



Original Article



Identification and Analysis of Gut Microbiota and Functional Metabolism in Decompensated Cirrhosis with Infection

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Abstract

Background and Aims: Intestinal dysbiosis play a role in the adverse outcomes of sepsis and septic shock. However, variations in bacterial diversity and microbiota-related functional metabolic alterations within the gut microbiome in decompensated cirrhosis (DC) patients with infection remain unknown. **Methods:** We conducted 16-srRNA sequencing on stool samples ($n=51$: sepsis, 27/no sepsis, 24) collected from consecutive DC patients upon admission. Bacterial diversity, significant taxa, and respective metabolic profiling were performed based on subgroup comparisons. Conet/Cytoscape was utilized to identify significant non-random patterns of bacterial copresence and mutual exclusion for clinical events. **Results:** Genera associated with pathogenicity in conditions of immune exhaustion (*Corynebacterium*, *Lautropia*) were predominant in patients with sepsis. Metabolic pathways associated with oxidative stress and endotoxemia [lipopolysaccharide (LPS) synthesis and sulfur relay] were significantly upregulated in sepsis. Specific taxa were associated with sites of infection in DC patients. Protective oxidant pathways that increase glutathione were upregulated in those without sepsis. Gammaproteobacteria family of sulfur-metabolizing bacteria, exaggeration of orally predominant pathogens (*Prevotella*), and pathways of severe LPS-related hyperinflammatory stress were notable in those with interleukin-6 levels $>1,000$ pg/dL. Pathogenic genera related to an immune deficient state was significant in DC with ≥ 2 infection episodes. Megamonas was associated with survival during the same admission. **Conclusions:** Specific gut microbiota and their metabolites were associated with sepsis and related events in patients with DC. Identifying beneficial strains that reduce immune exhaustion and supplementation of favorable metabolites

could improve therapeutics for DC and sepsis, for which larger prospective, well controlled population-based studies remain an unmet need.

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Introduction

The human microbiome, a collective genome of millions of bacteria, viruses, and fungi, have a sophisticated, multidirectional, and mutualistic relationship with their human host. At the core of this interaction is the gastrointestinal tract, which contains trillions of bacteria that create a complex ecosystem called the bacterial gut microbiota (BGM). The BGM affects host health and play major role in acute as well as chronic disease conditions. Autochthonous BGM impede pathogens through selective and controlled expansion, beneficial metabolite and nutrient production, and endocrine interactions while closely corresponding with the local and systemic immune system. Disruption in these mechanisms interact with other factors such as environmental (occupation and drug/toxin exposure), host (genetic predisposition, alcohol, and tobacco), and disease states (metabolic syndrome and cirrhosis). That leads to initiation of both quantitative (diversity reduction) and qualitative (perturbed functional metabolism) changes in the BGM, a process known as dysbiosis. Dysbiosis worsens existing disease (e.g., cirrhosis progression) or causes new clinical events (e.g., infections).¹ The BGM plays a central role in the etiology and progression of various liver diseases (e.g., alcohol associated hepatitis) and clinical events in cirrhosis (e.g., hepatic encephalopathy), and its modulation ameliorates adverse patient outcomes.²⁻⁴ Recent evidence had demonstrated that dysbiosis of the BGM and its functions promote sepsis, "a life-threatening organ dysfunction caused by dysregulated host response to infection," and associated organ dysfunction in affected patients. New research has shown that microbiota modulation may help improve clinical outcomes and organ dysfunction associ-

Keywords: Microbiome; Portal hypertension; Sepsis; Acute-on-chronic liver failure; Multidrug resistance.

Abbreviations: BGM, bacterial gut microbiota; DC, decompensated cirrhosis; iDC, infected decompensated cirrhosis; niDC, non-infected decompensated cirrhosis; IL, interleukin; HE, hepatic encephalopathy; MDR, multidrug resistant; RA, relative abundance; SCFA, short-chain fatty acids; UTI, urinary tract infection.

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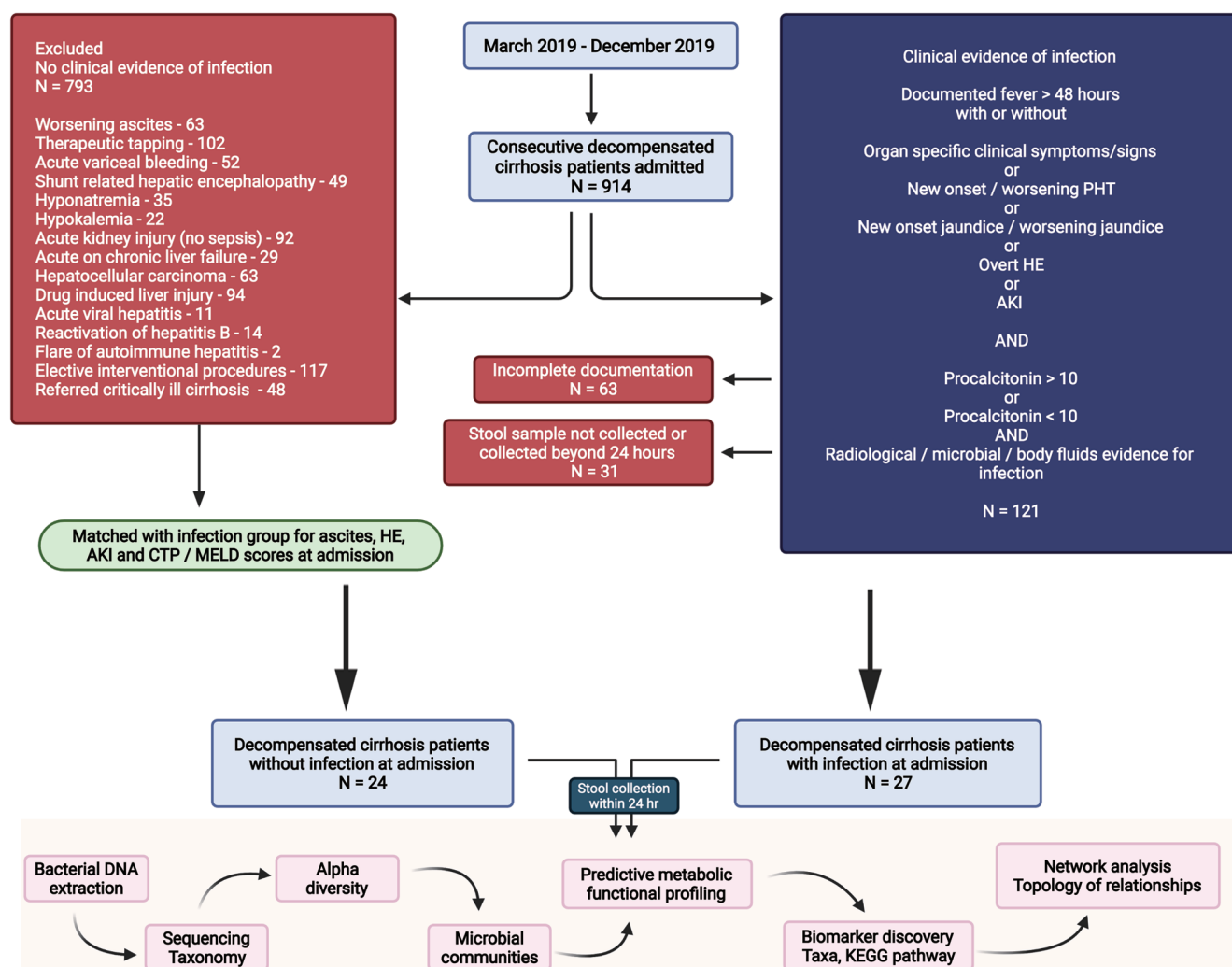


