HHS Public Access

Author manuscript

Gastroenterology. Author manuscript; available in PMC 2022 August 01.

Published in final edited form as:

Gastroenterology. 2021 August ; 161(2): 508–521.e7. doi:10.1053/j.gastro.2021.04.013.

Impact of Antibiotic Resistance Genes in Gut Microbiome of Patients With Cirrhosis

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Abstract

BACKGROUND AND AIMS: Cirrhosis is associated with changes in intestinal microbiota that can lead to hepatic encephalopathy (HE) and infections, especially with antibiotic-resistant organisms. However, the impact of gut microbial antibiotic resistance gene (ARG) burden on clinical outcomes is unclear. The aims of the study were to determine the impact of ARGs in cirrhosis-related gut metagenome on outcomes and disease progression, study the effect of rifaximin on ARG burden, and compare ARGs in cirrhosis with chronic kidney disease (CKD) and diabetes.

METHODS: In outpatients with cirrhosis who underwent metagenomics, we evaluated change in ARG abundances with progression and their multivariable impact on 90-day hospitalizations

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Conflict of interest

Jasmohan S. Bajaj's institution has received grants from Bausch health (USA), Grifols, and Kaleido. No other conflicts exist.

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Supplementary Material

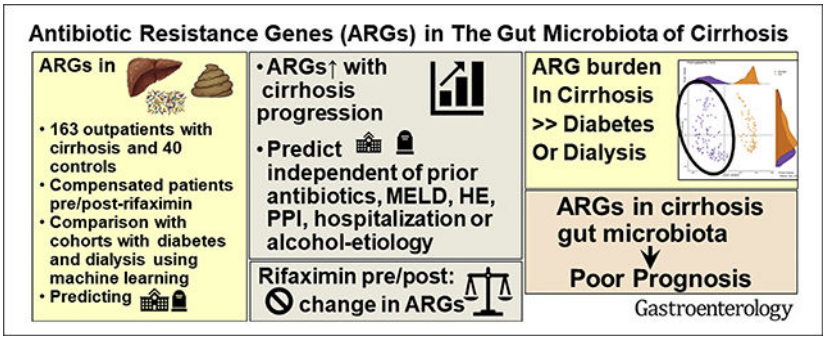
Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2021.04.013.

and deaths over 1 year. We also studied ARGs pre- and 8 weeks post-rifaximin in patients with compensated cirrhosis in an open-label trial. Finally, ARGs from CKD and diabetes studies were compared with cirrhosis on machine learning.

RESULTS: A total of 163 patients with cirrhosis (43 compensated, 20 ascites-only, 30 HE-only, 70 both) and 40 controls were included. ARG abundances were higher in cirrhosis versus controls and worsened with advancing cirrhosis severity; 44 patients were hospitalized and 14 died. ARG abundances were associated with hospitalizations and mortality while controlling for cirrhosis complications, medications, and demographics. *Rifaximin trial:* ARG abundance patterns were minimally affected in 19 patients post-rifaximin. *CKD/diabetes comparison:* ARG abundance patterns in cirrhosis are distinguishable on machine learning and include more gram-positive ARGs.

CONCLUSIONS: Cirrhosis is associated with high gut microbial ARG gene burden compared with controls, which worsens with disease progression and may be different from CKD and diabetes. ARGs are not affected by rifaximin and are associated with hospitalizations and death.

Graphical Abstract



Keywords

Ascites; Hepatic Encephalopathy; Rifaximin; Infections; Machine Learning

Patients with cirrhosis have a high likelihood of antibiotic resistance carriage and higher relative abundance of bacteria with pathogenic potential or “pathobionts” that is associated with poor outcomes.[1–4] These poor outcomes and higher mortality could be related to hepatic encephalopathy (HE) and infections, especially with drug-resistant organisms.[5,6] The summation of all antibiotic resistance genes (ARGs) and their precursors within a microbial community is termed a “resistome”; however, its clinical evaluation is currently limited to culture-based techniques.[7,8] Prior studies in cirrhosis have shown altered composition and function of intestinal microbiota, which can affect clinical outcomes.[9–11] With advancing cirrhosis, there is a higher relative abundance of pathobionts[12] that could express ARGs.[13–15] In addition, these ARGs can represent survival and quorum-sensing strategies that can predate antibiotic exposure,[16] regulate the microbial ecological dynamics in the gut, and determine survival in complex multiorganismal mucosal surfaces[17]; however, whether these are unique to cirrhosis or are a sequelae of chronic diseases per se is unknown. The knowledge of species with ARGs that are associated with poor outcomes

could be used as prognosticators and facilitate development of novel or targeted therapeutic strategies directed toward them rather than the broad-spectrum antimicrobial treatments that dominate clinical practice today.[18] However, the impact of rifaximin, a nonabsorbable, gut-specific antibiotic needs to be studied in the overall metagenomic context.[19]

We hypothesized that ARG burden would increase with advancing cirrhosis and provide prognostic information regarding hospitalizations and death independent of disease severity and prior antibiotic exposure. We also hypothesized that unlike traditional broad-spectrum antibiotics, rifaximin therapy would not increase the ARG burden.[20,21] Last, we hypothesized that the gut microbiota ARG burden is different in cirrhosis as compared with other chronic diseases associated with dysbiosis, such as chronic kidney disease on dialysis (CKD) and diabetes.

Materials and Methods

Subjects

Cross-sectional HE and hospitalization/death study.—Outpatients with cirrhosis and healthy controls between 21 and 75 years of age underwent informed consent before sample collection (Supplementary Figure 1, n = 203). Cirrhosis was defined by liver biopsy, endoscopic or radiological evidence of varices or porto-systemic shunting in chronic liver disease, frank decompensation, or through transient elastography. Patients unable to provide consent or samples, those with HIV infection, prior transplantation, those with alcohol abuse or probiotic use within the prior 8 weeks, with other organ failures (chronic kidney disease on dialysis [CKD], congestive heart failure, chronic obstructive pulmonary disease), cancer, those with other gastrointestinal diseases, or those in whom the diagnosis of cirrhosis was unclear were excluded. Patients were divided into compensated (no prior or current history of HE or ascites) and decompensated (ascites-only, HE-only, or both). Those with HE were further subdivided into those on lactulose only (Cirr-L), and those on lactulose and rifaximin (Cirr-LR). Hospitalizations and antibiotic exposures 6 months before sample collection were analyzed. Healthy controls were recruited through word of mouth and through community advertising and were all Virginia-based following a Western diet. Only individuals who were free of chronic diseases, including metabolic syndrome, autoimmunity, and intestinal disorders, and were not on prescription medications or chronic over-the-counter medications, including proton pump inhibitors (PPIs), were considered healthy controls. In addition to fecal samples, data pertaining to cirrhosis severity and concomitant medications were also recorded. All patients with cirrhosis were followed for 90 days for development of nonelective hospitalizations and for 1 year for risk of death using chart review. Details of hospitalizations were also noted. In keeping with clinical practice, all included patients were seen in clinic at least every 6 months. Based on our prior study using 16S ribosomal RNA (rRNA) and logistic regression, we were able to predict the role of microbiota on 90-day hospitalizations that occurred in 25% of subjects with 145 patients with cirrhosis.[22] This was used as our minimal sample size.

