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Clinical outcomes and associated bacterial and fungal microbiota changes after high dose probiotic therapy for severe alcohol-associated hepatitis

An observational study

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Abstract

Alcohol-associated hepatitis (AH) is a critical condition with high mortality rates and is worsened by infections. Organ failure is strongly associated with intestinal dysbiosis. Emerging research suggests that gut microbiota modulation with probiotics can improve AH outcomes. This study investigated the clinical and microbiome effects of high-dose probiotic infusion (HDPI) compared with corticosteroid therapy (CST) and fecal microbiota transplantation (FMT) in severe AH. Patients with biopsy-proven severe-AH were enrolled from March 2019 to June 2020 and matched for age and disease severity. The patients received HDPI (n = 20), FMT (n = 16), or CST (n = 14). HDPI consists of a potent probiotic mix delivered via a nasoduodenal tube for 6 days. The primary outcome was survival at 90-days. Stool samples were subjected to 16S and 18S rRNA sequencing to assess significant bacterial and fungal taxa and their interactions at baseline and post treatment. At 90-days, survival rates were 55%, 64.3%, and 87.5% (HDPI, CST, respectively). HDPI did not beneficially impact bacterial alpha-diversity but significantly altered beta-diversity. Notably, the number of pathogenic bacteria, such as *Bilophila* and *Roseburia* increased. Fungal analysis revealed no significant changes in alpha diversity, but significant dissimilarities in beta diversity post-HDPI. New fungal genera such as Basidiomycota and Phragmoplastophyta have emerged, with significant deleterious expansion in fungal communities and damaging modifications between fungal–bacterial interactions. HDPI failed to outperform CST in improving the clinical outcomes of patients with severe AH. While HDPI influenced both bacterial and fungal microbiomes, it also led to the persistence of pathogenic communities. FMT showed superior survival outcomes, highlighting the urgent need for further controlled trials.

Abbreviations: AH = alcohol-associated hepatitis, ALD = alcohol-related liver disease, CST = corticosteroid therapy, FMT = fecal microbiota transplantation, GM = gut microbiota, HDPI = high-dose probiotic infusion, LEfSe = linear discriminant analysis effect size, QIIME = Quantitative Insights into Microbial Ecology.

Keywords: acute on chronic liver failure, alcohol related liver disease, cirrhosis, dysbiosis, gut microbiome, mycobiome, portal hypertension

1. Introduction

Alcohol-associated hepatitis (AH) is associated with increased short-term mortality in the presence of infections and rapid onset of extrahepatic organ failure driven by intestinal dysbiosis.[1] Deleterious modifications of the gut microbiota (GM) which include bacteria, fungi, and viral groups in the presence of alcohol are well documented across multiple studies globally.[2] Gut microbiota contributes to individual susceptibility to alcohol-related liver disease (ALD) and AH

All patients provided written informed consent.

The authors have no funding and conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

This study was approved by the Institutional Review Board of Rajagiri Hospital, and was performed in conformance with the Helsinki declaration of 1975 and its pertinent revisions.

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through detrimental changes to immune signaling, functional metabolism, and the gut–brain axis.[3] Specific bacterial taxa have been shown to affect the promotion, progression, and clinical events associated with ALD and AH. For example, in the presence of alcohol, pathogenic bacteria, such as *Acinetobacter baumannii* enhance their virulence. Alcohol use reduces bacterial diversity, decreases the abundance of beneficial Bacteroidetes, and increases that of pathogenic Proteobacteria, as demonstrated by a significant decrease in Ruminococcaceae and an increase in both Lachnospiraceae and Enterobacteriaceae.[4,5] Alcohol-induced GM dysbiosis leads to liver damage owing to the proliferation of harmful microorganisms that produce exotoxins. One such exotoxin, cytolysin, secreted by *Enterococcus faecalis*, can directly harm hepatocytes. A higher presence of cytolysin-positive *Enterococcus faecalis* correlates with greater severity of liver disease and elevated mortality rates in patients with AH.[6] Specific GM, their interactions, and metabolites are associated with complications of AH and the corresponding treatment outcomes.[7] Similarly, fungal dysbiosis and fungal metabolites affect the severity of AH. The burgeoning field of research on fungal dysbiosis and proliferation of specific fungal species in ALD has revealed significant findings. Elevated serum levels of anti-*Saccharomyces cerevisiae* immunoglobulin G antibodies, which indicate a systemic immune response to fungi and their byproducts, are observed in patients with alcohol use disorder and ALD. In patients with AH, heightened serum anti-*Saccharomyces cerevisiae* immunoglobulin G antibodies levels are associated with increased mortality. Additionally, a higher relative abundance of specific fungal species, such as *Candida albicans* and *Malassezia restricta* in the gut, correlates with more severe liver injury and increased mortality in ALD patients.[8–10] Preliminary studies have shown that patients with ALD and AH can benefit from GM modulation. Clinical trials have explored the use of fecal microbiota transplantation (FMT) to alter the gut microbiome of patients with AH. Studies have demonstrated that AH patients who received FMT from healthy donors exhibited lower rates of alcohol relapse, infections, or hospitalizations; higher rates of resolution of hepatic encephalopathy and ascites; and improved survival in both the short and long term when compared to standard medical care, including pentoxifylline therapy.[11–13] Another randomized controlled study included 120 patients with AH who received either prednisolone or a healthy donor FMT. Patients receiving FMT had better 90-day survival rates and a reduced incidence of infections compared to those receiving prednisolone.[14] Probiotics have been shown to improve clinical outcomes in alcohol-associated cirrhosis, and foundational evidence supports their positive effects on GM. Preliminary evidence also supports the beneficial effects of probiotic therapy on GM in AH.[15,16] Nonetheless, the validated and replicated benefits of probiotic therapy and their impact on survival in the context of GM modulation remain unknown. Similarly, probiotic therapy-related modifications within fungal communities, fungal–bacterial interactions, and associated clinical outcomes in AH are enigmatic. We aimed to study the clinical outcomes and GM changes in AH patients receiving high-dose probiotic infusion (HDPI) compared to corticosteroid therapy (CST). Additionally, survival outcomes compared with matched patients undergoing healthy donor FMT were also analyzed. We also studied mycobiome (fungal) community changes and their interactions among patients with AH in the context of HDPI and CST therapies.