Fig. 1. Study design, inclusion and exclusion and patient enrollment.

ated with sepsis.^{5,6} Nonetheless, the role and function of BGM in patients with advanced cirrhosis and sepsis remain unknown. The current work therefore aimed to characterize the BGM and its functions associated with specific clinical and biochemical events among decompensated cirrhosis (DC) patients who were infected (iDC) or noninfected (niDC).

Study objectives

The study objectives were to characterize significant bacterial communities and their functional metabolism between iDC and niDC patients and distinguish and identify significant bacterial groups and associated functional metabolites among iDC patients grouped into the following sepsis specific categories: (1) infection sites, (2) infection episodes (0, 1, or ≥ 2), (3) Systemic inflammation in sepsis evaluated by interleukin (IL)-6 (<100, 100–1,000, and >1,000 pg/dL), (4) outcome during hospital stay, and (4) outcome 180 days after admission. We also described the topology of the relationships between pertinent bacterial taxa associated with grouped parameters and events in DC with and without infections using network analysis.

Methods

Patients

From March 2019 to December 2019, consecutive patients with decompensated cirrhosis admitted to the Liver Unit through the emergency and outpatient departments were included (Fig. 1). This study protocol was approved by the Institutional (hospital) review board and was performed in accordance with the Helsinki declaration of 1975 and its revisions. All participants provided informed consent for the use of de-identified fecal samples. A comprehensive clinical and drug history was obtained at admission, including events occurring over the preceding 3 months. This included details on prior admission for infection, exhaustive evaluation during the previous admission and discharge summaries when available, and decompensation events. Decompensation was defined as the presence of either jaundice, ascites, kidney injury, hepatic encephalopathy, or a combination of those. Patients with suspected drug-induced liver injury (including complementary and alternative drugs), those listed for liver transplantation, and those with index presentation as acute on chronic liver failure were excluded. Similarly,

patients with acute variceal bleeding, those with hepatic and extrahepatic malignancies, those undergoing interventional vascular and invasive hepatobiliary procedures, those with severe cardiopulmonary disease, those with advanced cirrhosis on multiple organ support, and those patients on hemodialysis were also excluded. Infection was suspected based on history and clinical and physical examination and confirmed either by radiological, biochemical, or microbiological evidence along with blood and body fluid analyses. All patients with serum procalcitonin ≥ 10 ng/mL were considered to harbor bacterial infection even without an identifiable source. When serum procalcitonin was < 10 ng/mL infection was confirmed when either radiological, blood and body fluid, or microbiological evidence supported the same.^{7,8} IL6 levels (normal < 7 pg/mL) were documented only to determine systemic inflammation and not to identify infection or sepsis. Decompensated cirrhosis patients with and without infections were matched for the presence of ascites, hepatic encephalopathy (HE), and acute kidney injury, Child Turcotte Pugh stage, and model for end-stage liver disease scores at baseline using the case-control matching function in MedCalc software (Ostend, Belgium). All included patients were started on intravenous third-generation cephalosporins, and antibiotics were modified per site of infection and culture sensitivity during the course of hospitalization.

Sample collection

Stool samples were collected within 24 h of hospital admission in sterile containers, immediately processed into aliquots within 1 h, and stored at -80°C in the in-house storage designated for the protocol. After collecting a minimum of 10 samples during a period, RNA/later (Ambion/Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was added to the aliquots, which were then transported to the main laboratory facility within 4 h while maintaining a cold chain -20°C for DNA extraction and further analysis.

DNA extraction and sequencing of bacterial communities

Approximately 200 mg of the provided stool sample was used for bacterial DNA extraction, using a defined and published protocol modification of the commercially available QIAamp DNA Stool Mini Kit1 (Qiagen, Venlo, Netherlands). Sequencing as per standardized validated methods at the V3-V4 regions was performed with an Illumina MiSeq next-generation sequencer (Illumina, San Diego, CA, USA) using the Illumina kit at 2×300 paired-end sequencing, after which taxonomic classification was performed according to the GreenGenes Database (version 13.8).

Metagenomic statistical methods

The alpha diversity, including phylogeny (i.e. summed evolutionary age of all the species in the community), was presented using phylogenetic diversity (PD or Faith's PD) measure, which uses phylogenetic distance to calculate the diversity of a given sample. Phylogenetic distance represents the number of changes that have occurred within a particular taxon (branch) ascertained within groups or between groups. Quantitative Insights into Microbial Ecology (QIIME version 2) was used to ascertain the quantitative and qualitative microbial communities.⁹ The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway was utilized to study functional metabolic interactions and relationships

within sequenced bacterial communities.¹⁰ Analysis of Similarity was used to test for a statistical difference between two or more microbial communities (alpha diversity). A p -value < 0.05 was considered to indicate statistically significant differences between grouped sample attributes.¹¹ The visualization tool Circos (v.0.69-9; tool version: 0.23) was used to facilitate the exploration and analysis of similarities and differences arising from comparisons of bacterial communities. The table viewer script in Circos tools was used to format abundance data. This method shows significant positive, negative, or neutral interactions between communities specified for the variable. Output data were presented using a circular chord ideogram layout that displayed the relationships among microbial communities concerning clinical or investigation variables at the phylum/class/order/family taxonomic level. To simplify the graphical output, tabulations were made by compounding all analyzed clinical or investigation variables.¹² Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, version 1.1.1) was used for predictive metabolic functional profiling of microbial communities using 16S rRNA marker gene sequences precalculated for protein-coding genes present in KEGG gene families and 16S rRNA gene copy number.¹³ Linear discriminant analysis effect size (LEfSe) combined with the Kruskal-Wallis and pairwise Wilcoxon tests were utilized to identify significant differences in abundance and functionality of microbial communities between groups. We used default significance (alpha value=0.05) and linear discriminant analysis thresholds (2.0) at all taxonomic levels between time points. To simplify the graphical output, tabulations on significant taxa and the associated functional metabolic pathways were provided.¹⁴

