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RESEARCH ARTICLE

Novel Microbial Signatures in the Faecal Microbiome Associated with Severe Alcoholic Hepatitis: *Bacteroides Finegoldii* and *Veillonella Dispar*

Rizwana Hasan^{1,2,#}, Ashish Kumar^{3,#}, Sudeep Bose², Rahul Roy¹, Anil Arora³, Praveen Sharma³, Sai Pawan Nagumantri¹, Debarati Paul² and Sangeeta Choudhury^{1,*}

¹Department of Research, Sir Ganga Ram Hospital, New Delhi 110060, India

²Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh 201313, India

³Institute of Liver Gastroenterology & Pancreaticobiliary Sciences, Sir Ganga Ram Hospital, New Delhi 110060, India

Abstract:

Background:

Alcoholic hepatitis is associated with dysbiosis, resulting in compositional changes in the gut microbiome, endotoxin transfer to the portal vein, and activation of inflammatory signalling pathways in the liver. However, the microbiome signature of severe alcoholic hepatitis (AH) patients of Indian origin is unknown.

Aim:

To investigate the microbiome diversity of Indian AH patients in comparison to non-alcoholic healthy controls (HC).

Methods:

16S rRNA amplicon-based metagenomics analysis of faecal samples at the time of AH diagnosis (n=12) was compared with HC (n=6), and correlation with survival status, co-occurrence networking, Linear Discriminant Analysis Effect Size (LEfSe), and the relationship between diet – microbiome were performed.

Results:

16S rDNA data showed a significant abundance of the *Enterobacteriaceae* family belonging to the phylum Proteobacteria and a depletion of the phylum Firmicutes in AH. A species-level classification identified *Klebsiella pneumonia*, *Klebsiella variicola*, and *Parabacteroides distasonis* as having the highest predictive performance value with reference to mortality at 60 days. Apart from this, a unique finding was the abundance of *Bacteroides finegoldii* and *Veillonella dispar* in AH patients. Moreover, the co-occurrence networking analysis revealed a higher level of connectivity and complexity within the microbial community of AH patients compared to HC. Thus, indicating the significant positive association (Spearman $r > 0.7$, $p < 0.01$) among members of the Firmicutes, Bacteroidetes, and Proteobacteria phyla in AH. Wilcoxon-Rank Sum test ($p < 0.01$) identified Firmicutes phyla occurrence associated with high carbohydrate intake and Actinobacteria phyla with low protein intake.

Conclusion:

The findings provide the baseline occurrence profile of the gut microbiome signature of liver damage and the diet-microbiome relationship in patients with severe AH. The study demonstrated an abundance of Proteobacteria phyla, indicating their role in inflammatory conditions, and further identified *Bacteroides finegoldii* and *Veillonella dispar* in severe AH patients. Additionally, *Klebsiella pneumonia*, *Klebsiella variicola*, and *Parabacteroides distasonis* were identified as mortality-predictive risk biomarkers.

Keywords: Alcoholic hepatitis, Faecal microbiome, Dysbiosis, Mortality rate, Diet-related microbiota diversity, Mucosal colon microbiome.

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1. INTRODUCTION

Long-term alcohol abuse exerts a spectrum of potent effects on different body systems, ranging from psychiatric symptoms, malnutrition, and chronic pancreatitis to alcoholic

liver disease, hepatocellular carcinoma, and cardiovascular disease. Alcohol modulates the human gut microbiota through the leaky intestinal barrier via the translocation of viable bacteria and their products to the liver [1]. One of the proposed mechanisms is the direct impact of the expression of pathogen-damage-associated molecular patterns, toll-like receptor 4 (TLR4), and NOD-like receptors (NLRs) [2 - 4]. More recently, Lang *et al.* (2020) observed altered intestinal

* Address correspondence to this author at the Department of Research, Sir Ganga Ram Hospital, New Delhi 110060, India; Tel: +91-97112 60058; E-mail: dr.sangeeta.sgrh@gmail.com

#These authors contributed equally for this work

microbial overgrowth and dysbiosis in patients with chronic alcohol consumption, which led to increased mortality risk [5]. So, maintenance of eubiosis and stabilizing the mucosal gut barrier may prevent liver damage progression. However, alcohol consumption-mediated microbiota patterns are unknown.

Studies by Mutlu and colleagues (2009) showed that rats fed continuously with ethanol for ten weeks caused dysbiosis of the mucosa-associated microbiome, indicating that the microbiota's composition is crucial in alcoholic liver disease (ALD) [6]. Furthermore, the same team studied gut microbiome alteration in human subjects (2012). Through PCR fingerprinting and pyrosequencing using 16S rRNA primers, they examined the mucosa-associated colonic microbiome in 48 alcoholics, both with and without alcoholic liver disease (ALD). Their findings suggested an increased abundance of Proteobacteria in comparison to Bacteroidetes, correlating with decreased microbial networking [7]. Yan *et al.* (2011) reported a similar finding of an increased ratio of Proteobacteria and a reduction in Bacteroides and Lactobacillus species [8]. A lower representation of the Lachnospiraceae and Ruminococcaceae families was reported in ALD [9].

Another altering factor is the diet, which can rapidly affect the community structure and functional potentials of gut microbiota [10], yet there is limited clarity on the types and/or patterns of the microbiome. A study by Graf *et al.* (2015) showed that the influence of short-/long-term diet effects, and gut microbiota composition were crucial factors along with inter- and intra-individual variability [11]. Indian diet is mainly composed of whole grains and plant-based products, which overall included high calorie and low protein intakes [12]. An extensive Indian study carried out by Dhakan *et al.* (2019), using 16S rRNA gene amplicon sequencing, whole-genome shotgun metagenomic sequencing, and metabolomics profiling, identified the gut microbiome composition among Central (Madhya Pradesh) and South (Kerala) Indians. This multiomics study identified 943,395 genes unique to the Indian gut microbiome, with *Prevotella* as the most dominant genus. *Prevotella* plays a critical role in the enrichment of branched-chain amino acid (BCAA) and lipopolysaccharide (LPS) biosynthesis pathways [13].

The present study, for the first time to our knowledge, has investigated the microbiota signature of AH patients in comparison to healthy non-alcoholic individuals in a tertiary care hospital based in New Delhi, India, using the amplicon-based metagenomics analysis of faecal samples. Concurrently, we carried out a multifactor statistical analysis of metagenomics data, including their effect on the composition of the diet.