2. Patients

We retrieved and analyzed hospital records of patients with severe AH from March 2019 to June 2020. After providing informed consent, all patients clinically suspected of having severe AH underwent liver biopsy for definitive diagnosis, and only patients with biopsy-proven severe AH were included in the study. Since there is no approved treatment for severe AH, the initial therapy of choice was provided on a case-by-case basis and as per the decision and informed consent provided by the patient or their authorized family member after discussing the pros and cons of the available therapeutic options. The patients chose CST, FMT, or HDPI. Patients with upper gastrointestinal bleeding within the past month, multiple organ failure requiring support, uncontrolled sepsis on inotropes, intestinal paralysis, hepatic or extrahepatic malignancy, or disseminated intravascular coagulation were excluded. Those willing to undergo liver transplantation at the outset or during medical treatment were referred to a transplant center for definitive management. Patients in whom consent for transjugular liver biopsy was not provided and those with a clinical and biochemical diagnosis of AH in whom a second etiology of liver injury was either confirmed or contemplated were also excluded. All patients were initiated on maximally tolerated beta-blocker therapy in the presence of clinically significant portal hypertension. For those with ascites, the lowest effective dose of diuretics was continued. In patients who developed hepatic encephalopathy after treatment completion, rifaximin was added as a secondary prophylaxis. Additionally, patients adhered to a regimen of salt restriction, diuretics, and a protein intake of 1 to 1.5 g/kg per day, as recommended by the overseeing nutritionist. Intravenous albumin administration was continued throughout the hospitalization period. Third-generation cephalosporins were universally administered upon admission, with antibiotic adjustments made according to individual patient needs based on culture and sensitivity results. Additionally, in patients who developed infections or acute kidney injury on follow-up and required admission, intravenous antimicrobial therapy, and albumin infusions with or without terlipressin were initiated. All participants (or their immediate family members) provided informed consent for the treatment and treatment-related procedures, and for the use of their de-identified stored fecal samples for future research. Stool samples were collected at baseline (within 24 hours of admission), at the end of 1 week and at the end of 1 month. The primary outcome measure was survival at the end of the 90 days. Secondary outcome measures included (a) analysis of GM changes (bacterial and fungal) from baseline, at 1 week and at 1 month after treatment with HDPI and identification of statistically significant taxa associated with the clinical outcome; (b) significant interactions between bacterial, fungal, bacterial, and fungal groups from baseline, at 1 week and end of 1 month after HDPI treatment network analysis; and (c) identification of significant species between the CST and HDPI (bacterial) groups as well as within the HDPI (fungal) group at the end of 1 month compared to the pretreatment stage. All participants provided written informed consent prior to participating in the study. This study was approved by the Institutional Review Board of Rajagiri Hospital (ID: CoEGIS/RAJH:06/2022) and performed in accordance with the 1975 Declaration of Helsinki and its pertinent revisions.

3. Methods

3.1. Corticosteroid therapy

Patients with severe AH who were eligible for corticosteroid therapy were administered oral prednisolone 40 mg once daily for 28 days. In those unable to consume orally (due to overt hepatic encephalopathy), injectable methylprednisolone 40 mg once daily was administered and switched to the oral route after resolution of cognitive failure. The response to CST was calculated at the end of 7 days using the Lille model score. In

responders, corticosteroid treatment was continued for 28 days. Only CST responders (Lille score, <0.45) were included in the final analysis.

3.2. Healthy donor FMT

For FMT, fresh donor stool samples (minimum mass, 30 g) were obtained daily, processed, and infused within 6 hours of collection. Donors were instructed to promptly provide fresh stool samples upon arrival at the hospital using sterile plastic collection containers. These samples were obtained 6 hours prior to the procedure. Each sample, weighing approximately 30 g (equivalent to approximately 2 cm^3 or roughly 3×10^{10} microbial load), was deemed sufficient for use. Following collection, 100 mL of sterile normal saline was added to the stool sample, which was then blended using a Philips® Hand Blender [Model: HR1363/04] for a duration of 2 minutes, utilizing 30-second pulses with 5-second intervals between each pulse. The resultant homogeneous suspension was filtered through sterile gauze pieces, and this process was repeated 3 times until the filtrate was free of solid matter. The personnel involved in the preparation of stool specimens were mandated to wear eye shields, masks, and fluid-resistant gowns for safety precautions. Once processed, 100 mL volume of meticulously filtered stool was administered through a naso-duodenal tube positioned under fluoroscopy guidance the day before the FMT procedure. The recipient was strictly prohibited from oral intake for a minimum of 4 hours before stool infusion. For a consecutive 7 days, a fresh stool suspension (100 mL was administered daily). Nonabsorbable antibiotics were avoided from the onset of the study and throughout the initiation of therapy. During stool infusion, patients were placed in a supine position at a 45-degree angle and remained in this posture for a minimum of 30 minutes after the post-procedure. The nasoduodenal tube was thoroughly flushed with normal saline (30 mL) following stool instillation. Food consumption resumed 2 hours post-FMT. While refraining from rifaximin and other nonabsorbable antibiotics, the patients were permitted disaccharides to facilitate the passage of 2 to 3 soft stools daily. Only patients who completed the FMT protocol and those who were corticosteroid responders (i.e., with Lille model score < 0.45 at the end of 1 week) were included in the study.

3.3. High-dose probiotic infusion therapy

High-dose probiotic infusion therapy involved immediate naso-duodenal infusion of 10 each of the mixed high-dose combinations of 5 commercially available fixed-dose probiotics:

(a) VSL3® (Sun Pharma, India), containing 4 strains of Lactobacillus (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subspecies *bulgaricus*), 3 strains of Bifidobacterium (*Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium infantis*), and one strain of Streptococcus (*Streptococcus salivarius* subspecies *thermophilus*).
(b) *Lactobacillus rhamnosus* (Unobiotics®, Cipla Ltd India).
(c) *Saccharomyces boulardii* (Econorm®, Dr Reddy's Laboratories, Hyderabad).
(d) *Clostridium butyricum* + *Bacillus mesentericus* + *Streptococcus faecalis* + *Lactobacillus sporegenes* (Bifilac®, Tablets Pharmaceuticals, India).
(e) *Bacillus clausii spores* (Enterogermina® Liquid, Sanofi Pharmaceuticals, India).

These were suspended in 200 mL of sterile water, and the suspension was administered once daily for 6 days through a naso-duodenal tube positioned under fluoroscopy guidance the day before the procedure. Post procedure protocols were similar to those of patients undergoing healthy donor FMT. The protocol and methodology are summarized in Figure 1.