Bioinformatics and network analysis

Networks were inferred using CoNet (v.1.1.1-beta) application within Cytoscape (v.3.7.2). The following measures, implemented by CoNet, were used to detect copresence/exclusion between bacterial communities: Pearson, Spearman, Mutual Information, Bray-Curtis dissimilarity, and Kullback Leibler dissimilarity. CoNet was also set up to determine the comparison metadata for each sample (i.e., the respective clinical or investigational variable grouping). Moreover, the following network measures were calculated using NetworkX (version 2.2): degree centrality, betweenness centrality, closeness centrality, and added to the node attributes in the graph. The centrality measure of *degree* assigns importance to a specific node (microbe at bacterial phylum/family/genus level) in the form of the number of direct connections to other nodes (microbes). The centrality measure of *betweenness* represents the number of times a microbe lies on the shortest path between other microbes, which identifies microbes that serve as the best "bridges" between other microbes in the network. This measure demonstrates the bacteria that influence flow around a system (in this case, the clinical or investigation variable). A high betweenness indicated the microbe that holds authority over different clusters in the specified network. The centrality measure of *closeness* represented microbial (nodal) closeness to all other nodes (microbes) in the network. These measure the shortest path between all nodes (microbes) and identifies the individual microbe best placed to influence the entire network more quickly or those who are good "broadcasters" of information (metabolic-cross talk). The topology of constructed networks and significant taxa, the top 2% of all taxa analyzed, were presented in a simplified tabular form based on these attribute indices of degree, closeness, and betweenness centrality measures.¹⁵⁻¹⁷ General statistical analysis was performed using MedCalc

Table 1. Clinical characteristics of patients enrolled into the study

	Infection absent (<i>n</i> =24)	Infection present (<i>n</i> =27)
Males	96.4%	97.8%
Age (years)	66.8±4.6	69.2±5.1
Etiology of cirrhosis	Alcohol: N=19, 79.2%	Alcohol: N=23, 85.1%
One episode of infection in past*	8.3%	62.9%
Two or more episodes of infection in past*	4.2%	37%
Active ascites at admission	70.8%	88.8%
Active hepatic encephalopathy at admission	33.3%	48.1%
Acute kidney injury at admission	37.5%	40.7%
IL6 level at admission (ng/dL)*#	23.5 (11.5–39.4)	85.7 (31.2–884.1)
Child Pugh Turcotte score at admission†	10 (8–12)	11 (8–13)
Model for end-stage liver disease score at admission†	20.5 (14–26)	21 (15–28)
Rifaximin use during last 90 days	86.8%	90.4%
Cephalosporin use during the last 90 days*	4.1%	48.7%
Piperacillin-tazobactam use during last 90 days*	4.1%	22.2%
Carbapenem use during the last 90 days*	0%	18.5%
Death during same admission*	4.1%	29.6%
Death during follow-up of 180 days	20.8%	40.7%

*Statistically significant at baseline, $p < 0.05$. #Third quartile range in brackets (25–75%). †Medians (minimum-maximum).

version 19.7 (Ostend, Belgium) and NCSS version 12 (Kayville, UT, USA) statistical software. Data were reported as means and standard deviation or as medians and interquartile range between brackets as applicable. The Shapiro-Wilk test was used to test normality and Levene's test was used for non-normal distributions. The Bartlett homogeneity test (for nominal variables) was utilized to assess the equality of variances. To decrease data variability and increase data conformity to a normal distribution, logarithmic transformation was applied. Chi-square and Fisher's exact tests were used to compare normally distributed variables; the Mann-Whitney *U* test was used to evaluate continuous variables. A simplified summary of patient inclusion and methodology is shown in Supplementary Figure 1.

Results

Patients and characteristics at baseline

This study included 51 patients (niDC=24 and iDC=27), with both groups having an equal male distribution. Spontaneous bacterial peritonitis (SBP) was most common in patients with infections ($n=8$, 29.6%), followed by pneumonia and urinary tract infection (UTI, both $n=5$, 18.5% each), skin and soft tissue infections (SSTIs; $n=4$, 14.8%), spontaneous bacteremia ($n=4$, 14.8%), and acute cholecystitis ($n=1$, 3.7%). Culture positivity was noted in three patients with SBP, two with *Escherichia coli* (one multidrug resistant, MDR) and one *Acinetobacter baumannii*, four with pneumonia, three with *Klebsiella pneumoniae* (two MDR) and one with *Staphylococcus aureus*, and three with *Escherichia coli* UTIs (two MDR) and one pan-drug resistant; two with SSTIs (*Escherichia coli* and *Klebsiella pneumoniae*), and four with bacteremia, three *Klebsiella pneumoniae* and one *Escherichia coli* (MDR). The iDC group had a significantly higher number of patients with at least one or two or more documented

infection episodes within the preceding 3 months. At admission, the iDC group had significantly higher IL6 levels compared with the niDC group (median 85.7 vs. 23.5 mg/mL respectively, $p < 0.001$). Moreover, the proportion of patients receiving an injectable cephalosporin (iDC 48.7% vs. niDC 4.1%; $p < 0.001$), piperacillin-tazobactam (22.2% vs. 4.1%; $p < 0.001$), or carbapenem (18.5% vs. 0%; $p < 0.001$) within the preceding 90 days was significantly higher in the iDC group than in the niDC group despite similar rifaximin use in both groups. A significantly higher proportion of patients with active infection compared to those without infection at admission died during the same admission (iDC 29.6% vs. niDC 4.1%; $p < 0.001$) but not at the end of follow-up. Pertinent baseline characteristics of both study groups are summarized in Table 1.

