflammation.⁴⁹ FXR-binding by BAs also regulates hepatic conversion of cholesterol to primary BAs via hepatic cholesterol 7 α -monooxygenase.⁵⁰ In dysbiosis, this production is decreased resulting in a decreased BA pool, further promoting expansion of gram-negative organisms, increasing the LPS burden, and promoting inflammation.^{51,52} Collectively, enterohepatic circulation of BAs plays an essential homeostatic role in hepatic immunity and metabolism with gut dysbiosis disturbing this balance in pathologic conditions. Mediators of gut-liver cross talk are summarized in Figure 1.

ALTERATIONS IN MICROBIOME POST-LT

LT recipients have multiple factors (eg, abdominal surgery, antibiotics, hospitalizations, and immunosuppression) that can further compound the baseline gut dysbiosis present in ESLD, underscoring the critical importance of understanding the impact of gut dysbiosis post-LT. Several longitudinal studies have now assessed gut dysbiosis in patients post-LT.^{15,53–57} An initial quantitative polymerase chain reaction (qPCR)-based study of 111 LT recipients with hepatitis B virus (HBV)-related liver disease established persistence of dysbiosis in LT recipients.⁵³ Both HBV cirrhotics and LT recipients (with HBV-related liver disease) showed quantitative changes in specific genera consistent with dysbiosis, though importantly, in LT recipients, there was further reduction in *Faecalibacterium*, a bacterial genus shown to have potent anti-inflammatory properties via nuclear factor κ B inhibition and induction of T_{REG}.⁵⁸ This coincided with an increase in pathogenic genera (*Enterococcus* and Enterobacteriaceae family), suggesting LT resulted in initial worsening of dysbiosis with subsequent partial recovery seen in patients 12 to 24 months posttransplant.⁵³

Two subsequent studies assessed perioperative (pre-transplant and within 3 mo of posttransplant) changes in gut microbial composition comparing pre-LT versus post-LT fecal samples in LT recipients using 16S ribosomal RNA gene sequencing. In a small pilot study by Sun et al¹⁵ that assessed perioperative changes in 8 LT recipients at 3 months posttransplant, there were no changes in microbial alpha diversity (richness) noted; however, there were differences in the relative abundance of specific bacterial taxa, notably increases in taxonomic groups that contain many putatively beneficial bacteria, such as *Clostridiales cluster XIVa*, *Blautia*, *Akkermansia*, and Eubacteriaceae family (includes *Eubacterium*), with a reduction in taxonomic groups that contain many pathogenic organisms, notably Enterobacteriaceae family (includes *Escherichia*). This finding underscores the potential limitations on diversity indices as markers of dysbiosis. Kato et al⁵⁵ studied 38 LT recipients and demonstrated for the first time, an initial decrease in SDI early post-LT (<7 d) with subsequent improvement in diversity nearing pretransplant levels by week 8 post-LT. While this study did not report genus-specific changes post-LT, it correlated for the first time low SDI to both acute cellular rejection (ACR) and blood stream infections.

In a study of 45 patients by Bajaj et al,⁵⁶ similar increases in SDI were observed at 6 months post-LT with reduction in the potentially pathogenic family Enterobacteriaceae and increases in potentially beneficial families Ruminococcaceae and Lachnospiraceae (*Clostridiales cluster XIVa*). Interestingly, in this study, there were no differences in SDI in post-LT patients compared with controls; however, there was a greater relative abundance of beneficial bacteria including Lachnospiraceae (*Clostridiales cluster XIVa*), Ruminococcaceae, and Eubacteriaceae in the healthy controls, indicating incomplete recovery of the microbiome post-LT. A follow-up study in a subset of 40 patients assessed functional consequences of these changes via metabolomics analysis.⁵⁷ Improvement in SDI post-LT was associated with reduction in serum endotoxin levels with taxonomic changes similar to those previously reported. Further metabolomic consequences of these compositional microbiota changes included increased microbial conversion of primary to secondary BAs and nontoxic iso- and oxo-BA post-LT, collectively generating an antimicrobial gut milieu (secondary BA) and decreased BA toxicity profile (iso- and oxo-

BA). Interestingly, improvement in diversity resulted in increased conversion of TMA to its atherogenic metabolite, TMAO, though the relevance of this functional consequence remains unclear. The compositional changes in microbiota post-LT are summarized in Figure 2.