2. MATERIALS AND METHODS

2.1. Subject Recruitment and Faecal Sample Collection

Patients diagnosed with alcoholic hepatitis and those who were admitted to the hospital (in-patient; n=12), aged 30 to 66 years were enrolled in the study. AH was defined and staged as per Maddrey discriminant function (DF) ≥ 32 , having portal vein hypertension and a MELD score of 27 (ranging from 24.3

- 31) with a history of alcohol abuse at least for the last 10 years. At the time of enrolment, patients were not on antibiotics for the last 7 days. AH patients positive for Hepatitis C virus (HCV), Hepatitis B virus (HBV), autoimmune diseases, those under 18 years of age, cancer or systemic diseases, hepatic cyst, those with hepatitis B surface antigen (HBsAg) positive were excluded. Faecal samples were taken within 24 hours post-admission.

The control healthy group included HC (n = 6) with no history of the disease at the time of enrolment, had normal clinical indices, and was not on antibiotics in the previous three months. Geographically, the patients and the controls shared a comparable standard of living as well as a similar socioeconomic background [Delhi National Capital Region (NCR)]. The duration of the study period was from June 2021 to July 2022. The Ethics Committee of Sir Ganga Ram Hospital, India (registered under the Drugs Controller General of India; ECR/20/Inst/DL/2013/RR-19), has approved the study (EC/06/19/1547) and informed written consent was obtained from those who voluntarily agreed to participate.

Routine dietary intake was noted, and their nutritional value was calculated in grams and energy in Kcal, based on ICMR criteria (ICMR, 2011; <https://www.nin.res.in/downloads/DietaryGuidelinesforNINwebsite.pdf>) [14]. Patients were segregated based on these nutritional factors into three groups: protein, carbohydrate, fat- high or low intake.

2.2. Sample Collection and DNA Isolation

Fresh solid faecal samples (about 250 mg) were collected in sterile collection containers and stored at -80°C in glycerol 1/1 (weight/volume). The total bacterial genomic DNA was isolated using the QiaAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. gDNA quality was confirmed by agarose gel electrophoresis and quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, USA) on the Qubit 2 instrument (ThermoFisher Scientific, USA).

2.3. Amplicon Library Generation and Sequencing

Hypervariable regions (V2-V9) of the 16S rRNA gene were PCR amplified using Ion 16S™ Metagenomics Kit (ThermoFisher Scientific, USA). In brief, 5 ng of gDNA were used for library preparation. The PCR reactions were performed with initial denaturation at 95°C/10 min, denaturation at 95°C/30 sec; annealing at 58°C/30 sec (18 cycles), and extension at 72°C/20 sec; extension at 72°C/7 min using a 9700 Thermocycler (ThermoFisher Scientific, USA). Amplicon libraries were purified using the AMPure XP reagent (Beckman Coulter, USA), quantified by the Qubit dsDNA HS Assay Kit, and verified on the Agilent 2100 Bioanalyzer (Agilent, USA) using the high-sensitivity DNA kit (Agilent, USA). Pooled equimolar concentrations (100 pM) of each library and further processed as described previously [15]. Sequencing was performed on the Ion S5 System using 400 base-read length chemistry in an 850 flow format.

2.4. Data Analysis and Statistics

The raw sequencing data were processed using Ion

Reporter Software (V-5.12), and the data implemented a filtering step to remove low-copy reads (those detected in fewer than 10 copies) from the sequencing data. The remaining reads were then assigned to operational taxonomic units (OTUs) at a 97% similarity for genus and 99% similarity for species threshold using the curated MicroSEQ 16S Reference Library v2013.1 and the curated Greengenes v13.5 sequence database. This assignment was performed using the Ion Reporter 5.18 software, a cloud-based platform, following the recommended workflow and pipelines described in the Ion Reporter Metagenomics 16S algorithms overview (<https://tools.thermofisher.com/content/sfs/brochures/ion-reporter-16s-metagenomics-algorithms-whitepaper.pdf>).

Additionally, to assess the alpha diversity metrics, we utilized an integrated QIIME pipeline within the Ion Reporter™ Software 5.18, as outlined in the QIIME2 documentation (<https://qiime2.org>). The alpha, and beta diversity were calculated according to the abundance-based coverage estimator, and significant differences were estimated using the R package “Bioconductor”. The Chao1 index (species abundance), Shannon and Simpson indices (species richness and evenness), and relative phyla abundance metrics were analyzed using the Paleontological Statistics Software Package (PAST). Beta diversity analysis was performed using UniFrac distance metrics and visualized via principal component analysis (PCoA) to investigate the structural variation of microbial communities. Statistically, a univariate frequency distribution was performed, and p values were obtained using the Mann-Whitney-U Test (Graph Pad Prism 9.3).

For clinical parameter analysis, p-values less than 0.05 were deemed statistically significant using SPSSv.28.0. The Receiver Operating Characteristic (ROC) curve illustrated the diagnostic ability of microbial species correlated with mortality risk in AH patients. The Kaplan-Meier test determined the total time (in days) from the confirmed diagnosis to the date of death.

DESeq2 analysis was performed to determine the differential expression pattern of species between AH and HC. Thereafter, taxon abundances at the levels of phylum, class, order, family, genus, and species were statistically compared among the groups. Heatmap of bacterial clusters generated using the normalized counts obtained from DESeq2 analysis, log-transformed, and Z-scaled using the package pheatmap in R (NG-CHM, GUI 2.20.2 BUILDER). The graphing package ggplot2 was used to plot these results. For the comparative diversity distribution of shared/unique OTUs at the species level, a Venn diagram was generated using Bioinformatics & Evolutionary Genomics. The volcano plot was derived from DESeq2 analysis using APEGLM.

The bacterial correlation network based on Spearman's rank analysis was performed with 100 permutations and represented at the genus level. OTUs with a prevalence of >10% and a frequency of >1% were used along with an interquartile filter for downstream analysis. Only strong correlations with Spearman's correlation coefficient $r > 0.07$ or $r < -0.70$ at P-values < 0.01 (Padjust, after correcting for false discovery) were considered for constructing the network.

To identify the most differential taxa associated with components of the diet (carbohydrates, proteins, and fat), the Linear discriminant analysis (LDA) effect size (LEfSe) algorithm (Wilcoxon rank-sum test, $p < 0.05$) and KEGG pathway contributions of predicted metagenomics data [16] were used.