Analysis of bacterial communities

General analysis of bacterial taxa associations between groups: Using the QIIME (v.2) and Greengenes microbial gene database, our analysis showed significant differences in baseline alpha and beta diversities and relative abundances (RA) of bacteria at the phylum, family, and genus levels between the niDC and iDC groups. Alpha diversity differed significantly between the niDC and iDC groups, regardless of infection site, between those with no infections (niDC) and those with two or more episodes of infection (iDC) and among all interleukin cutoffs (iDC). No significant difference in alpha diversity was seen among the iDC group patients who survived or died during same admission or on overall follow-up (Supplementary Figs. 2 and 3). QIIME and Circos analysis showed that the RA of specific bacterial taxa were associated with clinical and investigation variables (Fig. 2 and Supplementary Table 1). Briefly, RAs of the beneficial butyrate-producing family Lachnospiraceae; "nitrogen fixer" family Acetobacteraceae; and the Ruminococcaceae family were found to be inversely correlated

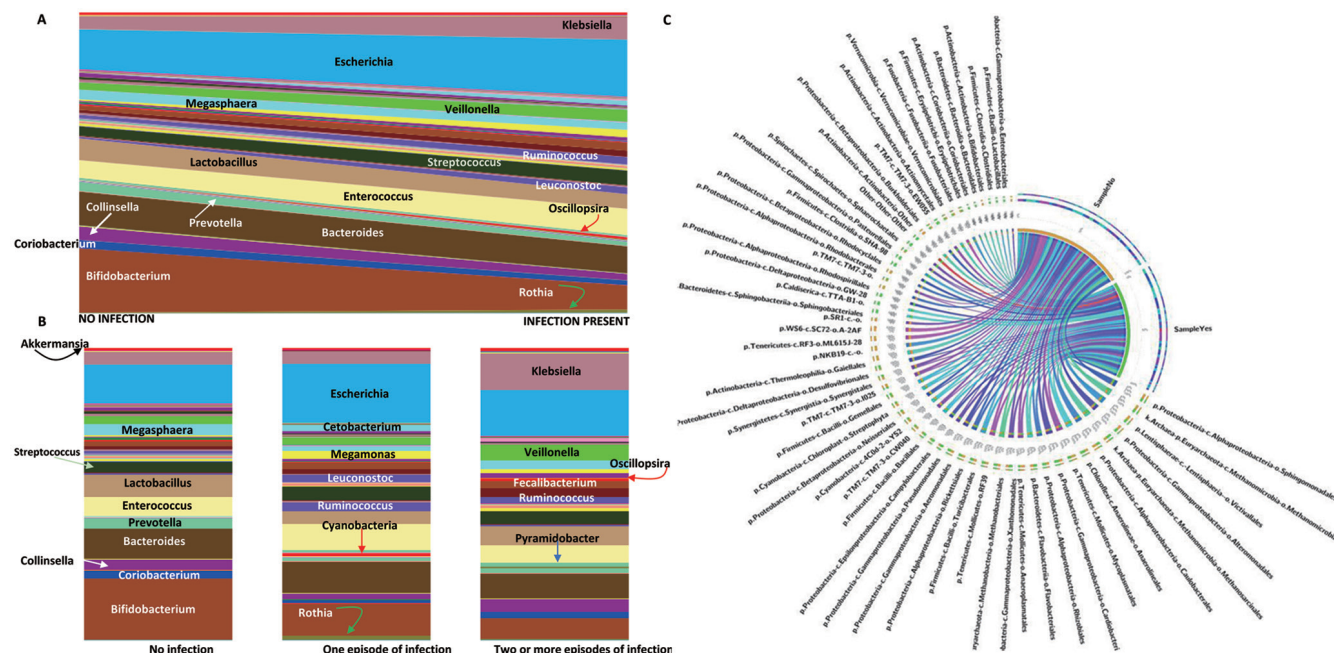


Fig. 2. Raw relative abundances of predominant bacterial genera in decompensated cirrhosis patients without infection compared to those with infections. (A) Area graph representational image output from QIIME analysis. (B) Bar graph representing differences in relative abundances of bacterial genera among decompensated cirrhosis patients without infection and those with one or two or more episodes of infection in the past. (C) Circular plot (Circos) representing bacterial taxa specifically associated with infections compared to those without infection at admission. For summary of the complete Circos output based on clinical and investigation variables, please see Supplementary Table 1.

with intestinal permeability in nIDC patients. Meanwhile, the predominantly pathogenic family Enterobacteriaceae, the oxidative stress-associated manganese oxidizing family Shewanellaceae, and the proinflammatory activity-associated family Oxalobacteraceae were specifically abundant in patients with infections at admission. Among the DC patients with repeated infections, opportunistic pathogens (generally skin predominant) associated with antimicrobial use-related expansion, such as Dermabacteraceae and Anaeroplasmataceae, were observed. Deviant, opportunistic, nonautochthonous phyla, such as Chromatiaceae, Brevibacteriaceae, and Intraspangiacae, were notably greater in patients with bacteremia compared to those with other sites of infection.

Significant bacterial taxa associations and their functional metabolism on multivariate analysis: After identifying the RAs of bacterial population and key bacterial families associated with specific clinical and investigation events, the multivariate LEfSe method utilizing standard tests for statistical significance with additional tests encoding biological consistency and effect relevance was utilized to determine significant bacteria and respective metabolic functions most likely to explain the differences between grouped variables. Briefly, genera associated with “gut barrier health,” such as Bifidobacterium (antimicrobial peptide synthesis, vitamin metabolism, and beneficial butyrate production via cross-feeding mechanism) and Coriobacterium (bile salt and steroid conversion and dietary polyphenol activation), were predominant in patients without infections. Metabolic pathways associated with negating oxidative stress (selenocompound, adipocytokine signaling, and cysteine and methionine metabolism pathways) were significantly upregulated in DC patients without infection at admission. In contrast, pathogenic genera (e.g., Leptotrichia, Lautropia, Neisseria, and Acinetobacter) and metabolic pathways associated with oxidative stress and gut barrier dysfunction (e.g., lipopoly-

saccharide biosynthesis, bacterial chemotaxis, and sulfur relay system) were significantly expressed in DC patients with infections at admission. Among infected patients with IL6 levels <100 and >1,000 pg/mL, Propionibacterium (with immunomodulatory properties, which has a role in human health by occupying niches that are colonized by other more pathogenic microorganisms) and Paraprevotella and Caldiserica, which participate in human disease by promoting chronic inflammation, were significantly increased. This was also associated with the upregulation of propanoate metabolism associated with beneficial short chain fatty acid (SCFA) generation in the former and pentose phosphate pathway associated with heightened oxidative stress in the latter. Similar specific bacterial taxa and metabolic pathways also differed significantly according to number, type, and sites of infection. DC patients who survived sepsis-related admission and were alive until the end of follow-up had significantly more Megamonas at baseline, whereas those who died on follow-up were Kingella and Neisseria predominant (Fig. 3 and Table 2).