IMPACT OF DYSBIOSIS ON IMMUNE-MEDIATED ALLOGRAFT INJURY

Animal models and human studies support a role for the gut microbiome as a modulator of both innate and adaptive liver allograft immunity, presenting a potential role for microbial-based therapies to reduce the risk of immune-mediated allograft injury.^{59,60} Ischemia-reperfusion injury (IRI) is universal following LT with initial ischemic injury leading to parenchymal metabolic disturbance and cell death. This results in the release of danger-associated molecular patterns that signal via PRR to activate innate immune cells (eg, Kupffer cells). Subsequent reperfusion potentiates this proinflammatory innate immune response that, if persistent, can impact the adaptive immune response.^{61,62} The severity of IRI predicts early allograft dysfunction and long-term graft survival and is of particular relevance to study the impact of the gut microbiome on early innate immune activation.^{63,64} Administration of probiotics, specifically Bifidobacterium and Lactobacillus, has been shown to ameliorate IRI in a mouse model via reduction in plasma endotoxin levels and restoration of intestinal barrier function.⁶⁵ Furthermore, in a rat model of LT, liver ischemic preconditioning (short periods of ischemia-reperfusion to condition tissue for a more prolonged IRI) restores gut microbial composition and ameliorates IRI, specifically increasing Lactobacillus, Bifidobacterium, and Clostridiales, with decreased Proteobacteria compared with untreated controls.⁶⁶ Gut bacteria-derived SCFA are potent immunomodulators and can inhibit macrophage activation, a critical mediator of IRI.^{39,61} Intravenous administration of butyrate alleviates IRI in mouse models and administration of acetate-producing Bifidobacterium probiotic strains alleviates kidney IRI in a mouse model, supporting a role for targeted administration of SCFA-converting bacteria.^{39,40}

BA signaling via FXR has also been shown to ameliorate IRI, and therefore targeting early restoration of autochthonous bacteria responsible for BA conversion to secondary BA, which include FXR agonists deoxycholic acid and lithocholic acid, is another potential therapeutic approach.^{57,67} While no studies are yet reported in humans, these findings support a role for microbial-based interventions to reduce IRI and subsequent early allograft dysfunction.

The microbiome can also shape adaptive immunity, and changes in the gut microbiota have been associated with ACR. Initial clinical trials investigating the impact of gut decontamination and administration of pre/probiotics showed no impact on liver allograft rejection.⁶⁸ However, subsequent animal models of LT have established a role for an altered microbiome in ACR, with initial observations demonstrating an altered microbiome as early as 1 week post-LT in rats with ACR.⁶⁹ In a more discrete analysis with serial fecal sampling in the first week following LT in rats, Ren et al⁷⁰ demonstrated that there was a dramatic shift in gut microbiome composition between rats that developed ACR and those that did not at post-LT day 3 (coinciding with early ACR) and day 7 (coinciding with severe ACR). The SDI was decreased in all rats post-LT compared with controls with a trend toward lower diversity at day 7 in rats with ACR. Quantification of dominant bacterial species by qPCR in rats with ACR at day 7 revealed reduction in beneficial bacteria *Faecalibacterium prausnitzii*

and *Lactobacillus* coinciding with an increase in *Clostridium bolteae*, an opportunistic pathogen. These findings support dysbiosis as a potential precursor to clinically apparent ACR post-LT.

Low-diversity dysbiosis has also been associated with ACR in humans post-LT. In a single longitudinal study, lower SDI was seen in 8 patients who developed ACR compared with those who did not develop ACR.⁵⁵ Specific bacterial families altered in patients who developed ACR included increases in Bacteroides, Enterobacteriaceae, Streptococcaceae, and Bifidobacteriaceae, with decreases in Enterococcaceae, Lactobacillaceae, Clostridiaceae, Ruminococcaceae (which includes *Faecalibacterium*), and Peptostreptococcaceae. Importantly, 5 patients had received antibiotics 7 days before the diagnosis, making generalization of these findings difficult. The impact of prebiotics on ACR has been assessed in a meta-analysis of 3 randomized controlled trials, all of which incorporated *Lactobacillus* into their probiotic formulation, though differences in specific species, dose, timing, and additional probiotics were present (Table 1).^{71–74} While there was a difference in ACR (20% in probiotic group vs 28% in control group), this was not statistically significant. Importantly, observational clinical trials are ongoing, and while no further data are yet available correlating rate of recovery of dysbiosis to ACR, initial reports from a longitudinal study from Columbia show that persistence of dysbiosis posttransplant is associated with 1-year mortality post-LT.⁷⁷