3. RESULTS

3.1. Clinical Findings

Demographic and clinical parameters of AH patients (n=12) and HC (n=6) are shown in Table 1. In addition, AH patients clinically had symptoms of scleral icterus, jaundice, and tender hepatomegaly, mostly with ascites (eleven out of twelve).

Table 1. Characteristics and laboratory parameters of AH patients and healthy controls.

Characteristics	Patients (n=12)	Controls (n=6)	P-value
-	Median (IQR)		
Age (years)	41 (35.5 - 50.5)	32 (29 - 37)	0.017*
Gender	Male (12/12)	Male (6/6)	-
Ascites	11/12	-	-
MELD	27 (24.3 - 31)	-	-
mDF	56.35 (43.1 - 72.18)	-	-
Mortality	8/12	-	-
Laboratory Parameters (Normal range)			
Hemoglobin (13 - 17 gm/dl)	9.25 (8.75 - 10.82)	14.5 (13.5 - 15.25)	0.001**
TLC (4 - 10 thous/ μ l)	11.82 (8.52 - 16.41)	8.6 (7.45 - 9.6)	0.061
Prothrombin Time (11-13sec)	20.4 (18.25 - 24)	12 (11 - 13.5)	0.001**
INR (1-1.5)	2.28 (1.65 - 2.68)	1.15 (1 - 1.35)	0.001**
Platelet (150 - 450 thous/ μ l)	190 (120 - 237.75)	220 (184.75 - 294.25)	0.24
Blood Urea Nitrogen (8.9- 20.6 mg/dL)	20.9 (12.07 - 37.79)	12.35 (9.28 - 15.08)	0.055
Creatinine (0.7 - 1.25 mg/dL)	0.83 (0.69 - 1.16)	0.95 (0.88 - 1.13)	0.261
Calcium (8.4 - 10.2 mg/dL)	8.2 (7.7 - 8.76)	9.40 (8.80 - 10.13)	0.007**
Phosphorus (2.3 - 4.7 mg/dL)	3.4 (2.9 - 4.1)	3.50 (2.80 - 4.08)	0.92

Characteristics	Patients (n=12)	Controls (n=6)	P-value
Sodium (136 - 146 mmol/L)	134 (130.0 - 140.0)	139.5 (138.75 - 145.25)	0.074
Potassium (3.5 - 5.1 mmol/L)	4.1 (3.61 - 4.69)	4.05 (3.75 - 4.83)	0.92
Total Bilirubin (0.20 - 1.20 mg/dL)	15.58 (10.88-19.19)	0.63 (0.23 - 0.99)	0.001**
Direct Bilirubin (0 - 0.5 mg/dL)	9.09 (6.19-15.46)	0.27 (0.10 - 0.40)	0.001**
Total Protein (6 - 8.3 gm/dL)	6.8 (6.35-7.08)	7 (6.73 - 7.43)	0.24
Albumin (3.50 - 5.20 gm/dL)	2.66 (2.43-3.10)	4.55 (3.88 - 4.95)	0.001**
SGOT (5- 34 IU/L)	119 (75.25 - 227.82)	25.5 (16.25 - 30.25)	0.001**

Note: IQR - interquartile range; TLC - total leukocyte count; INR -international normalized ratio; BUN - blood urea nitrogen; SGOT - serum glutamic-oxaloacetic transaminase, ; $p \leq 0.05$ was accepted as significant, (* $P < 0.05$, ** $P \leq 0.001$)