Network analysis of bacterial communities’ interaction with respect to clinical variables: Network analysis for interactions using NetworkX revealed striking differences and interactions between bacterial communities when grouped according to clinical and investigational parameters. The main advantage of network topology analysis is that it allows us to understand the type of interaction or association between bacterial communities. Accordingly, cirrhosis patients with and without infections had lower interactions of mutual exclusion (negative association) and higher interactions of co-occurrence (positive) at baseline, respectively (Fig. 4A). Similarly, the core bacterial taxon (at the family level) influencing other bacterial communities differed when grouping cirrhosis patients according to episodes of infection: Erysipelotrichaceae in those without infection, Verrucomicrobiaceae in those with a single infection, and Ruminococ-

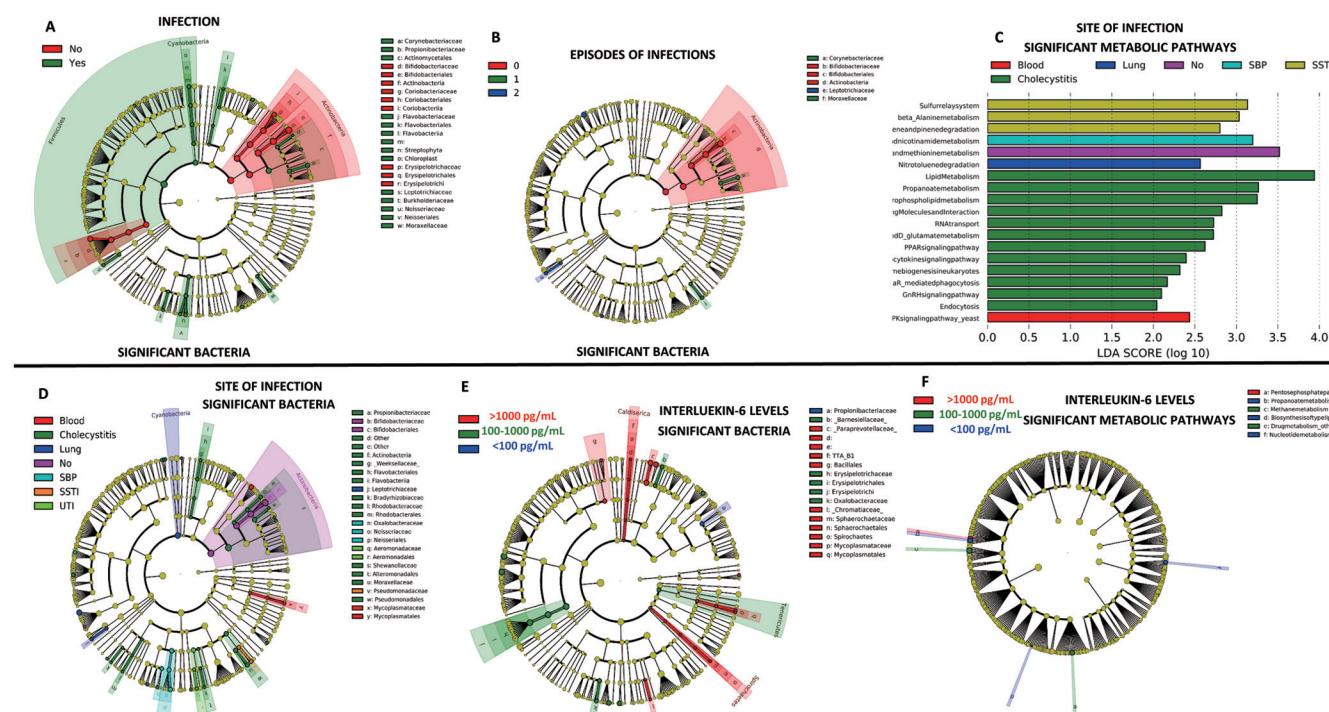


Fig. 3. Characterization of microbiomes between decompensated cirrhosis patients with and without infection. (A) Presence of infections. (B) Episodes of infection. (C, D) Site of infection and associated functional metabolism. (E, F) IL6 levels and associated functional metabolism in pg/ml. Data is presented from linear discriminant analysis effect size (LEfSe) analysis and linear discriminant analysis (LDA) scores of the 16S RNA sequences. The LEfSe method identifies the significantly different ($p < 0.05$, Kruskal-Wallis test, LDA score > 2.0) taxa or functional metabolic pathway at multiple taxonomic levels. All significant bacterial taxa are illustrated using cladograms, except (C), represented as histogram of the LDA scores (log10). For a complete summary of the LEfSe and LDA output between groups (clinical and investigation variables) see Table 2.

caceae in those with two or more episodes of infection (Fig. 4B). Further network analysis using circular attribute function showed that various bacterial taxa at the phyla and family levels interacted with each other differentially in cirrhosis patients when grouped according to presence or absence of infection, episodes of infection, and severity of systemic inflammation based on IL6 levels (Fig. 4C, D).

After analyzing the centrality measures of degree (interaction within the network), betweenness (influence within the network), and closeness (broadcast within the network), interesting changes were observed in the various topological attributes of the constructed networks among groups with respect to different grouping attributes. For instance, *Blautia* was most abundant in patients with sepsis, whereas *Gemella* was most interactive, *Leptotrichia* was most influential, and *Lactobacillus ruminis* was most central to the metabolic-cross talk within the network. Similarly, in patients with lung infection, *Klebsiella pneumoniae* was most abundant, whereas *Streptococcus* was most interactive and *Bifidobacterium longum* and *Enterococcus* were most influential and central to crosstalk, respectively. Meanwhile, in those with bacterial peritonitis, *Bifidobacterium*, *Leptotrichia*, *Streptococcus*, and *Lactobacillus* was most abundant, interactive, influential, and central to crosstalk, respectively. Supplementary Table 2 depicts the network topology in a simplified tabular form for comparison between various groups, based on attribute indices of degree, closeness, and betweenness centrality measures.