IMPACT ON INFECTIOUS POSTTRANSPLANT COMPLICATIONS

Modulation of the gut microbiota has been shown to impact the risk of infection post-LT; however, specific qualitative and functional predictors of infection remain elusive. Single-center studies have consistently shown reduction in post-LT infectious complications with administration of probiotics ± prebiotics.⁷⁸ In a systematic review and meta-analysis examining the pooled outcomes of 4 controlled studies that are assessing the impact of probiotics, findings revealed a reduction in infection rate (7% in probiotics group vs 35% in controls) and decreased length of hospitalization and intensive care unit stay (mean reduction 1.41 d for both in probiotic group).⁷¹ All 4 studies incorporated *Lactobacillus* into their probiotic formulations and 2 incorporated *Bifidobacterium*, both genera reduced in post-LT dysbiosis (Table 1).^{53,57,72–75} A subsequent single-center study showed similar reduction in posttransplant infectious complications with *Lactobacillus lactus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum* probiotic administration (4.8% in probiotic group vs 34.8% in controls; $P=0.02$).⁷⁶ Furthermore, in a small cohort of human LT recipients, low-diversity dysbiosis defined by low SDI was associated with blood stream infections when compared with time-matched controls, supporting the importance of dysbiosis as a predictor of infection and as a target for intervention.⁵⁵ Additional studies are needed to identify specific microbial-based targets for intervention as well as to define mechanisms by which probiotic use (with or without prebiotics) abrogates infection risk.

IMPACT OF DYSBIOSIS ON REGENERATION FOLLOWING PARTIAL GRAFT TRANSPLANTS

Innate immune activation is known to be a critical regulator of hepatic regeneration, and therefore targeting the microbiota to promote hepatic regeneration following transplantation with partial liver grafts (including living donor LT) is promising. Early mouse studies demonstrated that LPS is a critical trigger of hepatic regeneration following partial hepatectomy via increased deoxyribonucleic acid synthesis.⁷⁹ Subsequent studies involving bowel decontamination with antibiotics have revealed differing effects on hepatic regeneration, likely owing to different spectrums of activity.^{80,81} Commensal bacteria that are ampicillin-sensitive, specifically Eubacteria, Lactobacillus, and Clostridium families, appear to be necessary for promotion of hepatic regeneration.⁸¹ Interestingly, loss of these commensal bacteria led to a loss of Kupffer cell tolerance and an increase in activation in natural killer T (NKT) cells, the latter resulting in impaired hepatic regeneration. Furthermore, partial hepatectomy itself induces dynamic changes in gut microbiota characterized by decreased Firmicutes families (Clostridiales, Lachnospiraceae, and Ruminococcaceae) with increased Bacteroidetes families (Bacteroidetes S24–7 and Rikenellaceae), mirroring changes seen in decompensated cirrhosis.^{5,82}

BAs play a critical role in hepatic regeneration and their homeostasis is regulated in large part by microbial modification in the gut. BA signaling via FXR is necessary for normal hepatic regeneration in mice.⁸³ In a cross-sectional study of cirrhotics, dysbiosis is associated with reduced bacterial conversion of primary to secondary BAs (via bacterial 7 α -dehydroxylase) with an overall reduction in circulating BA pool.⁸⁴ Importantly, loss of autochthonous Firmicutes families (Ruminococcaceae and Lachnospiraceae) correlated with reduced conversion of primary to secondary BAs. Priming mice with retinoic acid reverses these maladaptive changes, increasing Ruminococcaceae and Lachnospiraceae, and leads to increased diversity of BAs with increases in enterohepatic circulation that promotes regeneration.⁸⁵

Human studies investigating the impact of dysbiosis and microbial-based interventions in the context of hepatic regeneration are limited, though one small single-center study did show improvement in liver function following right hepatectomy in patients receiving synbiotics (probiotics plus prebiotics; *Pediococcus pentosaceus* 5–33:3, *Leuconostoc mesenteroides* 77:1, *Lactobacillus paracasei* species paracasei F19, and *Lactobacillus plantarum* 2362 plus beta-glucan, inulin, pectin, and resistant starch) with uncomplicated postoperative courses.⁸⁶ Additional cross-sectional and therapeutic studies are needed to better understand the impact of microbial-based therapies on hepatic regeneration for both partial liver graft recipients as well as living liver donors.

IMPACT OF DYSBIOSIS ON DISEASE RECURRENCE

Nonalcoholic Fatty Liver Disease

Approaches aimed to facilitate recovery of dysbiosis and subsequent maintenance of eubiosis may help reduce the risk of recurrent disease post-LT. Gut dysbiosis is central to the