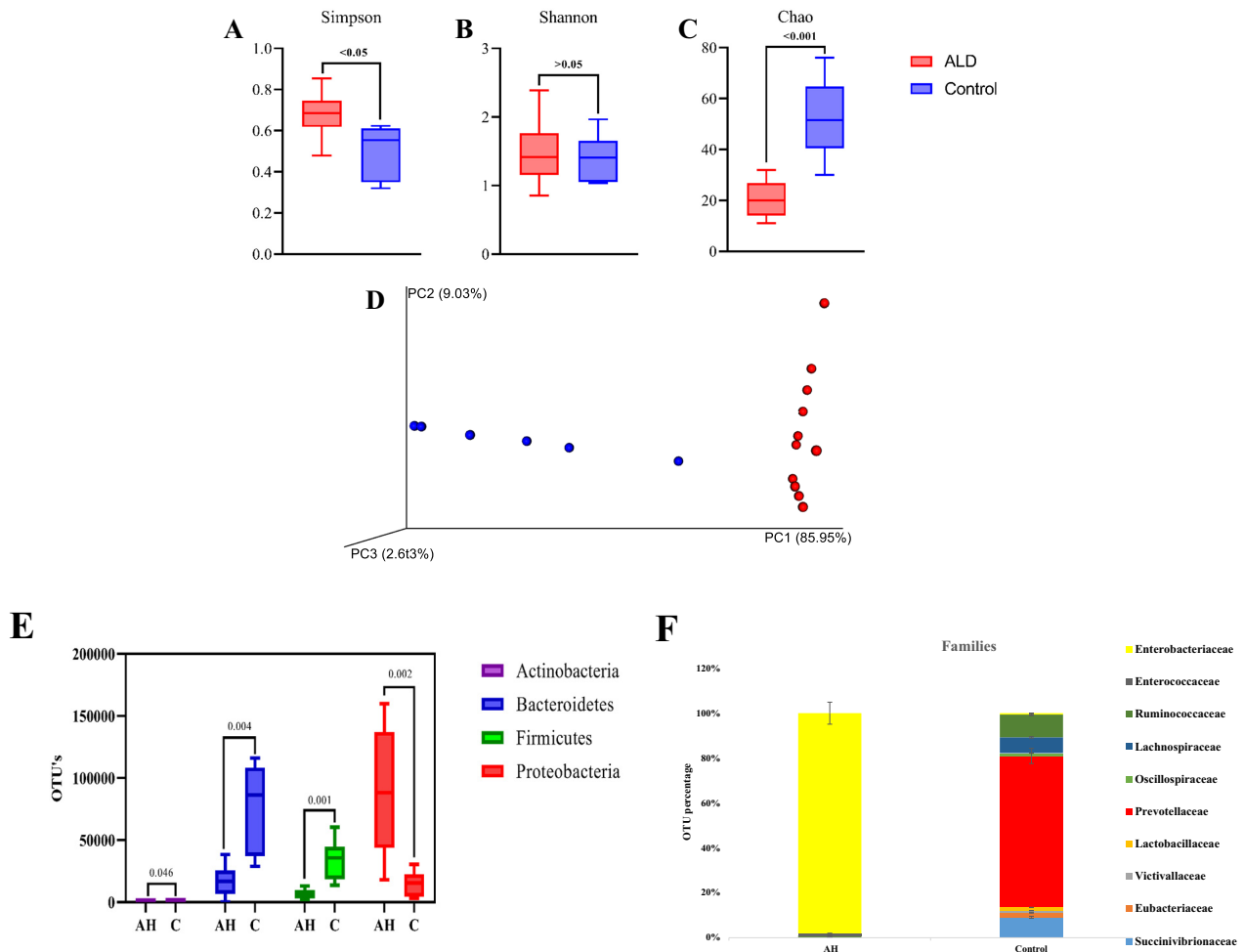


Fig. (1). Microbial diversity in alcoholic hepatitis patients (AH; n=12) and controls (C; n=6). Box & Whisker plot calculated using Mann-Whitney U Test to measure the α -diversity indices represented by Simpson index [$p < 0.05$] (A), Shannon [$p > 0.05$] (B), and Chao 1 [$p < 0.001$] (C). (D) Shows β -diversity using the Principal-coordinate analysis (PCoA), 3D coordination as per QIIME2 calculation [each dot represents one patient; red dots for AH and blue dots for healthy controls]. (E). Phylum level abundance represented by box & whisker plot. Distribution of four major phyla among AH patients versus HC. $p < 0.05$ (Mann-Whitney U Test) was taken as a significant value. (F). Bar graphs represent microbiome distribution at the family level in AH and healthy individuals.

3.2. Microbial Diversity, Abundance, and Distribution

The diversity of microbial communities was determined by alpha-diversity analysis. AH patients showed a more diverse dominant species ($p < 0.05$) than healthy controls, as calculated by the Simpson index that determines the highly abundant common or dominant species (Fig. 1A). Although higher species diversity existed in both AH and HC, no significant difference ($p > 0.05$) was observed between the two (Shannon

index; Fig. 1B). Fig. (1C), represented by the Chao index, observed significantly increased species abundance ($p < 0.001$) among the HC. Beta diversity analysis represents the similarity or dissimilarity of microbial composition between groups. Euclidean PCoA plots (Fig. 1D) showed a shift in relative abundance from 9.03% (PC2, AH) to 85.95% (PC1; HC), denoting the low level of similarity among AH. This indicates the difference in the faecal microbiome of HC and AH patients.

The 16S rDNA data identified three major phylogenetic abundances, Firmicutes (13.4%), Bacteroidetes (29.2%), and Proteobacteria (56.2%), comprising 99% of total sequences. Chlamydiae, Cyanobacteria, Verrucomicrobia, Tenericutes, Nitrospinae, Synergistetes, Lentisphaerae, Actinobacteria, and Cyanobacteria accounted for 1% of the remaining sequences (Supplement Information; Fig. S1). Compared to HC, Proteobacteria were significantly abundant in AH ($p=0.002$), whereas Firmicutes ($p=0.001$), Bacteroidetes ($p=0.004$), and Actinobacteria ($P=0.046$) were observed to be significantly decreased in AH patients (Fig. 1E).

Fifty-eight microbial families were identified, of which 40 families were significant, as identified by DESeq2 ($p \leq 0.005$). After a mean cut-off of ≥ 100 OTU, Enterobacteriaceae was dominantly observed in AH; Prevotellaceae, Ruminococcaceae, and Succinivibrionaceae were enriched in HC (Fig. 1F). Further, the DESeq2 data was represented as a Volcano plot, illustrating the quantitative pattern at species level between AH and HC ($p < 0.05$) with a fold change of ≥ 2 (Fig. S2).

3.3. Differentially Enriched OTUs

The 143 species present in both AH and HC, represented in the Venn diagram (Fig. SI-3) showed 21 of 143 were common to both; thirty-six out of one hundred forty-three were distinct in AH patients, and the rest 85 were present in HC.

Using DESeq2 normalized data, we identified seven species significantly abundant in the AH group with a cut-off >500 . These were *Klebsiella pneumoniae*, *Klebsiella variicola*,

Parabacteroides distasonis, *Bacteroides finegoldii*, *Bacteroides thetaiotaomicron*, *Veillonella dispar*, and *Clostridium aldenense*. On the other hand, *Prevotella copri*, *Succinivibrio dextrinosolvens*, *Prevotella stercorea*, *Dialister succinatiphilus*, *Sutterella sp.*, *Bacteroides plebeius*, *Gemmiger formicilis*, *Victivallis vadensis*, and *Desulfovibrio piger* were significantly abundant in HC and non-detectable in AH (Table 2).

Furthermore, the DESeq2 data comprising of significant ($p < 0.05$) OTUs was log normalized logarithmically and represented as a Heatmap (Fig. 2). The analysis showed differential species abundance in AH and HC, where enrichment of *K.pneumoniae*, *K.variicola*, *B. thetaiotaomicron*, and *P.distasonis* was found in AH, and *P. copri*, *S. dextrinosolvens*, and *P. stercorea* were found in HC. *P. distasonis* is a reference type of strain for the species Parabacteroides (ATCC 8503). Reduction in Lactobacillus species was noted among AH (Table S1).

3.4. Microbiota at the Species Level Correlates With Mortality Predictive Risk Stratification

Kaplan–Meier analysis showed 66% (8/12) of AH patients died within 60 days of diagnosis (Fig. 3A). To determine the mortality predictive performance of the seven abundant species identified earlier in AH patients (Table 2), the area under ROC curves (AUCs) from logistic regression analysis, was used. Fig. (3B) indicates *K. variicola*, *K. pneumoniae*, and *P. distasonis* could serve as a predictor for survival [AUC=0.938, 0.906 and 0.844 respectively; sensitivity = 1.00, 0.875 and 0.750 respectively] (Table S2).

Table 2. Distinct species in AH and HC.

