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# Gut microbiome dynamics and Enterobacterales infection in liver transplant recipients: A prospective observational study

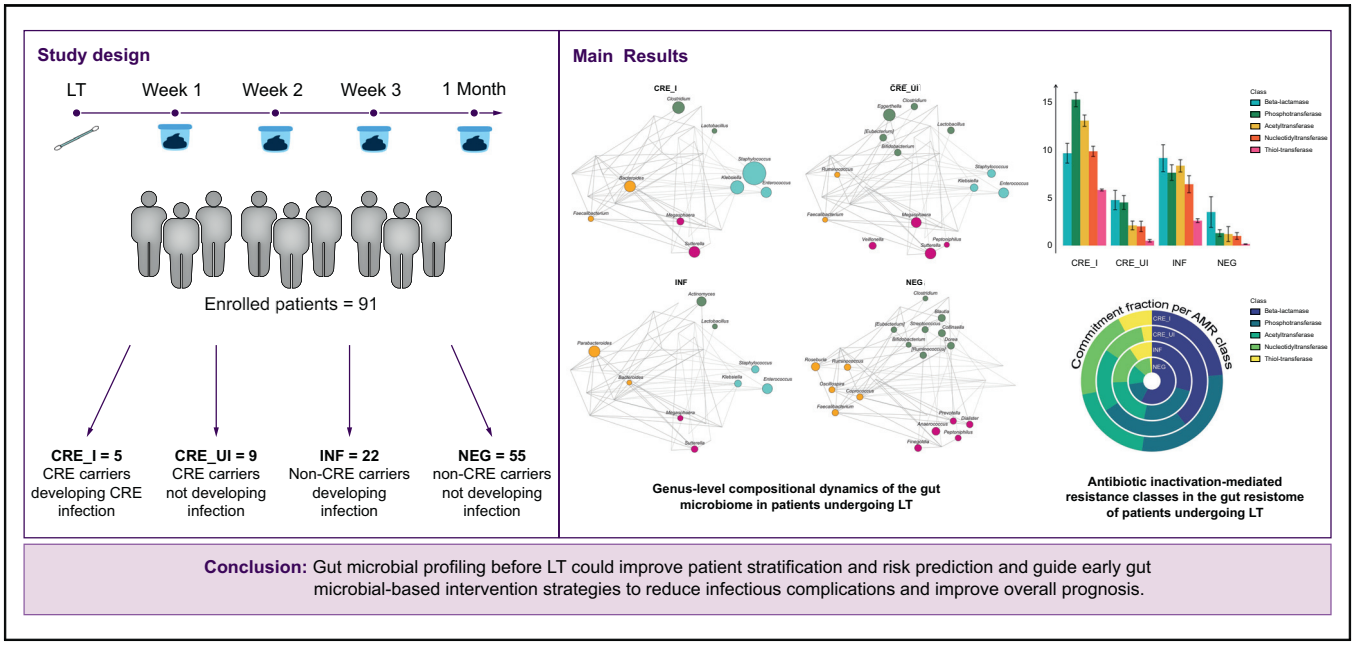
## Authors

**Federica D'Amico, Matteo Rinaldi,** Renato Pascale, Marco Fabbrini, Maria Cristina Morelli, Antonio Siniscalchi, Cristiana Laici, Simona Coladonato, Matteo Ravaioli, Matteo Cescon, Simone Ambretti, Pierluigi Viale, Patrizia Brigidi, Silvia Turroni, Maddalena Giannella

## Correspondence

renato.pascale2@unibo.it (R. Pascale).

## Graphical abstract




## Highlights

- Among liver transplant recipients, gut microbiome dynamics could improve patient stratification for infection risk.
- CRE-infected patients showed an over-representation of *Klebsiella*, serving as an early marker of subsequent CRE infection.
- CRE carriers had a stable and diverse GM, whose compositional dynamics tended to overlap with those of non-colonized.
- Changes in gut microbiome could guide intervention strategies to reduce infectious complications and improve prognosis.

## Impact and implications

Little is known about the temporal dynamics of gut microbiome (GM) in liver transplant recipients associated with carbapenem-resistant Enterobacterales (CRE) colonization and infection. The GM structure and functionality of patients colonized with CRE and developing infection appeared to be distinct compared with CRE carriers without infection or patients with other microbial infection or no infection and CRE colonization. Higher proportions of antimicrobial-resistant pathogens and poor representation of bacteria and metabolic pathways capable of promoting overall host health were observed in CRE carriers who developed infection, even before liver transplant. Therefore, pretransplant GM profiling could improve patient stratification and risk prediction and guide early GM-based intervention strategies to reduce infectious complications and improve overall prognosis.

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# Gut microbiome dynamics and Enterobacterales infection in liver transplant recipients: A prospective observational study

**Federica D'Amico**,[1,2,†] **Matteo Rinaldi**,[3,4,†] Renato Pascale,[3,4,*] Marco Fabbrini,[1,2] Maria Cristina Morelli,[5] Antonio Siniscalchi,[6] Cristiana Laici,[6] Simona Coladonato,[3,4] Matteo Ravaioli,[7] Matteo Cescon,[3,7] Simone Ambretti,[8] Pierluigi Viale,[3,4] Patrizia Brigidi,[1] Silvia Turroni,[2,‡] Maddalena Giannella[3,4,‡]

[1]*Microbiomics Unit, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy;* [2]*Unit of Microbiome Science and Biotechnology, Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy;* [3]*Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Bologna, Italy;* [4]*Department of Integrated Management of Infectious Risk, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola, Bologna, Italy;* [5]*Internal Medicine Unit for the Treatment of Severe Organ Failure, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola, Bologna, Italy;* [6]*Division of Anesthesiology, Department of Anesthesia and Intensive Care, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy;* [7]*General Surgery and Transplant Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola, Bologna, Italy;* [8]*Microbiology Operative Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola, Bologna, Italy*

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**Background & Aims:** The aim of this study was to investigate gut microbiome (GM) dynamics in relation to carbapenem-resistant Enterobacterales (CRE) colonization, CRE infection, and non-CRE infection development within 2 months after liver transplant (LT).

**Methods:** A single-center, prospective study was performed in patients undergoing LT from November 2018 to January 2020. The GM was profiled through 16S rRNA amplicon sequencing of a rectal swab taken on the day of transplantation, and fecal samples were collected weekly until 1 month after LT. A subset of samples was subjected to shotgun metagenomics, including resistome dynamics. The primary endpoint was to explore changes in the GM in the following groups: (1) CRE carriers developing CRE infection (CRE_I); (2) CRE carriers not developing infection (CRE_UI); (3) non-CRE carriers developing microbial infection (INF); and (4) non-CRE carriers not developing infection (NEG).

**Results:** Overall, 97 patients were enrolled, and 91 provided fecal samples. Of these, five, nine, 22, and 55 patients were classified as CRE_I, CRE_UI, INF, and NEG, respectively. CRE_I patients showed an immediate and sustained post-LT decrease in alpha diversity, with depletion of the GM structure and gradual over-representation of *Klebsiella* and *Enterococcus*. The proportions of *Klebsiella* were significantly higher in CRE_I patients than in NEG patients even before LT, serving as an early marker of subsequent CRE infection. CRE_UI patients had a more stable and diverse GM, whose compositional dynamics tended to overlap with those of NEG patients.

**Conclusions:** GM profiling before LT could improve patient stratification and risk prediction and guide early GM-based intervention strategies to reduce infectious complications and improve overall prognosis.

**Impact and implications:** Little is known about the temporal dynamics of gut microbiome (GM) in liver transplant recipients associated with carbapenem-resistant Enterobacterales (CRE) colonization and infection. The GM structure and functionality of patients colonized with CRE and developing infection appeared to be distinct compared with CRE carriers without infection or patients with other microbial infection or no infection and CRE colonization. Higher proportions of antimicrobial-resistant pathogens and poor representation of bacteria and metabolic pathways capable of promoting overall host health were observed in CRE carriers who developed infection, even before liver transplant. Therefore, pretransplant GM profiling could improve patient stratification and risk prediction and guide early GM-based intervention strategies to reduce infectious complications and improve overall prognosis.



