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Received: 12 December 2025

Accepted: 13 July 2026

Published online: 13 August 2026

Cite this article as: Trivett H., Dalby M.J., Peel N. *et al.* Decoding the microbiome and resistome of advanced chronic liver disease through long-read metagenomics. *Genome Med* (2026). <https://doi.org/10.1186/s13073-026-01724-9>

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Decoding the microbiome and resistome of advanced chronic liver disease through long-read metagenomics

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Abstract

Background - Patients with advanced chronic liver disease (ACLD) and underlying cirrhosis frequently require repeated courses of antimicrobial therapy, with both the frequency and spectrum of antimicrobial exposure increasing alongside disease progression. In this population, impaired immune function and increased intestinal barrier dysfunction contribute to a heightened susceptibility of multidrug-resistant bacterial infections.

Methods - To comprehensively characterise the gastrointestinal microbiome and antimicrobial resistance gene (ARG) landscape across the clinical spectrum of ACLD, and to identify microbial and resistome signatures associated with disease severity. We employed long-read metagenomic sequencing (Oxford Nanopore Technologies) to profile the gastrointestinal microbiome and resistome across distinct ACLD stages: acute-on-chronic liver failure (ACLF), decompensated cirrhosis (DC), and stable cirrhosis, compared to healthy controls.

Results - ACLF patients had pronounced levels of *Enterococcus faecium*, with six samples out of 28 showing over 95% relative abundance, suggesting its potential as a bacterial biomarker for advanced cirrhosis. We reconstructed 28 high-quality MAGs of *E. faecium* from cirrhosis patients, 17 of which originated from ACLF cases. This dominance of *E. faecium* correlated with substantially reduced microbial diversity and a marked depletion of key commensal taxa, including *Blautia*, *Akkermansia*, *Faecalibacterium*, and *Bifidobacterium*. Resistome analysis revealed significant enrichment of clinically relevant ARGs in DC and ACLF, including those conferring resistance to aminoglycosides, beta-lactams, and glycopeptides, correlating with prior antimicrobial exposure.

Conclusion - Long-read metagenomics enables high-resolution characterisation of microbial and resistome dynamics across ACLD severities. By capturing taxonomic shifts, functional potential, and ARG enrichment, this approach provides valuable insights into microbiome trajectories linked with disease severity, informing mechanistic research and potential clinical interventions.

Key words

- Advanced chronic liver disease (ACLD)
- Cirrhosis

- Long-read metagenomics
- Oxford Nanopore Technologies
- Gut microbiome
- Resistome
- Antimicrobial resistance (AMR)
- *Enterococcus faecium*
- Metagenome-assembled genomes (MAGs)
- Acute-on-chronic liver failure (ACLF)

Background

Advanced chronic liver disease (ACLD) represents a major global health burden, encompassing a spectrum from compensated cirrhosis to decompensated cirrhosis (DC) and acute-on-chronic liver failure (ACLF). These stages are associated with high morbidity and mortality, with ACLF having a high short-term mortality of 90 days in over 50% of patients (1,2).

Complex interactions between host factors, microbiome perturbations, and systemic inflammation drive disease progression in ACLD and increase susceptibility to bacterial infections (3). In cirrhosis, disruption of the intestinal barrier facilitates bacterial translocation from the gut into ascitic fluid and systemic circulation, leading to complications such as sepsis and spontaneous bacterial peritonitis (4).

Emerging evidence indicates that the gut microbiome plays a central role in the pathophysiology of ACLD, influencing immune regulation, intestinal barrier integrity, and susceptibility to infections (5,6). Progressive disease stages are characterised by microbiome perturbations, including the depletion of beneficial commensals such as *Faecalibacterium*, *Ruminococcus*, and *Bacteroides*, alongside reduced levels of key metabolites, including short-chain fatty acids, that maintain epithelial integrity and gut permeability. These shifts coincide with the expansion of potentially pathogenic taxa, i.e. pathobionts, that can dominate the gastrointestinal tract, including *Enterococcus faecium*, *Streptococcus thermophilus*, and *Ruminococcus lactaris* (7). In patients with ACLD, increasing overlap of the oral and gut microbiomes with progressive disease severity spanning SC, DC and ACLF is accompanied by a marked expansion of

virulence-factor and antimicrobial resistance gene (ARG) carriage (oral >1,200 ARGs; gut >670 ARGs), highlighting resistome evolution in cirrhosis-associated perturbations (8). However, most existing studies have relied on short-read or targeted sequencing approaches, limiting resolution of microbial genomes and their functional attributes, including ARGs.

Antimicrobial resistance is a critical concern in ACLD, where frequent antibiotic use for prophylaxis and infection management may select for multidrug-resistant organisms (9). It is estimated that approximately one-third of bacterial infections in patients with cirrhosis are caused by multidrug-resistant bacteria, resulting in a fourfold increase in mortality risk compared to infections caused by non-resistant bacteria (10). In clinical settings, culture-based pathogen detection remains the standard practice; however, these methods are limited by slow turnaround times, poor detection of fastidious species, and low throughput, often resulting in the missed detection of polymicrobial infections (11,12). The increasing adoption of multiplex PCR has improved diagnostic speed; however, as these assays rely on predefined primer targets, they are restricted to known organisms. Consequently, both culture-based and molecular methods may fail to identify clinically relevant or novel pathogens and overlook broader genomic context, such as gene function, antimicrobial resistance (AMR), and secondary microbial contributions to infections (13).

Metagenomic sequencing, particularly long-read technologies, offers unique advantages for resolving complex microbial communities and their potential metabolic pathways, virulence factors, and resistome. Crucially, it enables the recovery of high-quality metagenome-assembled genomes (MAGs) and accurate ARG profiling, which conventional diagnostic methods can miss. Applying this approach to ACLD could uncover microbial signatures and resistance determinants associated with disease severity, providing insights into host-microbe and microbe-microbe interactions that underpin clinical outcomes (14). Indeed, with the appropriate infrastructure, metagenomics can yield actionable results with a rapid turnaround from sample collection to pathogen identification in less than 24 hours (15–17).

In this study, Oxford Nanopore Technologies (ONT) long-read metagenomics was employed to characterise the gut microbiota and resistome across the clinical spectrum of ACLD - stable cirrhosis, DC, and ACLF, comparing them to healthy controls. We aimed to identify taxonomic profiles and functional changes associated with ACLF progression, with a focus on MAGs and ARG enrichment profiles. Our findings show that liver disease progression is marked by pronounced gut microbiota disruptions and enrichment of ARGs, highlighting distinct microbial and resistome signatures that could inform clinical monitoring and therapeutic strategies.

Methods

Study cohorts and sample collection

This cross-sectional study included 76 adult participants recruited at King's College Hospital between 2013 and 2019. Participants were categorised into four cohorts: healthy controls (HC; n = 12), stable cirrhosis (SC; n = 20), decompensated cirrhosis (DC; n = 16), and acute-on-chronic liver failure (ACLF; n = 28), giving a total advanced chronic liver disease (ACLD) cohort of 64 patients.

Patient participants, or their family nominee as consultees in the case of lack of capacity, provided written informed consent within 48 hours of presentation. Patients were managed according to standard evidence-based protocols and guidelines (18). Main exclusion criteria included pregnancy, hepatic or non-hepatic malignancy, pre-existing immunosuppressive states, replicating hepatitis B or C or HIV infection, and known inflammatory bowel disease.

Participant faecal samples were collected for downstream microbiome and resistome analyses and at the time of sampling, demographic, clinical, and biochemical metadata were collected at the time of biological sampling. Clinical data was collected from electronic patient records using standardised study case report forms with pre-defined clinical parameters by delegated members of the study team. This clinical metadata was then entered into a secure, password-encrypted electronic database utilising a pseudo-anonymisation approach. Clinical metadata included participant age and sex, body mass index, aetiology of liver disease, type and severity of hepatic decompensation, physiology, clinical lab parameters (haematology, coagulation,

biochemistry, metabolic) clinical microbiology readouts, concomitant antibiotic and other pharmacotherapies, degree of organ failure and mortality/transplant-free survival. Major hepatic prognostic and disease severity scores including Child-Pugh score (19), model for end-stage liver disease (MELD) (20), United Kingdom model for end-stage liver disease (UKELD) (21), Chronic Liver Failure Consortium-acute decompensation (CLIF-C AD) were calculated.

ACLD aetiology spanned 17 distinct diagnostic categories, with the most common being alcohol-related liver disease (ALD) in abstinent individuals (n=26), ALD in active drinkers (n=8), and metabolic dysfunction–associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease (n=7). Participants receiving routine antimicrobial therapy as part of standard clinical care remained eligible for inclusion.

Healthy controls (HCs) aged >18 years were recruited to establish reference values for the various assays performed. Exclusion criteria for healthy controls were body mass index <18 or >27; pregnancy or active breastfeeding, a personal history of thrombotic or liver disease; chronic medical conditions requiring regular primary or secondary care review such as inflammatory bowel disease, and/or prescribed pharmacotherapies. The healthy controls were not age matched. During the original recruitment period, older healthy individuals suitable as ‘negative controls’ were less available for recruitment and biological sample collection.

Faecal sample acquisition

Faecal samples were obtained within 48 hours of admission to the hospital and collected into non-treated sterile universal tubes (Alpha Laboratories™), without any additives. Faecal samples were kept at 4°C without any preservative and, within 2 hours, were homogenised, pre-weighed into 200mg aliquots in FastPrep tubes (MP Biomedicals™), for storage at -80°C for subsequent DNA extraction.

DNA extraction and long-read metagenomic sequencing

The FastDNA Spin Kit for Soil (MP Biomedicals™) was used to extract DNA from faecal samples, following the manufacturer’s instructions, with an extended one-minute bead-beating using the FastPrep-24 (MP Biomedicals™), with a speed setting of 6.0. DNA concentrations of each sample were determined using the Qubit Broad

Range assay (Life Technologies), and molecule length was analysed on an Agilent TapeStation using Genomic Tape and reagents (Agilent Technologies).

Libraries were constructed with the Ligation Sequencing kit (SQK-LSK109) and Native Barcoding Expansion kit (EXP-NBD104) according to the manufacturer's instructions (NBE_9065_v109_revAD_14Aug2019). Samples were pooled, with up to 400 ng of DNA input per sample. The pooled library was sequenced on a GridION using a FLO-MIN106D flow cell with the standard 72 hour run script (MinKNOW v21.11.7).