Species	AH (Mean \pm SD)	HC (Mean \pm SD)	p-value
<i>Klebsiella pneumoniae</i>	8669.08 \pm 10783.87	31.5 \pm 22.16	9.13E-11
<i>Klebsiella variicola</i>	5706.83 \pm 6001.46	23.34 \pm 26.28	7.90E-09
<i>Parabacteroides distasonis</i>	4898.92 \pm 6909.72	147.5 \pm 238.04	1.61E-05
<i>Bacteroides finegoldii</i>	2133.92 \pm 2989.83	ND	3.50E-12
<i>Veillonella dispar</i>	1466 \pm 1892.91	ND	7.90E-13
<i>Bacteroides thetaiotaomicron</i>	1377.09 \pm 2428.69	183.34 \pm 326.7	0.002605
<i>Clostridium aldenense</i>	506.25 \pm 690	ND	9.13E-11
<i>Prevotella copri</i>	ND	59941.34 \pm 21661.94	3.37E-135
<i>Succinivibrio dextrinosolvens</i>	ND	5113.84 \pm 4761.5	2.92E-21
<i>Prevotella stercorea</i>	ND	2146.84 \pm 1798.9	1.60E-26
<i>Dialister succinatiphilus</i>	ND	1312.5 \pm 1183.09	2.83E-18
<i>Sutterella sp.</i>	ND	907 \pm 881.08	6.32E-18
<i>Bacteroides plebeius</i>	ND	860 \pm 1644.40	1.1E-10
<i>Gemmiger formicilis</i>	ND	621.5 \pm 903.34	1.32E-17
<i>Victivallis vadensis</i>	ND	590.67 \pm 507.6	3.26E-17
<i>Desulfovibrio piger</i>	ND	512.34 \pm 446.23	9.28E-17

Note: ND – not detected

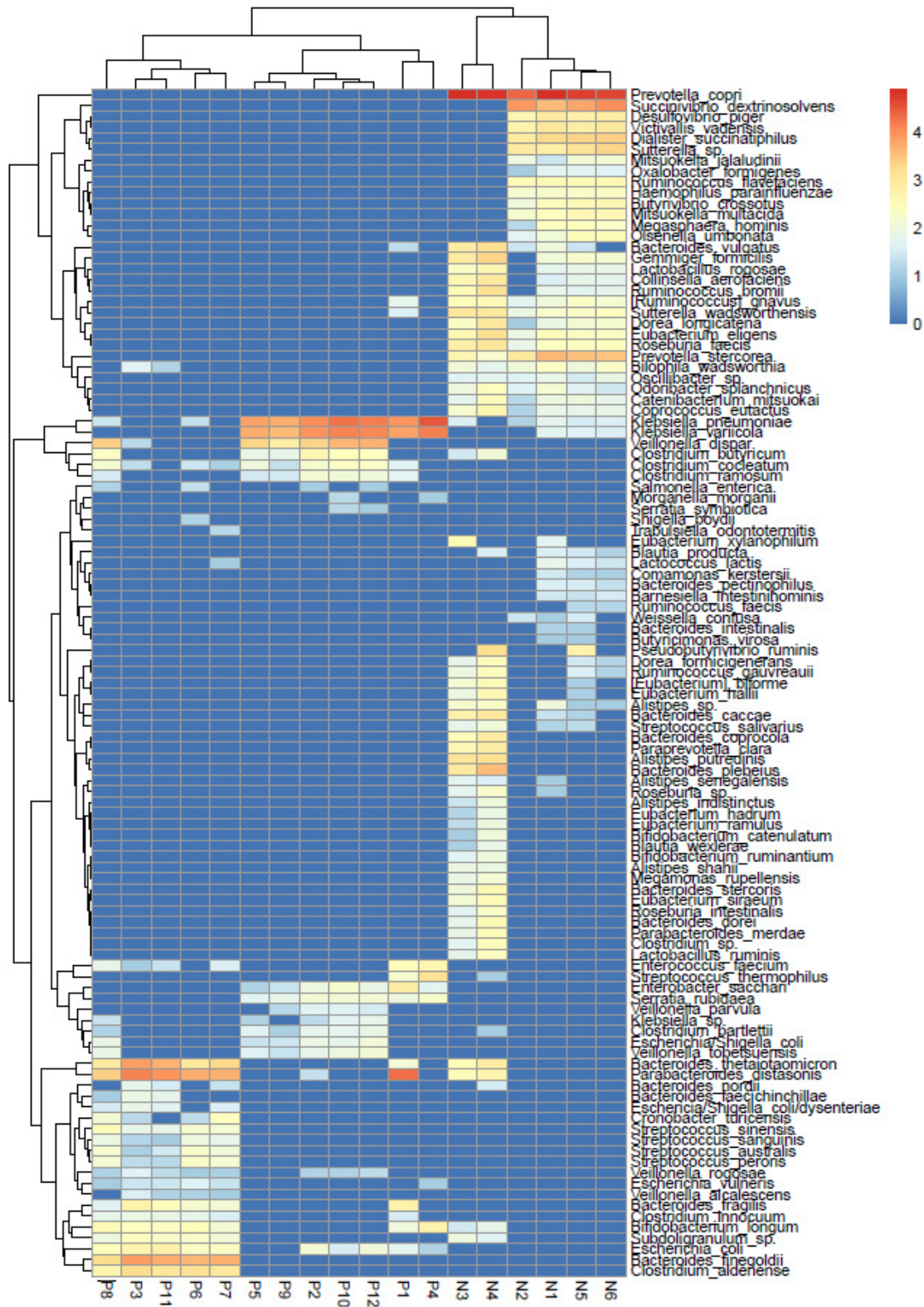


Fig. (2). Heatmap of OTU identified by the 16S rRNA genes between AH and HC. The counts per sample (blue: represents a low percentage of OTUs in the sample; red: represents a high percentage of OTUs). Vertical clustering (hierarchical clustering) indicates the similarity in the richness of different species among different samples. The closer the Euclidean distance between the two species, the shorter the branch length, indicating a more significant similarity in richness between the two species. The heatmap was built using NG-CHM BUILDER. The scale on the right indicates the breakpoints associated with the colors.