Pre/post-rifaximin study.—Compensated outpatients with cirrhosis between 18 and 65 years of age without prior or current HE, with cognitive impairment or minimal

HE on paper-pencil tests and without allergies to rifaximin or current/prior therapy for HE, prior episodes of HE, prior transjugular intrahepatic porto-systemic shunting, those unable to give informed consent, were recruited after informed consent in an open-label trial (Supplementary Figure 1). The clinical trial results and microbiome profile of these participants as measured by 16S rRNA gene profile are previously published.[23] Cirrhosis-associated clinical details and stool for microbiota were collected at baseline. They were administered 550-mg capsules of rifaximin twice a day for 8 weeks, at which point adherence was evaluated and repeat stool sample was collected.

The protocols and biorepository were approved by the institutional review boards at the Virginia Commonwealth University and Richmond VA Medical Center, and all subjects gave written informed consent before participation for the procedures and for the biorepository. Remaining methods are in the supplement.

Analyses of ARGs

Metagenomic analyses were performed at Diversigen (www.diversigen.com) and reads after quality trimming were mapped against antimicrobial resistance (AMR) accessions available CARD v.3.1.0 (Comprehensive Antibiotic Resistance Database https://card.mcmaster.ca/).[24,25] The CARD includes well-characterized, peer-reviewed resistance determinants and associated antibiotics, which is updated monthly. This database includes 88 pathogens, 9560 chromosomes, 21,362 plasmids, 102,181 whole-genome sequencing assemblies, and 222,011 alleles. The outputs are organized by the Antibiotic Resistance Ontology (ARO) and AMR gene detection models. The database also determines computer-generated resistome predictions for the sequenced genomes, plasmids, and whole-genome shotgun assemblies available at the National Center for Biotechnology Information for these 88 pathogens. These resistomes include sequence variants beyond those reported in the scientific literature, as predicted by the Resistance Gene Identifier.[25]

Bio-informatics Analysis

We analyzed ARG changes between compensated, HE-only, ascites-only, and patients with both on false discovery rate (FDR)-corrected Kruskal-Wallis tests. Using BiomMiner, which used DESeq2 and Kruskal-Wallis tests (FDR-corrected), we compared patients with cirrhosis with controls, then HE versus no-HE, and finally those who required hospitalizations at 90 days and death in 1 year[26,27] (Supplementary Materials). We compared ARG patterns (ARO terms, resistomes, and AMR genes). Then we performed these analyses using MaAsLin2 for hospitalization and death for the ARG patterns including clinical variables such as age, gender, alcohol etiology, diabetes, PPIs, lactulose, rifaximin, decompensation status (compensated/HE-only, ascites-only, both) and model for end-stage liver disease (MELD) score.[28] Separate analyses were also performed for PPI use. Finally, a similar analysis of bacterial species and ARG patterns were performed for patients pre- and post-rifaximin.[29]

Comparison With Papers on CKD and Diabetes

We analyzed metagenomic outputs from 2 other articles: Qin et al[30] for type 2 diabetes (T2D; n = 170), and Wang et al[31] for CKD on dialysis (n = 223) that had similar

demographic profiles and metagenomic analyses details (Supplementary Material). Last, an Orange cross-validation modeling and prediction workflow (Supplementary Figures 2 and 3) was used to differentiate the study outputs based on these ARG patterns.[32] Specifically, we developed a prediction pipeline using the Orange data mining tool to determine the predictive powers of the best performing classifiers. The ARG samples were split using a WEKA workflow into training datasets (80%) for modeling and a naïve hold-out datasets (20%) to test the predictive accuracy of the trained model. The model was trained using 5-fold cross-validation on the training dataset and then the Orange prediction function was used on each blinded sample in the naive hold-out set to classify it.

Results

Forty healthy controls and 163 patients with cirrhosis (43 compensated, 30 HE-only, 20 ascites-only, and 70 with both, Table 1) were included. When comparing vis-à-vis HE, 63 were without prior HE, and 100 had prior HE, of whom 43 were Cirr-L and 57 were Cirr-LR (Supplementary Table 1). Patients with Ascites+HE had a higher MELD and greater alcohol-related etiology, PPI use, spontaneous bacterial peritonitis (SBP) prophylaxis, lactulose and rifaximin use, and prior hospitalizations/antibiotic use compared with the rest. Demographics, dietary characteristics, and diabetes were similar. All patients were seen in clinic as standard of care at least once in 6 months before and 59 patients had required an upper endoscopy for variceal surveillance or eradication within the past year. Of the 49 people hospitalized 6 months before sample collection, most were in the ascites+HE group who were admitted a median of 1 (interquartile range 0–2) times. Most hospitalizations were due to liver-related reasons (ascites n = 13, HE n = 17, acute kidney injury n = 11, others n = 8). Exposures to antibiotics were also highest in ascites+HE, equivalent across the HE-only/ascites-only and lowest in compensated patients. Most antibiotics were administered for short courses (<14 days) within hospitalizations. The remaining were administered for outpatient urinary tract infections or suspected upper respiratory tract infections. None were diagnosed with *Clostridioides difficile* infection or required vancomycin; 14 patients received fluoroquinolones, 16 received cephalosporins, 3 amoxicillin-clavulanate, 3 metronidazole, 3 macrolides, and 3 trimethoprim-sulfamethoxazole. Patients with HE had a greater rate of PPI use and alcoholic etiology of cirrhosis and were more likely to be men (Supplementary Table 1). Age and diabetes prevalence were similar regardless of HE/no-HE. When Cirr-L and Cirr-LR groups were compared, we did not find significant differences on demographics, PPI use, MELD score, or alcohol-related etiology. All participants were Virginia-based and were on similar Western diet and on 7-day dietary recall had similar caloric intake (Table 1). On follow-up, 44 patients were hospitalized over 90 days and 14 died over 1 year (details later in this article). A separate group of patients with compensated cirrhosis were included in the rifaximin trial (Supplementary Figure 1).

Cirrhosis Is Associated With Greater Burden of ARGs Relative to Healthy Controls

Bacterial species that were highest in controls, which reduced over the disease spectrum, belonged to *Faecali-bacterium, Alistipes, Eubacterium,* and other short-chain fatty acids producers, such as *Dorea, Subdoligranulum,* and *Roseburia* (Supplementary Table 2). Patients with cirrhosis had greater abundance of resistomes associated with pathobionts

belonging to Enterobacteriaceae, as well as *Streptococcus, Enterococcus,* and *Acinetobacter* spp (Supplementary Tables 3–5). These resulted in greater abundance of resistance patterns focused on beta-lactamases, macrolide, quinolone, glycopeptide, fosfomycin, and tetracycline resistance, and those focused on generic AMR pathways compared with controls (Supplementary Tables 3–5, Figure 1). There was a statistically significant difference in the species distribution based on the Bray-Curtis permutational multivariate analysis of variance (PERMANOVA) analysis ($P = .001$).