## Introduction

Gram-negative pathogens, particularly Enterobacterales, which are natural inhabitants of the gut microbiome (GM), have emerged as the leading cause of bacterial infection after liver transplant (LT).[1] In centers endemic for carbapenem-resistant Enterobacterales (CRE), colonization and infection with CRE after LT may occur with associated high morbidity and mortality rates.[2,3] Several studies have shown that CRE colonization is an important risk factor for developing CRE infection after LT.[3–5] In addition,

Keywords: Gut microbiome; Carbapenem-resistant Enterobacterales; Liver transplant; Antibiotic resistance; Gut resistome.

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† These authors contributed equally to this work and share the co-first authorship.

‡ These authors jointly supervised this work as co-senior authors.

* Corresponding author. Address: Infectious Diseases Unit, IRCCS Policlinico Sant'Orsola, Department of Medical and Surgical Sciences, University of Bologna, Via Massarenti 11, 40138 Bologna, Italy.

*E-mail address:* renato.pascale2@unibo.it (R. Pascale).

a correlation between the relative abundance of carbapenemase-producing *Klebsiella pneumoniae* in the GM and the risk of developing bloodstream infection (BSI) was shown,[6] suggesting a crucial role for a dysregulated GM favored by exposure to carbapenems. In addition, GM dysbiosis and antimicrobial exposure are key determinants of the risk of BSI by any microorganism.

Few studies have explored the GM compositional structure in patients undergoing LT, mainly focusing on disease-related dysbiosis before transplant,[7,8] suggesting that reduced pre-LT alpha diversity could be a wake-up call for subsequent colonization by multidrug-resistant organisms. In addition, Macesic *et al.*[9] proposed GM-based strategies to study multidrug-resistant organism colonization dynamics after LT to improve infection control policies, inform empiric treatment regimens, and/or consider fecal microbiota transplantation as proposed by some experts.[10,11] However, little is known about the temporal dynamics of the GM composition in LT recipients associated with CRE colonization and infection development. Furthermore, as far as we know, no information is yet available on changes in the pattern of genes harbored by the GM, including those conferring antibiotic resistance (*i.e.* the resistome).

The aim of our study was to longitudinally profile the GM of patients undergoing LT, from the day of transplant and weekly until 1 month, in relation to CRE colonization status and the development of a CRE or non-CRE infection within 60 days post LT. Fecal samples collected over time were analyzed by 16S rRNA amplicon sequencing for diversity assessment and compositional trajectory reconstruction. A subset of samples was subjected to shotgun metagenomics for functional insights, including resistome dynamics.

## Materials and methods

### Study design and population

We performed a prospective cohort study of all consecutive adult (≥18 years) patients undergoing LT from 1 November 2018 to 31 January 2020. Exclusion criteria included multivisceral transplantation, re-transplantation, and refusal to participate in the study.

### Setting

The study was conducted at IRCCS Azienda Ospedaliero-Universitaria di Bologna (Italy), a 1,420-bed tertiary-care hospital located in northern Italy, with a LT program performing more than 90 procedures each year.

### Study procedures

During the study period, all LT candidates were screened for CRE carriage using a rectal swab at the time of inclusion in the waiting list, in case of hospital admission, and at the time of transplant. After LT, rectal swabs were performed once weekly until hospital discharge and in case of hospital readmission. Isolation and contact precautions were activated for CRE carriers, but no pharmacological interventions, including selective intestinal decontamination, targeted surgical prophylaxis, or antimicrobial treatment, were administered to asymptomatic patients. For GM analysis, rectal swabs were collected immediately before transplant, whereas stool samples were collected weekly for up to 1 month after LT. Interchangeability of fecal sampling and rectal swabbing for assessment of the GM structure has been demonstrated elsewhere.[12]

### GM profiling

Microbial DNA was extracted from rectal/fecal samples using the repeated bead-beating protocol[13] with only a few modifications.[14] Library preparation and sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) were performed following the manufacturer's instructions. Raw sequences were processed using QIIME 2.[15] A subset of samples was further processed through shotgun metagenomics (Fig. 1). DNA libraries were prepared using the QIAseq FX DNA Library Kit (QIAGEN, Hilden, Germany) and sequenced on an Illumina NextSeq

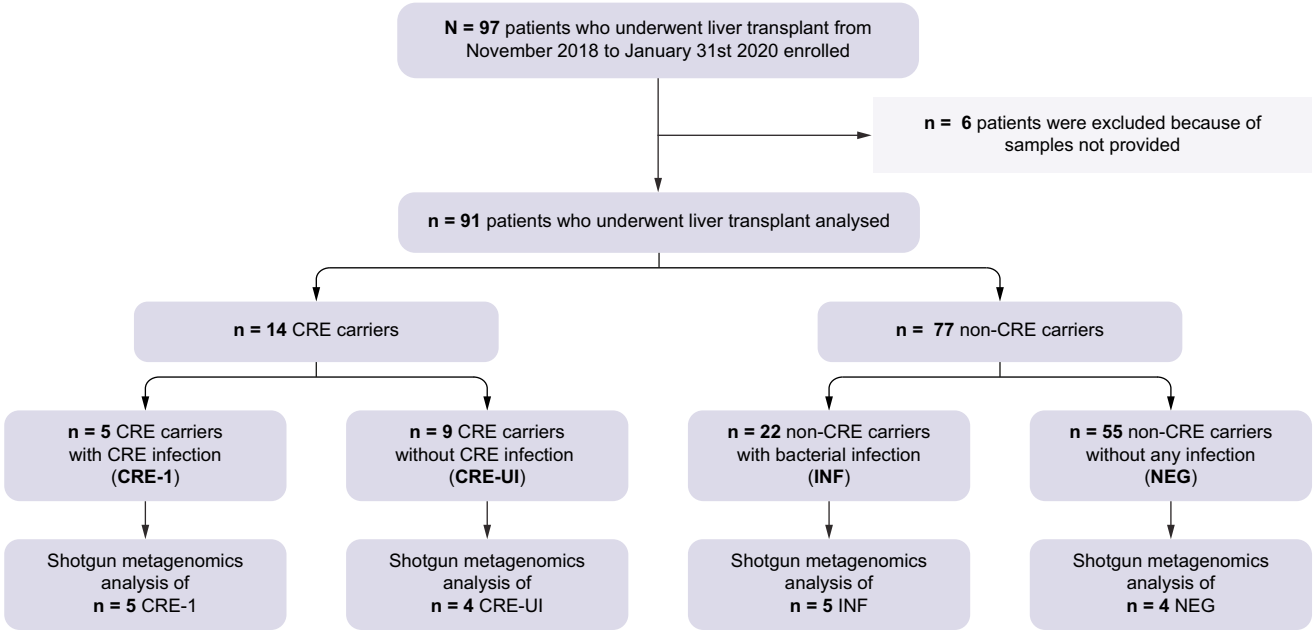


**Fig. 1. Study flowchart.** CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with microbial infection; NEG, without CRE colonization and without infection.

**Table 1. Characteristics of the study population.**

| | Total (N = 91) (%) |
|---|---|
| Demographic data | |
| Age (years), median (IQR) | 58 (49–63) |
| Sex male, n (%) | 65 (71.4) |
| Underlying liver disease, n (%) | |
| Viral hepatitis | 53 (58.3) |
| HCV infection | 29 (31.9) |
| HBV infection | 24 (26.4) |
| Alcohol | 28 (30.8) |
| Metabolic disease | 21 (23.1) |
| Autoimmune disease | 8 (8.8) |
| Primary sclerosing cholangitis | 1 (12.5) |
| Primary biliary cirrhosis | 2 (25.0) |
| Non-specific autoimmune diseases | 5 (62.5) |
| Fulminant hepatitis | 2 (2.2) |
| HCC | 49 (53.8) |
| MELD at inclusion in waiting list, median (IQR) | 14 (10–19) |
| Time in waiting list (days), median (IQR) | 234 (69–577) |
| Events within 90 days before LT, n (%) | |
| Antibiotic exposure | 48 (52.7) |
| Bacterial infections | 17 (18.7) |
| Hepato-renal syndrome | 3 (3.3) |
| Ascites grade III/refractory | 14 (15.4) |
| Bleeding | 6 (6.6) |
| Encephalopathy | 10 (11) |
| ACLF | 12 (13.2) |
| Hospital admission | 26 (28.6) |
| ICU admission | 3 (3.3) |
| MELD at transplantation (days), median (IQR) | 16 (10–24) |
| CMV serostatus mismatch, n (%) | |
| Donor–recipient mismatch (D+/R-) | 16 (17.6) |
| Induction regimen, n (%) | |
| Bolus of steroids | 91 (100) |
| Antithymocyte globulins | 2 (2.2) |
| Rituximab | 2 (2.2) |
| Intraoperative complications, n (%) | |
| Prolonged operation (>8 h) | 31 (34.1) |
| Intraoperative bleeding | 18 (19.8) |
| Coledocojejunostomy | 14 (15.4) |
| Postoperative complications, n (%) | |
| Acute renal failure | 37 (40.7) |
| Renal replacement therapy | 18 (19.8) |
| Prolonged mechanical ventilation (>48 h) | 10 (11) |
| Poor graft dysfunction | 17 (18.7) |
| Primary graft non-function | 2 (2.2) |
| Biliary procedure | 3 (3.3) |
| Re-intervention | 14 (15.4) |
| Rejection | 5 (5.5) |
| Outcome | |
| Length of hospital stay (days), median (IQR) | 17 (12–31) |
| All cause 180-day mortality, n (%) | 3 (3.3) |