pathogenesis of NAFLD and severity of dysbiosis correlates with both disease onset and progression.^{16,87–91} Therefore, the gut microbiome is a potential target for prevention of recurrent NAFLD post-LT. Low-diversity dysbiosis characterized by an increase in *Escherichia* and *Enterococcus* and decrease in *Lactobacillus* and *Bifidobacterium* is present in animal models of NASH progression, suggesting probiotic interventions beneficial to allograft outcomes may also address dysbiosis related to NASH recurrence.^{92,93} A small study in humans with NASH assessing the impact of probiotics that included *Lactobacillus* and *Bifidobacterium* showed reduction in aspartate aminotransferase and intrahepatic triglyceride content, however, with no change in abundance of *Lactobacillus* or *Bifidobacterium* in the gut.^{94,95} Additional observational studies have demonstrated conflicting results about relative abundance of specific genera in adult patients with NASH, likely owing in large part to dietary and disease severity differences, and underscore the complexity of microbial-based interventions for recurrent NASH post-LT.⁸⁷ There is a growing body of evidence for metabolic consequences of dysbiosis, both in the context of NAFLD and independent of liver disease, suggesting that microbial-based therapies may not only prevent the risk of de novo or recurrent NAFLD but also reduce the risk of metabolic complications associated with immunosuppressant side effects.³⁶

Hepatocellular Carcinoma

The gut microbiome has been shown to impact hepatocarcinogenesis in disease models; therefore, microbial-based therapies may be useful to reduce the risk of recurrent HCC post-LT. Gut bacteria-derived LPS has been demonstrated to promote inflammation and accelerate hepatocarcinogenesis in mouse models via its action on both parenchymal cells and nonparenchymal cells, supporting a central role for the gut-liver axis in the development of HCC.^{96,97} In a small cohort of 15 patients with cirrhosis and HCC, culture-based analysis revealed an increase in *Escherichia coli* compared with patients with cirrhosis but without HCC, supporting this hypothesis.⁹⁸ Subsequent culture-independent analysis in 20 patients with NAFLD-related HCC revealed relative decreases of *Bifidobacterium* and *Blautia* compared with NAFLD-related cirrhosis without HCC coinciding with decreased circulating inflammatory markers and peripheral mononuclear cell activation.⁷ Interestingly, *Bifidobacterium* has been shown to modulate antitumor immunity and administration of probiotic formulation containing *Bifidobacterium* strains ameliorated tumor size and progression in a rat model of HCC.^{99,100} Taken together, these data support a possible role for *Bifidobacterium*-based therapy to reduce the risk of recurrent HCC posttransplant.

Additional microbial-based mechanisms of HCC have been recognized. Using qPCR, *Faecalibacterium prausnitzii* was noted to be reduced in a cohort of cirrhotic patients with HCC compared to those without, suggesting a potential additional role for SCFA as a risk modifier via maintenance of intestinal barrier function and systemic anti-inflammatory effects.⁵³ Modulation of gut microbiota may also impact antitumor surveillance as evidenced by a mouse model that demonstrated that antitumor NKT cell recruitment is dependent on microbial conversion of primary to secondary BAs.¹⁰¹ While more studies are needed, there is growing evidence that microbial-based therapies can impact recurrence of HCC in LT recipients.

Cholestatic Liver Disease

Low-diversity dysbiosis has been associated with cholestatic liver diseases and microbial-based therapies may hold promise to reduce the risk of recurrence of PSC and primary biliary cholangitis (PBC) post-LT. Mouse models of PSC have established the importance of dysbiosis in disease progression, in part through alterations in microbial-mediated BA metabolism.^{102,103} Alterations in BA composition and gut microbial composition have also been noted in patients with both PSC and PBC.^{104–108} PSC has been consistently associated with low-diversity dysbiosis with enrichment in *Veillonella* as well as *Enterococcus*.^{105,106,109,110} Similarly, in a single study of PBC patients, PBC was associated with reduced microbial diversity, increases in 8 genera including *Veillonella*, and decreases in 4 genera including *Faecalibacterium*, when compared with healthy controls. These changes were partially reversed with administration of ursodeoxycholic acid, a secondary BA.¹⁰⁸ Multiple antibiotic and probiotic trials have demonstrated biochemical improvement in patients with PBC and PSC,¹¹¹ though ongoing studies are needed for targeted interventions that may be applicable to prevention of recurrent disease post-LT. Interestingly, a small single-center study described alterations in the gut microbiota in post-LT patients with nonanastomotic strictures compared to those without, notably increased *Enterococcaceae*, *Streptococcaceae*, and *Enterobacteriaceae* with decreased *Bacteroidaceae*, suggesting a potential role for microbial-based interventions to prevent post-LT biliary complications independent of cholestatic liver disease.¹¹²

OPERATIONALIZING FUNCTIONAL MICROBIOMICS IN LT

Moving forward, several issues warrant attention to ensure sustained progress in translating our evolving wealth of knowledge on the microbial changes described into clinically meaningful interventions. Diversity indices, while helpful as coarse measures to associate dysbiosis with an outcome or specific patient population, do not incorporate taxa-specific changes that mechanistically may be essential to causation of a specific clinical end point. For instance, *Enterococcus* species have been associated with severity of PSC and, given their abundance in bile, may be of particular importance for biliary complications. Alternatively, loss of butyrate-producing species, such as *Faecalibacterium*, may be specifically relevant to immune-mediated allograft injury given their association with regulatory T-cell induction. Therefore, future studies in LT recipients will need to be designed and powered to focus on specific posttransplant complications.