Metagenomic read profiling using MARTi

ONT sequencing data in FAST5 format was converted to POD5 format using POD5 v0.3.15 (<https://github.com/nanoporetech/pod5-file-format>). Basecalling and demultiplexing of the raw signal data were performed using Dorado v0.8.3 (22) with the dna_r9.4.1_e8_sup@v3.6 model. Taxonomic classification and AMR gene detection were performed with Metagenomic Analysis in Real Time (MARTi) v0.9.28 (23). For each sample, analysis was restricted to the first 100,000 basecalled reads that passed MARTi's internal quality-control filters. This read cap was selected to provide sufficient sensitivity for taxonomic profiling and AMR gene detection while minimising variation in detection power between samples and reducing overall analysis time. Importantly, this approach reflects the intended rapid, real-time use case of MARTi, where informative analyses may be performed on early sequencing output prior to completion of a full sequencing run. MARTi performs quality-control filtering internally. A minimum read length of 150 bp was applied because the minimum alignment length within the MARTi pipeline was also set to 150 bp; shorter reads would therefore be unable to produce informative alignments. This alignment length threshold was used to reduce spurious taxonomic classifications arising from matches to residual nanopore adapter or barcode sequence contamination present in reference assemblies. A minimum read quality score of Q8 was retained, corresponding to the MARTi default setting.

Additional read filtering steps (e.g. filtlong) were intentionally not applied within the MARTi workflow, as the analysis was designed to mimic a minimal-preprocessing, rapid diagnostic scenario. In contrast, more stringent quality filtering and host read

removal were applied as part of the full metagenomic assembly workflow, reflecting its distinct aims and downstream analyses.

For taxonomic classification, we used MARTi's BLAST-based Lowest Common Ancestor pipeline against NCBI's core nucleotide (core_nt) database (October 2024 release). Where specified, an LCA threshold of 0.1% was used, this means that any taxa representing fewer than 0.1% of the classified reads will be moved up a taxonomic level (e.g. from species to genus) until the threshold is reached. AMR gene detection was performed using the CARD database (24) v4.0.0 (nucleotide_fasta_protein_homolog_model.fasta). Read-based AMR gene detection was performed separately within the MARTi pipeline, which uses the CARD database as part of its internal workflow. CARD was applied exclusively for MARTi analyses, while AMRFinderPlus was used for all assembly-based resistome analyses, as described below. Resulting taxonomic and AMR classifications were explored and visualised using the MARTi graphical user interface.

Sequencing read quality control and metagenomic assembly

Long read metagenome raw reads (FAST5) were converted into POD5 files using POD5 v0.3.15 (25). The converted files for each sequencing run were basecalled and demultiplexed using Dorado v0.8.3 as described above. Next, raw FASTQ reads produced by Dorado were trimmed and quality-filtered (q15) using fastplong (v0.2.2) (26). Subsequently, host-associated contamination (human reads) was removed using Hostile v2.0.0 (27) under default parameters.

Cleaned metagenome reads were assembled using metaFlye (v2.9.5) (28). The assembled contigs then underwent three rounds of polishing using Medaka v2.0.0 (29) with the model r941_e81_sup_g514.

Metagenome-assembled genome binning

The MetaWRAP pipeline (v1.3) (30) generated metagenome bins using the metagenome assemblies and filtered clean reads described above. Bins were generated using three binning software tools within the binning module: CONCOCT (v1.1.0) (31), MetaBAT (v2.12.1) (32,33), and MaxBin (v2.2.6) (34). The bins were refined to generate a final set of bins using the bin refinement module within MetaWRAP, based on quality assessment using CheckM (v1.1.3) (35) with parameters

of >70% completeness and <10% contamination. Metagenome bins were characterised using GTDB-Tk (v2.4.0) via the classify_wf module (36).

In total, 389 bins were produced across the metagenomes, including 27 bins identified as *E. faecium*. Of the 389 bins, two bins were characterised as archaea, specifically *Methanobrevibacter smithii*. All MAGs were dereplicated using dRep (v3.4.2) (37) with an ANI 99.9% as the strain-level inference cut-off, producing 382 dereplicated MAGs.

A neighbour-joining tree was produced for the dereplicated genomes using Mashtree (v1.4.6) (38) with 1000 bootstrap replicates and --min_depth 0. The tree was then mid-point rooted and visualised in iTOL (v7.2) (39).

Taxonomic profiling

Following the quality filtering steps, taxonomic profiling of the filtered metagenomic reads (in FASTQ format) was performed using Sourmash (version 4.8.12) (40). Analyses were conducted with a k-mer size of 51, utilising the GTDB-rs214 database. The workflow incorporated the sketch, gather, and tax annotate commands for taxonomic classification, enabling the estimation of taxa across the metagenomes. Using R (V4.4.2) (41), within RStudio (version 2025.05.1) (42), the taxonomy-annotated output was converted into a Phyloseq object using Sourmashconsumr (43) (v0.1.0). Phyloseq (44) (v1.50.0) was utilised to calculate diversity metrics, including the relative abundance of taxa and alpha diversity. This was visualised using ggplot2 (45).

AMR profiling of the metagenome

Antimicrobial resistance gene profiles were generated for the assembled metagenomes using AMR Finder-Plus (v4.0.19) with the database version 2024-12-18.1 to identify antimicrobial resistance genes, employing the parameters --ident_min 0.95 and --coverage_min 0.9 (46). ARG analyses were conducted on polished metagenome assemblies, plasmid-associated contigs, and MAGs to enable resistance profiling at both community and individual genome levels. MAGs were reconstructed via MetaWRAP (30). ARG detection was applied consistently across all assembled contig and genome datasets using AMRFinderPlus.

Statistical analyses were conducted in R (version 4.4.2) (41) using RStudio (version 2025.05.1) (42). Faecal AMR gene data were analysed as presence/absence

matrices. Community-level differences in faecal AMR gene profiles were explored using non-metric multidimensional scaling (NMDS) based on Jaccard dissimilarities, implemented in the R package *vegan* (47) v2.6-10 using `metaMDS()` with `distance = "jaccard"` and `k = 3`. NMDS site scores were extracted using `scores()`, ordination fit was assessed by inspecting the NMDS stress value and the Shepard plot generated using `stressplot()`. For visualisation, group centroids were calculated from the NMDS1 and NMDS2 coordinates and displayed on the two-dimensional NMDS plot. Pairwise Euclidean distances between group centroids were calculated separately using the three-dimensional NMDS coordinates, NMDS1, NMDS2, and NMDS3. Jaccard dissimilarity matrices for downstream analyses were calculated using `vegdist()` with `method = "jaccard"` and `binary = TRUE`. Homogeneity of group dispersions was evaluated using `betadisper()`, with significance assessed using `permutest()` with 999 permutations. Because group dispersions differed significantly, group differences were tested using analysis of similarity (ANOSIM), implemented with `anosim()` using 999 permutations. Pairwise ANOSIM comparisons were performed by applying `anosim()` to each pairwise subset of groups, with false discovery rate (FDR) correction for multiple testing.

Differences in the presence/absence of individual AMR genes across the four groups were assessed with Fisher's exact test, or Chi-squared tests where all cell counts exceeded five. Genes with 10 or fewer total occurrences across all samples were excluded.

Differential relative abundance of bacterial species between groups was tested using the Kruskal–Wallis rank-sum test, appropriate due to the absence of some abundant species from certain participant groups. Relative abundances were compared across groups for each species, with significant results ($P < 0.05$) followed by post-hoc pairwise Dunn's tests with FDR correction. Kruskal–Wallis P values across all taxa were also corrected using FDR.

For all analyses, statistical significance was defined as $P < 0.05$ after adjustment for multiple testing where applicable.

Plasmid contig identification and AMR profiling

Metagenomic contigs were characterised as plasmid, virus or chromosome using GeNomad (v1.11.0) (48) using the end-to-end classification module. Plasmid assigned

contigs were profiled using AMR Finder-Plus (v4.0.19) (46) as described above, to predict AMR genes present on plasmid contigs. In addition, plasmid identification was predicted using ABRicate (v1.0.1) (49) via PLSDB (v2024_05_31_v2) (50) with options --minid=90 and --mincov=90.

***Enterococcus faecium* profiling**

Those classified as *E. faecium* were oriented using Circulator (v1.5.5) to orient genomes to begin at *dnaA* (51). The average nucleotide identity was calculated between the *E. faecium* MAGs using FastANI (v1.34) using default parameters (52). Additionally, antimicrobial resistance gene profiles were generated for the *E. faecium* MAGs using AMR Finder-Plus (v4.0.19) as described above.

For comparative purposes, 22 *E. faecium* genome sequences were retrieved from NCBI from Lebreton et al (53), as representatives of clade A1 (n=11) and clade A2 (n=11). Based on the associated metadata, genomes were selected that originated from human isolates and were distributed across clades A1 and A2. Given that clade A2 includes both animal- and human-associated isolates, only human-derived genomes were included in this analysis. These reference genomes were used to contextualise cirrhosis-derived *E. faecium* MAGs within established human-associated clade A lineages. MLST was called using PubMLST, under default parameters against the *E. faecium* typing scheme, providing allelic profiles, sequence type and estimations of clonal complexes for the *E. faecium* MAGS and reference genomes (54).

Phylogenetic core gene SNP tree generation

The reference genomes along with the MAGs were then annotated using Bakta (v1.11.0) under default parameters using the full version 6 database, which consists of 10 publicly available reference databases (55,56). A core gene alignment was generated using Panaroo (v1.5.2) (57), which merged paralogs, while SNP variants were extracted with snp-sites (v2.5.1) (58). A phylogenetic tree was generated from the core SNP alignment using IQ-TREE (59). ModelFinder (60) within IQTREE was employed to find the best-fitting model for the species, selecting the GTR+F+I+R6 model to construct the phylogenetic tree using 1000 ultrafast bootstrap replicates (61). ClonalFrameML (v1.13) was employed to account for recombination when inferring the phylogenetic relationships among the genomes using a guide tree generated from

the core SNP alignment and the full core gene alignment generated by Panaroo as the input sequence alignment (62). A SNP distance matrix was generated from the ClonalFrameML alignment file using snp-dists (v0.8.2) (63). iTOL (v7.2) was used for tree visualisation and annotation (39).