ACLF, acute-on-chronic liver failure; CMV, cytomegalovirus; D, donor; HCC, hepatocellular carcinoma; ICU, intensive care unit; MELD, model of end-stage liver disease; R, recipient; LT, liver transplant.

**Table 2. Characteristics of CRE carriers.**

| | Value, n (%) |
|---|---|
| Pre-LT | 5 (5.5) |
| Time from the first CRE carriage detection to LT (days), median (IQR) | 249 (166–612) |
| CRE infection post-LT | 1 (20) |
| Any other bacterial infection post-LT | 1 (20) |
| All-cause 180-day mortality | 1 (20) |
| Post-LT | 9 (9.9) |
| Time from LT to first CRE carriage detection (days), median (IQR) | 31 (19–62) |
| CRE infection post-LT | 4 (44.4) |
| Any other bacterial infection post-LT | 3 (33.3) |
| All-cause 180-day mortality | 1 (11.1) |

Unless otherwise indicated, for each variable, the number of cases and the percentage of the total are reported.
CRE, carbapenem-resistant Enterobacterales; LT, liver transplant.

platform. Filtered reads were processed using the HUMAnN3 pipeline[16] for functional insights and StrainPhlAn for strain phenotyping. The resistome profile was obtained using reference data from the Comprehensive Antibiotic Resistance (CARD) database.[17] Detailed information is reported in the Supplementary Methods.

### Study variables

To explore changes in the GM after LT according to CRE colonization status and infection development, patients were classified as follows:

(1) CRE carriers who developed CRE infection (CRE_I);
(2) CRE carriers who did not develop infection (CRE_UI);
(3) non-CRE carriers who developed a microbial infection (INF); and
(4) non-CRE carriers who did not develop severe infection or BSI (NEG).

CRE carriage was defined as the isolation of CRE from a rectal swab in the absence of symptoms and signs of infection. For CRE infection, criteria set by the Centre for Diseases Control and Prevention were followed.[18] For non-CRE infection, all microbial infections were included. The overall occurrence of microbial infection was assessed within 60 days after LT. Only the first episode was considered.

GM variables included the following: alpha and beta diversity, and relative abundance of phyla, families, and genera during the first month after LT. For a sample subset (Fig. 1), the proportions of microbial pathways and genes, including those of the resistome, were considered as additional variables.

### Data collection

Data sources were clinical charts and hospital electronic records. Data were prospectively gathered anonymously and managed using the REDCap electronic data capture tool hosted by Alma Mater Studiorum – University of Bologna.[19] The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and approved by the local ethics committee (Comitato Etico Indipendente di Area Vasta Emilia Centro, no. 279/2018/Sper/AOUBo).

### Statistical analysis

For descriptive analysis, categorical variables were presented as absolute numbers and relative frequencies. Continuous variables were presented as mean and SD if normally distributed or as median and IQR if non-normally distributed.

For 16S rRNA gene analysis, alpha diversity was computed using Faith's phylogenetic diversity index. Beta diversity was estimated based on UniFrac distances, which were used to build principal coordinate analysis (PCoA) plots. All GM statistical analysis and graphs were obtained using R 4.0.3. Permutational multivariate analysis of variance (PERMANOVA) for PCoA plots was performed using the 'Adonis' function of the 'vegan' R package (https://cran.r-project.org/package=vegan). Differences in alpha diversity, the GM compositional structure, and relative

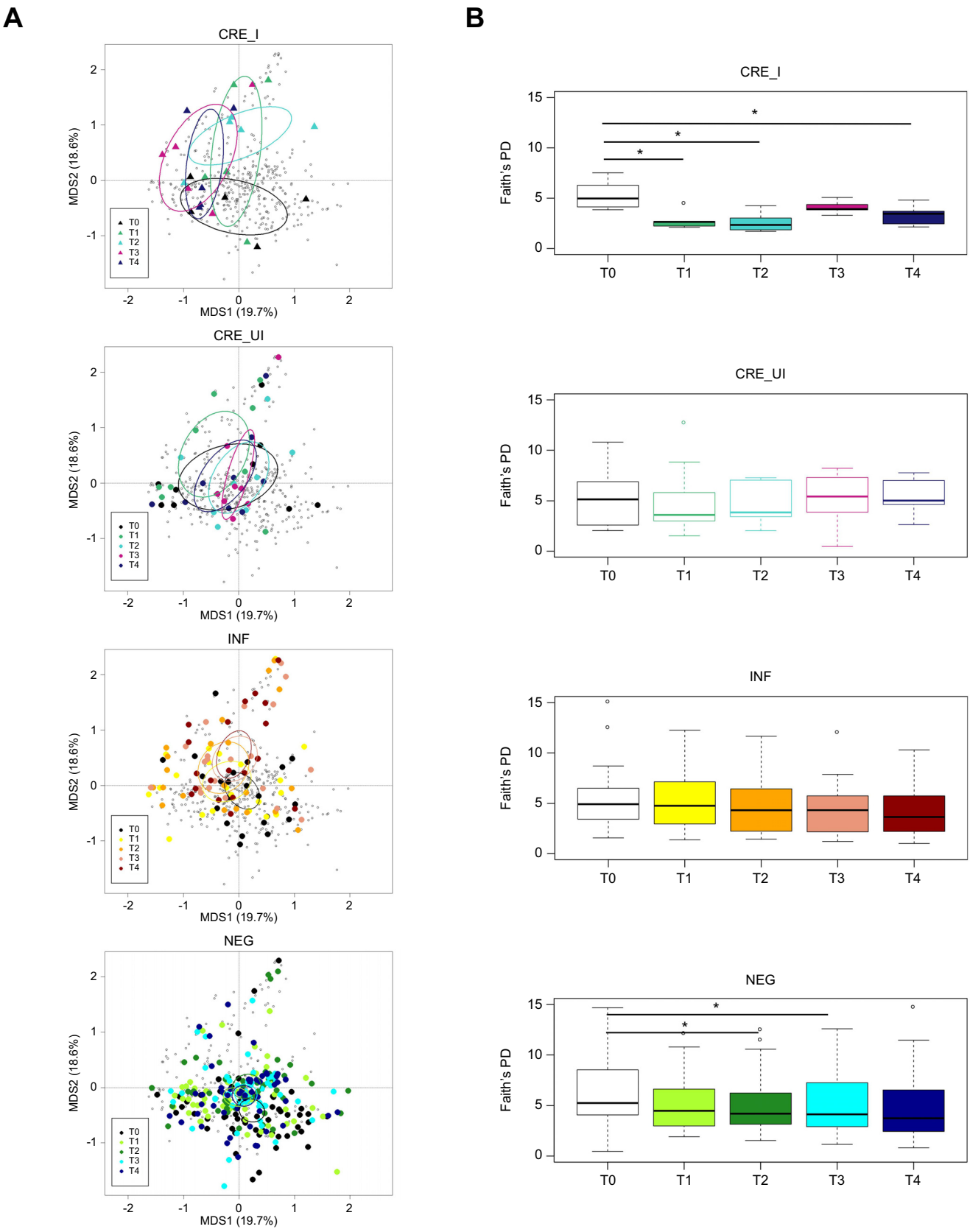


**Fig. 2. Gut microbiome diversity in patients undergoing liver transplantation.** (A) PCoA based on weighted UniFrac distances between the gut microbiome profiles of samples collected before transplant (T0) and weekly up to 1 month post transplant (T1 to T4). Patients were stratified according to CRE colonization status and infection development at 2 months post transplant as follows: (1) CRE_I, (2) CRE_UI, (3) INF, and (4) NEG. Ellipses include 95% confidence area based on the SE of the weighted average of sample coordinates. Significant segregation between groups was found (Adonis, $p \leq 0.05$; see Results). (B) Boxplots showing

pathway abundance among groups were detected using a Kruskal–Wallis test followed by a *post hoc* Wilcoxon test (paired or unpaired, as needed). Only the differentially represented pathways were considered for heatmap construction and correlations. Heatmaps were plotted using the 'heatmap.2' function of the 'gplots' R package (https://cran.r-project.org/package=gplots).