In addition, ongoing microbiota-based studies in LT recipients will require integration of microbiota, metabolomics, functional data, and confirmatory experiments in *in vitro* and *in vivo* model systems to move beyond clinical associations and establish causative roles and associated mechanisms. This will require multicenter collaboration for comprehensive clinical and biological phenotyping to elucidate the multiscale interactions at play across the gut-liver axis. The FLORINASH (the role of intestinal microflora in nonalcoholic fatty liver disease) study, a multicenter European clinical registry and biorepository, serves as model for this approach, integrating clinical; fecal 16S ribosomal RNA and metagenomics microbiome assessment; liver transcriptome; serum metabolomics, proteomics, and lipidomics; and urinary metabolomics data. The power of this resource is evident in the

recent identification of the novel role of microbial-driven branched-chain amino acid metabolism in hepatic steatosis.¹¹³ Discoveries such as this can then provide specific targets for intervention, via either induction or inhibition of specific microbial pathways. For instance, the latter approach has recently been used to inhibit gut microbial TMA production from choline to reduce atherogenic TMAO production and atherosclerotic disease burden in a mouse model.¹¹⁴ While limited, single-center prospective clinical trials investigating the impact of microbiota on LT outcomes are ongoing, there is a critical need for comprehensive, multicenter studies to refine specific microbial targets for intervention.

Importantly, the magnitude of impact of changes in microbiota on post-LT clinical outcomes must also be considered. For instance, is the relative abundance of *E coli* posttransplant a more powerful prognostic tool for HCC recurrence than the presence of viable tumor in the explant? Can microbiome composition data be considered together with well known predictors of HCC recurrence post-OLT, such as Milan/University of California, San Francisco criteria and microvascular invasion, to improve prognostics? These prognostic inferences will require more robust prospective studies and incorporation of currently validated clinical predictors to determine the true impact of dysbiosis on clinical outcomes in LT recipients. Collectively, using these principles will eliminate the heterogeneity observed in nontargeted pre- and probiotic interventional studies to date and provide novel, targeted, and impactful microbial-based interventions moving forward.

FUTURE DIRECTIONS

Microbiome-based research has to date focused on bacteria; however, the microbiome includes fungi and viruses, both of which may have additional metabolic and immunologic consequences on the liver allograft. For instance, chronic alcohol ingestion results in increased intestinal fungi, notably *Candida* species, coinciding with increased portal 1,3- β -d-glucan that leads to activation of hepatic macrophages. This finding correlated with severity of alcoholic liver disease in a small cohort, suggesting this may be a critical mediator and potential target to ameliorate hepatic inflammation.¹¹⁵

The interplay between immunosuppression agents and gut microbiome is another evolving area of interest. Tacrolimus has been associated with dysbiosis, decreasing overall gut microbiota diversity associated with loss of butyrate-producing species. Furthermore, when compared with everolimus and mycophenolate mofetil, patients on tacrolimus and mycophenolate mofetil had a distinct bacterial metagenome, suggesting functional differences in gut microbiota based on immunosuppression regimen were present independent of taxonomic changes.^{116,117} Bacterial species may also impact drug absorption and metabolism and, conversely, can ameliorate drug-specific side effects as has been shown with *Lactobacillus* and tacrolimus-induced hypertension.^{116,118}

Given the abundance of donor passenger lymphocytes and liver-resident innate immune cells transferred from the donor to recipient in LT, the donor microbiome may also be of clinical significance. Interestingly, there is evidence that the donor microbiome may impact alloimmunity in a cohort of patients following bone marrow transplant with increased donor

microbial diversity associated with lower rates of acute graft-versus-host disease in recipients, supporting a possible role for donor-based interventions for the liver allograft.¹¹⁹

Ultimately, understanding the evolution of the functionality of the microbiome both pre- and post-LT will be critical to translate our current knowledge into therapeutic targets. While clinical investigation has thus far focused largely on administration of pre- and probiotics, more sophisticated and focused approaches, including bacterial gene editing to modify specific metabolic pathways are being developed.¹²⁰ Combined with ongoing usage of gnotobiotic animal models to delineate specific functional consequences of individual bacterial taxa, more targeted, microbial-based interventions are likely to evolve (Figure 3).¹²¹

CONCLUSIONS

The gut microbiome is a central participant in regulating hepatic metabolism and immunity and our understanding of its impact on post-LT physiology is evolving at an increasingly rapid pace. As we continue to obtain data from longitudinal studies of post-LT patients, therapeutic targets for microbial-based interventions will continue to evolve to improve the allograft function and to reduce the risk of post-LT complications. Our hope is that microbial-based therapies will be used in concert with our current standard of care to improve clinical outcomes in post-LT patients.