4. Analysis

4.1. Statistical analysis

Statistical analyses were conducted using the MedCalc Statistical Software (Ostend, Belgium). Data presentation was standardized with means and standard deviations or medians and ranges, as applicable. Categorical data were evaluated using either Fischer exact or chi-square tests, while the Wilcoxon Rank test facilitated pairwise comparisons between baseline and post-interventional data. For parameters exhibiting a normal distribution, means were utilized to assess statistical significance, whereas for non-normally distributed data (such as log-normal or similar distributions), median values were employed. One-way analysis of variance was used to discern differences in investigational variables at baseline among the groups' means. Statistical significance was set at $P < .05$. Prior to conducting one-way analysis of variance, Levene Test for Equality of Variances was administered, and if the results were positive ($P < .05$), data underwent logarithmic transformation. Survival analysis was performed using the Kaplan–Meier method, and survival time curves were graphically depicted. The log-rank test facilitated the comparison of survival curves, with a P value < .05, denoting significant differences in survival outcomes.

4.2. Microbiota analysis

The stool samples were meticulously divided into 500 µg aliquots and promptly preserved at -80 °C until subjected to processing and DNA isolation procedures. Using an Illumina MiSeq next-generation sequencer (Illumina, CA), we conducted fecal 16S rRNA amplicon sequencing targeting the V3 to V4 region of bacterial DNA extracted from approximately 200 mg of collected stool specimens. For the extraction of bacterial DNA, we implemented a validated protocol adaptation of the widely used QIAmp DNA Stool Mini Kit1 (Qiagen, Venlo, The Netherlands). Quantitative Insights into Microbial Ecology (QIIME2 version 2021.4) were utilized for bioinformatics analysis of the gut microbiota, encompassing the comprehensive processing of all reads derived from 16S rRNA gene sequences. Briefly, the initial step involved importation of raw data FASTQ files into a format compatible with subsequent processing within QIIME2. Subsequently, the DADA2 algorithm was deployed for a multifaceted quality control process, encompassing trimming, denoising, and assembly of raw sequences to expunge phiX, chimeric, and erroneous reads. Following this, the representative sequences of the Amplicon Sequence Variants underwent aligned with the pretrained GREENGENES database (version 13.8), utilizing a 99% similarity threshold facilitated by the QIIME2 feature-classifier plugin. This step culminated in the generation of a classification information table, providing insight into taxonomic composition at the species level. Similarly, 18S rRNA for high-resolution taxonomic studies of fungi was also performed using the QIIME2-DADA2 pipeline.[17] Alpha diversity, the within-sample diversity to analyze and compare the number of different organisms (richness), and how evenly distributed these organisms were in terms of abundance (evenness) were computed using the *scikit-bio* tool in QIIME2.[18] Beta diversity and between-sample diversity were measured using distance and dissimilarity metrics, which included the *core-metrics-phylogenetic* pipeline plug-in that automatically produced Bray-Curtis, Jaccard, weighted UniFrac, and unweighted UniFrac metrics. Methods to reduce dimensionality and visualize trends in the data were performed using Principal Coordinate Analysis, which was included by default in the *core-metrics-phylogenetic*

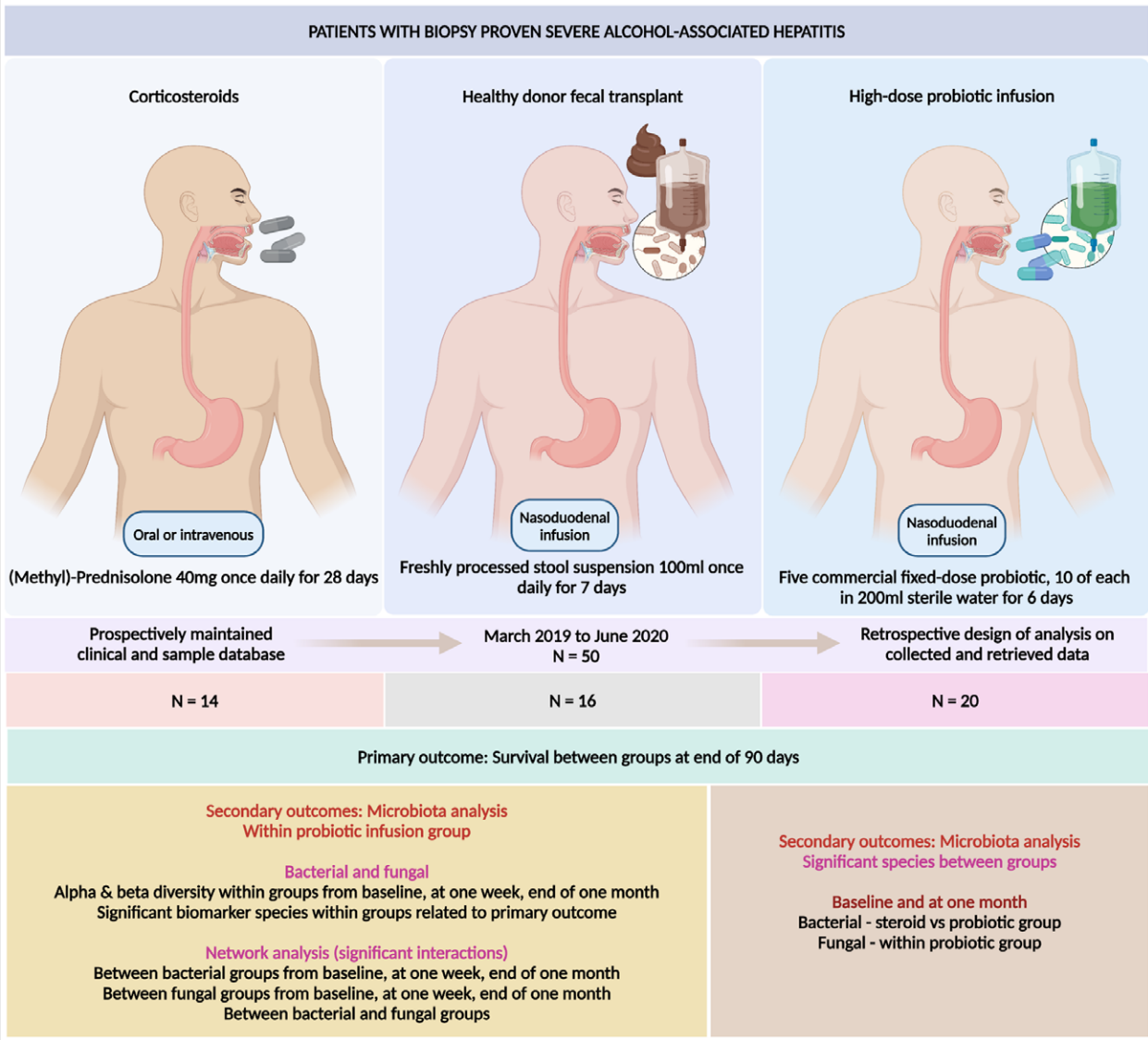

Figure 1. A brief representation of patients and methods within the study design and related clinical and basic science-associated outcome measures.