For the generation of co-abundance groups (CAGs), only the bacterial genera present in at least 10% of the samples were considered. Associations between genera were assessed using the Kendall correlation test, visualized using hierarchical Ward-linkage clustering based on Spearman correlation coefficients, and used to define CAGs.[20] Wiggum plot networks were created using Cytoscape software, with the circle size proportional to the relative bacterial abundance and the connections between nodes representing significant Kendall correlations between genera.[21] Kendall correlations were also sought between pathway proportions and species-level relative abundances to link functional and compositional data. Only species with a relative abundance of >0.3% in at least 5% of metagenomic samples were included in the correlation analysis. Radar charts were plotted using the 'fmsb' R-package (https://cran.r-project.org/package=fmsb), and differences in total counts of Antibiotic Resistance Ontologies (AROs) and count-associated resistance mechanisms were tested using pairwise Wilcoxon tests. Values of $p$ were corrected for multiple comparisons using the Benjamini–Hochberg method. A false discovery rate (FDR) of ≤0.05 was considered statistically significant. FDR ≤0.1 was considered a trend.

## Results

### Study cohort description

During the study period, 97 patients underwent LT, with six excluded because samples were not provided. Thus, 91 patients were analyzed. The characteristics of the study population are shown in Table 1. Sixty-five (71.4%) were male, and the median age was 58 (IQR 49–63) years. Viral hepatitis was the primary indication for LT in 53/91 participants (58.3%), followed by alcohol in 28/91 patients (30.8%). About half of the patients (49/91 [53.8%]) had hepatocellular carcinoma (HCC). The median model of end-stage liver disease (MELD) score at LT was 16 (IQR 10–19), without differences between patients with and without CRE colonization. All CRE were KPC-type carbapenemase producers, except one that was a producer of New Delhi Metallo-beta lactamase (NDM). The immunosuppressive induction regimen at the time of LT and cytomegalovirus (CMV) serostatus is reported in Table 1. CMV events were observed in five patients, two with CMV reactivation and three with CMV pneumonia. Among all patients, 17 (18.7%) had poor graft dysfunction and 14 (15.4%) underwent reoperation. All-cause 180-day mortality was observed in three patients (3.3%). Up to one-third of LT recipients developed at least one episode of microbial infection (31 [34%]) within 60 days after LT.

Overall, 14 (15.4%) patients were identified as CRE carriers during the study period. Of these, five (5.5%) were found to be colonized at the time of transplantation, whereas the remaining nine (9.9%) acquired colonization within a median of 31 (IQR 19–62) days from LT. Among CRE carriers, five of 14 (35.7%) developed CRE infection within a median of 31 (IQR 3–46.5) days after LT. These infections included two primary BSI, two intra-abdominal infections, and one lower respiratory tract infection (data shown in Table 2). At least one episode of microbial infection was diagnosed in 22 non-CRE carriers. Among these, 10 patients had Enterobacterales BSI with the isolation of *E. coli* in three cases, *Klebsiella* spp. in three cases, *Enterobacter* spp. in three cases, and *Citrobacter* spp. in one case. However, in the remaining 55 non-CRE carriers, no microbial infection was diagnosed within 60 days after LT (Fig. 1).

For each of the 91 patients who provided rectal/fecal samples, sampling occurred on the day of transplantation (T0) and weekly for up to 1 month after LT (T1 to T4), for a total of 430 samples for GM profiling. Twenty-five samples were missing.

### GM diversity in patients undergoing LT

The GM profile of the 91 LT patients was first characterized at baseline in relation to pre-transplant conditions and main complications. PCoA based on weighted UniFrac distances showed no separation by HBV and HCV infection, presence of HCC and alcohol abuse, or the occurrence of acute-on-chronic liver failure (ACLF; pairwise Adonis, $p$ >0.05) (Fig. S1).

Next, the GM dynamics were reconstructed over time according to CRE colonization status and microbial infection development in the four groups of patients: CRE_I, CRE_UI, INF, and NEG (see Materials and methods). As for beta diversity, the four patient groups showed a distinct temporal trajectory in the weighted UniFrac-based PcoA plot (Fig. 2A). In particular, for the NEG group, significant segregation was observed between baseline and all post-LT samples ($p$ = 0.001). In contrast, in the remaining three groups (*i.e.* CRE_I, CRE_UI, and INF), the samples generally overlapped, with the only exception of T4 of the INF group, which was significantly separated from the respective T0 ($p$ = 0.006). Regarding intergroup comparisons, it should be noted that the NEG and CRE_I samples segregated from each other at all time points ($p$ ≤0.05), whereas for the CRE_UI and INF groups, some differences emerged only later in time (CRE_UI *vs.* NEG at T1 and INF *vs.* NEG at T2 to T4, $p$ ≤0.01) (data not shown). When focusing on alpha diversity (Fig. 2B), we found an immediate post-LT reduction for CRE_I patients that persisted over the sampling period (Wilcoxon test, $p$ ≤0.04). A similar decline over time was observed in the NEG group ($p$ ≤0.05), albeit by far less and with greater interindividual variability (NEG *vs.* CRE_I at T1 and T2, $p$ = 0.03). In contrast, no significant differences were found in the CRE_UI and INF groups.

### Compositional dynamics of the GM in patients undergoing LT

The compositional layouts of the GM were profiled over time in the four patient groups defined earlier (Fig. 3 and Figs. S2 and S3). In line with the alpha diversity data, the GM of CRE_I patients underwent post-LT de-structuring, with a general impoverishment of the community. In particular, we observed an overabundance of pathobionts, namely Enterobacterales, whose relative abundance increased over time, specifically at T3 and T4,

the temporal distribution of alpha diversity estimated with Faith's PD index in liver transplant patients grouped as above. Wilcoxon test, *$p$ ≤0.05. CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with microbial infection; MDS, multi dimensional scaling; NEG, without CRE colonization and without infection; PCoA, principal coordinate analysis; PD, phylogenetic diversity.