Discussion

This study defined and demonstrated the quantitative, qual-

itative, and interactive roles of the BGM in the presence and absence of infections. To the best of our knowledge, no study has comprehensively analyzed and characterized the BGM with respect to clinical events associated with infections in cirrhosis. Our study aimed to provide insights on the role of BGM in promoting sepsis and associated events among patients with cirrhosis. Accordingly, our findings showed that pathogenic genera, such as *Leptotrichia*, *Neisseria*, and *Erwinia*, were predominant in cirrhosis patients with infection, whereas beneficial bacteria, such as *Bifidobacterium* and *Coriobacteria*, were predominant in those without infection. Similarly, the functional metabolism of the predominant BGM included upregulation of pathways associated with oxidative stress, endotoxemia, and proinflammatory process in those with infections and endogenous antimicrobial, antioxidant, and cytokine signaling pathways in those without infections. Specific pathogenic bacterial taxa were associated with different sites of sepsis (e.g., *Mycoplasmataceae* in bacteremia and *Aeromonas* in UTIs). Repeat infections and high systemic inflammation were associated with rare, multidrug-resistant, and immune exhaustion-related intestinal bacterial groups. This change in BGM was significantly notable in those without infections (*Bifidobacterium*) who subsequently developed their first (*Lautropia*) and multiple episodes of infection (*Leptotrichia*). Specific bacterial genera with high propanoate functional metabolism, such as *Megamonas*, were associated with survival in infected patients with cirrhosis during admission and after follow-up. Network analysis showed that noninfected cirrhosis patients had richer and more diverse interactions, comprising mutual exclusions and co-occurrence between various bacterial taxa, compared with those with infections.

Host-microbiota interactions can predispose individuals

Table 2. Significant bacterial taxa enriched on biomarker discovery analysis between clinical, investigational, disease severity and outcomes grouped into variables between patients with cirrhosis with or without sepsis

Infection at admission (n=27)		No infection at admission (n=24)	
Significant bacterial genera			
Propionibacterium, Moraxella, Acinetobacter, Capnocytophaga, Lautropia, Leptotrichia, Pediococcus, Neisseria, Porphyromonas, Erwinia, Streptophyta		Eggerthella, Coriobacteria, Bifidobacterium	
Significant functional metabolism pathways			
Porphyrin and chlorophyll metabolism		Adipocytokine signaling pathway	
Lipopolysaccharide biosynthesis		Restriction enzyme pathway	
Bacterial chemotaxis		Selenocompound metabolism	
Glycosyltransferases		Bacterial toxins pathway	
Sulfur relay system		Nicotine and nicotinamide metabolism	
Glutathione metabolism		Streptomycin biosynthesis	
Calcium signaling pathway		Cysteine and methionine metabolism	
Glycerophospholipid metabolism		Histidine metabolism	
Cell motility and secretion		Galactose metabolism	
Riboflavin metabolism		Alanine, aspartate and glutamate metabolism	
Tetracycline biosynthesis			
IL6 levels at admission (n=51)			
Significant bacterial genera			
IL6 <100 (n=36)	IL6 >100 but <1,000 (n=9)	IL6 >1,000 (n=6)	
Propionibacterium	Barnsiella, Erysipelotrichi, Oxalabacteraceae	Paraprevotella, Sphaerochaetaceae, Chromatiaceae, Mycoplasmatales, TTA_B1 (Caldiserica)	
Significant functional metabolism pathways			
IL6 <100	IL6 >100 and <1,000	IL6 >1000	
Propanoate metabolism, biosynthesis of type II polyketides, nucleotide metabolism	Methane metabolism, drug metabolism (other enzymes)	Pentose phosphate pathway	
Sites of infection			
Significant bacterial genera			
No infection (n=24)	Actinobacteria, Bifidobacteria, Sharpea		
Bacteremia (n=4)	Eggerthella, Mycoplasmataceae		
Pneumonia (n=5)	Lautropia, Cyanobacteria, Leptotrichia, SHD-231		
SBP (n=8)	Oxalabacteraceae, Neisseriaceae, Anaerotruncus		
SSTIs (n=4)	Pseudomonadaceae, Pseudomonas		
UTI (n=5)	Aeromonadaceae, Aeromonas		
Cholecystitis/Cholangitis (n=1)	Paludibacter, Arthrobacter, Chryseobacterium, Shewanella, Arcobacter, Paracoccus, Pseudomonas, Acinetobacter, Schwartzia, Flavobacteria		
Significant functional metabolism pathways			
No infection	Cysteine and methionine metabolism		
Bacteremia	MAPK signaling pathway_yeast		
Pneumonia	Nitrotoluene degradation		
SBP	Nicotinate and nicotinamide metabolism		
SSTIs	Sulfur relay system, beta-alanine metabolism, limonene and pinene degradation		
UTI			

(continued)

Table 2. (continued)

Sites of infection	
Cholecystitis/Cholangitis	Propanoate metabolism, glycerophospholipid metabolism, D-glutamine and D-glutamate metabolism, PPAR signaling pathway, adipocytokine signaling pathway, MAPK signaling pathway_yeast, ribosome biogenesis, FC-gammaR mediated phagocytosis, GnRH signaling pathway
Number of episodes of infection	
<i>Significant bacterial genera</i>	
No infections (n=21)	Bifidobacteria, Actinobacteria
One episode of infection (n=17)	Corynebacteriaceae, Moraxellaceae
Two or more episodes of infection (n=10)	Leptotrichiaceae
<i>Significant functional metabolism pathways</i>	
No infections	Protein digestion and absorption, ribosome biogenesis, proteasome pathway, cysteine and methionine metabolism, starch and sucrose metabolism
One episode of infection	RNA transport signaling
Two or more episodes of infection	
Same admission death (n=8)	Same admission survived (n=19)
<i>Significant bacterial genera</i>	
Sphingobacteria, tta_b1, Aerococcus, Neisseriaceae, Chromatiaceae, Anaeroplasmataceae, Mollicutes, Sphaerochataceae	Megamonas
<i>Significant functional metabolism pathways</i>	
No significant biomarker found	No significant biomarker found
Overall outcome-death (n=11)	Overall outcome-survived (n=16)
<i>Significant bacterial genera</i>	
Neisseria	Dorea
Kingella	Megamonas

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v 1.1.1) was used for predictive metabolic functional profiling of microbial communities using 16S rRNA marker gene sequences (precalculated for protein-coding genes present in KEGG gene families and 16S rRNA gene copy number). The linear discriminant analysis effect size (LEfSe) combined with the Kruskal-Wallis and pairwise Wilcoxon tests were utilized to identify significantly different microbial communities in their abundance and functionality between groups. We used default significance (alpha value=0.05) and linear discriminant analysis thresholds (2.0), at all taxonomic levels in the patients between time points. The table summarizes the graphs sourced from analytical output associated with significant biomarker discovery with regards to clinical or biochemical variable group in question using this methodology.