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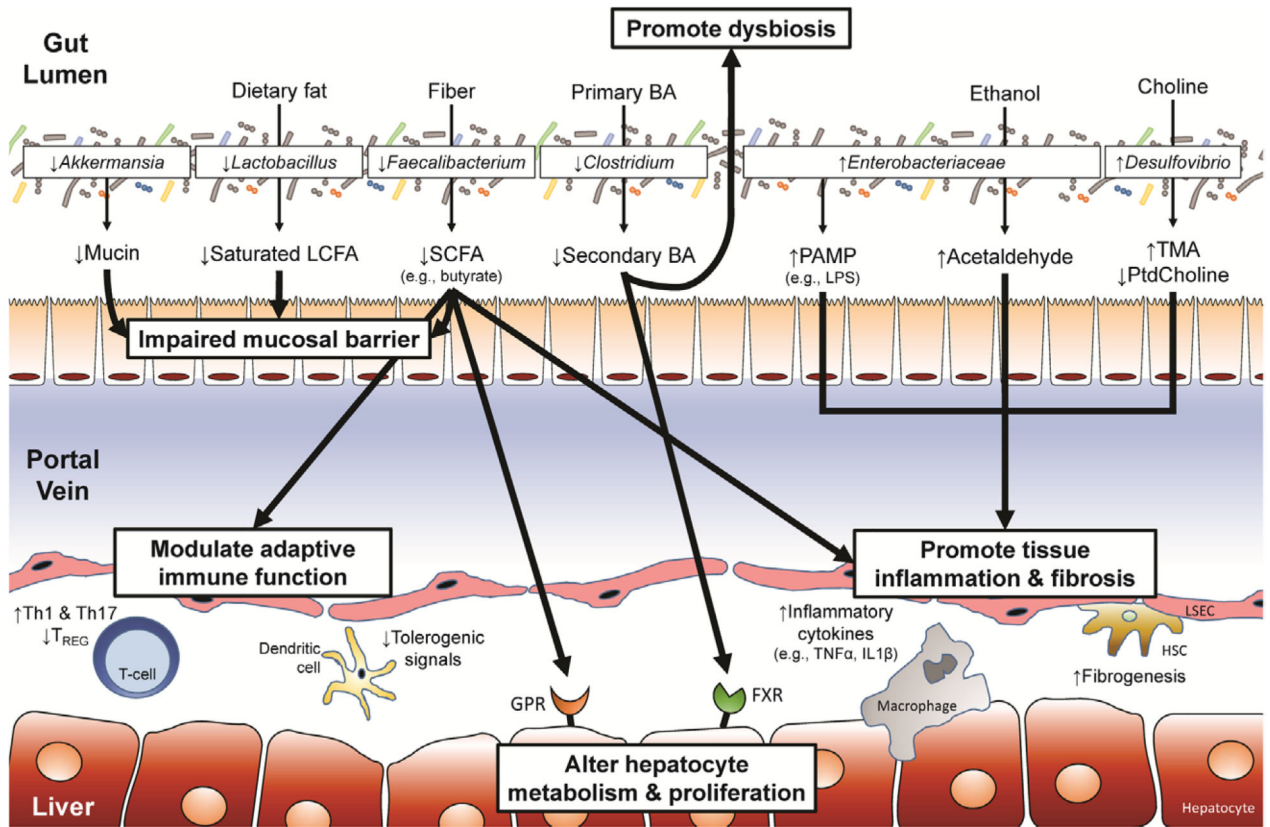