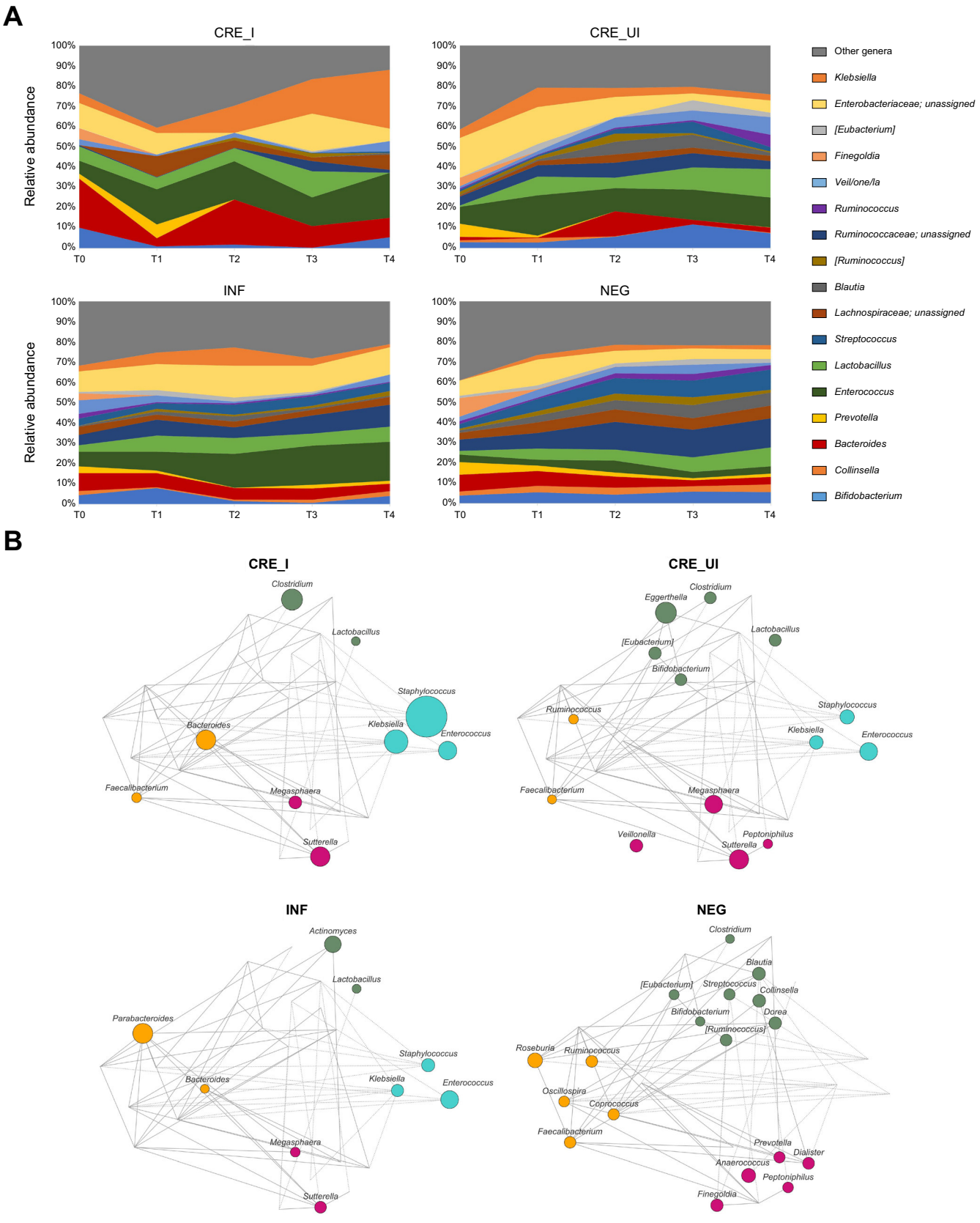


**Fig. 3. Genus-level compositional dynamics of the gut microbiome in patients undergoing liver transplantation.** (A) Area plots showing the relative abundance of major genera in the gut microbiome of liver transplant patients before transplant (T0) and weekly up to 1 month post transplant (T1 to T4). Patients were stratified according to CRE colonization status and microbial infection development at 2 months post transplant as follows: (1) CRE_I, (2) CRE_UI, (3) INF, and (4) NEG. Only taxa with a mean relative abundance of >10% in at least 5% of the samples of the total dataset are shown. (B) Wiggum plots showing the pattern

compared with those in all other patient groups (Wilcoxon test, $p \leq 0.05$). This increase was attributable to *Klebsiella,* the proportions of which reached 29% at T4 (*vs*. T0, $p = 0.03$). An increase in *Klebsiella* was also found over time for NEG patients ($p \leq 0.05$), although considerably lower than that in the CRE_I group (mean post-LT relative abundance, 2.3%; $p \leq 0.001$). Notably, the proportions of *Klebsiella* (and Enterobacterales) at baseline were already significantly higher in the CRE_I group (4.8% *vs*. 0.08% in the NEG group, $p = 0.03$). In addition, the NEG group showed significant variations in the proportions of most of the dominant genera (*i.e.* those with mean relative abundance >10% in at least 5% of all samples; $p \leq 0.05$) while maintaining some level of diversity. Similar temporal changes while keeping the overall community representation were also observed in CRE_UI patients. Interestingly, Enterobacterales levels were higher at baseline, as well as at T1 and T2, in these patients than in NEG patients ($p \leq 0.05$) but decreased sharply at later time points. *Klebsiella* levels in CRE_UI patients were far lower than those in CRE_I patients (mean post-LT relative abundance, 5.1%; $p \leq 0.02$) while being significantly higher than those in NEG patients ($p \leq 0.001$). As for the INF group, the most distinctive signature was the increase in *Enterococcus,* whose relative abundance increased over time to 19.1% at T4 (*vs*. T0, $p = 0.03$). Again, pre-LT *Enterococcus* levels were already significantly higher in INF patients than in NEG patients (7.2% *vs*. 3.8%, $p = 0.05$). It should be noted that 1 month after LT (T4), *Enterococcus* proportions were >10% in the other patients as well, except for NEG ones (NEG *vs*. CRE_I *vs*. CRE_UI, 3.6% *vs*. 22.3% *vs*. 14.7%; $p \leq 0.05$).

When looking at the GM structures, in terms of networks of correlated genera, we found four CAGs named *Streptococcus*, *Bacteroides*, *Prevotella*, and *Enterococcus*, based on the dominant genus within each of them (PERMANOVA, $p < 0.05$; Fig. S4). The Wiggum plots showing CAG relationships (Fig. 3B) highlighted the clear prevalence of the *Enterococcus* CAG in the CRE_I group, with the co-abundance of *Enterococcus*, *Klebsiella*, and *Staphylococcus*. This CAG was also represented in CRE_UI and INF patients, whereas it was underrepresented in NEG patients. It should be noted that the proportions of *Staphylococcus* were significantly higher in the CRE_I group than in the NEG group at T1 through T4 (Wilcoxon test, $p \leq 0.001$) (Fig. S2). In contrast, CRE_UI patients showed a fair representation of all four CAGs, with some overabundances shared only with NEG patients. Except for *Enterococcus* CAG, the NEG group showed the greatest over-representation of CAGs, with the co-abundance of several health-associated taxa, including the well-known short-chain fatty acid (SCFA) producers *Blautia*, *Dorea*, *Roseburia*, *Coprococcus*, *Faecalibacterium*, *Ruminococcus*, and *Oscillospira.*

## Functional dynamics of the GM in patients undergoing LT and *Klebsiella pneumoniae* strain heterogeneity

The functional analysis was conducted on a subset of five patients in the CRE_I group, four patients in the CRE_UI group, five patients with carbapenem-susceptible Enterobacterales BSI in the INF group, and four patients in the NEG group (Fig. 1). As

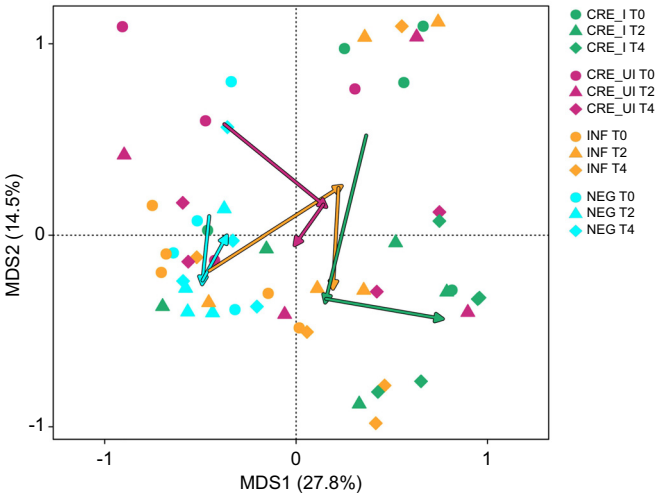


**Fig. 4. Functional dynamics of the gut microbiome in patients undergoing liver transplantation.** PCoA based on Bray–Curtis dissimilarity between the functional profiles of the gut microbiome before transplant (T0) and at 2 and 4 weeks post transplant (T2 and T4, respectively). Patients were stratified according to CRE colonization status and BSI development at 2 months post transplant as follows: (1) CRE_I, (2) CRE_UI, (3) INF, and (4) NEG. The arrows connect the centroids of each patient group over time. Significant segregation between groups was found (Adonis, $p \leq 0.05$; see Results). BSI, bloodstream infection; CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with carbapenem-susceptible Enterobacterales BSI; NEG, without CRE colonization and without infection; PCoA, principal coordinate analysis.

shown in the PCoA plot based on Bray–Curtis dissimilarities between functional profiles (Fig. 4), the baseline samples of CRE_I patients were significantly separated from those of NEG and INF patients (pairwise Adonis, $p < 0.05$), suggesting a different functional GM potential already before LT. One month after LT, the CRE_I samples clustered separately even with respect to the CRE_UI ones ($p < 0.05$), supporting the establishment of distinct functional dynamics related to the onset of CRE infection.