ARG Patterns Across Decompensating Events

On Kruskal-Wallis (Table 2), AMR genes higher in ascites and both belonged to aminoglycoside *(ANT, APH),* sulfonamide resistance and selected beta-lactamases *(SHV, CTX-M, SRT)* and efflux pumps. Others were found only in decompensated patients regardless of complication (porins). Aminocoumarin-resistant parY, lincosamide resistance, ileS, RpoB, and PDC beta-lactamase were higher in patients with HE regardless of ascites. These patterns were reflected in ARO terms with greater membrane fusion pump efflux complex *(Mex)* and *Klebsiella*-related genes in decompensated patients. *Streptomyces* spp, *Lactococcus, Bifidobacterium,* and *Staphylococcus aureus* resistomes were higher were higher in patients with HE, whereas *Klebsiella* and *Shigella* spp were lower. Ascites, regardless of HE, was associated with greater abundance of *Pseudomonas, Serratia,* and *Clostridium perfringens.*

ARG Patterns in Patients With Prior HE Compared With Those Without HE

Specific microbial changes showed lower abundance of species belonging to Lachnospiraceae and Ruminococcaceae in those with HE, whereas *Streptococcus, Lactobacillus, Enterococcus,* and *Escherichia* spp were higher in those without HE (Supplementary Table 6, Supplementary Figure 4). When ARGs were analyzed, patients with HE had greater abundance of resistomes focused on *Staphylococcus, Listeria, Streptococcus, Pseudomonas,* and *Bifidobacterium* spp relative to no-HE patients (Supplementary Tables 7–9, Supplementary Figure 4). ARG abundance of beta-lactamase, vancomycin resistance, as well as RbpA bacterial RNA polymerase binding protein were higher in HE, whereas quinolone resistance genes were higher in those without HE. These patterns were also followed when ARO terms were analyzed between patients with and without HE. Despite these changes on DESeq2, species distribution based on the Bray-Curtis PERMANOVA analysis did not show significant differences in ARG patterns ($P = .11$, ARO term, $P = .113$ AMR gene family, and $P = .12$ resistomes) between patients with/without HE.

ARG Patterns in Patients on PPI Compared With Those Without PPI

Because patients on PPIs had more advanced cirrhosis vs the rest (Table 1, Supplementary Table 1), we performed MaAsLin2 for ARG terms. We found that PPI use was associated with *Enterococcus faecalis*- and *Enterococcus faecium*-related genes, that is, higher Vancomycin resistance (VanYB, VanRB, VanHB, VanB) ARO terms and VanH, VanX, VanY AMR gene families. However, none of the resistomes survived FDR.

Hospitalizations

Forty-four patients needed hospitalizations at 90 days, which was most frequent in more advanced patients (Table 1). The major reasons were HE (n = 19) followed by acute kidney injury and electrolyte disturbances (n = 7), infection (n = 11), gastrointestinal bleeding (n = 3), and others (n = 9). Of the 11 infections, methicillin-resistant *Staphylococcus aureus* was found in 3 patients (2 bacteremia and 1 SBP), *Candida* spp in 3 patients, *Streptococcus viridans* bacteremia in 1 patient, and no organism isolated in 4 patients (cellulitis and pneumonia in 2 each). Three patients had 2 infections during the same hospitalization (SBP followed by urinary tract infections).

Bacterial species distribution based on the Bray-Curtis PERMANOVA analysis was not significantly different ($P = .121$) and there was no difference in the Shannon diversity between groups (2.87 ± 0.75 not hospitalized vs 2.83 ± 0.69 hospitalized, $P = .178$). Potentially beneficial taxa belonging to species in Ruminococcaceae and Lachnospiraceae associated with lower risk of hospitalizations (Supplementary Figure 5, Supplementary Table 10 and 11). Pathobionts belonging to *Enterobacter*, *Pseudomonas*, *Yersinia,* and *Enterococcus* spp remained associated with a greater risk of hospitalizations despite controlling for clinical factors by MAAsLin2 (Supplementary Table 11). Also, using MAAsLin2 (Table 3), aminoglycoside-2-O-nucleotidyltransferase (*ANT [2])* gene, one of the most common determinants of enzyme-dependent aminoglycoside resistance prevalent in in gram-negative bacteria were associated with higher hospitalization risk, whereas generic AMR genes related to rifamycin, aminocoumarin, and lincosamide ribosomal RNA methyltransferase were associated with lower hospitalization risk. ARO term associated with hospitalizations independent of clinical factors were *dfrA12, InUA, MexE, OXY* beta-lactamase, and *VanVB.* Of these, *dfrA12* is present in several pathogenic gram-negative species *(Acinetobacter baumannii, Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Shigella,* and *Morganella* spp), *MexE* is present in *Pseudomonas* spp, and is a multidrug efflux complex whereas *OXY 1–6* beta-lactamase is found in *Klebsiella* spp. *VanVB* is a vancomycin-resistant ARG found in *E faecalis,* whereas *InuA* is a plasmid-mediated nucleotidyl-transferase found in several pathobionts belong to *Enterococcus, Staphylococcus, Listeria,* and *Escherichia* spp. These data were found in resistomes as well, where gram-negative bacteria, including *Citrobacter* were associated with hospitalization, whereas *Streptomyces* spp were protective independent of clinical factors. Regardless of whether composite of decompensation (compensated, ascites-only, HE-only, or both) or no-HE or HE were considered, the ARG analyses contribution toward hospitalizations was similar (Table 3, Supplementary Table 12).

Deaths Over 1 Year

Fourteen patients died; all of whom had both HE and ascites. All deaths occurred due to liver-related reasons: infections in 10, variceal bleeding in 2, and the rest with cancer. As shown in Supplementary Table 13, patients who died were more likely to have pathobionts *(Pseudomonas, Serratia, Klebsiella, Proteus* spp) with *Lactobacillus* spp, and relatively lower autochthonous taxa (*Lachnospira* spp, *Prevotella copri;* Supplementary Figure 5). Bray-Curtis PERMANOVA analysis demonstrated $P = .05$ for bacterial species between those who died vs survived but no changes in Shannon diversity were seen

(survived 2.86 ± 0.71 vs died 2.77 ± 0.89, $P = .75$). Microbial changes focused on *Clostridium, Pseudomonas, Lactobacillus,* and *Neisseria* spp were associated with death, whereas *Streptococcus*, *Veillonella,* Lachnospiraceae, and *Bacteroides* spp were associated with protection on MAAslin2 (Supplementary Table 14).

Similar to hospitalizations, *ANT* (2") was the AMR gene associated with death (Table 4). *ANT, MexE/M,* and *dfrA12* were higher in those who died, with beta-lactamase *(TEM), TriB (P aeruginosa), LnUp* (Lincosamide resistance), *tetS* (tetracycline ribosomal protection protein in *E faecalis),* and *tet(B)* present in gram-negative bacteria (Supplementary Table 15). Only *CfxA6,* which is a beta-lactamase from an uncultured bacterium, was associated with lower death. As expected, MELD score and greater decompensation were associated with death. When resistomes were considered, MELD score, HE, and lactulose use were associated with death, along with *Legionella*, gram-negative bacteria, and *Enterobacter* spp. Firmicutes members belonging to *Desulfitobacterium* spp were protective. Similar to hospitalizations, ARG patterns associated with death were similar regardless of whether composite of decompensation or no-HE or HE were considered (Table 4, Supplementary Table 15).