FIGURE 1. Mechanisms of altered hepatic immunity and metabolism secondary to gut dysbiosis. Gut dysbiosis leads to an imbalance in bacterial microbes that results in alterations in microbial metabolites delivered to the liver via the portal vein that ultimately lead to changes in hepatic immunity and metabolism. Depicted are examples of specific bacteria that have been shown to be altered in gut dysbiosis related to end-stage liver disease or postliver transplant complications in human and mouse models. Corresponding alterations in microbial metabolites are also noted. Collectively, these changes further promote dysbiosis (via reduced secondary bile acid [BA] conversion), impair mucosal barrier function (via reduction in mucin, long-chain fatty acid [LCFA], and short-chain fatty acid [SCFA]), promote tissue inflammation (via increased pathogen-associated molecular pattern [PAMP], acetaldehyde, and trimethylamine (TMA)/trimethylamine-N-oxide (TMAO) with reduced SCFA and phosphatidylcholine [PtdCholine]), impair adaptive immune tolerance (via reduced SCFA and increase in inflammatory cytokines/chemokines), and alter hepatic metabolism and cellular proliferation (via G-protein coupled receptor [GPR] and farnesoid X receptor [FXR] signaling by SCFA and BAs, respectively). These functional changes due to gut dysbiosis highlight the mechanisms by which an altered microbiome can potentially impact allograft function. HSC, hepatic stellate cell; IL, interleukin; LSEC, liver sinusoidal endothelial cell; LPS, lipopolysaccharide; TNF, tumor necrosis factor; T_{REG}, regulatory T cells.

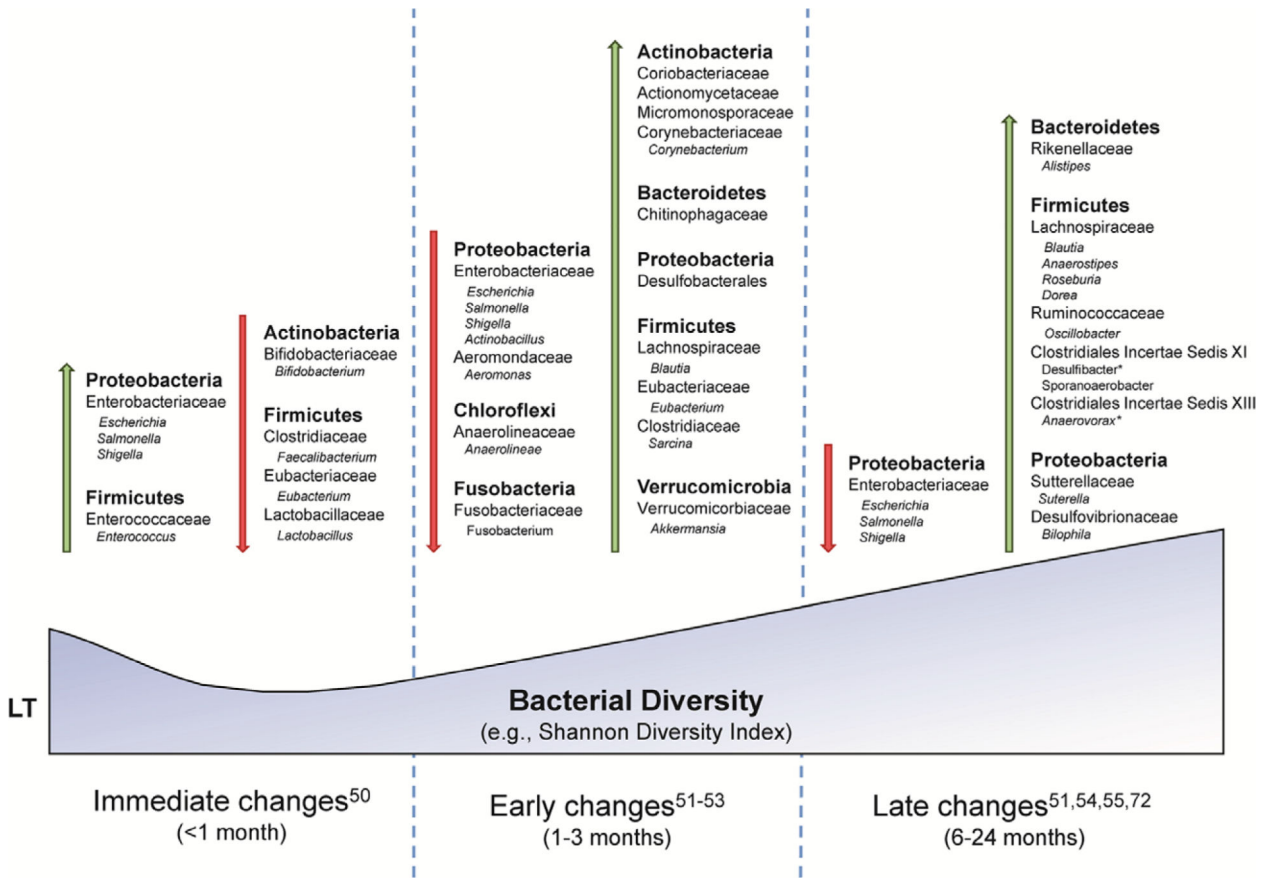


FIGURE 2. Changes in gut microbiome in liver transplant (LT) recipients. All changes in gut microbiome are relative to pre-LT cirrhotic patients at the designated time point post-LT. All bacteria are identified by phyla and further classified by family and genus (with the exception of desulfobacterales).