Results

Baseline demographics and clinical characteristics

This cross-sectional study included 76 participants categorised into four cohorts: HC, SC, DC and ACLF. Of these, 32 participants identified as female and 44 as male. The HC group comprised 12 hospital staff members and served as a reference for comparative analyses and establishing baseline values. Among the 64 participants diagnosed with ACLD, the distribution by stage was as follows: SC (n = 20), DC (n = 16), and ACLF (n = 28). The mean age for the HC cohort was 36 years, compared to 53 years for patients with cirrhosis overall (SC = 56 years, DC = 52 years, ACLF = 49 years). Antimicrobial exposure was frequent across the ACLD cohort, with 62.5% receiving treatment as part of standard clinical infection management regimens. ACLF patients, as expected, had the highest exposure, with 27 of 28 patients (96.4%) receiving antimicrobials, often as combination therapy. Fourteen ACLF patients were treated with two or more agents, including antibacterial and antifungal combinations. Additional cohort characteristics, disease aetiologies, and antimicrobial treatment regimens are provided in Additional file 1: Table S1.

Progression of ACLD is associated with reduced taxonomic diversity

Alpha diversity showed a progressive decline across ACLD severity groups. The Chao1 index, which estimates species richness, was highest in the HC cohort and decreased sequentially in the SC, DC, and ACLF groups (Figure 1A). Similarly, the Shannon index, which accounts for both richness and evenness, revealed a stepwise reduction in microbial diversity, with the lowest values observed in ACLF patients (Figure 1B). The Inverse Simpson diversity index indicated a significant difference in microbial diversity between HC and each disease state. A Kruskal-Wallis test confirmed significant differences in microbial diversity across groups ($\chi^2 = 48.13$, df = 3, $p < 0.001$). Pairwise Wilcoxon tests with the Benjamini-Hochberg correction showed

all group comparisons were significant ($p < 0.001$), suggesting a progressive decline in diversity from HC to ACLF (Figure 1C). Non-metric multidimensional scaling (NMDS) showed that SC clustered closely with HC, while DC had an intermediate position between SC and ACLF, exhibiting partial overlap with ACLF. PERMANOVA analysis based on Bray-Curtis dissimilarity revealed significant differences in microbial community composition among groups ($R^2 = 0.08$, $p = 0.001$) (Figure 1D).

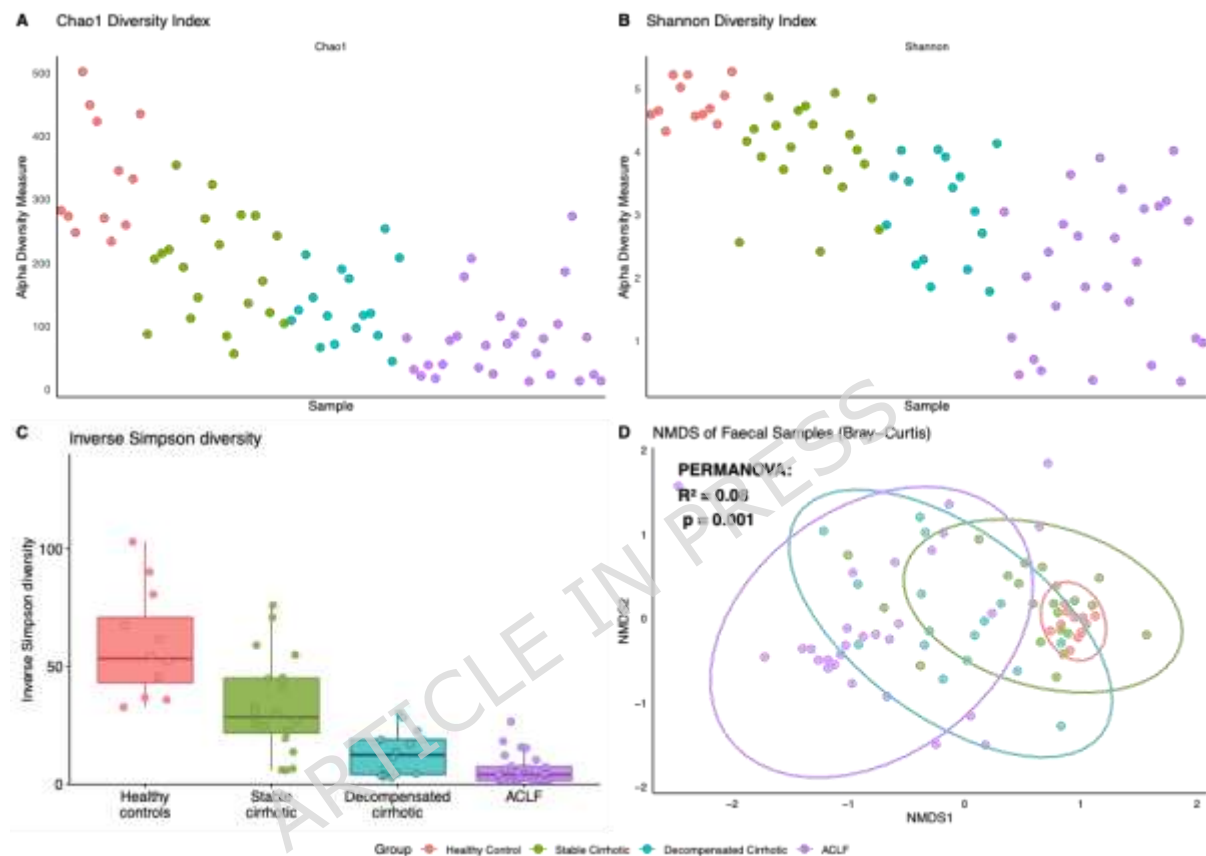


Figure 1- Comparison of microbial diversity profiles of faecal samples from healthy controls and cirrhosis patients.

A) Chao1 diversity index representing species richness. **B)** Shannon diversity index accounting for species richness and evenness. **C)** Inverse Simpson's Index measuring microbial diversity across cohorts, post hoc pairwise Wilcoxon tests (Benjamini-Hochberg adjusted) revealed significant differences between the HC cohort and each disease state. **D)** NMDS plot based on Bray-Curtis dissimilarity with PERMANOVA significance ($R^2 = 0.08$, $p = 0.001$) overlaid and 95% confidence ellipse shown (HC, $n = 112$; SC, $n = 20$; DC, $n = 16$ ACLF, $n = 28$).

The gut microbiome differed significantly between HC and ACLD cohorts when comparing bacterial diversity using taxonomic profiling (Figure 2A). Stacked bar plots revealed a progressive decline in the relative abundance of beneficial bacterial genera, including *Anaerostipes*, *Agathobacter*, *Bifidobacterium*, *Blautia*, *Faecalibacterium*,

Fusicatenibacter, and *Ruminococcus*. In parallel, an increase in pathogenic and opportunistic taxa was observed, including *Enterococcus*, *Escherichia*, and *Klebsiella*. These changes were more pronounced in ACLF, where *Enterococcus* dominated microbial communities. Detailed species-level analysis (Figure 2B) showed that HC samples species were significantly more diverse than those of cirrhosis patients, characterised by several keystone taxa such as *Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, *Agathobacter rectalis*, *Bifidobacterium longum*, *Blautia_A wexlerae* and *Ruminococcus_E bromii_B*. Post hoc pairwise comparisons using Dunn's test revealed significant differences between the HC and ACLD cohorts for *A. rectalis*, *B. wexlerae*, and *F. saccharivorans*, with their abundances progressively declining in samples as cirrhosis severity increased (Figures 2C, 2D, and 2E). Conversely, pathogenic bacteria dominated the gastrointestinal microbiome in the later stages of liver disease, replacing beneficial taxa, most notably *Enterococcus faecium*, *Escherichia coli*, and *Klebsiella pneumoniae*. The *Enterococcus* genus was absent in HC but showed a stepwise increase in both relative abundance and prevalence with advancing disease severity, particularly *E. faecium*. In the ACLF cohort, *E. faecium* was present in the majority of samples, with 10 individuals exhibiting a relative abundance of more than 50%. This marked overrepresentation underscores the shift towards a pathogen-enriched community and the potential clinical relevance of *E. faecium* overgrowth in ACLD progression. Differential abundance testing using the Kruskal-Wallis test revealed significant differences in *E. faecium* abundance among study groups (FDR-adjusted p-value < 0.001). Post hoc Dunn's tests showed *Enterococcus* was significantly enriched in ACLF compared to HC, SC, and DC; however, no significant difference was found between HC and SC (FDR-adjusted p-value = 0.267) (Figure 2F).