pipeline. Because these were longitudinal data, axis customization to include the outcome variable was performed. To identify statistical differences in longitudinal metadata applied to the beta-diversity metrics, we performed a permutational analysis of variance nonparametric test and the analysis of similarity, which compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups, run using *qiime diversity beta-group-significance* pipeline. An *R* value close to "1.0" suggested dissimilarity between groups while an *R* value close to "0" suggested an even distribution of high and low ranks within and between groups. R values below "0" suggest that dissimilarities were greater within groups than between groups.[19,20] To discern significant differences in microbial communities' post-therapy among survivors, we employed a robust multivariable biomarker discovery approach known as the linear discriminant analysis effect size (LEfSe). This method synergistically integrates the Kruskal–Wallis and pairwise Wilcoxon tests. Statistical significance was determined using default parameters, maintaining an alpha value of 0.05, with a linear discriminant analysis threshold of 2.0 at all taxonomic levels, both between different time points and across various groups.[21] The outcomes of our analysis are depicted in bar charts, which effectively illustrate the distribution of significant bacterial taxa across the studied cohorts.

4.3. Network analysis

Networks (bacterial, fungal, and bacterial–fungal interactions) were elucidated through robust CoNet® (v.1.1.1-beta) applications integrated within Cytoscape™ (v.3.8.2), employing computational techniques to ascertain the significance of associations between individual nodes. CoNet®, a sophisticated analytical tool, possesses the unique capability to consider taxonomic intricacies and generate abundance counts for higher taxonomic levels by aggregating data from lower branches, thereby enabling the exploration of associations at various taxonomic levels. This functionality broadens the scope of the analysis. Implemented within CoNet are several measures, including Pearson, Spearman, Mutual Information, Bray-Curtis dissimilarity, and Kullback–Leibler dissimilarity, all aimed at detecting copresence/exclusion relationships. To ascertain the significance of associations between node pairs, each network underwent multiple passes: initially, a "null distribution" was computed by permuting the data to assess the effect of random shuffling of sample-id labels for each operational taxonomic unit or OTU count. Thereafter, bootstrapping with the ReBoot feature of CoNet was performed to rigorously compute these associations, ensuring a thorough analysis. Only edges with a *P*-value below the

stringent threshold of .05 were retained, thus delineating truly significant connections. To delve deeper into network dynamics, pivotal network measures, including degree centrality, betweenness centrality, and closeness centrality, were meticulously calculated using NetworkX® (version 2.2). These metrics were then seamlessly integrated into the node attributes within the graph, enriching the analysis with invaluable insights into network structure and dynamics. The assessment of network topology was executed with precision using the radial analysis feature embedded in Cytoscape™. This methodological approach served to pinpoint the central taxa exerting a profound influence on core pathways. The resultant output is vividly depicted through radial-networked interactions, facilitating a comprehensive understanding of network architecture and function.[22,23]

5. Results

5.1. Clinical outcomes

After applying the inclusion and exclusion criteria during a retrospective review of prospectively maintained electronic medical records in the Hepatology Department of our hospital from March 2019 to June 2020, we identified 50 patients with a clinical diagnosis of biopsy-proven severe AH. Of these, 14, 16, and 20 patients completed the CST, healthy donor FMT, and HDPI therapy, respectively. All patients were male and matched for age and liver disease severity scores. All patients were followed up for 3 months post therapy to analyze survival outcomes after treatment. The investigational profile of the patients in each group is shown in Figure 2A. At the end of 3 months, 55% (N = 11) patients on HDPI survived compared to 64.3%[9] on CST [hazard ratio (95% confidence interval): 1.4 (0.4–4.8), $P = .53$] and 87.5%[14] on healthy donor FMT [4.4 (1.4–14.1, $P = .04$)] (Fig. 2B).

5.2. Microbiota analysis and outcomes

The16S rRNA sequencing at V3 to V4 analysis for bacterial communities and 18S rRNA for high-resolution taxonomic studies of fungi along with network analysis to study interactions using DADA2-Amplicon Sequence Variant clustering were performed on stored stool samples at baseline (N = 18), 1 week (N = 15), and at the end of 1 month (N = 9) in HDPI patients. GM analysis in the HDPI group did not reveal significant changes in bacterial alpha-diversities from baseline to both follow-up periods at the end of 1 week as well as the end of 1 month (Fig. 3A). However, significant changes in total and weighted beta diversities between the end of 1 week and 1 month post therapy were notable in those receiving HDPI (Fig. 3B and C). In the biomarker analysis, those receiving HDPI demonstrated an increased relative abundance of potentially pathogenic taxa (*Bilophila*, *Roseburia*) at the end of 1 month compared to baseline (Fig. 4A and B). LEfSe analysis to demonstrate significant taxa end of 1 month between probiotic and steroid-treated groups revealed that Tissierellaceae, *Clostridium* and *Finegoldia* and *Butyricoccus* and *Weissella*, the former well-known opportunistic pathogens, were significantly increased in patients receiving HDPI and CST, respectively (Fig. 4C).

Fungal alpha diversities did not significantly change from the baseline post HDPI. The phylum Ascomycota was predominant at baseline, while Basidiomycota and Phragmoplastophyta were newly introduced at 1 month post HDPI. At the genus level, *Yueomyces* was the most abundant at baseline, whereas the genera *Myrothecium* and *Magnoliophyta* increased 1 month after high probiotic infusion (Fig. 5A and B). Significant dissimilarity within fungal beta-diversities was notable between the end of 1 week and 1 month post therapy with high-dose probiotics (permutational analysis of variance $P = .02$) [Fig. 5C]. Multivariate biomarker discovery using LEfSe revealed that the fungi *Bagnisiella* and *Talaromyces* were significant after HDPI therapy at the end of 1 week and 1 month, respectively (Fig. 5D).