Pre/post-Rifaximin Trial Did Not Show Major Changes in ARG Patterns

Rifaximin was well-tolerated and safe as published.[23] One sample could not be located so we analyzed samples of 19 subjects. Shannon diversity or beta-diversity in bacterial species (PERMANOVA $P = .199$) was not changed from baseline. On DESeq2, post-rifaximin there was a significant increase in autochthonous species such as *Blautia, Butyricimonas, Lactobacillus,* and *Eubacterium* and lower *E faecium* and *Clostridium scindens* after rifaximin therapy (Figure 2). Rifaximin did not significantly change AMR gene abundance and only reduced *Klebsiella oxytoca* resistome abundance. On ARO terms, there was a reduction in gram-negative resistance patterns *(Escherichia coli acrA, marA,* and *H_NS,* which are involved in antibiotic efflux) and beta-lactamases *(SRT2* and *TEM219).* Only *VanI* marginally increased post-rifaximin.

Cirrhosis Is Associated With Higher Burden of ARGs Relative to CKD, and Diabetes, Whereas Controls Are Largely Similar

Type 2 diabetes.—Qin et al[30] studied 170 Chinese patients with T2D with similar age as our patients. Figure 3 shows a significant separation on principal coordinates analysis between the groups, which were significant on PERMANOVA (all $P < .001$) for ARG pattern comparisons. This separation was maintained even when patients with cirrhosis with or without diabetes were compared with patients with diabetes alone (Supplementary Figures 6 and 7). Patients with cirrhosis had a higher number of ARGs compared with diabetes, with resistomes being higher in cirrhosis belonging to a wide range of gram-positive and negative microbes with pathogenic potentials, whereas patients with diabetes had a relatively narrower range of resistome representation (Supplementary Figures 8 and 9, Supplementary Tables 16–18). AMR gene families and ARO terms were relatively similarly spread between the 2 conditions spanning vancomycin, beta-lactamase, and quinolone resistance. There were only 6 ARGs different between the cirrhosis controls and the T2D controls (Supplementary Figure 10A).

Chronic kidney disease.—Wang et al[31] studied 223 patients with CKD on dialysis. The authors excluded recent antibiotic use and those with major nonrenal diseases including liver disease. Although patients were from China, their demographics and nonvegetarian diet intake were largely similar to our cohort. As shown in Figure 3, there was a significant separation on principal coordinates analysis between cirrhosis and CKD with PERMANOVA $P < .001$ for all 3 comparisons. Compared with CKD, patients with cirrhosis had a greater number of AMR, ARO, and resistome log-fold changes (Supplementary Figures 8 and 9, Supplementary Tables 19–21). Despite this, several important pathobionts were higher in CKD, such as *Klebsiella pneumoniae, Acinetobacter, Enterobacter,* and *Legionella*. Patients with cirrhosis had higher *Escherichia, Staphylococcus, Enterococcus, C difficile, Klebsiella oxytoca,* and *Streptomyces* spp. Reflecting these, there were beta-lactamase genes in both conditions but glycopeptide, vancomycin, cephalosporinase, and rifamycin resistance genes were higher in cirrhosis. Patients with CKD also had greater ARO abundances belonging to a broad spectrum of gram-negative and -positive pathobionts. There were only 7 ARGs different between the cirrhosis controls and the CKD controls, indicating minimal confounders (Supplementary Figure 10B). On Kruskal-Wallis analyses, vancomycin and efflux pumps were seen higher in cirrhosis on AMR and ARO terms (Supplementary Figures 11 and 12). Several ARO terms belonging to multidrug efflux pumps along with beta-lactamases, macrolide, aminoglycoside, quinolone, and tetracycline resistance were uniquely higher in cirrhosis. Resistomes higher in cirrhosis were gram-negative pathobionts, *Streptococcus* spp, and *C difficile. Streptomyces* spp were also increased in cirrhosis compared with diabetes and CKD (Supplementary Figure 12).

Naïve machine-learning prediction model.—The average accuracy for all the naïve samples was for each class is presented in Supplementary Table 22 and Supplementary Figure 13). For ARO terms, AMR gene families and resistomes, there was an excellent separation from samples derived from our patients compared with the other study outputs based on models created. However, random forest was the best method to separate the groups and true positivity rate in cirrhosis on the naive samples was 97.9% for AMR gene families, 99% for ARO terms, and 100% for resistomes. It should be noted that the blinded naïve samples are not used in the model training and thus represent the true accuracy of the prediction model and could be used as an accurate diagnostic of new naïve samples.

Discussion

The results of the current study demonstrate that ARG abundances are higher in cirrhosis compared with healthy controls, increase with worsening disease regardless of ascites and HE, and unlike previously described with absorbable antibiotics, are not affected by rifaximin therapy. We also found that greater abundance of ARG is related to hospitalizations and death independent of cirrhosis severity, prior antibiotic exposure, hospitalizations, or concomitant medications. Moreover, the ARG profile of cirrhosis is distinct compared with outputs from 2 articles studying CKD and diabetes.

The underlying immune deficits, exaggerated inflammatory response, liver dysfunction, and multiple hospitalizations make cirrhosis a prime candidate for suffering these negative consequences.[6,33] Therefore, the carriage rate and potential impact and determinants of

ARGs needs to be defined in cirrhosis. Metagenomic and 16S rRNA gene analyses have consistently demonstrated a higher proportion of pathobionts in cirrhosis compared with controls, which worsen with progression of disease.[1,9,10,12,34–36] However, not all strains of potential pathobionts have ARGs associated with them, which is why we focused on those in the CARD database that have ARG genes mapped.

We confirmed prior metagenomic studies of patients with cirrhosis and determined that the relative abundances of pathobionts were higher compared with controls and worsened with advancing cirrhosis complexity. We extended prior studies by defining AMR gene families and their corresponding resistomes that were associated with this progression. In cirrhosis compared with controls, there was a higher abundance of beta-lactamase, vancomycin resistance, and quinolone resistance. This trend worsened with development of ascites, HE, and progression of cirrhosis.[37] As expected, decompensated patients had relatively higher ARO and AMR gene abundance, along with resistomes belonging to pathobionts. These could be due to exposure to the health care environment, because over the past 6 months they had been hospitalized and/or exposed to antibiotics or because of the greater abundance of organisms with these genes that may be used as a survival mechanism independent of antibiotics.[16]

In addition, most of the decompensated patients were on rifaximin and some on SBP prophylaxis. This is important because unlike a prior study in which ciprofloxacin, amoxicillin, and metronidazole exposure over 5 days significantly increased the ARG burden,[21] the use of rifaximin per se was not associated with this. This confirms and extends prior studies of rifaximin that demonstrate a low resistance footprint into the cirrhosis realm as well.[38,39] We also found that rifaximin was associated with greater abundance of potentially beneficial taxa, and reduction in resistomes of *Klebsiella* spp as well as gram-negative ARG abundance in the small trial. This was reiterated by finding similar changes in cross-sectional subjects with HE-only or HE+ascites patients who showed lower *Shigella* and *Klebsiella* and higher *Streptomyces* resistomes compared with ascites-only patients. The *Streptomyces* spp resistome increase in cirrhosis with HE, and in cirrhosis compared with CKD and diabetes is interesting because these organisms are the source of rifamycin, from which rifaximin is derived.