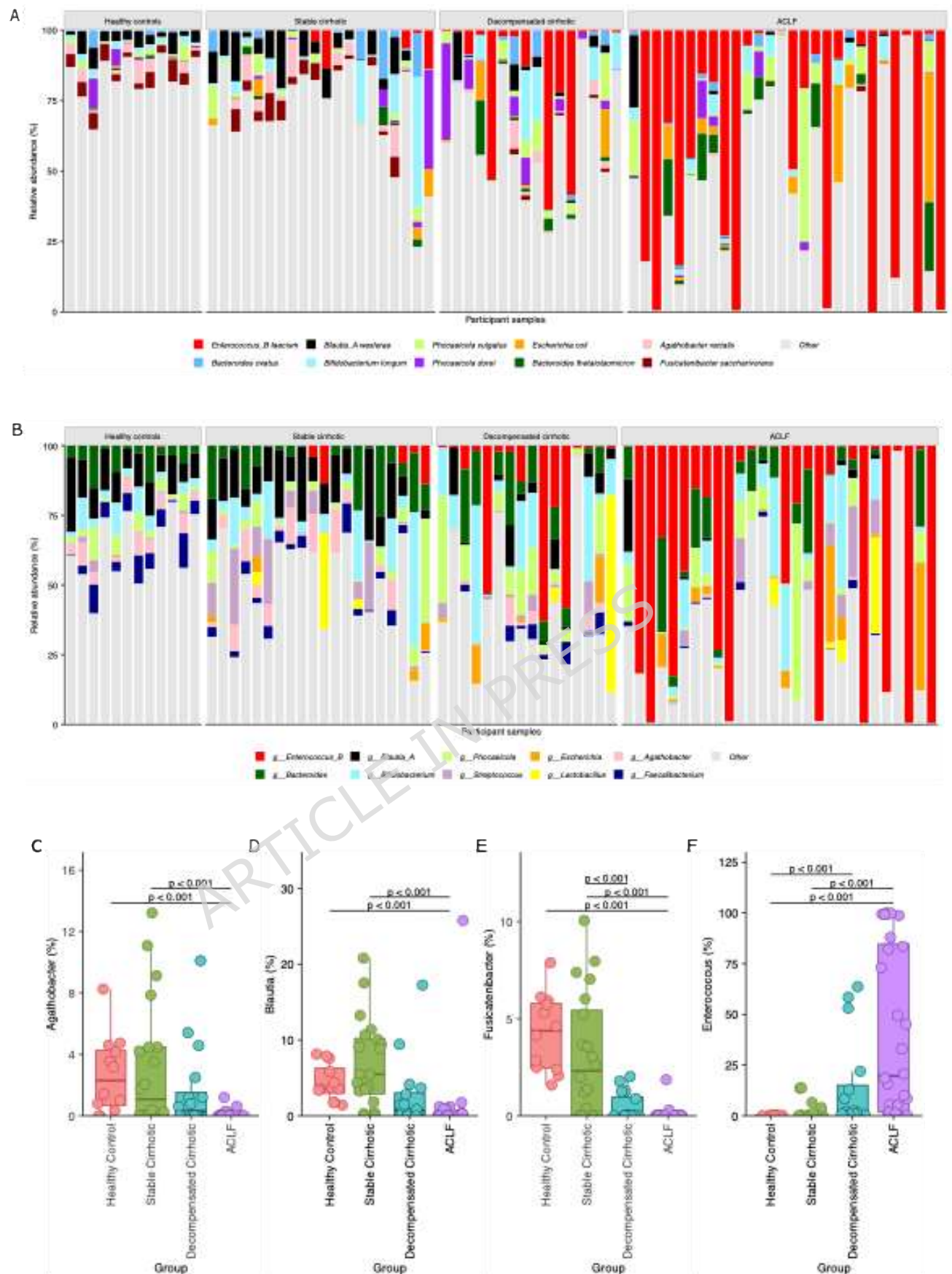


Figure 2- Microbial composition in cirrhosis patients compared to healthy controls.

A) Stacked bar plot showing the relative abundance of the top ten most abundant bacterial genera across the study cohort, ordered by disease severity (HC, n = 112; SC, n = 20; DC, n = 16 ACLF, n = 28). All remaining genera outside the top ten are grouped and displayed as “other.” **B)** Stacked bar plot showing the relative abundance of the top ten most abundant bacterial species across the study cohort, ordered by disease severity (HC, n = 112; SC, n = 20; DC, n = 16 ACLF, n = 28). All remaining species outside the top ten are grouped and displayed as “other.” **(C-F)** Box plots showing relative abundance of species significantly enriched between cohorts: **(C)** *Agathobacter rectalis*, **(D)** *Blautia_A wexlerae*, **(E)** *Fusicatenibacter saccharivorans*, **(F)** *Enterococcus_B faecium*. Post hoc Dunn’s tests revealed significant enrichment patterns, depicted by lines above boxplots.

The gut microbiome of ACLD patients represents an enriched reservoir of antimicrobial resistance

Antimicrobial resistance profiling was conducted on metagenomic assemblies to assess the prevalence of ARGs across study groups. Notably, ARG abundance was significantly higher in ACLF compared to other cohorts. These ARGs spanned 20 unique antimicrobial classes, including beta-lactams, aminoglycosides, glycopeptides, and tetracyclines (Figure 3A, Additional file 1: Table S2). HC and SC harboured a limited number of genes associated with tetracycline, sulphonamide, and macrolide-lincosamide-streptogramin within their resistome (Figure 3A). These may reflect intrinsic resistance genes that occur naturally across several taxa and contribute to baseline resistome levels, independent of antimicrobial exposure.

However, a stepwise expansion of ARG burden was observed for multiple antimicrobial classes. For example, beta-lactam resistance genes were present in low abundance in HC and SC but were enriched in DC and ACLF. Similar trends were observed for quinolone, phenicol, and tetracycline resistance determinants, which were significantly enriched in DC and ACLF. This enrichment may be partially driven by clinical exposure to antimicrobials such as ciprofloxacin, norfloxacin and doxycycline (Additional file 1: Table S1), as well as increased healthcare contact in advanced disease stages, which may increase exposure to and acquisition of antimicrobial-resistant organisms. Community-level differences were explored using NMDS based on Jaccard dissimilarities (Figure 3B). HC formed a tight cohesive cluster, whereas SC, DC, and ACLF displayed progressively greater dispersion with increasing disease severity. Pairwise centroid distances calculated from NMDS coordinates indicated progressive divergence from HC to advanced disease states, with the largest separation observed between HC and ACLF (0.98), and smaller

distances between adjacent disease stages such as SC and DC (0.33) (Additional file 1: Table S3). Testing for unequal dispersion using `betadisper()` confirmed heterogeneity ($P = 0.003$). To account for this, non-parametric ANOSIM confirmed significant differences between patient groups and HC ($P=0.002$).

At the gene level, differential distribution was assessed using Fisher's exact test or Chi-squared test, depending on counts. Six ARGs showed statistically significant differences: *aac(6')-Ie/aph(2'')-Ia*, *tet(O)*, *tet(W)*, *msr(C)*, *aac(6')-I*, and *tet(L)* (Figure 3C). Of these genes, four genes (*tet(O)*, *tet(W)*, *msr(C)*, and *tet(L)*) were more frequently observed in HC. In contrast, aminoglycoside-modifying genes *aac(6')-Ie/aph(2'')-Ia* and *aac(6')-I*, exhibited a stepwise increase in prevalence, enriched in DC and ACLF cohorts. Similar enrichment patterns were observed for ARGs within the glycopeptide class, including *vanA* and *vanB*, as well as their variants.

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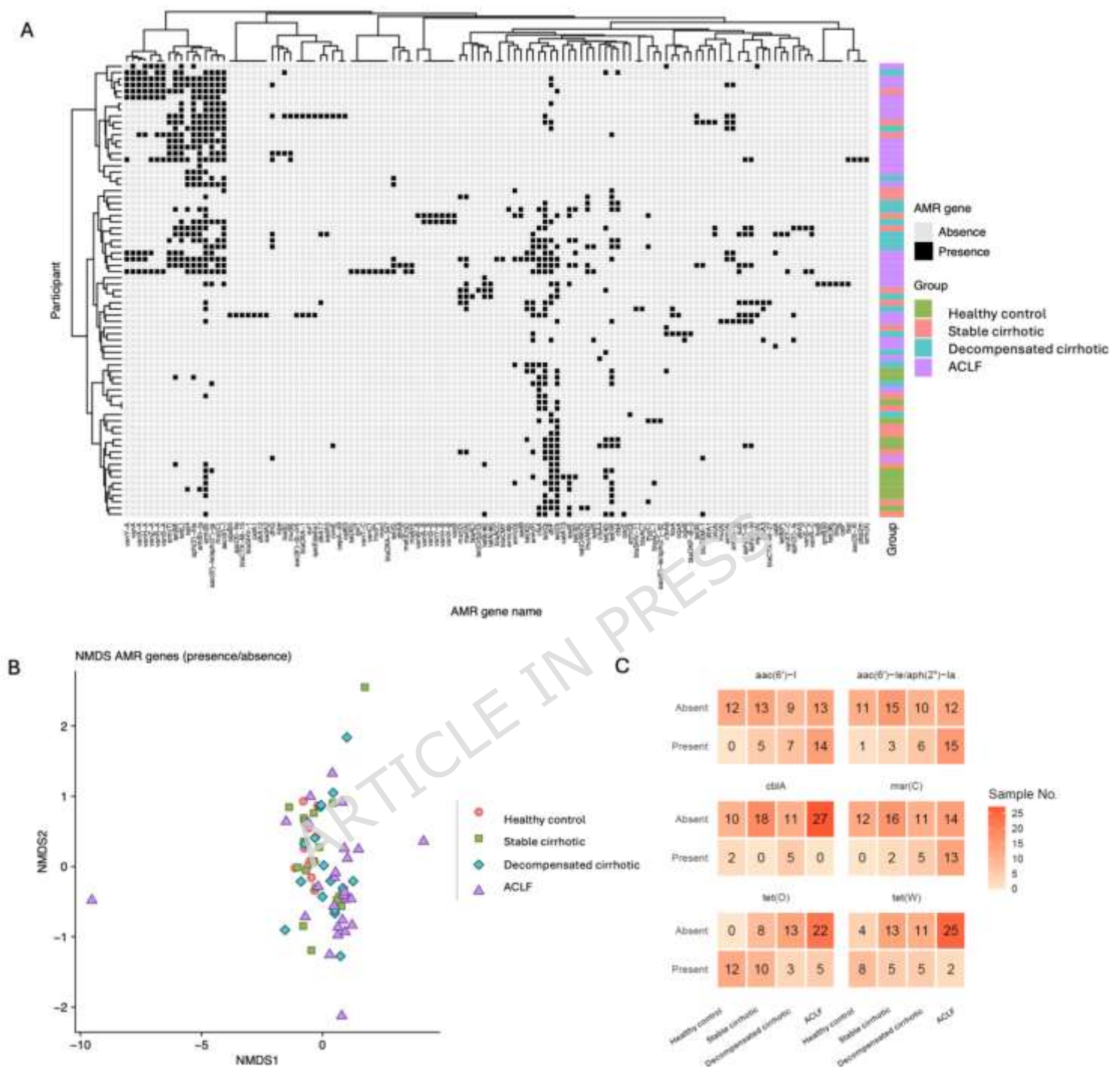


Figure 3- Distribution and clustering of antimicrobial resistance genes.