Regarding functional pathways (Fig. 5), at baseline, CRE_I patients showed greater representation of pathways enhancing carbon source retrieval (*e.g.* sulfoquinovose degradation I, threalose degradation V, and glucarate degradation) and electron transfer chain (*e.g.* superpathways for the biosynthesis of menaquinol-6 to -13; demethilmenaquinol-6, -8, and -9; and ubiquinol-7 to -10), as well as pathways related to lipopolysaccharide (LPS) production (lipid IV A biosynthesis and superpathway of Kdo2-lipid A biosynthesis). Some of these, particularly those involved in quinol and LPS biosynthesis, and glucarate degradation, were shared by the CRE_UI samples, which also showed reduced proportions of pathways involved in translation accuracy (*i.e.* queuosine biosynthesis I and the *de novo* biosynthesis of a common intermediate named preQ0). In

of variation of the four identified CAGs in the four patient groups across the entire dataset (*i.e.* including T0 to T4 time points). CAGs were named according to the most abundant genera and color coded as follows: *Streptococcus* (green), *Bacteroides* (orange), *Prevotella* (violet), and *Enterococcus* (cyan). Each genus is depicted as a node whose size is proportional to the overabundance relative to background. Positive and significant Kendall correlations between two or more genera are indicated with lines connecting the nodes ($p < 0.05$). See also Fig. S4. CAG, co-abundance group; CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with microbial infection; NEG, without CRE colonization and without infection.

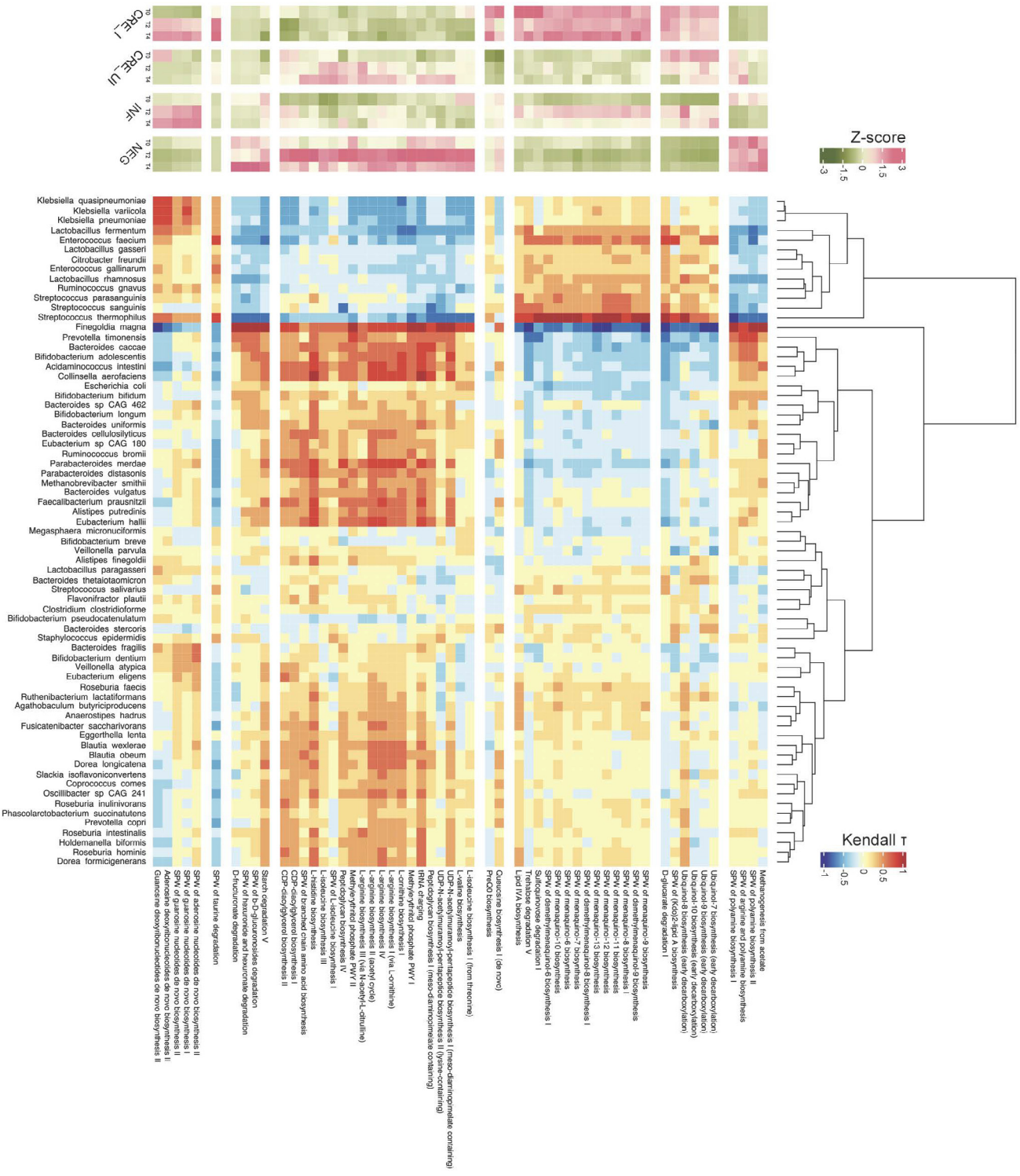


**Fig. 5. Gut microbiome functional signatures of CRE infection in patients undergoing liver transplantation.** (Left; green/pink) Heatmap representing pathways with Z-scores indicative of differences in the distribution of CPMs scaled by rows for each patient group (CRE_I, CRE_UI, INF, and NEG). Only pathways showing significant differences (Kruskal–Wallis test, $p$ <0.05) among groups across time points (before transplant [T0] and at 2 and 4 weeks post transplant [T2 and T4, respectively]) were plotted. Pathways are sorted and divided according to their Z-score. (Right; blue/red) Heatmap showing the Kendall correlations between the same pathways and species-level compositional information. Only species with a relative abundance of >0.3% in at least 5% of the samples were included in the correlation and plotted. BSI, bloodstream infection; CPM, copies per million; CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization without CRE infection; INF, without CRE colonization but with carbapenem-susceptible Enterobacterales BSI; NEG, without CRE colonization and without infection; SPW, superpathway.

contrast, before LT, non-colonized patients (*i.e.* NEG and INF) were characterized by a higher capacity for polyamine biosynthesis and SCFA production (as supported by the increased representation of methanogenesis from acetate).

At T2, most functional signatures were retained, but the NEG samples were distinguished by an overall higher arginine biosynthesis potential (*e.g.* via L-ornithine, acetyl cycle, and *N*-acetyl-L-citrulline), still supporting a high polyamine production. Methyleritritol phosphate (a precursor for isoprenoid biosynthesis) pathways I and II, and tRNA charging functions, also showed higher copies per million in the NEG group, as did pathways related to cell wall constituents (*e.g.* UDP-*N*-acetylmuramyl-pentapeptide and peptidoglycan biosynthesis) and membrane stability (*e.g.* CDP-diacylglycerol biosynthesis I and II), with the latter shared with the CRE_UI samples.

Interestingly, 1 month after LT, the CRE_I samples showed an increased level of the superpathway of taurine degradation, typically used for growth by various pathogens, including *Klebsiella*.[22,23] Furthermore, they shared with the INF samples an enrichment in pathways and superpathways associated with *de novo* nucleotide biosynthesis. In contrast, in addition to maintaining a high production potential of arginine, polyamines, and SCFAs, the NEG samples showed an increase in copies per million associated with the degradation of compounds related to hepatic detoxification processes, such as β-D-glucuronosides, D-fructuronate, hexuronide, and hexuronate, possibly suggesting a remodeling of the GM based on the recovery of the function of the transplanted liver.