The potential beneficial effect of rifaximin against hospitalizations that has been noted in HE and other gut-derived outcomes, such as SBP, could be the reason why *Streptomyces* resistomes,[40] even though higher in cirrhosis and HE, were associated with protection from hospitalization.[20,41] The favorable effect on gram-negative resistomes with rifaximin could potentially be one of the reasons behind the reduction in hospitalizations because of HE and potentially other complications of cirrhosis with rifaximin use, and association with protection against *C difficile* infection[42] and traveler's diarrhea.[43] It also clarifies that the worsening ARG burden with cirrhosis progression reflects the underlying disease process and is not a rifaximin-related epiphenomenon.

The occurrence of ARGs could be due to exposure to health care systems and antibiotics and/or the selection of these genes as a means to enhance trans-kingdom and quorum-sensing communications.[16] These are supported by studies in antibiotic-unexposed and

natural systems and in organisms exposed to sub-MIC antibiotic concentrations of antibiotics show ARG expressions that are distinct from the effect seen after exposure to adequate antibiotic concentrations.[40,44–46] Therefore, despite controlling for prior antibiotic exposure and hospitalizations, ARG patterns were associated with poor outcomes in cirrhosis but most of these outcomes were not antibiotic-resistant infections. However, the gut remains a major reservoir of these organisms.[15,47] Unique patterns for HE and ascites-related ARG carriage and PPI use were found but on multivariable analysis, several of the genes found to be higher in decompensated patients on Kruskal-Wallis tests were also associated with hospitalizations and death. Prominently aminoglycoside resistance (*ANT2*) and membrane fusion pump efflux complex *(MexE)* found in gram-negative taxa were higher in those with negative outcomes while those potentially associated with rifaximin use (rpoB) and *Streptomyces* spp were protective against negative outcomes. Therefore, the presence of specific ARGs are additive prognosticators of a hostile gut milieu that can predict negative infectious and noninfectious outcomes despite controlling for clinical factors.

The focus on cirrhosis is necessitated by the comparison with several other diseases that are often comorbid or complicate the course of this disease. Diabetes is often found in cirrhosis, and cirrhosis can result in renal impairment and requirement for dialysis.[48] None of our patients with cirrhosis were on dialysis. Notwithstanding differences in cohorts, the greater ARG burden in cirrhosis as well as major separation between the CKD compared with cirrhosis likely reflects the major role of liver in the regulation of the gut-liver axis and gastrointestinal immune response.[49] Our finding of higher gram-positive resistomes and vancomycin resistance ARGs in cirrhosis extends prior studies of alcohol-related liver disease into the cirrhosis realm and could reflect the key role of the liver in clearing grampositive bacterial translocation.[49–51] The higher ARG burden in cirrhosis vs CKD is striking because these conditions are associated with high use of antibiotics, impaired systemic immune response,[52,53] and high carriage of resistant organisms.[54,55] We found that ARG patterns were different between cirrhosis and diabetes but, unlike that in CKD, this was spread out. The frequent coexistence of diabetes and cirrhosis may make this differentiation less relevant patho-physiologically.[48] Regardless of the comparison, we found a unique signature of ARGs in cirrhosis consisting of several gram-negative rods and *C difficile* and *Streptococcus* spp that are associated with infections and poor prognosis.[56–58]

These findings as well as the contribution of ARGs toward negative outcomes in cirrhosis demonstrate that this burden is clinically relevant and could be harnessed to enhance prognostication. Therapies that beneficially modulate the gut microbiota, such as fecal microbiota transplant, can reduce the ARG burden in patients with and without cirrhosis.[18,21] Therefore, focusing on patients with cirrhosis that have a high ARG burden can not only improve the prognostication but also potentially select them for therapeutic options.

Our study is limited by the cross-sectional sampling, relatively small number of patients pre/post-rifaximin, and using previously published metagenomic datasets for comparison. However, our recent short-term longitudinal follow-up of patients over 15 to 30 days who were randomized to placebo or standard-of-care arms in fecal microbiota transplant trials

did not show appreciable changes in ARG abundance.[21] Also, several factors such as HE, prior or current antibiotics, PPIs, and other medications can affect the ARG burden. We controlled for these using individual comparisons, FDR, and multivariable analyses and found consistent changes across groups. This increases confidence in the generalization of these results into the clinical population, which often have all these factors as part of their treatment regimen. Although patients in CKD and diabetes studies were from China, the use of metagenomic libraries was similar and their demographics and other data were largely comparable to our dataset and there were few differences between their controls and ours. This was like earlier US and China data on 16S rRNA sequencing in cirrhosis vs controls[59,60]; however, systematic differences, including diet and socio-cultural impacts cannot be excluded. Finally, these data demonstrate association but not causation of the role of ARGs in disease progression.

We conclude that patients with cirrhosis have a high burden of ARGs compared with controls, which worsen with disease progression. Rifaximin modulates ARGs favorably, unlike absorbable antibiotics. ARGs focused on gram-negative rods are associated with 90-day hospitalizations and death over 1 year independent of clinical factors, which could refine prognostication. This ARG burden in cirrhosis is different and may be higher from that found in diabetes and CKD based on outputs from 2 previous studies. Strategies that focus on detection of ARGs for prognosis and predicting outcomes and targeting them for therapy in this era of rampant antibiotic overuse could improve the prognosis in cirrhosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Microbial sequences, which are the basis for the figures, will be deposited in a publicly accessible database before publication. The SRA IDs are as follows; current study: PRJNA678582, Qin et al Type 2 diabetes: PRJNA422434, Wang et al chronic kidney disease: PRJNA449784. Due to institutional review board restrictions, metadata that are potentially identifiable according to US law are not available from our dataset.

Funding

This work was partly supported by VA merit review 2I0CX00176, NCATS R21TR002024, and R21TR003095.

Abbreviations used in this paper:

AMR	antimicrobial resistance
ARG	antibiotic resistance gene
ARO	antibiotic resistance ontology
CKD	chronic kidney disease
FDR	false discovery rate
HE	hepatic encephalopathy

MELD	model for end-stage liver disease
PERMANOVA	permutational multivariate analysis of variance
PPI	proton pump inhibitor
rRNA	ribosomal RNA
T2D	type 2 diabetes
SBP	spontaneous bacterial peritonitis

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Cirrhosis is associated with gut microbial dysbiosis and a growing burden of antibiotic-resistant infections. However, the impact of antibiotic resistance genes on cirrhosis-related outcomes is unclear.

NEW FINDINGS

Cirrhosis is associated with high burden of gut microbial antibiotic resistance genes abundance compared with controls, which worsens with disease progression and may be different from other diseases. Antibiotic resistance genes, which are impacted by most common antibiotics, are not affected by rifaximin therapy and are associated with hospitalizations and death independent of clinical factors.

LIMITATIONS

Cross-sectional analysis of cirrhosis and small sample size in patients pre- and post-rifaximin. Comparisons with other diseases based out of studies from geographically disparate populations.