The topology of the radial network analysis revealed the persistence of potentially pathogenic central bacterial interactions in HDPI-treated patients, showing non-beneficial host interactions with probiotic use in severe AH. Briefly, core taxa interactions at baseline and at the end of 1 week and 1 month were represented by Staphylococcaceae (*Staphylococcus*), Enterococcaceae (*Enterococcus*), and Streptococcaceae (*Streptococcus luteciae*). Similarly, radial network analysis revealed striking central network influence modifications among fungi from the baseline and bacteria-to-fungi interactions after HDPI (Fig. 6A). At baseline, *Bagnisiella examinans* had a central influence within the mycobiome network, followed by *Talaromyces* (a central interaction node with maximum interactions with *Colletotrichum nicotianae* at the end of 1 week post treatment). At the end of 1 month in HDPI-treated patients, *Cladosporium iridis* became a central influencer. Fungal–bacterial interactions also diversified after HDPI therapy during the longitudinal follow-up (Fig. 6B). Mutually exclusive interactions included those between the fungus *Phascolarctobacterium*

A

Variables	Group														
	HIGH DOSE PROBIOTIC INFUSION (N=20)					FMT (N=16)					CORTICOSTEROID THERAPY (N=14)				
	Min	Max	Mean	Median	SD	Min	Max	Mean	Median	SD	Min	Max	Mean	Median	SD
Age	32	78	48.4	47.5	11.7	36	60	47.5	45.5	8.3	29	57	43.8	44	9.4
Hemoglobin	6.8	14.2	10.3	9.9	1.9	8.7	12.6	10.2	9.8	1.2	8.4	14.2	10.7	10.2	1.8
WBC count	4.9	27.6	11.4	10.5	5.6	3.7	16.9	11.5	12.3	4.1	4.5	27.6	12.9	12.5	6.3
Platelet	0.4	2.5	0.8	0.7	0.5	0.4	1.9	0.9	0.9	0.3	0.4	2.5	0.9	0.7	0.7
Total bilirubin	5	39	19	18.7	10.7	6.3	27.4	14.7	14	6.9	4	33.6	16.3	15.3	8.4
AST	56	512	174.8	161	97.8	46	313	140.4	132.5	67.3	47	512	186.9	171.5	108.9
ALT	20	213	64.9	53.5	47.6	18	185	60.1	43.5	42.1	16	213	69.3	55	51.5
ALP	45	172	97.1	94.5	36.5	99	287	169	143.5	64.5	65	238	140.3	129.5	52.7
Albumin	1.5	3.6	2.7	2.8	0.4	1.8	28.9	4.1	2.3	6.6	1.8	3.2	2.8	2.9	0.4
INR	1.7	3.8	2.4	2.4	0.5	1.6	2.8	2.1	1.9	0.3	1.4	3.7	2.2	2.1	0.6
Blood urea	10	99	34.5	20.5	28.4	11	90	30.8	27	19.7	10	52	28.3	26.5	11.8
Creatinine	0.4	2.1	0.9	0.8	0.5	0.7	3.2	1.1	0.9	0.6	0.4	1.2	0.7	0.7	0.2
Serum sodium	115	137	127.2	128	5.3	119	133	128	128.5	3.7	115	137	128.6	129.5	6.1
MDF	45.1	163	89.2	77.9	34.1	53.4	109.6	73.4	71.8	15.1	48.3	140	75.7	68.1	25.2
CTP	11	14	12.4	12	0.8	12	14	13.2	13	0.6	10	14	12.3	13	1.3
MELD	19	40	27.8	26	6.1	20	35	25.5	25	3.8	21	33	26.6	26	3.1

Footnote: Age in years, hemoglobin (g/L), WBC (white cell count) x 109/L, platelet count x 105/mL, AST – aspartate aminotransferase (U/L), ALT – alanine aminotransferase (U/L), albumin (g/dl), INR – international normalized ratio, blood urea (mg/dL), creatinine (mg/dL), serum sodium (mmol/L), MDF – Maddrey's discriminant factor, CTP – Child Turcotte Pugh class, MELD – model for end stage liver disease.

B

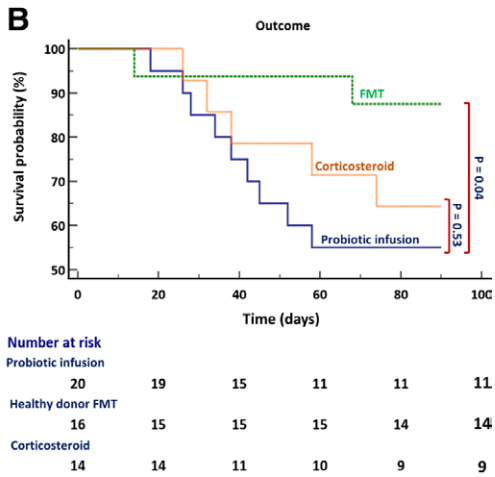

Figure 2. (A) Investigational details of all patients enrolled into the 3 groups [high-dose probiotic infusion therapy (HDPI), healthy donor fecal microbiota transplantation (FMT), and corticosteroid therapy (CST)], (B) the survival curve analysis (using log-rank test) between groups at end of 90 days follow up.