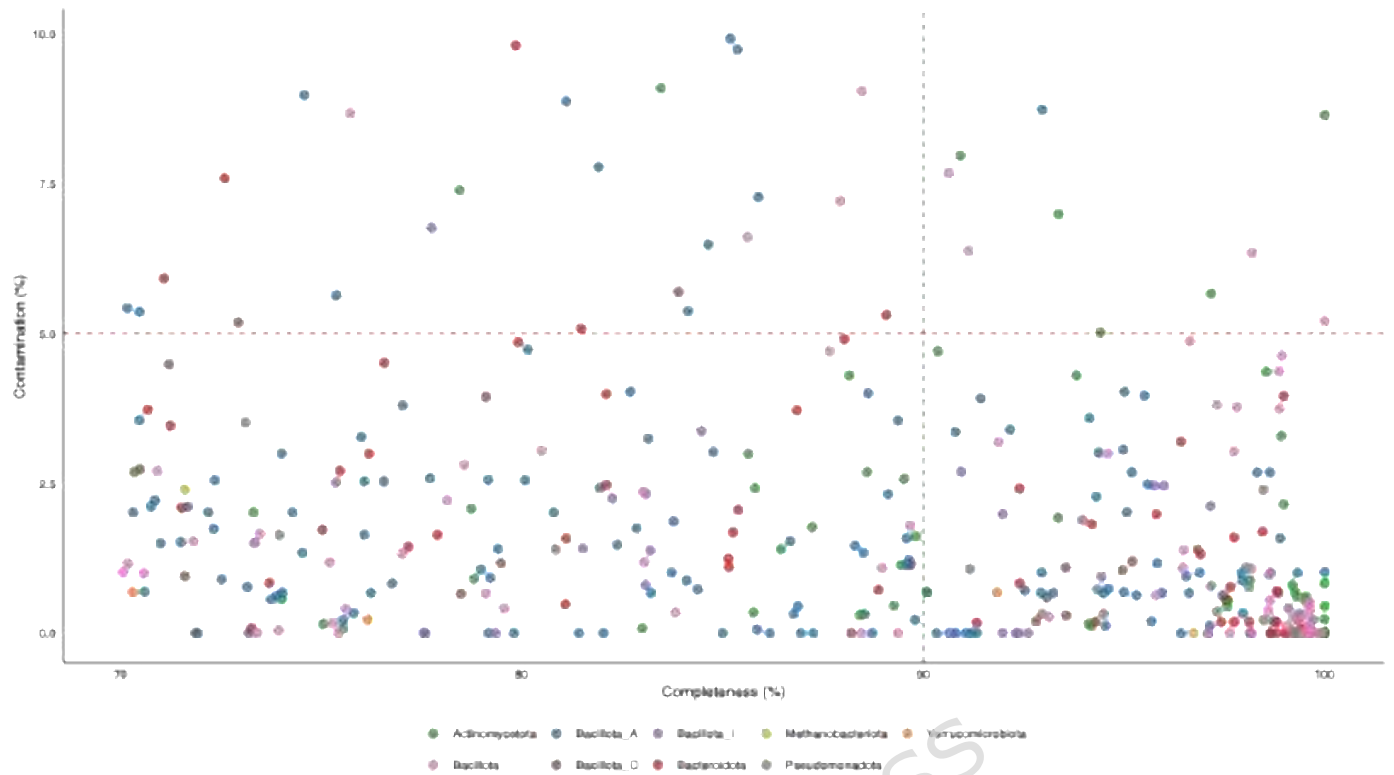
A) Clustered heatmap showing presence (black) and absence (grey) of ARGs within cohort samples. **B)** NMDS plot based on Jaccard dissimilarities of ARG presence/absence, displaying clustering patterns by disease severity. **C)** Differentially distributed ARGs across cohorts for, *aac(6')-I*, *aac(6')-Ie/aph(2'')-Ia*, *cbIA*, *msr(C)*, *tet(O)*, and *tet(W)*. Numbers represent sample counts with ARGs present within each cohort.

Recovery of metagenome assembled genomes from clinical samples highlights strain-specific resistomes

Draft genomes were generated from the metagenomes by grouping contigs based on their shared properties, resulting in MAGs. In total, 389 MAGs were generated with completeness > 70 % and contamination < 10 %. Three samples were excluded due to the absence of high-quality bins, as defined by CheckM quality metrics (completeness $\geq$ 70% and contamination $\leq$ 10%). Dereplication at 99.9% ANI yielded 382 nonredundant strain representative MAGs, excluding seven genomes identified as *E. faecium*. Completeness ranged from 70.07 % to 100 %, and contamination ranged from 0 % to 9.918 % (Figure 4A and Additional file 1: Table S4). The MAGs represented 10 phyla, of which Bacillota was the most abundant phylum (n=255), Actinomycota (n=54) and Bacteroidota (n=50). The most prevalent species within the dereplicated MAGs were *E. faecium* (n=22) followed by *B. longum* (n=17) (Figure 4B).

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A



B

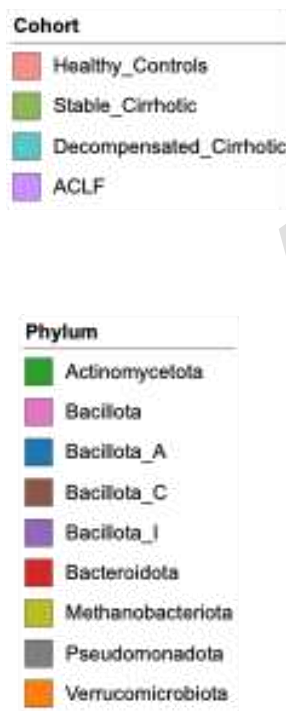


Figure 4- Phylogeny and quality assessment of metagenome-assembled genomes

A) Scatter plot of MAG quality showing completeness (x-axis) versus contamination (y-axis). High-quality MAG thresholds, as defined by MIMAG criteria (64) are indicated by dotted lines: green for completeness and red for contamination. Individual MAGs are color-coded by phylum. **B)** Strain level neighbour-joining tree of bacterial strains (n=382) colour coded by cohort and phylum, with species IDs annotated in outer rings.

Given previous reports, including our own, linking *E. faecium* to advanced cirrhosis, we examined this species in greater detail. Twenty-eight MAGs were retrieved from SC, DC, and ACLF cohorts and placed within a midpoint-rooted maximum-likelihood tree, alongside reference genomes from Lebreton et al (53) (Figure 5). Most *E. faecium* MAGs clustered tightly with A1 clade reference genomes, except for one MAG from a SC patient, which grouped within the A2 clade (Figure 5). MLST analysis revealed that A1 cluster MAGs belonged to clonal complex 17 (cc17), with predominant sequence types (STs) of ST78, ST80, ST117, and ST192, which are lineages previously associated with hospital-acquired transmission and multidrug resistance. Overall, these MAGs displayed extremely high similarity over 97 %, with several pairs sharing over 99.9 % ANI (strain-level cut-off) (Additional file 1: Table S5), suggesting potential strain sharing within patient groups, with persistence of *E. faecium* strains across individuals. Consistent with this, core-genome SNP analysis demonstrated a broad range of pairwise distances, from as few as 13 SNPs to 7245 SNPs, indicating the presence of both closely related strains and more genetically distinct lineages within the dataset (Additional file 1: Table S6).

Phylogenetic analysis of 28 *E. faecium* MAGs and 22 reference genomes revealed distinct patterns of ARG distribution. Vancomycin resistance genes (*van*) were largely absent from MAGs, including those within the A1 cluster associated with hospital-acquired infections, despite belonging to cc17 based on MLST (Figure 5). The macrolide/streptogramin resistance gene *msr(C)* was detected in nearly all genomes, being absent from only eight MAGs. This widespread detection is consistent with *msr(C)* being an intrinsic macrolide resistance determinant in *E. faecium* and part of the species' core resistome (65). Aminoglycoside resistance gene *sat(6')-I* was absent in three MAGs; however, *aac(6')-Ie/aph(2'')-Ia*, *ant(6)-Ia*, and *apt(3'')-II* were consistently present in reference genomes but largely absent in MAGs. Notably, *van* and *aac(6')-Ie/aph(2'')-Ia* were detected in the metagenomic dataset, coinciding with

an increase in *E. faecium* abundance, suggesting that resistance determinants may be associated with species expansion.

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Figure 5- Phylogenetic tree of *E. faecium* MAGs and 22 reference genomes.

A mid-rooted phylogenetic tree of 28 *E. faecium* MAGs, highlighted in pastel blue, and 22 reference genomes, highlighted in pastel yellow, was constructed and visualised using iTOL v7. Sequence types called using PubMLST are indicated alongside each genome. Sample sites are denoted by

coloured symbols. The presence or absence of ARGs is shown as a matrix alongside the tree, with red indicating presence and white indicating absence.

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Multiple ARGs are encoded on plasmid like contigs within the ACLD gut microbiome

Whilst MAGs provided species-specific insights into ARG presence, metagenomic binning does not capture plasmids, which are major contributors to ARG dissemination within bacterial hosts. To address this limitation and overcome the underrepresentation of plasmid-associated ARGs in the MAG analysis, plasmid contigs were identified within the metagenomes and profiled to examine the distribution of ARGs. Across the 76 faecal samples, 64 contained plasmid-assigned contigs that harboured ARGs (Figure 6A). Although long-read ONT sequencing was employed, the use of R9 chemistry and the lack of consistently circularised plasmids limited base-level accuracy and completeness of plasmid backbones, precluding confident replicon or mobility typing. The clustered heatmap of plasmid-associated ARGs revealed distinct resistance signatures across disease stages. HC plasmid-like contigs encoded a limited number of ARGs, such as β -lactam and tetracycline resistance determinants, without significant enrichment by disease stage. Several β -lactamase genes were detected across all groups, including *bla*TEM-1, *bla*CTX-M-27, *bla*OXA-1, and *bla*SHV-11, alongside tetracycline resistance genes *tet*(O), *tet*(W), and *tet*(Q), which are more frequently observed in HC and SC. In more advanced cirrhosis patient samples, increases in *erm* genes (conferring resistance to lincosamide/macrolide/streptogramin) and aminoglycoside resistance determinants were detected in DC and ALCF (Figure 6A and Additional file 1: Table S7).

Differential distribution analysis confirmed five ARGs with significant group-specific differences: *aac*(6')-Ie/*aph*(2'')-Ia, *ant*(6)-Ia, *aph*(3')-IIIa, *sat*4, and *tet*(O) (Figure 6B). Aminoglycoside resistance genes, including *aac*(6')-Ie/*aph*(2'')-Ia, *ant*(6)-Ia, *aph*(3')-IIIa, and *sat*4, were strongly enriched in ACLF patients, with the first three detected in 14 of 24 ACLF samples and *sat*4 present in nine, highlighting a marked expansion of aminoglycoside resistance in advanced cirrhosis (Figure 6B). In contrast, tetracycline resistance was more frequent in HC and SC (e.g., *tet*(O) in 5/15 HC and 6/18 SC) but largely absent in ACLF samples (Figure 6B).