As for species potentially responsible for functional disparities, several pathobionts correlated with both CRE_I and INF functional signatures, such as *Klebsiella* spp. (*K. quasipneumoniae*, *K. pneumoniae*, and *K. variicola*), *Enterococcus* spp. (*E. faecium* and *E. gallinarum*), *[Ruminococcus] gnavus*, and *Streptococcus* spp. (*S. sanguinis* and *S. parasanguinis*). In contrast, NEG-related functions were positively correlated with the presence of typically health-associated taxa, such as *Prevotella timonensis*, *Bifidobacterium* spp. (*B. adolescentis*, *B. bifidum*, and *B. longum*), *Bacteroides* spp. (*B. caccae*, *B. uniformis*, and *B. vulgatus*),

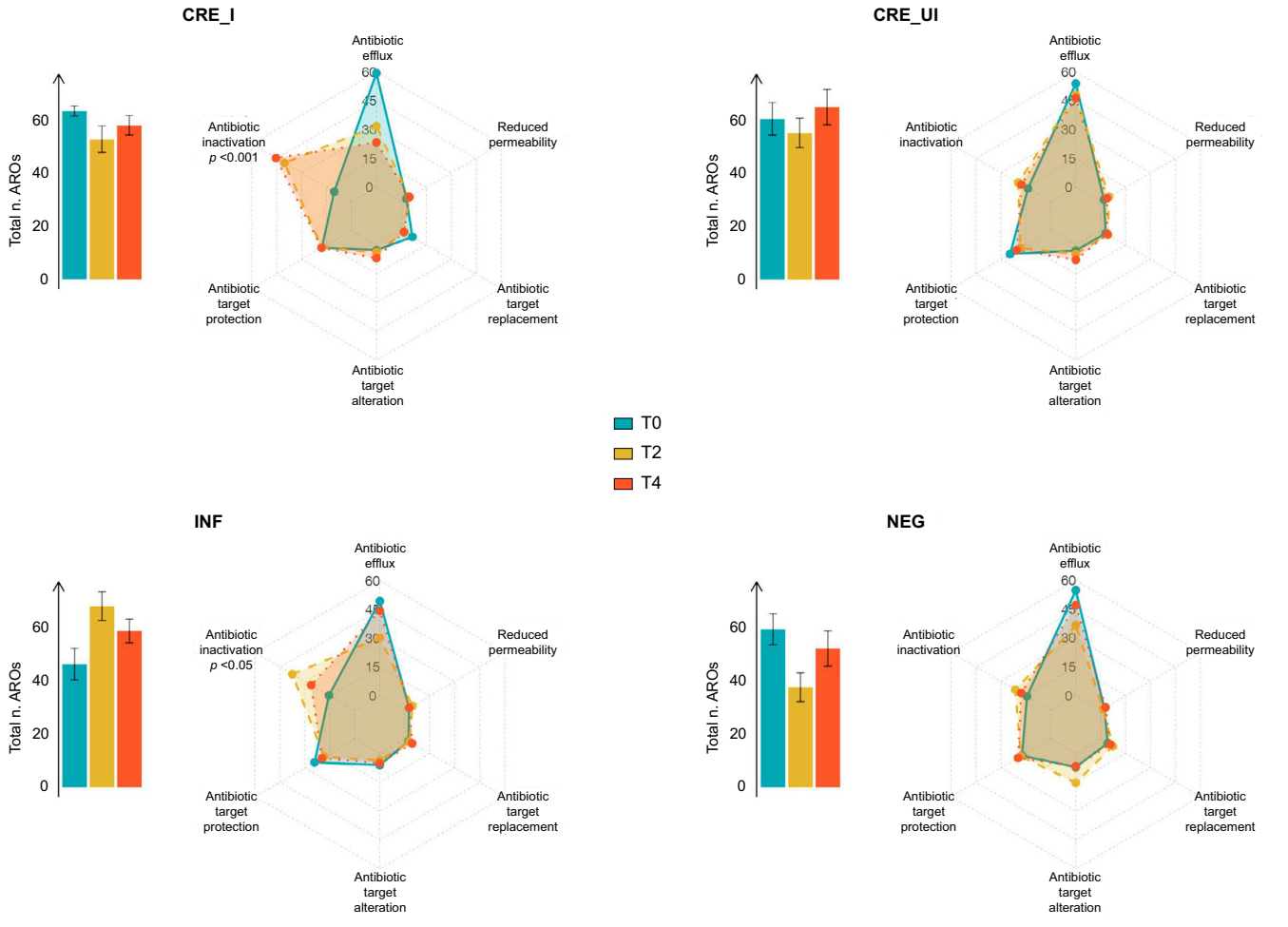


**Fig. 6. Dynamics of the gut resistome in patients undergoing liver transplantation.** Bar plots showing the average number of AROs and radar charts showing the percentage contribution of antibiotic resistance genes to each resistance mechanism are reported for each patient group (CRE_I, CRE_UI, INF, and NEG) before transplant (T0), and at 2 and 4 weeks post-transplant (T2 and T4, respectively). Whiskers represent the SEM. Significant differences were found in the distribution of resistance mechanisms (Wilcoxon test, $p$ <0.05). ARO, Antibiotic Resistance Ontology; BSI, bloodstream infection; CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with carbapenem-susceptible Enterobacterales BSI; NEG, without CRE colonization and without infection.

*Ruminococcus bromii*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Blautia* spp. (*B. wexlerae* and *B. obeum*), and *Dorea* spp. (*D. longicatena* and *D. formicigenerans*).

Finally, considering the significant variation in the relative abundance of *K. pneumoniae* across sample groups, we compared its strain diversity against a dataset of 1,000 reference genomes (Fig. S5). According to our results, the *K. pneumoniae* strains found in the CRE_I and INF groups were located in a distant branch of the phylogenetic tree compared with those in the CRE_UI and NEG groups, thus suggesting different intrinsic characteristics.

### Gut resistome in patients undergoing LT

Finally, the dynamics of the gut resistome were evaluated through the detection of AROs (*i.e.* the number of antibiotic resistance genes and mutations; see Materials and methods and Supplementary methods) (Fig. 6). No significant differences were observed over time for any patient group in terms of the number of AROs identified. However, when focusing on the resistance mechanisms, we found some interesting patterns. In particular, we observed a similar distribution of mechanisms at baseline in all groups, mainly involving antibiotic efflux. The representation of this mechanism declined over time in the gut resistome of CRE_I patients, parallel to the increase in antibiotic inactivation-mediated resistance, with a final contribution of 45.4% of all identified AROs (Wilcoxon test, $p$ <0.001). A similar shift was observed at T2 for INF patients ($p$ <0.05), with a trend toward recovery to initial proportions at T4 ($p$ = 0.06). As expected, patients who developed no infection (*i.e.* CRE_UI and NEG) did not show significant temporal variations in resistance mechanisms. The increase in antibiotic inactivation-mediated resistance for CRE_I and INF patients during the transplant window was attributable to various genes, including those encoding beta-lactamases, phosphotransferases, acetyltransferases, nucleotidyltransferases, and thiol-transferases (Fig. 7 and Table S1). Notably, all these genes, except those coding for beta-lactamases, were over-represented in the CRE_I resistome compared with the INF resistome ($p$ ≤0.004). Overall, AROs were mostly located in the bacterial chromosome (average AROs per sample ± SEM, 9.72 ± 0.371) compared with the plasmidome (0.247 ± 0.0215) (Fig. S6).