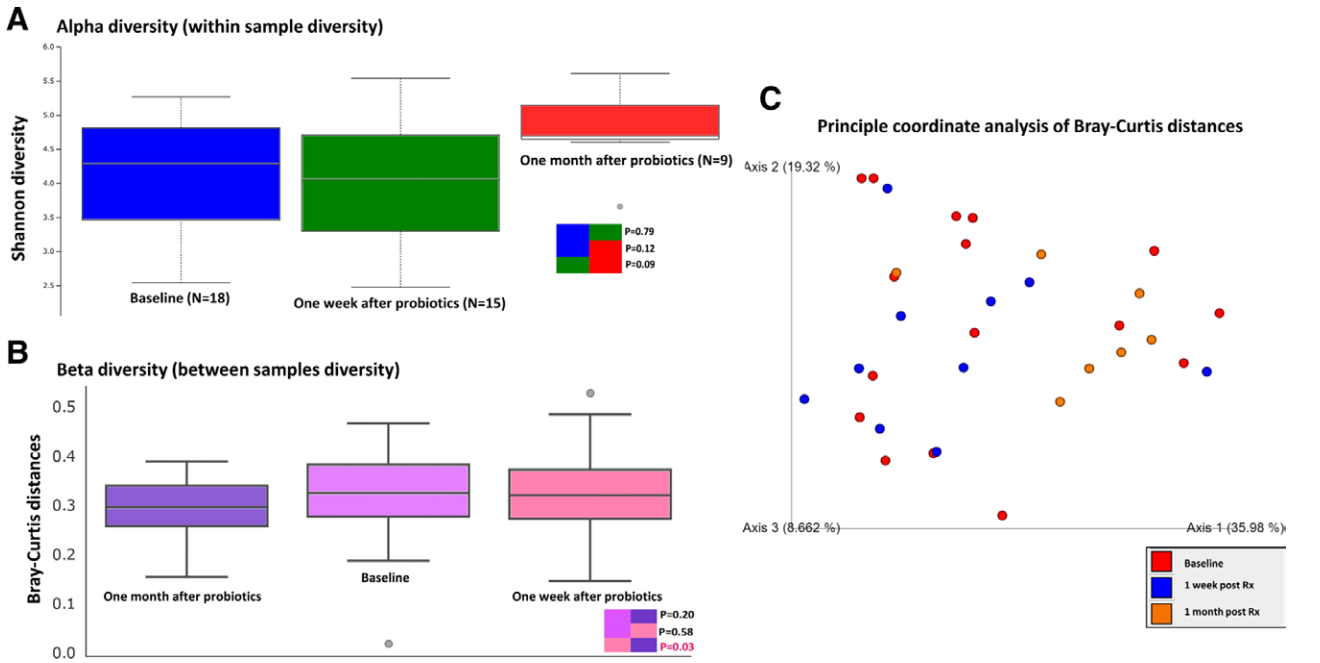

Figure 3. (A) Box-plots representation of the alpha (A, within samples) and beta (B, between samples) diversity comparisons (bacterial taxa) of the gut microbiomes in the group that received high dose probiotic infusion therapy at various time points at baseline to end of 1 month. Analyses were performed on genus-level taxa tables using Wilcoxon test. Please note that only beta-diversities between 1 week and 1 month was significantly different ($P = .003$). The principal coordinate analysis of Bray-Curtis distances (C) on bacterial taxa beta diversities showing different time periods analyzed post HDPI therapy according to the legend. There was no significance between groups.

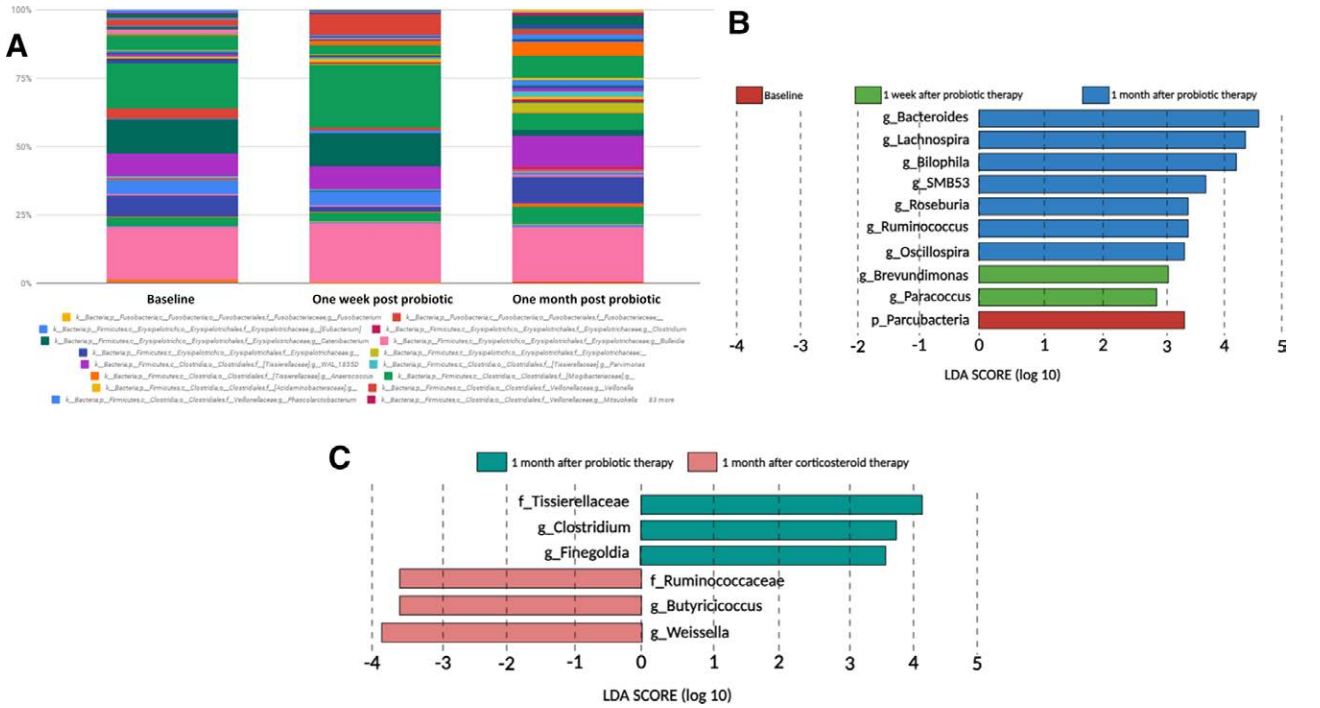

Figure 4. (A) The stacked bar representation of changing bacterial taxa from baseline and at end of 1 week and 1 month after treatment with high dose probiotic infusion (HDPI); (B) shows the significant bacterial taxa that were relatively abundant from baseline and at the end of 1 month after HDPI therapy, as represented by the Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis. The relative abundance is significant when $P < .05$, and logarithmic LDA score ≥ 2.0; (C) shows the bar graph of LDA for LEfSe analysis results of significantly abundant bacterial taxa at the end of 1 month follow up after treatment with either corticosteroids or HDPI.

and *Enterococcus*, *Mycospherellaceae* and *Prevotella* at baseline, and *Cladosporidium* and *Bagnisiella* on *Lactobacillus helveticus* at 1 week and end of 1 month after HDPI treatment, respectively.