In metagenomes where *E. faecium* was present, there was an increased presence of ARGs, particularly glycopeptides and aminoglycoside resistance determinants (including streptothricin resistance), consistent with the intrinsic resistance profile of

E. faecium (Figure 6A). Screening plasmid-derived contigs identified only one contig classified as an *E. faecium* plasmid carrying a *van* gene cluster, using $\geq 90\%$ identity and coverage thresholds

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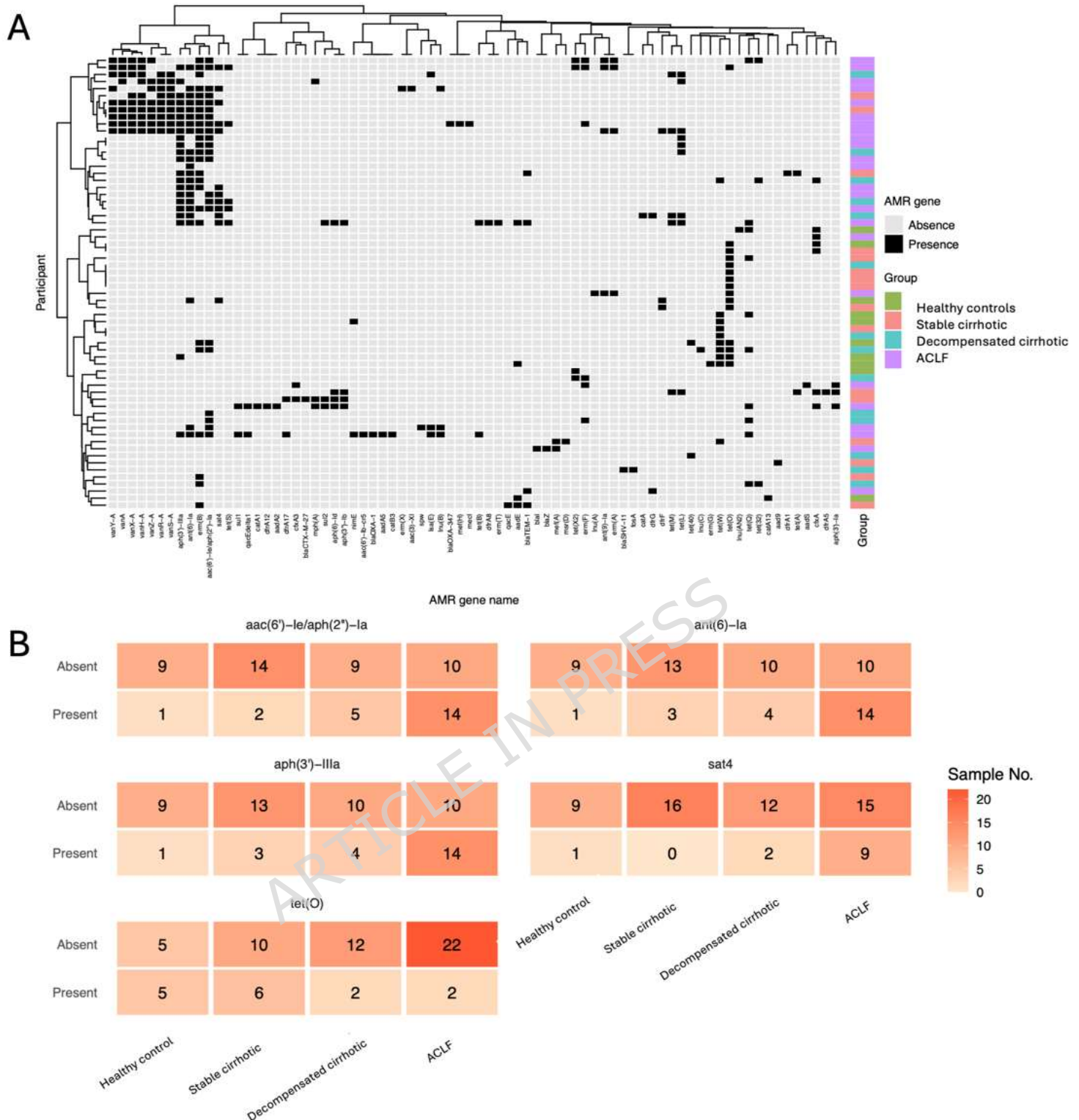


Figure 6- Clustered presence/absence heatmap of putative plasmid-associated ARGs grouped by drug class.

A) Heatmap showing ARG distribution on plasmid-derived contigs identified by GeNomad across samples. ARGs grouped by antimicrobial class; black = presence, grey = absence. Group annotations denote participant categories: HC, SC, DC, ACLF. **B)** Statistically significant, differentially distributed ARGs across cohorts for *aac(6')-Ie/aph(2'')-Ia*, *ant(6)-Ia*, *aph(3')-IIIa*, *tet(O)*, and *sat4*. Numbers represent sample counts with ARGs present on plasmid contigs.

Using MARTi (Metagenomic Analysis in Real Time) open-source software to profile metagenomes

To explore the feasibility of rapid, read-based metagenomic profiling, we supplemented the results gathered using traditional bioinformatic methodologies with *MARTi*. We applied *MARTi* to a subset of 35 samples to evaluate its potential for rapid, open-source metagenomics profiling (23). *MARTi* employs a read-based approach to classify sequencing data, enabling simultaneous taxonomic profiling, detection of ARGs, and attribution of ARGs to specific taxa. This read-level resolution is particularly advantageous for complex clinical samples, where accurate characterisation of both microbial diversity and resistance determinants is essential. Taxonomic profiling of the top 25 genera highlighted a progressive decline in diversity from SC, through to ACLF (Figure 7). ACLF samples were dominated by opportunistic taxa, including *Escherichia* and *Enterococcus*, accompanied by marked depletion of keystone beneficial genera such as *Faecalibacterium*, *Blautia*, and *Anerostipes*. Alpha diversity metrics calculated for the *MARTi* subset (Additional file 1: Table S8) showed lower diversity in ACLF compared with DC samples. Interpretation across the full disease spectrum is limited by the inclusion of only a single SC sample in this subset, precluding assessment of a progressive decline from SC through DC to ACLF. In contrast, analysis of the full cohort presented earlier in the manuscript demonstrates statistically significant reductions in diversity with increasing disease severity

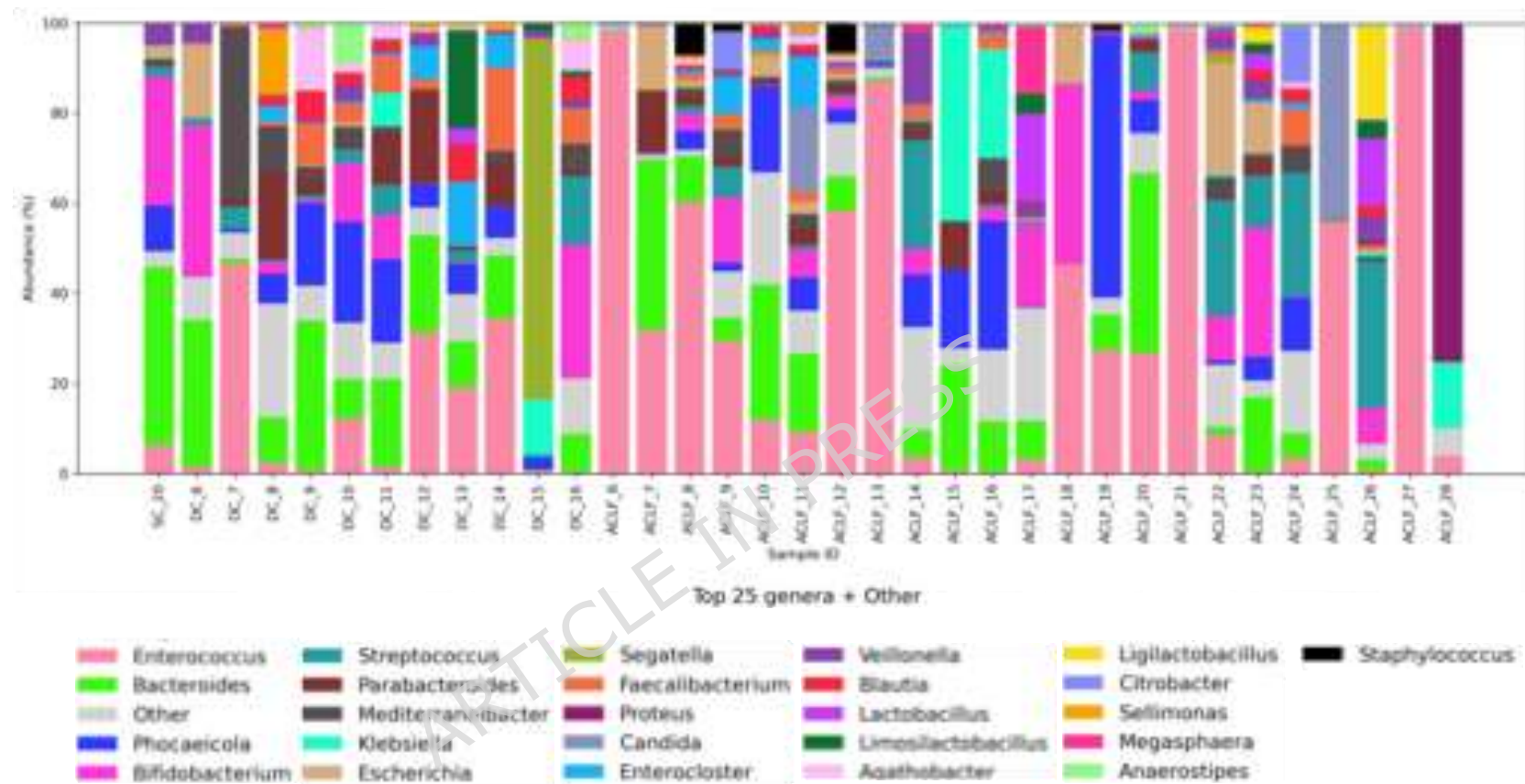


Figure 7- Comparative analysis of reads for taxonomic profiles produced by MARTi

Taxonomic relative abundance plot using LCA minimum abundance threshold of 0.1%. Top 25 taxa are shown, all others are collected under “Other”.