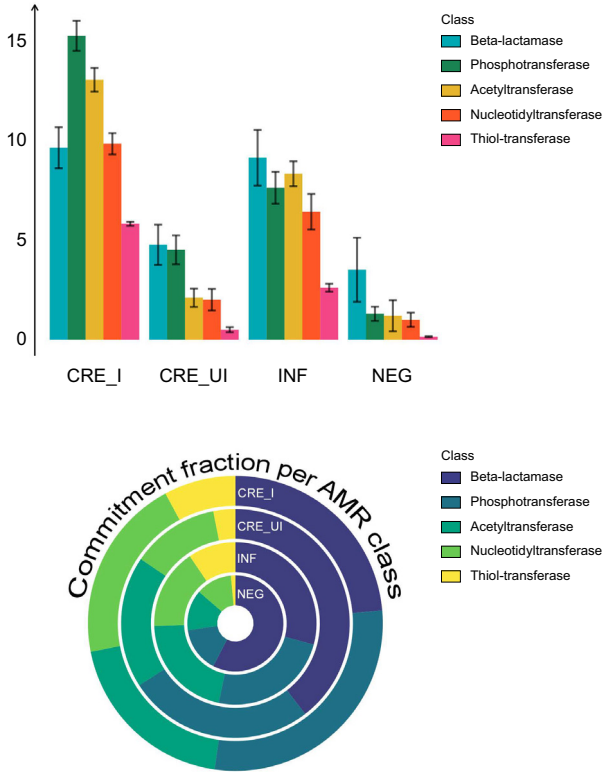


**Fig. 7. Antibiotic inactivation-mediated resistance classes in the gut resistome of patients undergoing liver transplantation.** (Top) Average number of AMR genes per patient for each antibiotic inactivation-mediated resistance class in each patient group (CRE_I, CRE_UI, INF, and NEG). Whiskers represent the SEM. As differences in resistance mechanisms were found at 2 and 4 weeks post transplant (Fig. 6), only these two time points were considered for analysis. (Bottom) Commitment fraction per antibiotic inactivation-mediated resistance class to the total number of AMR genes in each patient group. AMR, antimicrobial resistance; BSI, bloodstream infection; CRE, carbapenem-resistant Enterobacterales; CRE_I, with CRE colonization and CRE infection; CRE_UI, with CRE colonization but without CRE infection; INF, without CRE colonization but with carbapenem-susceptible Enterobacterales BSI; NEG, without CRE colonization and without infection.

## Discussion

To the best of our knowledge, this study is the first to longitudinally characterize the GM structure, including its functionality, in patients undergoing LT, up to 1 month post transplant, in relation to CRE carriage and infection. As previously shown by Annavajhala *et al.*,[8] CRE carriers who developed CRE infection (*i.e.* CRE_I) exhibited a number of distinctive features of the GM composition, some of which were already present before transplantation. First, they showed an immediate and sustained post-LT decrease in alpha diversity, which was reflected in a general depletion of the GM structure with a gradual over-representation of *Klebsiella* and *Enterococcus* (up to approximately one-third and one-quarter of the whole ecosystem at 1 month post LT, respectively). Notably, the proportions of *Klebsiella* were significantly higher in CRE_I patients than in NEG patients even before LT, likely serving as an early marker of subsequent CRE infection. In contrast, CRE carriers who did not develop infection (CRE_UI) were characterized by an overall more stable and diverse GM, whose compositional dynamics tended to overlap with those of NEG patients. The main exception was represented by the higher percentages of *Klebsiella* and *Enterococcus* compared with those in NEG patients, although far from the dominances found in CRE_I patients. Again, these findings confirm the available literature that identifies these potential pathogens as signatures of poor GM health and thus increased risk of complications in the LT setting.[8,10,11] However, it is worth noting that the *Klebsiella* strains found in CRE_I and CRE_UI patients (with particular regard to *K. pneumoniae* species) were markedly distinct. This suggests that the development of infection does not depend solely on the achievement of certain relative abundances but rather on strain-specific determinants. However, NEG patients were predictably characterized by clusters of typically health-associated, SCFA-producing microbes such as *Blautia*, *Dorea*, *Roseburia*, *Coprococcus*, *Faecalibacterium*, *Ruminococcus*, and *Oscillospira*, which may help restore or maintain an overall healthier gut environment, providing protection against pathogens, bacterial translocation, and infectious manifestations.[7]

Notably, discriminating GM features for CRE colonization and infection in LT patients were also identified for the first time at the functional level. Again, some of these features were already present at baseline, potentially acting as early predictors of later complications. In particular, even before LT, the GM of CRE_I patients was enriched in functions for microbial growth and LPS production and showed a progressive increase in genes for taurine degradation, paralleling the increase in taurine utilizers, such as *Klebsiella*.[22,23] In contrast, during the entire study window, the GM of NEG patients showed a number of characteristics typical of a eubiotic ecosystem, particularly increased levels of pathways involved in the generation of health-associated metabolites such as SCFAs, polyamines, and amino acids (particularly arginine). It is worth noting that arginine levels are typically low in LT recipients and its supplementation has been shown to have a protective role in the transplanted liver graft.[24–26] Arginine is also a precursor of polyamines, which are involved in several aspects of host homeostasis and have recently been proposed as a novel therapeutic option in the LT setting to attenuate liver ischemia/reperfusion injury and promote liver regeneration.[27] It is therefore tempting to speculate that GM may help improve LT outcomes, by compensating for arginine deficiency and providing a range of metabolites that have a beneficial impact on the gut–liver axis. Furthermore, 1 month after LT, NEG patients also showed increased GM capability of degrading compounds related to hepatic detoxification processes, suggesting GM remodeling upon restored liver functionality.

Finally, when looking at the gut resistome, we did not find differences in the number of antibiotic resistance genes across patient groups over time, but in resistance mechanisms, with antibiotic inactivation-mediated resistance becoming prevalent in the CRE_I and INF resistome. However, these resistomes were distinguishable by a higher representation of genes encoding phosphotransferases, acetyltransferases, nucleotidyltransferases, and thiol-transferases, but not beta-lactamases (the main mediators of carbapenem resistance),[28] in CRE_I patients, which persisted up to 1 month after LT. Of course, the ability of GM to inactivate antibiotics is a major concern, as it can allow pathogens to proliferate and disseminate beyond the gastrointestinal tract, thereby compromising patient prognosis and LT success. Meanwhile, it should be noted that the antimicrobial resistance genes identified in our study were mostly located in bacterial chromosomes, thus reducing the risk of plasmid-related horizontal gene transfer.

There are several limitations in this study. Although we report the first characterization of GM structure and functionality in relation to CRE carriage and infection development in patients undergoing LT, the limited sample size (especially for the CRE_I group) and number of events may have affected the statistical power. The application of shotgun metagenomics to a subset of samples may have further biased the results. In addition, the single-center design could have influenced some key issues related to epidemiology and patient management, thus limiting the generalizability of our findings. However, it should be noted that the reported figures on CRE infection rates among colonized patients are similar to those observed in larger multicenter studies,[29] as is the all-cause 6-month mortality rate.[1] Finally, the study is descriptive and predominantly associative in nature.

In conclusion, the GM structure and functionality of CRE_I patients were distinct compared with both uncomplicated patients and those who were CRE carriers but did not develop infection or those with non-CRE infection, up to 1 month after LT. Such differences were mostly reflected in higher proportions of potential antimicrobial-resistant pathogens and functions conferring them selective advantages, with poor representation of bacteria and metabolic pathways capable of promoting overall host health, particularly that of the gut–liver axis. The fact that these features were partly detectable even before LT suggests that GM profiling could improve the accuracy of patient stratification and risk prediction algorithms. In addition, our data pave the way for the development of GM-based intervention strategies (*e.g.* using prebiotics, probiotics, and postbiotics) to 'correct' unfavorable traits before LT. This approach has the potential to reduce LT-related complications and improve the overall prognosis of these patients. Further studies should be conducted in larger cohorts, especially for CRE_I and CRE_UI patients, and possibly use techniques such as metabolomics to validate GM functionality and its actual contribution to short- and long-term clinical outcomes of LT.

## Abbreviations

ACLF, acute-on-chronic liver failure; ARO, Antibiotic Resistance Ontology; BSI, bloodstream infection; CAG, co-abundance group; CARD, Comprehensive Antibiotic Resistance; CMV, cytomegalovirus; CRE, carbapenem-resistant Enterobacterales; CRE_I, CRE carriers who developed CRE infection; CRE_UI, CRE carriers who did not develop infection; FDR, false discovery rate; GM, gut microbiome; HCC, hepatocellular carcinoma; INF, non-CRE carriers who developed a microbial infection; LPS, lipopolysaccharide; LT, liver transplant; MELD, model of end-stage liver disease; NDM, New Delhi Metallo-beta lactamase; NEG, non-CRE carriers who did not develop severe infection or BSI; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; SCFA, short-chain fatty acid.

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## Conflicts of interest

The authors of this study declare that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

Conceptualization and design of the study: FD, MRi, RP, MG, ST, PB, MG. Acquisition of data: RP, MRi, SC, MF, MC, MRa, SA. Analysis and interpretation of data: MRi, RP, FD, MF. Writing – original draft: FD, MRi, RP. Review and editing: MCM, ST, PB, MG. Supervision: PV, PB.

## Data availability statement

Data supporting this study will be available upon reasonable request.

## Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101039.

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*Author names in bold designate shared co-first authorship*


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