to infections, promote more severe infections, and even prevent their occurrence, as demonstrated in insects, small animals, and humans. Microbe-arbitrated host protection from infections can occur through the maintenance of host immune homeostasis, interference competition with pathogenic taxa, and competitive resource utilization. A decline in host health, as seen in development and progression of cirrhosis, can cause dysbiosis, which promotes the perturbation and transition of commensal microbiota toward pathogenicity and further predisposes the host to infections.¹⁸ Reduced microbial diversity and loss of colonization resistance due to perturbation caused by dysregulated local and systemic immune homeostasis promote systemic infections originating in the gut, as demonstrated with *Clostridioides difficile* infection.¹⁹ Gut microbiota can be used to develop a pathogenic profile with the onset of dysbiosis driven by illness and its management or immune compromise, such as antibiotic treatments and liver disease, respectively.^{20,21} Liu *et al.*²² found that changes in gut microbial patterns promoted necrotizing enterocolitis and late-onset sepsis in babies born prematurely and that changes in gut bacteria

were probably the causative factors of the development of infectious complications in predisposed patient groups. Moreover, changes in gut microbiota composition can potentially predispose patients to a state of immunosuppression, thereby increasing the risk of sepsis. Indeed, Hyoju *et al.*²³ clearly demonstrate how a selective high-fat, sucrose-rich Western diet, antibiotic exposure, and surgical injury converge on the microbiome, causing lethal sepsis and multiorgan failure without exogenous pathogens. In immunosuppressed patients, such as those undergoing bone marrow transplantation, antibiotic-associated dysbiosis promoted a five- to nine-fold increase in the risk of bloodstream infection and sepsis. Similarly, a strong dose-response relationship between dysbiosis-causing events and subsequent severe sepsis-related hospitalization had been observed among elderly patients. Furthermore, a previous study showed that prolonged antibiotic exposure and utilization of additional antibiotic classes and broader-spectrum antibiotics during hospitalization were associated with dose-dependent increases in the risk of subsequent sepsis. This suggested that the association between antibiotic exposure

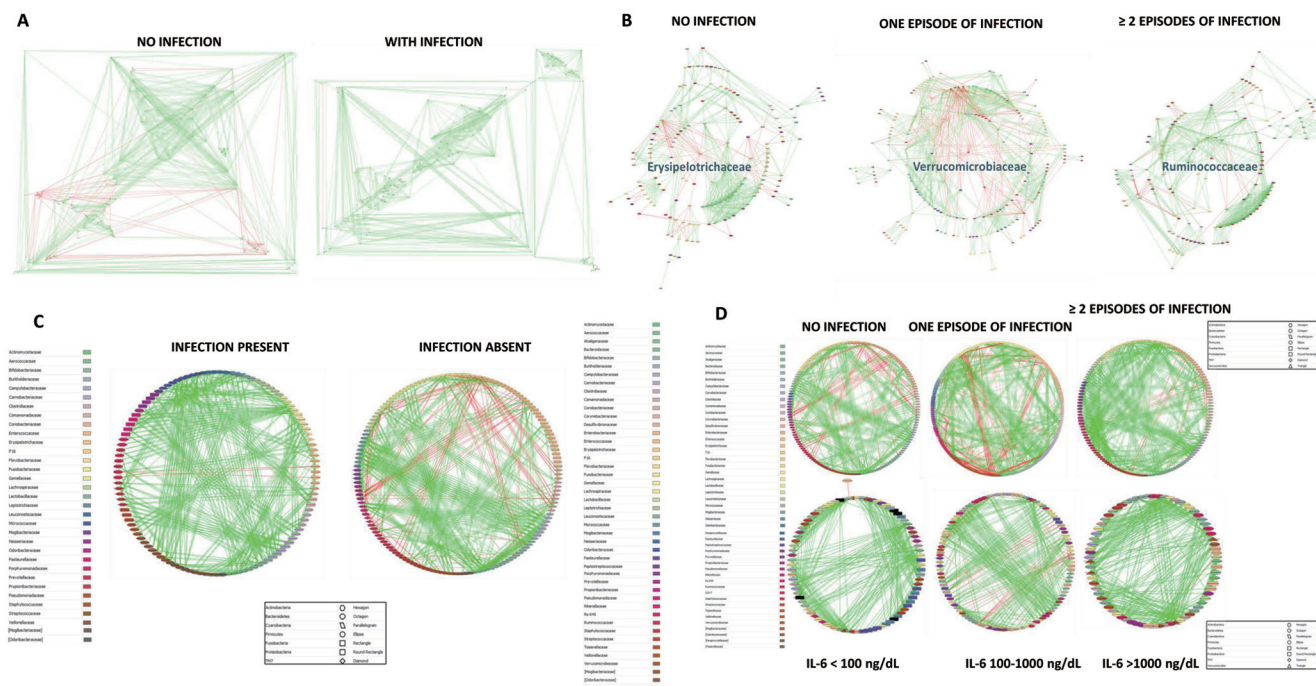


Fig. 4. Network analysis showing topology and interactions between bacterial taxa in patients with cirrhosis grouped by clinical or investigational variables. (A) Loss of mutual exclusivity between bacterial taxa (depicted by each node, between connections) in cirrhosis patients with infections compared with those without infections (red lines indicate negative influence or mutual exclusion, green lines indicate co-occurrence). (B) Radial layout visualization which demonstrates bacterial taxa that predominate (centrally placed) and influence other bacterial communities in cirrhosis patients with no infections compared to those with single or two or more episodes of infections. (C) Detailed bacterial taxa interactions in cirrhosis patients with and without infections as per attribute circle layout visualization. Each colored node is represented by a bacterial class, while mutually exclusive and co-occurrence interactions are depicted via red and green lines, respectively. (D) Loss of mutual exclusivity and change in taxa and associated interactions is evident in cirrhosis patients developing infections demonstrates attribute circle layout visualization between cirrhosis patients grouped according to number of infection episodes and IL6 cutoffs. The changing taxa and associated co-occurrence and mutual exclusivity are evident with increasing infection episodes and severity of inflammation. In (C) and (D), the phyla are depicted as "shape forms" and family as "shades of colors." For a complete summary of the network topology of bacterial taxa interactions, please see Supplementary Table 2

and subsequent sepsis was associated with microbiome depletion and not illness severity.^{24–26}