ARG analysis demonstrated broad resistance diversity across the subset, with aminoglycosides, glycopeptides and tetracyclines representing the top three drug classes (Figure 8A). These patterns likely reflect previous exposure to antibiotics (Additional file 1: Table S1). Four ACLF samples (ACLF6, ACLF7, ACLF13 and ACLF27) had very high ARG read counts (>5,000), highlighting a substantial resistance burden. Species-level attribution identified *E. faecium* and *E. coli* as the predominant ARG carriers (Figure 8B). Notably, *E. faecium* showed particularly high ARG loads in ACLF 6, ACLF 7, ACLF 13, ACLD 21 and ACLF 27, each exceeding 1,000 ARG reads. This may reflect selective pressures from antimicrobial exposure, alongside increased acquisition of multidrug-resistant strains associated with frequent hospitalisation, invasive procedures and prolonged healthcare contact in advanced cirrhosis.

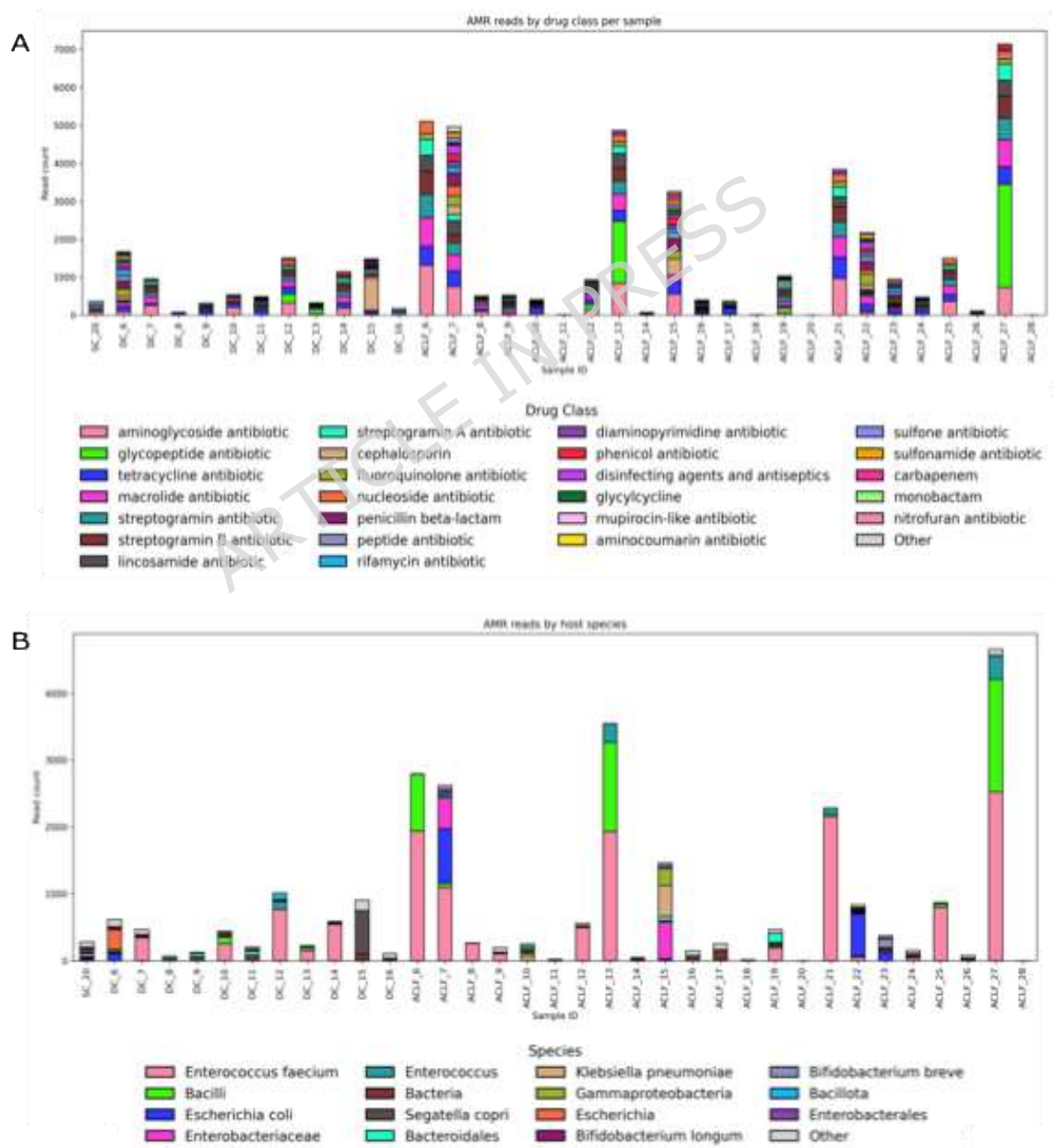


Figure 8- Comparative analysis of reads for AMR profiles produced by MARTi

A) AMR read count bar plot for top 25 drug classes. Read counts mapping to AMR genes were aggregated and grouped by their associated resistance drug class. Because a single AMR gene may have multiple associated mechanisms or classes, reads can be counted in more than one category. **B)** Potential host taxa of AMR genes for top 15 bacterial taxa. Host taxa of AMR genes identified using walkout analysis within MARTi using a minimum threshold of 10%, reporting a potential host of an AMR gene if at least 10% of the AMR-matching reads were assigned to that taxon.

Discussion

This study used ONT long-read metagenomic sequencing to characterise the gut microbiome and resistome in patients with ACLD, providing insights into microbial community structure and AMR spanning different ACLD severities. The untargeted nature of metagenomic sequencing enabled strain-level identification without prior assumptions, overcoming the limitations of culture-based diagnostics (66). Historically, such approaches required advanced bioinformatic expertise; however, the emergence of plug-and-play bioinformatic workflows has significantly improved the accessibility and usability of metagenomic sequencing, with data analysis now integrated into intuitive online platforms that generate clear and concise outputs (67,68).

Metagenomic profiling of the ACLD patient faecal samples revealed striking and progressive alterations in microbial community structure across disease severities. Diversity indices (Chao1, Shannon, and Inverse Simpson) revealed a stepwise decline from HC to ACLF, supported by PERMANOVA and NMDS clustering. This progressive loss of richness and evenness coincided with a compositional shift from beneficial taxa, such as butyrate-producing taxa like *Faecalibacterium*, *Blautia*, and *Agathobacter*, towards opportunistic pathogens, most notably *Enterococcus* and *Escherichia*. These changes were most pronounced in ACLF, where *E. faecium* frequently dominated (>50% relative abundance in multiple samples), especially among patients with more advanced cirrhosis and a history of multiple antibiotic treatments. This expansion reflects disruption of the gastrointestinal ecosystem, consistent with previous reports. It is likely linked to reduced colonisation resistance and compromised mucosal integrity, factors that facilitate pathogen overgrowth and translocation (7,69,70). In contrast, *E. faecium* was not detected in HC samples. While this cross-sectional dataset cannot determine the source of colonisation, the absence of *E. faecium* in healthy participants who served as negative controls in this study

suggests that healthcare staff are unlikely to act as reservoirs within this cohort, or indeed are themselves colonised with nosocomial strains. However, hospital-associated acquisition in and between patients remains possible from other hospital reservoirs, such as hospital sinks, medical equipment, bedding, and the air (71,72).

The depletion of beneficial, particularly butyrate-producing, taxa is both ecologically and clinically significant. Microbes, such as *Faecalibacterium*, *Roseburia*, *Ruminococcus*, and *Agathobacter*, play key roles in gut homeostasis by fermenting nondigestible carbohydrates into short-chain fatty acids, such as butyrate (73). Butyrate supports epithelial barrier function, modulates immune responses, and exerts anti-inflammatory effects, and its loss may therefore exacerbate (74) gut permeability and inflammation, creating conditions that favour opportunistic pathogens such as *Enterococcus*, and facilitating bacterial translocation into the bloodstream (75). Loss of these beneficial taxa is a common feature of ACLD, often linked to altered bile acid metabolism, repeated exposure to broad spectrum antibiotics, and dietary changes (69). A caveat to this observation was of *Bifidobacterium* in DC and ACLF cohorts. This genus is usually susceptible to broad spectrum antibiotics, displaying high antimicrobial sensitivity (76); however, *Bifidobacterium* persisted, albeit at lower abundances than in HC, in advanced disease stages, potentially reflecting the influence of lactulose therapy, which has prebiotic effects and promotes its growth, which is an observation supported by cohort metadata and previous reports.

The overgrowth of *E. faecium* was significant in both DC and ACLF patients, consistent with previous findings (69,70). Genomic analysis revealed high strain diversity (97 – 99.9 % ANI) with 27 of 28 genomes clustering within the A1 clade, a lineage commonly associated with hospital-acquired infections. This clade links to nosocomial persistence, biofilm formation, and accumulation of multiple ARGs (77), reflecting strong selective pressures within the cirrhotic gut, driven by frequent antibiotic exposure, compromised mucosal epithelial barriers, and immune dysfunction, which collectively favour the expansion of multidrug-resistant, *E. faecium* lineages, and possible strain sharing between patients. In a prospective cohort of ACLD patients, circulating *E. faecium* DNA was associated with heightened systemic inflammation and renal dysfunction, proposing *E. faecium* translocation as a key consequence of gut barrier failure in ACLF and providing further clinical context for microbiome signatures centred on this pathobiont (70). However, it is also possible that the strains represent

endogenous populations of *E. faecium*, which exploit the selective conditions presented as they evolve and expand within the host (78). The genomic diversity observed across MAGs indicates potential for independent within-host emergence of these particular strains under similar selective pressures, rather than from a single nosocomial source.

In keeping with the multidrug-resistant characteristic of A1 and cc17 lineages, vancomycin resistance genes (*vanA*, *vanB*) were enriched in the metagenomes from DC and ACLF patients. The *van* gene complexes, typically carried on mobile genetic elements (MGEs), mediate high-level vancomycin resistance and facilitate horizontal gene transfer (HGT) across taxa, and have been attributed to the overprescription and reliance on vancomycin to treat infections (79). Their presence aligns with global concerns over the emergence of vancomycin-resistant *E. faecium*, recognised by the WHO as a critical priority pathogen (80). Further genomic analysis confirmed the predominance of cc17-associated A1 clade MAGs; however, *van* genes were largely plasmid-borne and absent from MAG assemblies, reflecting both their extrachromosomal localisation and shallow sequencing depth in this study. The ONT R9 chemistry, though valuable for long-read sequencing, has lower accuracy than newer versions. Combined with limited metagenomic depth, this likely reduced strain-level resolution and plasmid recovery, contributing to the apparent underrepresentation of some ARGs in MAG-based analyses.