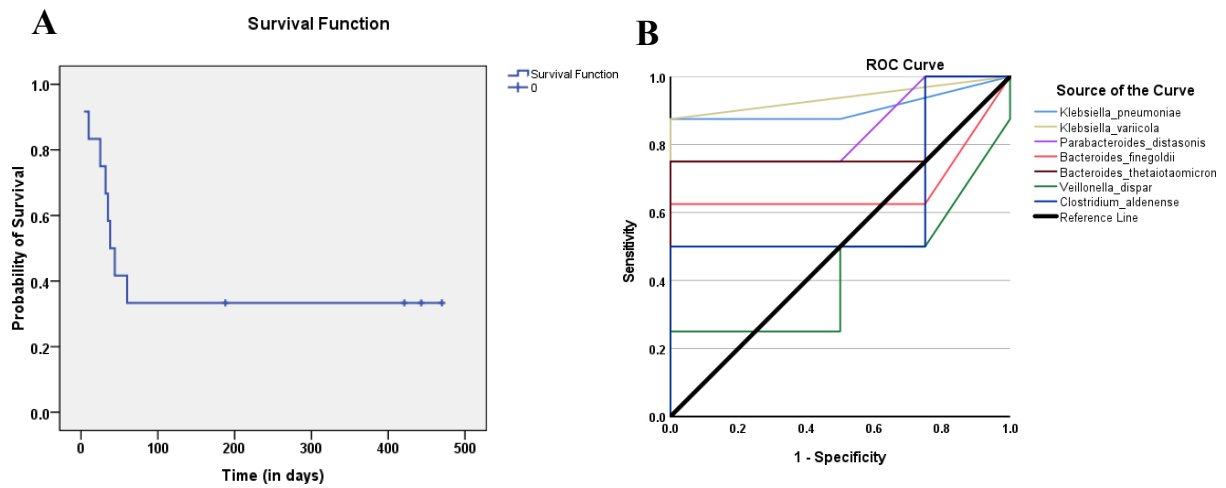


Fig. (3). (A). Kaplan-Meier survival analysis in AH patients. X-axis represents the survival time in days; Y-axis shows the probability of survival. (B). Receiver operating characteristic (ROC) curves for mortality based on sensitivity and specificity of pathogenic species classification using OTUs at the species level. 95% confidence intervals are shown as coloured ribbons. ROC curves of species are shown on the right for classification.

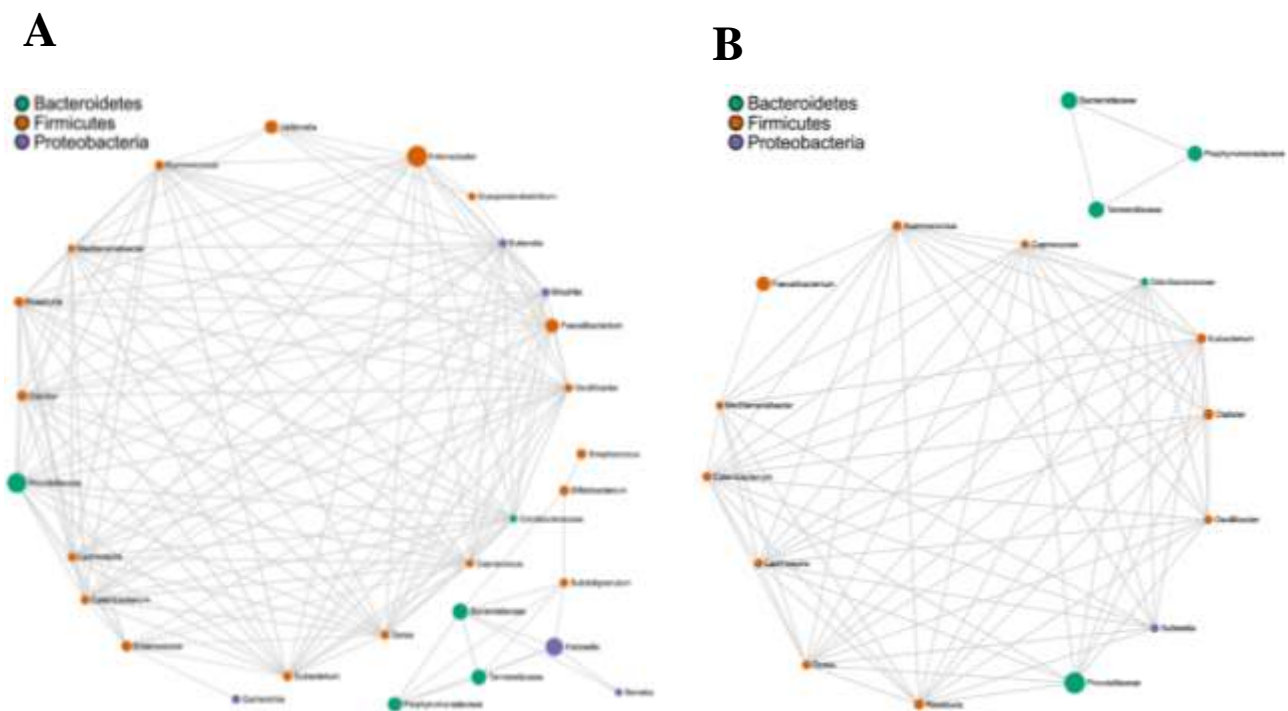


Fig. (4). Co-occurrence network of bacteria at the genera level for the AH group (A) and control (B). Spearman rank analysis was used with 100 permutations. Only p value < 0.01 and the correlation coefficient $r > 0.7$ were represented in the network. The size of each node is proportional to the number of connections, whereas the color of each node correlates with taxonomy. The family names referred to the entity for which genus names were unidentified and/or inconclusive.