6. Discussion

Our work highlights that while HDPI showed potential modulatory action on GM in the context of diversification, it did not significantly outperform corticosteroids in improving clinical

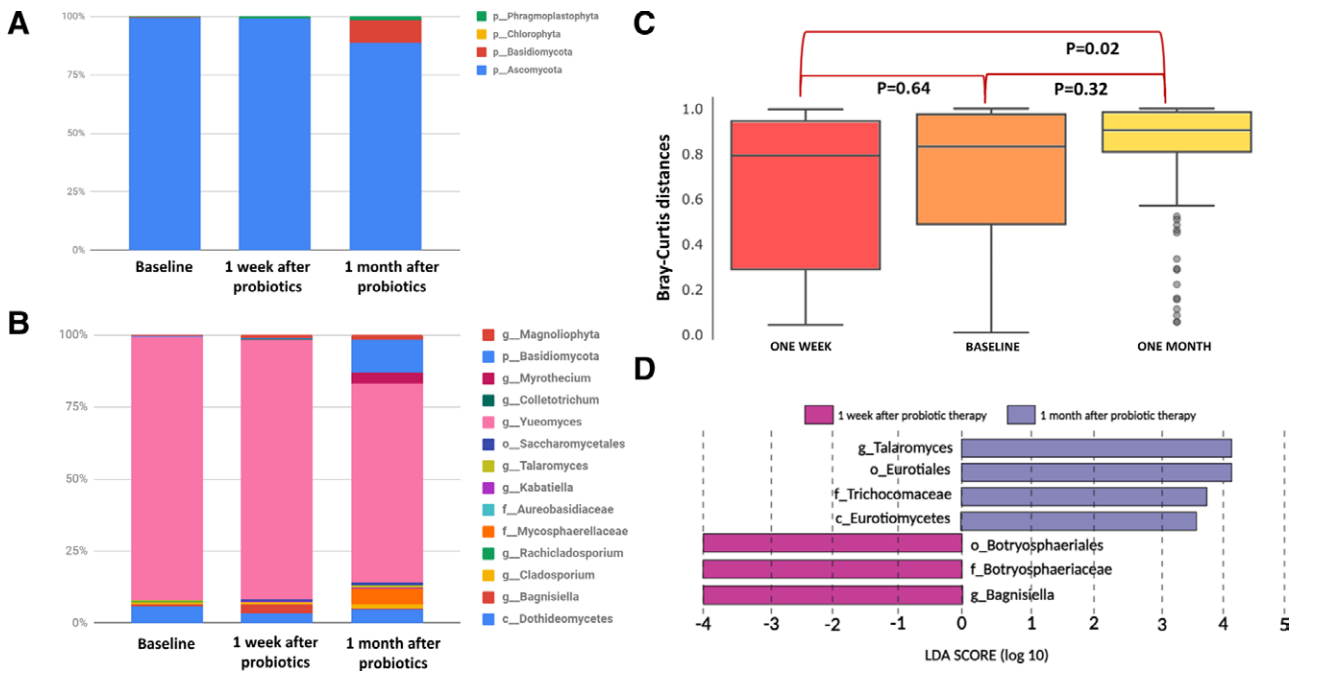

Figure 5. (A and B) It showed the stacked bar representation of changing fungal taxa from baseline and at the end of 1 week and 1 month after treatment with high dose probiotic infusion (HDPI); (C) box-plots representation of the beta diversity comparisons (fungal taxa) within the gut microbiomes in the group that received high dose probiotic infusion therapy at various time points at baseline to end of 1 month. Please note that only fungal beta-diversities between 1 week and 1 month was significantly different ($P = .002$); (D) shows the significant fungal taxa that were relatively abundant between the end of 1 week and 1 month after HDPI therapy, as represented by the Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis. The relative abundance is significant when $P < .05$, and logarithmic LDA score ≥ 2.0.

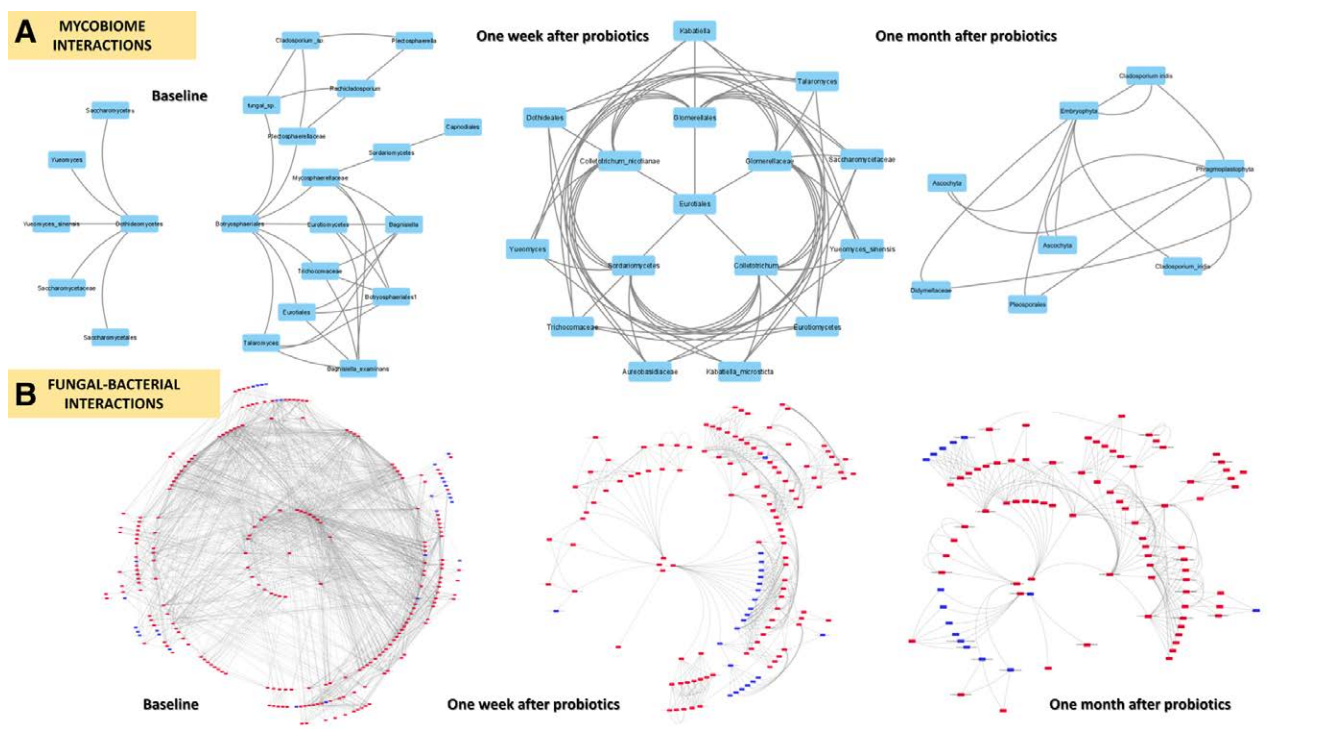

Figure 6. (A) The various changing significance of fungal taxa central influencers at baseline, compared with end of 1 week and 1 month end after treatment with high dose probiotic infusion therapy (HDPI) as demonstrated using radial network representation of CoNet® and NetworkX® analysis. Similar changes to diversity and exclusive interactions between fungal and bacterial groups from the baseline to 1 month after treatment with HDPI is also notable (B). Bipartite co-occurrence networks were generated in CoNet® and visualized in Cytoscape™.