Notably, vancomycin-resistant *E. faecium* is also multidrug resistant and is often co-resistant to gentamicin (81). As highlighted above, aminoglycoside resistance genes were detected within the wider resistome; however, the bifunctional aminoglycoside acetyltransferase gene *acc(6')-Ie-aph(2'')-Ia* could not be directly linked to *E. faecium*. This gene was frequently identified on plasmid-like contigs and significantly enriched in ACLF faeces. The current dataset lacked sufficient resolution to confidently associate plasmids with their bacterial hosts, constraining the ability to resolve MGE dynamics within the microbiome. Although long-read ONT sequencing was employed, the use of R9 chemistry and the absence of consistently circularised plasmids limited accurate reconstruction of complete plasmid backbones. Future studies using higher-accuracy long-read or hybrid assembly approaches will be required to enable robust plasmid characterisation, including replicon and mobility typing. Whilst specific bacterial hosts could not be conclusively linked to these ARGs, the increased

prevalence correlates with the abundance of *E. faecium*, supporting its role as a likely reservoir of glycopeptide resistance. The co-localisation of multiple ARGs on MGEs is particularly concerning, as it underscores the potential for co-transfer of multidrug resistance, increasing dissemination within the gut microbiome, and limiting effective treatment options for severely ill patients, such as those with ACLF.

Taking a broader look at the total microbiome resistome, AMR analysis revealed a clear correlation between ACLD severity and the escalation of resistance burden and has been recently reported to affect not only the gut microbiome but also the oral cavity (8). ARGs spanned multiple classes, including β -lactams, aminoglycosides, glycopeptides, and tetracyclines. While HC and SC harboured limited ARGs, ACLF samples exhibited extensive resistomes, with aminoglycoside-modifying genes (*aac(6')-Ie/aph(2'')-Ia*) strongly enriched. Plasmid-associated ARGs were common in advanced cirrhosis samples, highlighting the potential for HGT and rapid dissemination of multidrug resistance within the gut ecosystem. However, this resistome analysis was limited to the detection of acquired ARGs and did not account for chromosomal mutations that can also confer clinically relevant resistance; therefore, the total resistance potential of key opportunistic pathogens may be incompletely represented. These resistome patterns, combined with impaired colonisation resistance due to depletion of beneficial taxa, align with clinical metadata showing frequent broad-spectrum antibiotic exposure in DC and ACLF patients. In DC and ACLF patients, broad-spectrum antibiotics such as piperacillin/tazobactam, meropenem and rifaximin were frequently administered. These antibiotics are often prescribed, prior to infection confirmation, because culture-based diagnostics can take up to a week to isolate and identify causative pathogens (82). Such selective pressures likely facilitated the expansion of resistant opportunistic pathogens, including *E. coli*, *K. pneumoniae* and *E. faecium*, while depleting commensal populations that normally confer colonisation resistance.

In addition to traditional methodologies used to survey the microbiomes and resistomes of patients with ACLD, we utilised MARTi, a real-time, open-source metagenomic analysis platform (23), which provides a comprehensive read-based taxonomic profiling and host attributions of ARGs. The platform's user-friendly interface greatly enhances accessibility, which is crucial for wider adoption in clinical settings, where bioinformatic training is often limited by complexity and expense (83).

It's ability to analyse reads progressively, updating results as sequencing continues, further enhances its potential as a diagnostic tool. While MARTi relies on reference databases and may therefore miss rare or complex taxa and ARGs, and has limited capacity for functional profiling beyond ARG detection, these gaps can be addressed by supplementing the analysis with conventional metagenomic approaches. Nevertheless, MARTi's accessible interface makes it well-suited for clinical applications, providing rapid, actionable insights where computationally intensive traditional pipelines may be impractical. These findings demonstrate the translational potential of metagenomic sequencing coupled with easy-to-use analysis platforms in clinical microbiology, enabling rapid pathogen detection and AMR surveillance, particularly for severely ill patient groups, such as those with ACLF.

While these findings highlight distinct microbial and resistome alterations in ACLD, the cross-sectional study design precluded the assessment of temporal dynamics, preventing evaluation of how microbial and resistome profiles evolve during disease progression and treatment. Future work should address these limitations by employing higher accuracy sequencing chemistries, deeper metagenomic coverage, and longitudinal sampling. These refinements would enhance MGE and plasmid recovery, enable robust plasmid-host interactions, and provide clear insights into the evolution of ARGs over time as ACLD progresses. Incorporating these capabilities into workflows like MARTi would also further enhance their translational and clinical applicability and diagnostic value. Together, such improvements would help define strain-specific microbial and resistome signatures of ACLD, informing therapeutic interventions and infection control, including surveillance strategies in this high-risk population.

Conclusions

Long-read metagenomic sequencing demonstrates substantial potential for characterising both the microbiome and resistome in patients with ACLD who are at very high risk of infections that require hospitalisation. The culture-independent approach provides a comprehensive view of the gut ecosystem, simultaneously

identifying pathogenic taxa, community composition, and circulating ARGs. The ability to detect clinically relevant species such as *E. faecium* and their associated ARGs directly from metagenomic data highlights its value as a diagnostic and surveillance tool. The findings also highlight how chronic antibiotic exposure and microbiome disruption in ACLD create selective pressures that promote the expansion of multidrug-resistant pathogens such as *E. faecium*. Integrating metagenomic approaches, particularly through user-friendly, real-time analysis platforms like MARTi, into clinical workflows could support rapid pathogen detection, guide antimicrobial stewardship, and improve therapeutic decision-making, ultimately enhancing outcomes for ACLD patients at high risk of infection.

Abbreviations

ACLD- Advanced chronic liver disease

ACLF- Acute-on-chronic liver failure

ALD- alcohol-related liver disease

AMR- Antimicrobial resistance

ANOSIM- Analysis of similarity

ARG- Antimicrobial resistance gene

CLIF-C AD- Chronic Liver Failure Consortium-acute decompensation

DC- Decompensated cirrhosis

FDR- False discovery rate

HC- Healthy control

HGT- Horizontal gene transfer

MAG- Metagenome assembled genome

MARTi- Metagenomic Analysis in Real Time

MASLD- metabolic dysfunction–associated steatotic liver disease

MELD- Model for end-stage liver disease

MGE- Mobile genetic element

NMDS- Non-metric multidimensional scaling

ONT- Oxford Nanopore Technologies

SC- Stable cirrhosis

UKELD-United Kingdom model for end-stage liver disease

van-Vancomycin resistance genes

Declarations

Ethics approval and consent to participate

The study was granted ethics approval by the national research ethics committee (12/LO/1417) and the local research and development department (KCH12-126) and performed in accordance with the Declaration of Helsinki. Patient participants, or their family nominee as consultees in the case of lack of capacity, provided written informed consent within 48 hours of presentation. Patients were managed according to standard evidence-based protocols and guidelines.

Consent for publication

Not applicable. All presented data are de-identified and anonymized.

Competing interests

VCP declares consultancy roles with Resolution Therapeutics, Emles Bioventures, AlfaSigma S.p.A., AstraZeneca, Norgine Pharmaceuticals Ltd, and Menarini Diagnostics Ltd. The remaining authors declare that they have no competing interests.

Acknowledgements

We are grateful to all the patient participants and healthy volunteers for agreeing to take part in this study, and to the clinical and liver research teams at King's College Hospital for facilitating recruitment, collecting metadata and sample collection.

Funding

This project was funded by the Wellcome Trust Investigator Award no. 220876/Z/20/Z to LJH, and a Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme, Gut Microbes and Health BB/R012490/1, and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/ 000PR10356, and by the BBSRC Institute Strategic Programme Food Microbiome and Health BB/X011054/1 and its constituent project BBS/E/F/000PR13631 to LJH. VCP is supported by the Foundation for Liver Research (Registered charity number: 268211/1134579) and the King's College Hospital Institute of Liver Studies and Transplantation Charitable Research Fund, King's College Hospital Charity (Registered charity number: 1165593). RL, NP and DH were funded by BBSRC Strategic Programme Grant Decoding Biodiversity BBX011089/1, and its constituent work package BBS/E/ER/230002A. NP was supported by the BBSRC-funded Norwich Research Park Biosciences Doctoral Training Partnership grant BB/M011216/1.

Author contributions

Conceptualisation: LJH, VCP, RL; Funding acquisition & supervision: LJH; Methodology & sequencing: DH; Sample processing: RK, AAG. Data analysis: MJD, NP, HT; QC, data processing, metagenomic assemblies, and data visualisation: HT; Clinical metadata curation: MM & GH; Clinical oversight & cohort design: VCP. Writing - original draft: HT, LJH; Writing - review & editing: All authors contributed to reviewing and editing the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1377628 and SRA SRP653470 (84). Custom scripts used in this study for figure curation and statistical analyses are available on GitHub and permanently archived in Zenodo (85)

Supplementary Information

Additional file 1 This Excel workbook contains eight supplementary tables referenced throughout the manuscript. The tables provide cohort metadata, antimicrobial resistance (AMR) profiles, genomic similarity and distance metrics, metagenome-assembled genome (MAG) quality statistics, plasmid-associated AMR gene data, and microbiome diversity measures.

Table S1: cohort metadata- Clinical and demographic metadata for all study participants.

Table S2: metagenome AMR- Antimicrobial resistance genes identified from metagenomic sequencing data.

Table S3: centroid distance centroid distance matrix used for comparative genomic analyses.

Table S4: MAG completeness vs contamination Quality assessment metrics for metagenome-assembled genomes, including completeness and contamination estimates.

Table S5: ANI *E. faecium* matrix for MAGs Pairwise average nucleotide identity (ANI) matrix for *E. faecium* metagenome-assembled genomes.

Table S6: SNP distances Single nucleotide polymorphism (SNP) distance matrix between genomes.

Table S7: Plasmid contig AMR antimicrobial resistance genes identified on plasmid-associated contigs.

Table S8: Alpha diversity Alpha diversity metrics calculated from metagenomic sequencing data Generated using MARTi

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