Critical illness, as noted in patients with decompensated cirrhosis, profoundly disturbs the gut microbiota due to low food intake and malnutrition, recurrent hospitalizations for portal hypertensive complications, worsening liver function and progressive decline in immune status, and multiple exposure to antibiotics. This phenomenon is evident in the current study, wherein patient with advanced cirrhosis requiring multiple admissions demonstrated worsening dysbiosis due to repeated infections, causing pathobiont generation, immune exhaustion, multiple organ failure, and poor survival. The effects of the gut microbiome on sepsis outcome have been clearly demonstrated in an animal model. Notably, when sepsis was introduced using the cecal ligation and puncture method in two different sets of mice purchased from different vendors, with different fecal microbiota beta diversities and immune phenotypes, higher mortality was notable in the first group. When both groups of mice were housed together, differences between beta diversities and immune phenotypes disappeared, and further sepsis introduction included similar survival outcomes. The aforementioned study highlighted the importance of the gut microbiome for survival from, and host immune response to, sepsis. Thus, the transition of a microbiome into a pathobiome could promote poor clinical outcomes and mortality from sepsis by modulating the host immune response, which was seen in our patients with repeated infections, severe unchecked inflammation, and higher mortality.²⁷ A prior study that evaluated the effects of gut microbiome dynamics in intensive care unit (ICU) patients

showed that changes in the gut microbiota were associated with patient prognosis. The proportions of Bacteroidetes and Firmicutes significantly changed during ICU stay, and extreme changes were observed in almost all patients with a poor prognosis, suggesting a correlation between qualitative and quantitative dysbiosis and sepsis outcomes. Similarly, a study conducted by our group demonstrated that progressive dysbiosis in advanced cirrhosis with repeated infections and hospitalizations promoted extreme changes in fecal bacterial communities and was associated with poor short- and long-term clinical outcomes.²⁸ Specific classes of antibiotics have been associated with fecal microbiota changes. Accordingly, penicillins, cephalosporins, and carbapenems were associated with a reduction in beneficial *Bifidobacteria* and *Lactobacilli* and an increase in pathogenic *Enterobacteriaceae/Enterococci*. Published faecal microbiota data suggest the development of resistance in *Enterobacteriaceae* following exposure to piperacillin-tazobactam. Moreover, antibiotic-associated microbiota disruption can occur as early as 3 days and last for 12 months as shown via molecular analytical methods in those receiving short courses of fluoroquinolones.²⁹

This study found that infections upon admission in DC patients were associated with deleterious changes in the fecal microbiome, which progressively worsened in those with repeated infections. The GBM of those receiving high-end antibiotics for worsening infections transitioned toward a pathobiont profile, and associated bacterial taxa were identified as opportunistic pathogens predominant in immunocompromised states. Prior studies on patients with and without sepsis demonstrated heterogeneous patterns of in-

testinal microbiota, including the disappearance of bacterial genera with important functions in host metabolism. Moreover, our fecal analysis of critically ill patients with sepsis at different sites revealed significant differences in GBM profiles, similar to our findings among patients with cirrhosis.³⁰ Agudelo-Ochoa *et al.*³¹ found that the microbiota of ICU patients with sepsis contained numerous microbes strongly associated with inflammation, such as *Parabacteroides*, *Fusobacterium*, and *Bilophila* species. Furthermore, a difference in the abundance of pathogenic species, such as *Enterococcus* spp., was observed in patients with sepsis who died. Among our patients, those with infections, those with repeated infection, and those who survived after matching for portal hypertension events, liver disease severity, and extrahepatic organ failure involvement, had a GBM that was preferentially and significantly associated with proinflammation, intestinal barrier dysfunction, and endotoxemia.

We chose to study IL6 in our patients for several reasons. Studies have shown that IL6 levels are increased in patients with cirrhosis and is linearly associated with the severity of underlying cirrhosis. IL6 was also found to play a role in the hyperdynamic circulation observed in patients with cirrhosis. In DC, plasma IL6 levels on admission were shown to provide the most sensitive and specific tool for the diagnosis of bacterial infection and were closely linked to the development of overt HE in patients with liver cirrhosis and was particularly useful in predicting mortality in patients with cirrhosis.^{32–35} In that context, our study revealed the important role of GM dysbiosis and specific bacterial taxa associated with varying IL6 levels in DC patients with infections. Short-chain fatty acid-producing *Propionibacterium* was relatively abundant in those with IL6 <100 and pathogenic taxa were associated with states of immune exhaustion and opportunistic infections predominated the gut in DC patients with IL6 >1,000. That demonstrated a plausible association of GM as a promoter and driver of systemic inflammation and eventually organ dysfunction in DC patients with sepsis.

This study is not without limitations. Apart from being a single-center, retrospective study, we did not match our patients in terms of other confounders that could have affected gut microbiome, as well as long-term outcomes, such as dietary habits, heterogenous environmental exposures, and other known or unknown chronic comorbidities. Nonetheless, the DC groups with and without infections were comparable with respect to liver disease severity, portal hypertension events, nonabsorbable antibiotic use, and extrahepatic organ dysfunction at admission. We did not assess dynamic changes in the BGM from admission to discharge or the end of follow-up, given that it could have been affected by heterogenous and possibly unmodifiable confounding factors. During follow-up, we did not account for the various causes of death in our patients. Further, the generalized clinical outcomes, such as survival or death, were based on a single point fecal microbiota analysis for ease in analysis, which could have oversimplified the role of gut dysbiosis in our patient population.

Among patients with cirrhosis, dysbiosis occurs as part of the etiology and development of liver disease and subsequently promotes infections in advanced cirrhosis due to portal hypertension and immune dysfunction. That worsens with subsequent hospitalization and therapeutic or symptom management interventions required in this special patient group, which prevents the complete recovery of the microbiota. Periods of dysbiosis predisposed DC patients to opportunistic infections, which further worsened dysbiosis, and predisposed them to sepsis, wherein the microbiota composition becomes extremely compromised and the risk of secondary infections, immunosuppression, and organ dysfunction increases. The study showed that gut microbiota changes in advanced DC patients induced a state of

prolonged immunosuppression, rehospitalization because of infections, and increased mortality. Similar to the feasibility and effectiveness of fecal microbiota transplantation as a treatment option for severe *C. difficile* infection in the ICU, the findings suggest that gut microbiota modulation using healthy stool transplants could be an important therapeutic option in the fight against multidrug resistant bacteria in vulnerable patient populations, such as those with advanced cirrhosis, pending further large quality, well controlled observations and clinical trials.

Future directions based on the current work includes defining subtypes of patients (independent from other confounders) based on the microbial signatures or metabolites that would benefit from targeted therapies; validation of our findings in small animal models or a second patient cohort (with a different geographic or ethnic background) prospectively, and analyzing longitudinal data (e.g., after antibiotic therapy, in patients with antibiotic prophylaxis for infections such as SBP).

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Conflict of interest

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

Author contributions

Acquisition of data, analysis and first draft (CAP), revision, analysis review and editing (RA, JKPA, SR), critical revision and data review (PA). All authors approved the final version of the manuscript.

Data sharing statement

At request, from the corresponding author, Dr Cyriac Abby Philips.

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