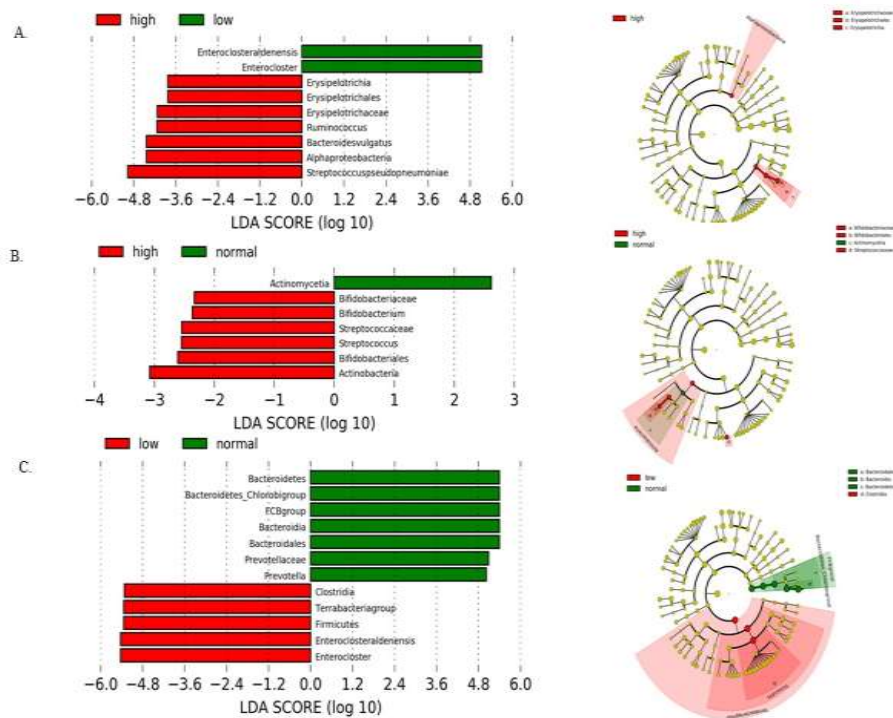


Fig. (5). Linear Discriminant Analysis (LDA) Effect Size (LefSe) plot of taxonomic biomarkers identified in the gut microbiome. Specific bacterial traits were found at different taxa levels according to the diet of carbohydrates, protein, and fat (A-C), respectively. The LefSe algorithm signifies statistical consistency and relevance of the bacterial classes in the AH and control gut microbiome. The taxonomic cladogram obtained from LefSe analysis of 16S sequences is represented on the right side. High is indicated by red and low and normal by green; circle sizes in the cladogram plot are proportional to bacterial abundance (Wilcoxon rank-sum test, $p < 0.05$). The circle represented from the inner to outer circle: phyla, classes, order, families, genus, and species. The brightness of each dot is proportional to its effect size.

3.5. Correlation Network Microbiome

Bacterial co-occurrence network analysis using Spearman's correlation values is a well-tested and utilized method to study the interaction between different bacterial members in a community. A total of 166 and 75 strong correlations/edges were found in AH and HC, respectively. In AH, 130 positive and 36 negative correlations/edges were noted (Fig. 4A), whereas no negative correlation was recovered in HC (Fig. 4B). In both groups, the co-occurring genera belonged to three phyla - *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (gamma- and beta-Proteobacteria). Among these, the phylum *Firmicutes* was dominant, indicating its key position in the hub taxa (Fig. S4). In AH, 146/166 (89%) edges had either one or more *Firmicutes* members with high centrality scores. In HC, 70/75 (93%) edges were associated with at least one of the nodes corresponding to *Firmicutes* phyla. The direction of the correlation (positive and negative), the exact number of edges, and correlation values are shown in Tables S3 and S4.

3.6. Microbiota Composition according to Dietary Nutrient Intakes

Distinct microbiome taxa were detected among AH with either low and/or high intake of carbohydrates, proteins, and fat, based on LefSe analysis (Table S5). Fig. (5A) depicts a significant increase in the number of *Bacteroides vulgatus*/ *Firmicutes* phylum ($p=0.04$) and *Erysipelotrichales*/ *Bacillota*

phylum ($p=0.04$) among those who had a high intake of carbohydrates. Most of the AH patients showed a low intake of protein diet. *Actinobacteria* phylum was predominant with a significant abundance of *Streptococcus* and *Bifidobacterium* at the genus level (p -value ranging from 0.0019 – 0.042; Fig. 5B). AH patients whose fat intake was low, showed a significantly decreased abundance of *Terrabacteria* group ($p \leq 0.0007$; Fig. 5C), but *FCB* group (*Fibrobacterota*, *Chlorobiota*, and *Bacteriodota* member phyla) was found to be at a normal level. A detailed diet chart and intake by participants along with statistical evaluation has been included as Table SI-6 (A) and (B).

4. DISCUSSION

Alcohol-specific gut microbiome signatures of Indian AH patients showed a differential signature compared to those of normal healthy individuals. By performing the bacterial co-occurrence network analysis, major hub interactions were identified, and further, a differential microbial pattern was delineated based on dietary components. Although the literature cites studies on faecal microbiome profiling in AH patients, the gut microbiota composition of Indian ethnic groups is lacking. Demographically, alcoholic hepatitis patients enrolled in the present study exhibited leucocytosis, thrombocytopenia, and hyper bilirubinaemia. An increase in mortality rate was observed due to septicemia, a commonly

observed syndrome among AH patients. Similar demographics were observed in other studies [16 - 20].

4.1. Microbial Signature

A significantly decreased alpha diversity (Shannon index/PCoA) was observed in AH patients, correlating with the disease stage. An abundance of Proteobacteria, especially the *Enterobacteriaceae* family, and lower numbers of Firmicutes in AH patients compared to an increased abundance of Bacteroidetes / *Prevotellaceae* family in HC, providing evidence to link bacterial translocation in severe hepatitis. Earlier studies in both alcohol-ingested mouse models and human subjects (ALD patients) concurred with a significantly increased Proteobacteria phylum [6]. Increased *Enterobacteriaceae* and reduced *Bacteroidetes*, *Lactobacillus*, and *Actinobacteria* were seen in patients with ALD and other chronic liver diseases [21]. A study carried out by Llopis *et al.* (2016) observed that severe AH patients harbour abundant Bifidobacteria and Streptococci [22]. Abundances of Proteobacteria have been implicated in chronic Crohn's disease and ulcerative colitis [23, 24], thereby supporting their presence in intestinal dysbiosis.

Sub-classification analysis at the species level identified the top seven microbes as *K. pneumonia*, *K. variicola* (Proteobacteria phylum), *P. distasonis*, *B. finnegoldii*, *B. thetaiotaomicron* (Bacteroidetes phylum), *V. dispar*, and *C. aldenense* (Firmicutes phylum) in AH patients. *B. finnegoldii* and *V. dispar* have not been reported earlier in AH patients. The top 3 microbial species [*K. pneumonia*, *K. variicola*, and *P. distasonis*] showed the highest predictive performance with reference to mortality. This bacterial panel can be used as a mortality risk predictive biomarker. A study by Nakamoto *et al.* (2019) suggests *K. pneumonia* disrupts the intestinal barrier, resulting in permeability and liver inflammation that is mediated by Th17 cells in primary sclerosing cholangitis [25]. Other studies have also observed *K. pneumonia* to be associated with high morbidity/mortality in AH patients with immunocompromised conditions like sepsis [26 - 28]. Similarly, we observed a significant increase in *K. variicola*, a common multidrug-resistant species in ALD [29]. Very recently, Ezeji *et al.* (2021) found *P. distasonis*, a new species belonging to the Bacteroidetes phylum. The authors herein described the emerging role of this microbe in antimicrobial resistance, in inflammatory signalling cascades, resulting in the production of IL-1 β activated by the HIF-1 α pathway that attenuates T-regulatory cell development and favours IL-17 secretion [30].