outcomes for patients with severe AH because of a lack of modulation quality within both bacterial and fungal communities. Moreover, the persistence of potentially pathogenic bacterial communities' post-therapy was notable, suggesting a complex interaction between the gut microbiota and treatment efficacy. We also investigated the interplay between fungal and bacterial

communities in AH patients undergoing HDPI and found striking changes in fungal communities and their interactions with bacteria after HDPI therapy. However, there was no significant improvement in clinical outcomes compared to corticosteroid therapy. In contrast, a matched group of similar patients undergoing healthy donor stool transplant fared significantly better than those on HDPI. Species diversity is essential for maintaining healthy organ and host function.[24]

In our patients, microbiome diversity, as measured using metrics for diversity, richness, and evenness, did not differ between treatments at baseline and were considered dysbiotic at the outset. After intervention, the insignificant improvements in bacterial and fungal diversity from baseline under most parameters tested showed a lack of GM modulatory effects, especially with HDPI. Since the observed differences in alpha-diversity between groups of patients may have important clinical implications and HDPI therapy did not beneficially affect bacterial communities, we did not find clinically translated outcomes in our study. In the context of beta diversities from baseline, HDPI use was associated with significant changes from the end of 1 week to 1 month. An in-depth analysis revealed that HDPI therapy led to a significant expansion of potentially pathogenic taxa at the end of 1 month in patients with severe AH. At baseline, the core taxa *Parcubacteria*, after the use of high-dose probiotics, underwent modulation to diversify and expand into potentially pathogenic species such as *Paracoccus* and *Brevundimonas* at the end of 1 week and *Roseburia*, *Bilophila* and *Lachnospira* at the end of 1 month after treatment. Parcubacteria lack biosynthetic capabilities and DNA repair and are rich in adhesion and attachment proteins which make them ectosymbionts and parasites of other microbes and their wide diversity of genes potentially mediating cell–cell contact suggests a broad range of partner/prey interactions.[25] The development and expansion of *Brevundimonas* at 1 week and *Bilophila* at end of 1 month signals a worsening "pathobiont" restructuring of the GM in those receiving HDPI. The former is emerging as an important multidrug-resistant opportunistic pathogen in advanced chronic illness, while the latter is known to reduce the production of secondary bile acids in the gut, resulting in decreased production of primary bile acids in the liver, contributing to disrupted microbiota and an increase in lipopolysaccharide release.[26,27] When we looked at significant taxa affecting outcomes at the end of 1 month between HDPI and steroid-treated groups, we found persistence of higher pathogenic bacterial species (*Tissierella* and *Finegoldia*) and interestingly, the beneficial bacterial genus *Weissella* in HDPI and steroid-treated patients, respectively. This potentially signals the absence of "beneficial" or positive actions on baseline dysbiotic GM in severe AH with both therapies. *Tissierella* genera are severely opportunistic pathogens that worsen clinical outcomes, and documented reports on bacteremia with fecal exposure in debilitated persons are well known.[28] More interesting was the expansion of *Weissella* genus at the end of 1 month in patients receiving CST. The *Weissella* genus includes bacteria that are commonly found in the environment and present as commensals in the gastrointestinal tract of healthy vertebrates. *Weissella confusa* and *W cibaria* have been the focus of extensive research because of their numerous health benefits as probiotics. However, there is emerging data on their potential opportunistic pathogenicity in individuals with underlying health conditions. Overall, the benefits of these species as probiotics are well documented, with a few reported cases of bacteremia, endocarditis, and meningitis, which occur mostly in immunocompromised individuals or those with medical comorbidities.[29] It is imperative to note that the diversity of intestinal fungi is positively correlated with disease progression in patients with different degrees of liver disease, such as chronic HBV infection.[30] The lesser the diversity of changes to diversity, the greater the severity of liver disease and progression of liver disease, which was striking in our study.

In our patients, even though a "beneficial taxon" expanded with an intervention such as CST, its potential benefits may have been hampered in the presence of advanced disease and immunosuppressive condition in the host which may help us understand that single beneficial taxa are not the norm for GM modulation, but, multiple beneficial taxa with healthy interactions supporting host functions—an outcome currently available only preliminarily with FMT sparking the need for larger prospective studies. In contrast, the use of HDPI therapy tactfully led to the expansion of pathogenic bacterial genera in patients, resulting in the absence of beneficial clinical outcomes.

From a mycobiome perspective, multiple outcomes within the GM communities were notable. The use of HDPI induced modulatory changes within the fungal taxa. Clinically relevant outcomes were not observed. Significant changes in fungal beta diversities between time points were observed in HDPI-treated patients. However, the significant fungal genera that expanded after the intervention at the end of follow-up, as noted in biomarker discovery, included *Bagnisella* and *Talaromyces*. The former belongs to Dothideaceae and shows coincidental pathogenic opportunism, whereby the infection is largely dependent on the portal of entry and the immune status of the host, as noted in our study outcome.[31] *Talaromyces* and its associated genera cause severe invasive infections in humans and are considered agents of a neglected tropical disease.[32] The use of HDPI preferentially increased host-damaging fungal taxa, which would have led to a lack of beneficial outcomes related to survival. Similarly, the persistence of ascomycota-related groups even after HDPI therapy and associated exclusion interactions by opportunistic fungal taxa on beneficial bacterial taxa as assessed through the network analysis also provided insights into the non-beneficial and possibly harmful role of probiotic therapy in severe alcohol-related liver disease.[10,33]

7. Summary and conclusion

High-dose probiotic infusion therapy does not yield better clinical outcomes than corticosteroids in patients with severe AH. The therapy was linked to minimal quantitative and qualitative changes in bacterial taxa, with potentially pathogenic bacterial communities persisting after posttreatment. In comparison, patients receiving healthy donor FMT had better 90-day survival rates. HDPI also caused significant alterations in fungal communities among AH patients, with notable changes in fungal taxa and specific bacterial-fungal interactions following therapy. However, these microbial changes, including expansion of opportunistic and pathogenic fungal taxa and deleterious and exclusive fungal–bacterial interactions, did not translate to improved clinical outcomes at the 3-month mark compared to standard care. The need for prospective controlled trials is pressing to better understand and validate these findings.

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