IMPACT

Strategies that focus on detection of antibiotic resistance genes for prognosis and predicting outcomes, encouraging use of nonabsorbable antibiotics, such as rifaximin, and development of therapeutic strategies to limit antibiotic resistance gene burden this era of rampant antibiotic overuse could improve the prognosis in cirrhosis.

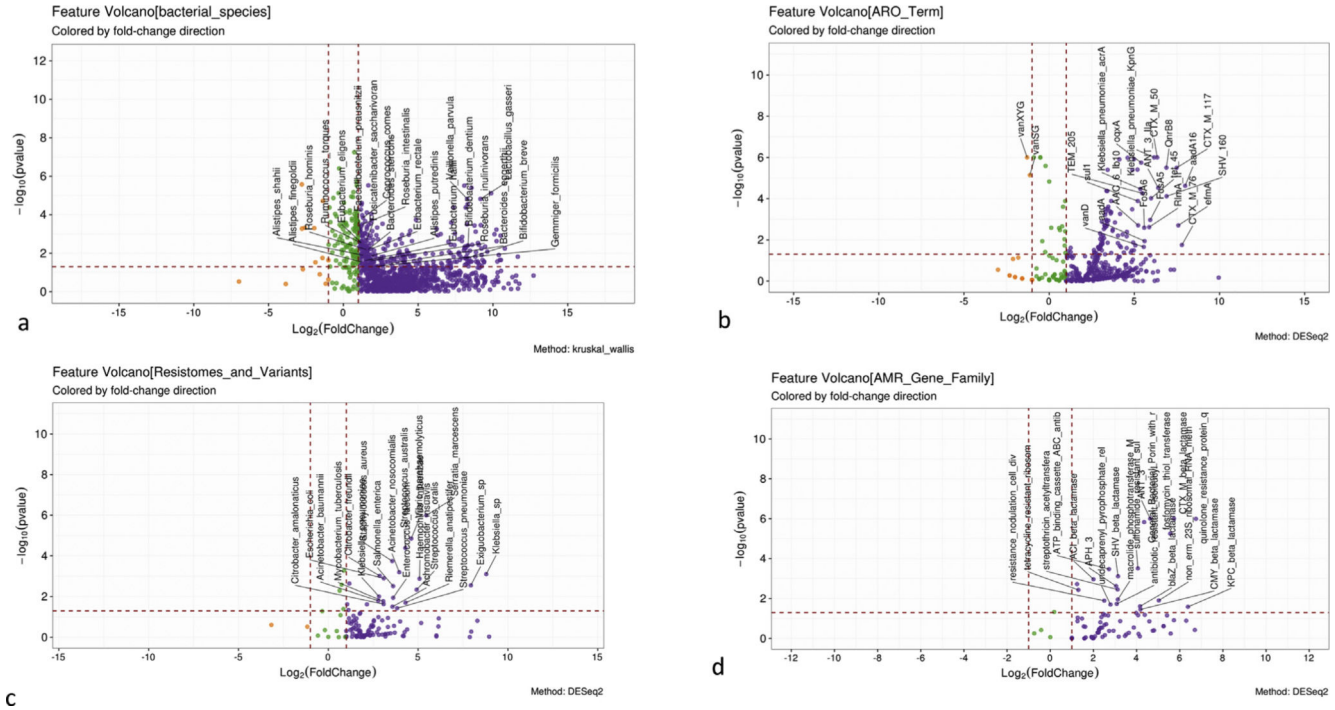

Figure 1.

Comparison of healthy controls and cirrhosis. For all comparisons, *purple* is higher in cirrhosis, *orange* is higher in controls. (*A*) Volcano plot of Kruskal-Wallis comparison of bacterial species. (*B*) Volcano plot of DESeq2 lineage of ARO terms. (*C*) Volcano plot of DESeq2 lineage of resistomes and variants. (*D*) Volcano plot of DESeq2 lineage of AMR gene families.

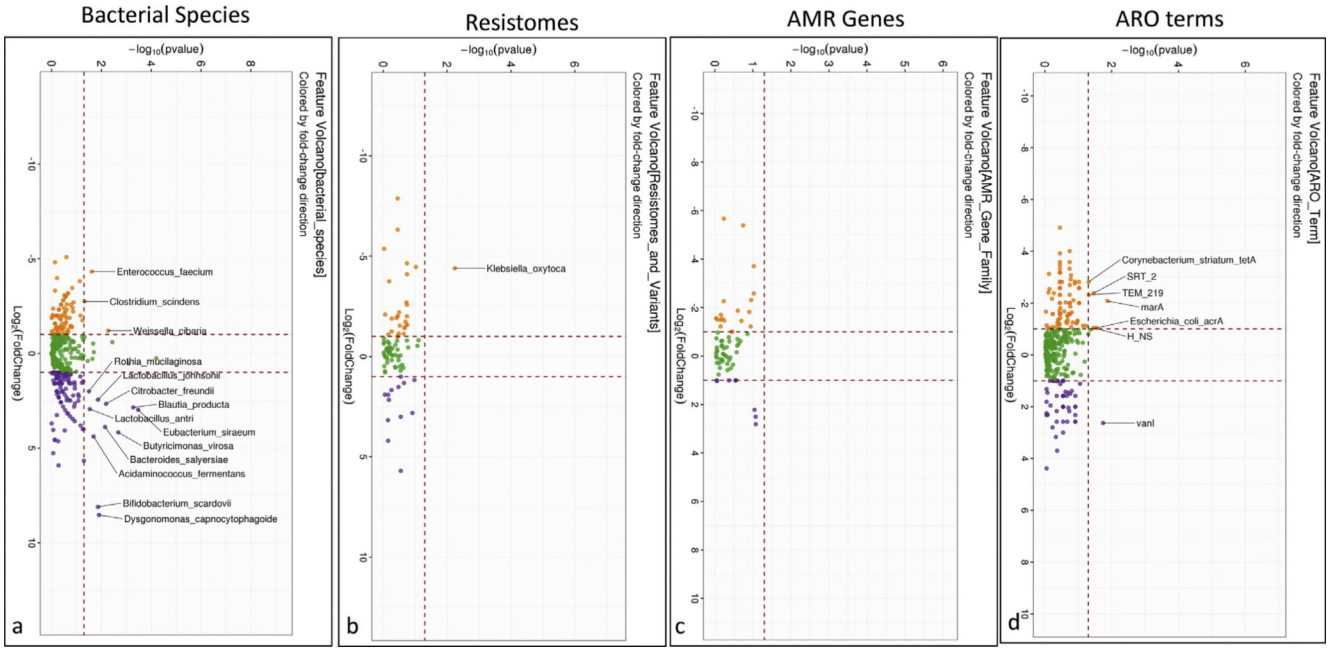

Figure 2.

Comparison of microbial and ARG changes before and after rifaximin. (*A*) Volcano plot of DESeq2 lineage of bacterial species compared between pre- (*orange*) and post-rifaximin (*purple*). (*B*) Volcano plot of Kruskal-Wallis comparison of resistomes that changed between pre- (*orange*) and post-rifaximin (*purple*) showing higher *Klebsiella oxytoca* pre, which was not found post-rifaximin. (*C*) Volcano plot of Kruskal-Wallis comparison of ARO terms that changed between pre- (*orange*) and post-rifaximin (*purple*) showing no significant change between the time-points. (*D*) Volcano plot of Kruskal-Wallis comparison of AMR gene families that changed between pre- (*orange*) and post-rifaximin (*purple*) showed reduction in baseline AMR gene expressions after rifaximin.