Taxonomically, *B. finnegoldii* and *B. thetaiotaomicron* belong to the Bacteroidales order. These species have been reported in inflammatory bowel disease (IBD) and colorectal cancer [31, 32]. Studies have also shown that *B. finnegoldii* helps in host metabolism and *B. thetaiotaomicron* is involved in nutrient absorption, epithelial cell maturation, and maintenance [33, 34]. In our study, both of these bacteria were significantly abundant in patients, yet their role in AH is yet to be determined. Another unique species identified is *V. dispar*, found in AH patients. Studies have reported a relative abundance of *Veillonella* at the genus level to correlate with

MELD score, serum bilirubin levels, and disease recurrence [5, 35 - 37].

A recent study by Prasoodanan *et al.* (2021) identified a high abundance of *Prevotella copri* among healthy individuals belonging to different geographical locations in India. This species plays a critical role in carbohydrate and fibre metabolism [38]. Similar data were observed in the HC group, whereas AH patients showed the absence or non-detectable levels of *Prevotella copri*, *Succinivibrio dextrinosolvens*, *Prevotella stercorea*, and *Dialister succinatiphilus*.

The correlation network analysis revealed strong interconnections between Firmicutes, Bacteroidetes, and Proteobacteria genera, indicating an imbalanced microbiome in AH. Enterocloster (Bacillota phyla), Prevotellaceae (Bacteroidota phyla), and Klebsiella (Proteobacteria phyla) were identified as key taxa in the network, similar to findings in NAFLD patients [39].

4.2. Diet and Microbiome

Dietary components are major factors that influence the gut microbiota both in healthy and diseased conditions. The three major macromolecules, fats, carbohydrates, and proteins, stimulate responses that modulate gut microbial diversity [18]. In the present study using LEfSe analysis, we found distinct taxa based on a higher or lower intake of macronutrients. AH patients with a high-carbohydrate rich diet showed significantly increased *Bacteroides vulgatus*/Firmicutes phylum and *Erysipelotrichales*/ Bacillota phylum; Actinobacteria phyla were predominantly associated with low-protein intake; patients with low-fat intake showed a significantly decreased abundance of Terrabacteria group, although the FCB group (Fibrobacterota, Chlorobiota, and Bacteroidota member phyla) was found to be at a normal level. Murphy *et al.* (2010) observed increased Firmicutes when mice were fed a high-fat diet [40]. Contrary to our data, studies have shown increased levels of Bifidobacteria in the faecal excretion of rats fed with high protein [41, 42]. In a study conducted by Nakayama *et al.* (2015), a comparison of faecal microbiota was carried out among 303 school-going children from Central Asian and Southeast Asian regions. The findings suggested that dietary factors may influence the abundance pattern of the *Bacteroides* and/or *Prevotella* genus [43]. Thus, diet-related microbial diversity can reflect changes in the gut microbiota structure and composition based on nutritional factors.

Studies have reported that the utilization of probiotics and prebiotics has been associated with clinical improvement in patients with alcoholic liver disease [44 - 46]. However, a comprehensive correlation between the gut microbial pattern and the composition of the diet is not well studied. Interestingly, a significant abundance of Bifidobacterium, a probiotic, was found among those who had a high-protein diet. Studies have reported, a shift in the relative abundance of Bifidobacterium in relation to dietary nutrients among AH patients [47 - 49]. Thus, suggesting increasing numbers of Bifidobacterium in AH patients could potentially mitigate liver damage and inflammation. While one cannot completely rule out the possibility that some of these taxa, could be transient food-associated microbes, there is a growing body of evidence

suggesting that levels of Bifidobacterium can increase in pathologies like AH [50]. Yet the possibility of exploring the use of pre-/probiotic as an intervention for AH or Faecal Microbiota Transplantation in AH could be tailored for a better prognosis [51].

Keeping in view the lack of evidence on gut-liver microbiota profiling in alcoholic hepatitis patients of a particular geographical location and its effect on Indian diet preferences, a strict exclusion criteria enabled only a limited number of patients to be enrolled during the study period. This preliminary study provides the foundation for further research in this field, and an in-depth understanding of their effects can lead to the development of newer medicinal options for alcohol-related liver diseases.

CONCLUSION

Consequences of chronic alcohol use cause overgrowth and/or altered taxonomic intestinal microbiomes, thus resulting in gut dysbiosis. Our findings observed that intestinal dysbiosis in AH harbours mainly Proteobacteria phyla, *Klebsiella pneumoniae*, *Klebsiella variicola*, and *Parabacteroides distasonis* can serve as predictors for survival. In addition, two novel species, *Bacteroides fingoldii*, and *Veillonella dispar*, could be used as potential diagnostic biomarkers of severe AH. Although studies have reported the effect of food and epigenetic factors on the composition of the microbiome in healthy and disease conditions, to date their interactive microbial mechanistic remains equivocal, thus necessitating further studies. Taken together, our findings illustrate the importance of the relationship between interacting microbes and their regulation based on disease status and/or alcohol composition.

AUTHORS' CONTRIBUTIONS

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. RH performed a collection of human samples/isolation/NGS assays, and analysis and framed the manuscript. AK conceptualized, and enrolled patients as per inclusion criteria, provided detailed demographics, and edited the manuscript. RR performed data analysis using the 'R' package. SB, SNP, and DP guided and framed the manuscript. AA and PS helped with clinical work details and proofreading. SC conceptualized, guided, analyzed the data, framed, and edited the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was reviewed and approved by Sir Ganga Ram Hospital Ethics Committee (Ethics approval code: EC/06/19/1547). The patients/participants provided their informed consent to participate in the study.

HUMAN AND ANIMAL RIGHTS

No animals were used in the research. The reported experiments were carried out as per the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed written consent was obtained from those who voluntarily agreed to participate.

STANDARDS OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIAL

The data and supportive information are available within the article.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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