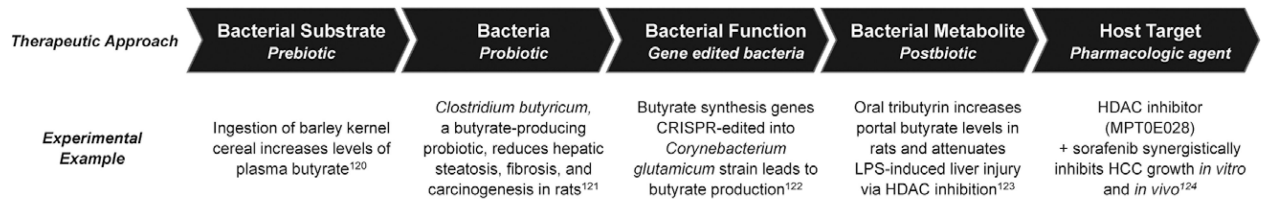


FIGURE 3.

Potential approaches to microbial-based therapy in the liver allograft. While clinical trials to date have focused on administration of pre- and probiotics, our physiologic understanding of the functional impact of an altered gut microbiome as well as our ability to therapeutically manipulate the gut microbiome and microbial metabolites is rapidly evolving.

Administration of pre- and probiotics is shifting to target taxa-specific microbial pathways; functional modification of bacteria via gene editing or pathway inhibition allows for modulation of specific metabolites; and targeted post-biotics and synthetic analogs present opportunities for novel therapeutics informed by microbial pathways. Depicted are experimental examples of targeting microbiota-mediated butyrate production at multiple levels along the microbial pathway.^{122–126} This construct can be applied to other microbial pathways for therapeutic intervention specific to the effects of individual microbiota and microbial metabolites. HCC, hepatocellular carcinoma; HDAC, histone deacetylase; LPS, lipopolysaccharide.

TABLE 1.

Summary of probiotic clinical trials in liver transplant recipients

	Probiotic	Dose, CFU	Duration	ACR (risk ratio)	Infection (risk ratio)	LOS (mean difference), days
Rayes et al ⁷²	<i>Lactobacillus plantarum</i> 299	1 × 10 ⁹	Day 1 to day 14	0.69 (0.37:1.29)	0.38 (0.13:1.05)	-1.00 (-2.26 to 0.26)
Rayes et al ⁷³	<i>Pediococcus pentosaceus</i> 5-33:3 <i>Leuconostoc mesenteroides</i> 77:1 <i>Lactobacillus paracasei</i> species <i>paracasei</i> FI9 <i>Lactobacillus plantarum</i> 2362	10 × 10 ⁹	Day 0 to day 14	0.86 (0.32:2.28)	0.06 (0.01:0.44)	-1.40 (-2.09 to -0.71)
Eguchi et al ⁷⁵	<i>Lactobacillus casei</i> strain Shirota <i>Bifidobacterium breve</i> strain Yakult	20 mg 15 mg	Day -2 to day 14		0.17 (0.02:1.29)	
Zhang et al ⁷⁴	<i>Lactobacillus acidophilus</i> (LA-14) <i>Lactobacillus plantarum</i> (LP-115) <i>Bifidobacterium lactis</i> (BL-04) <i>Lactobacillus casei</i> (LC-11) <i>Lactobacillus rhamnosus</i> (LR-32) <i>Lactobacillus brevis</i> (LB-35)	27 × 10 ⁹	Day 1 to day 7+	0.67 (0.12:3.65)	0.29 (0.09:0.96)	-2.00 (-3.44 to -0.56)
Grat et al ⁷⁶	<i>Lactococcus lactis</i> PB411 <i>Lactobacillus casei</i> PB121 <i>Lactobacillus acidophilus</i> PB111 <i>Bifidobacterium bifidum</i> PB211	3 × 10 ⁹	Day of listing to day 0 (16.7% <2 weeks)		0.18 (0.02:1.37)	
Cumulative data				0.73 (0.44:1.22)	0.20 (0.11:0.38)	-1.41 (-1.97 to -0.86)

The findings of interventional probiotic clinical trials in liver transplant recipients are summarized. All patients received enteral nutrition with fiber supplementation in addition to the listed probiotics with the exception of Eguchi et al⁷⁵ (enteral nutrition alone) and Grat et al⁷⁶ (no additional nutritional supplementation).

ACR, acute cellular rejection; CFU, colony-forming units; LOS, length of stay.