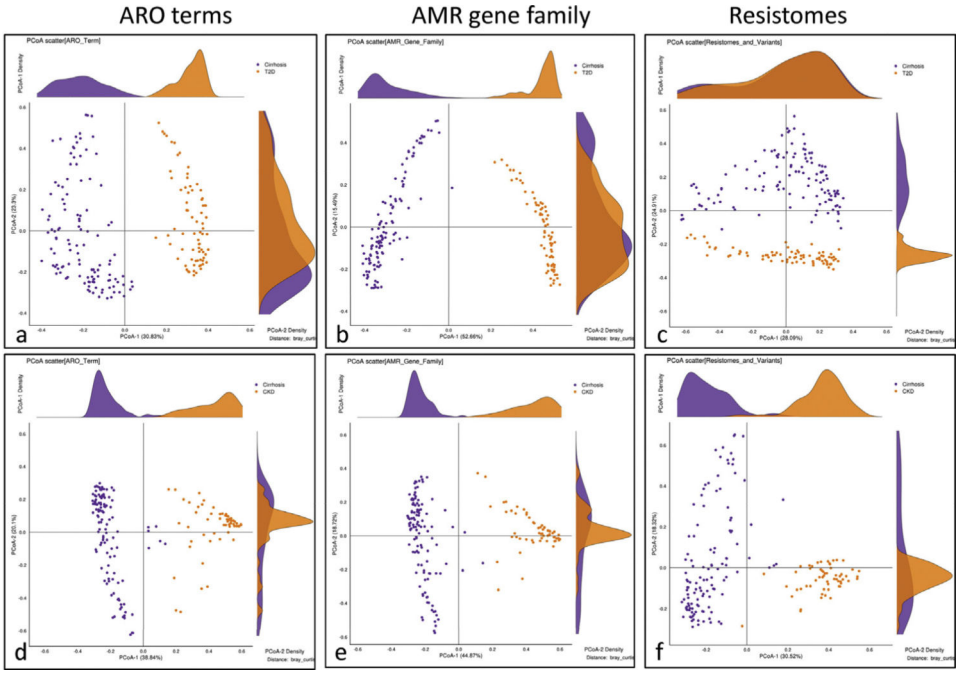

Figure 3.

Principal coordinate analysis of cirrhosis compared to other chronic diseases. (*A-C*) Comparison of cirrhosis (*purple*) with T2D (*orange,* Qin et al[30]) showing clear separation between the groups on resistome, AMR gene family, and ARO term abundances. (*D-F*) Comparison of cirrhosis (*purple*) with CKD on dialysis (*orange,* Wang et al[31]) showing clear separation between the groups on resistome, AMR gene family, and ARO term abundances.

Table 1.

Characteristics of Subject Groups

		All cirrhosis (n = 163)				
			Decompensated cirrhosis (n = 120)			
	Controls (n = 40)	Compensated (n = 43)	HE only (n = 30)	Ascites only (n = 20)	Both (n = 70)	*P* value
Age (*y*)	58.6±10.3	60.3±7.2	61.0±11.4	59.2±10.1	60.1±8.9	.12
White/Black/Other	26/13/1	31/12/0	23/7/0	15/5/0	49/18/3	.85
Latinx/Not	5/35	2/41	3/27	2/18	7/63	.46
Gender (male)	24 (60)	36 (84)	15 (50)	16 (80)	59 (84)	.30
Proton pump inhibitor	0 (0)	16 (37)	14 (47)	12 (60)	49 (70)	<.0001
Type 2 diabetes	0 (0)	22 (51)	9 (30)	7 (35)	23 (33)	.35
Hospitalized 6 mo prior	0 (0)	3 (7)	6 (20)	2 (10)	38 (54)	<.0001
Antibiotic exposure 6 mo prior	0 (0)	3 (7)	5 (17)	4 (20)	32 (46)	<.0001
MELD score	NA	8.3±2.6	9.7±3.4	12.4±4.5	14.4±4.8	<.0001
Lactulose	NA	0 (0)	30 (100)	0 (0)	70 (100)	<.0001
Rifaximin	NA	0 (0)	16 (53)	0 (0)	41 (59)	<.0001
SBP prophylaxis	NA	0 (0)	0 (0)	1 (5)	6 (9)	1.0[a]
Alcohol-related etiology	NA	7 (16)	8 (27)	6 (30)	35 (50)	<.0001
Daily caloric intake	2229±239	2120±421	2094±406	2010±522	2151±510	.57
Future hospitalizations in 90 d	0 (0)	2 (5)	2 (10)	6 (30)	34 (48)	<.0001
Death in 1 y	0 (0)	0 (0)	0 (0)	0 (0)	14 (20)	<.0001

NOTE. Data presented as raw number (%) or mean±SD. ANOVA, χ^2 tests as appropriate.

MELD, model for end-stage liver disease; NA, not applicable; SBP, spontaneous bacterial peritonitis.

[a]Comparison within both/ascites only using Fisher's exact test.

Table 2.

Kruskal-Wallis Comparisons of ARG Patterns Between Groups

Variables	*P* value	Log-2-fold changes			
		Comp	HE only	Ascites only	Both
AMR gene family					
PDC beta-lactamase	4.55E-04	−0.68	0.48	−1.14	1.34
Llm 23S ribosomal RNA methyltransferase	7.59E-04	−0.16	0.73	−0.4	−0.16
Multidrug toxic compound extrusion transporter	9.19E-04	−0.72	0.18	0.35	0.19
CMY beta-lactamase	.001	−3.43	1.11	1.33	1
sulfonamide resistant sul	.005	−1.41	−0.5	0.95	0.96
CTX-M beta-lactamase	.006	−1.9	−1.93	3.13	0.7
SRT beta-lactamase	.007	−2.74	−2.08	4.12	0.7
DHA beta-lactamase	.008	0.23	−1.11	1.99	−1.11
APH(6)	.009	1.5	−1.81	−1.24	1.55
ANT(2”)	.011	−1.35	−0.87	1.25	0.96
SHV beta-lactamase	.012	−0.95	−1.7	2.57	0.07
Aminocoumarin resistant parY	.016	−0.81	1.04	−0.68	0.45
Major facilitator superfamily antibiotic efflux pump	.020	−0.3	−1.02	0.36	0.96
TEM beta-lactamase	.022	−0.11	−0.42	0.13	0.4
Antibiotic resistant isoleucyl-tRNA synthetase	.036	−0.89	0.33	−0.9	1.47
General Bacterial Porin with ↓permeability to β-lactams	.043	−2.21	0.23	1.23	0.75
APH(3”)	.044	−0.96	−0.64	1.03	0.57
Rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	.045	−1.09	1.88	−0.88	0.1
ARO terms		Comp	HE only	Ascites only	Both
arlS	.007	−1.11	0.8	−0.35	0.66
CTX-M-141	.013	−1.97	2.81	−1.97	1.13
MexE	.013	−1.21	0.55	0.12	0.55
Klebsiella pneumoniae KpnE	.016	−1.37	0.94	0.39	0.04
vanSG	.016	1.06	1.06	−1.06	−1.06
cdeA	.022	−0.69	0.52	0.11	0.07

CTX-M-3	.023	−0.33	1	−0.33	−0.33
MexJ	.024	−0.65	0.25	0.29	0.68
SHV-126	.027	−0.08	−0.26	0.12	0.22
MexC	.030	−0.58	0.39	0.91	1.09
Resistomes and variants		Comp	HE only	Ascites only	Both
Staphylococcus aureus	.002	−0.78	0.35	−1.17	1.6
Klebsiella sp	.002	−0.44	−0.44	1.33	−0.44
Pseudomonas fluorescens	.003	−0.35	−0.1	0.09	0.36
Serratia marcescens	.007	−2.13	−1.15	1.86	1.42
Shigella flexneri	.01	−1.58	0.82	−0.21	0.98
Lactococcus lactis	.02	−2.32	1.1	−0.21	1.42
Streptomyces rishiriensis	.02	−0.81	1.04	−0.68	0.45
Bifidobacterium bifidum	.03	−0.86	0.32	−0.91	1.45
Clostridium perfringens	.04	−0.69	−1.74	2.13	0.3
Streptomyces niveus	.04	0.53	1.21	−1.36	0.38

NOTE. *P* values are FDR corrected, Comp: compensated, Log2fold changes: negative value indicates lower presence in that specific category and vice-versa.

ARG, antibiotic resistance gene; ARO, antibiotic resistance ontology.

Table 3.

Hospitalizations According to ARG Patterns Using MaAsLin2

AMR gene family	Coefficient	SE	*P* value	Q value
MELD score	0.412904688	0.064851856	2.00E-09	2.18E-07
Composite score of decompensation	0.69654277	0.124371937	9.26E-08	5.05E-06
ANT(2__)	3.962029525	0.965979207	6.55E-05	0.002
Lactulose use	0.554979205	0.161860073	.000773425	0.021
Llm_23S_ribosomal_RNA_methyltransferase	−1.362069117	0.446233892	.002664409	0.028
Aminocoumarin resistant parY	−0.667160276	0.23270564	.004709751	0.029
SBP prophylaxis	1.949305738	0.823698373	.019167732	0.045
Rifamycin resistant beta subunit of RNA polymerase (rpoB)	−0.563369541	0.234559872	.01747475	0.048
ARO term	Coefficient	SE	*P* value	Q value
MELD score	0.412904688	0.064851856	2.00E-09	2.48E-06
Composite score of decompensation	0.69654277	0.124371937	9.26E-08	5.73E-05
dfrA12	5.219724894	1.174708864	1.66E-05	0.0068
aadA9	3.466531159	0.841747734	6.14E-05	0.0115
ANT(2__)	3.962029525	0.965979207	6.55E-05	0.0115
lnuA	3.758216836	0.892344383	4.25E-05	0.0115
MexE	5.602615552	1.356726994	5.87E-05	0.0115
cfrC	5.603541801	1.423029531	.000123069	0.019
CMY_48	5.525229197	1.422226328	.000150225	0.0207
vanVB	3.698116695	0.992895908	.000271825	0.034
OXY_1_6	5.02332093	1.359142234	.000301604	0.035
OXY_1_4	4.706700245	1.288924643	.000353835	0.037
Resistomes	Coefficient	SE	*P* value	Q value
MELD score	0.412904688	0.064851856	2.00E-09	3.60E-07
Composite score of decompensation	0.69654277	0.124371937	9.26E-08	8.34E-06
Clostridium_botulinum	5.982816577	1.341559342	1.55E-05	0.0009
Lactulose use	0.554979205	0.161860073	.000773425	0.035

Gram_negative_bacterium	4.238461957	1.356837413	.002124458	0.04
Citrobacter_freundii	1.048760055	0.359106171	.004006676	0.042
Nocardia_farcinica	−0.601249962	0.207076231	.004218298	0.043
Streptomyces_niveus	−1.483564927	0.518466411	.004788398	0.045
Streptomyces_rishiriensis	−0.66716	0.232706	.00471	0.048

NOTE. Composite score of decompensation: 0 = compensated, 1 = HE only, 2 = Ascites only, 3 = both HE and ascites.

AMR, antimicrobial resistance; ARG, antibiotic resistance gene; ARO, antibiotic resistance ontology; HE, hepatic encephalopathy; MELD, model for end-stage liver disease; SBP, spontaneous bacterial peritonitis.

Table 4.

Death According to ARG Patterns Using MaAsLin2

AMR gene family	Coefficient	SE	*P* value	Q value
ANT(2)	4.947526707	0.800392205	5.20E-09	5.66E-07
MELD score	0.486367013	0.106357407	9.66E-06	0.0005
Composite score of decompensation	0.686274301	0.164688078	5.06E-05	0.002
ARO term	**Coefficient**	**SE**	***P* value**	**Q value**
TriB	17.01623506	1.918959351	1.51E-15	1.87E-12
ANT(2__)_Ia	4.947526707	0.800392205	5.20E-09	2.14E-06
MexE	6.358167931	1.01571941	3.48E-09	2.14E-06
dfrA12	6.455393429	1.057135985	7.60E-09	2.35E-06
cfrC	6.839210335	1.269810454	2.57E-07	5.30E-05
tetS	2.932463416	0.541967397	2.29E-07	5.30E-05
MELD score	0.486367013	0.106357407	9.66E-06	0.0017
AAC(6_)_Ia	4.948180187	1.107617659	1.50E-05	0.0023
tet(B)	2.384584309	0.54127897	1.94E-05	0.0027
Composite score of decompensation	0.686274301	0.164688078	5.06E-05	0.006
LnuP	3.651573402	0.871787447	4.65E-05	0.006
CfxA6	−3.140165729	0.852606443	.000316043	0.03
TEM_48	4.580083552	1.254330205	.000354129	0.03
mexM	3.608731994	1.005280679	.000441217	0.039
TEM_219	1.776788748	0.500650074	.000509585	0.042
ACT_38	3.59641316	1.026742741	.000599018	0.046
mdsB	2.13115354	0.61161402	.000638267	0.046
Resistomes	**Coefficient**	**SE**	***P* value**	**Q value**
MELD score	0.486367013	0.106357407	9.66E-06	0.002
Composite score of decompensation	0.686274301	0.164688078	5.06E-05	0.004
Legionella pneumophila	2.128854381	0.574804015	.000293338	0.014
Gram negative bacterium	3.925435914	1.152970009	.000839916	0.03

Desulfitobacterium hafniense	−3.209096819	0.988378431	.001425081	0.043

NOTE. Composite score of decompensation: 0 = compensated, 1 = HE only, 2 = Ascites only, 3 = both HE and ascites.

AMR, antimicrobial resistance; ARG, antibiotic resistance gene; ARO, antibiotic resistance ontology; HE, hepatic encephalopathy; MELD, model for end-stage liver disease.