Research Article
Cirrhosis and Liver Failure

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Oral-gut microbiome interactions in advanced cirrhosis: characterisation of pathogenic enterotypes and salivatypes, virulence factors and antimicrobial resistance

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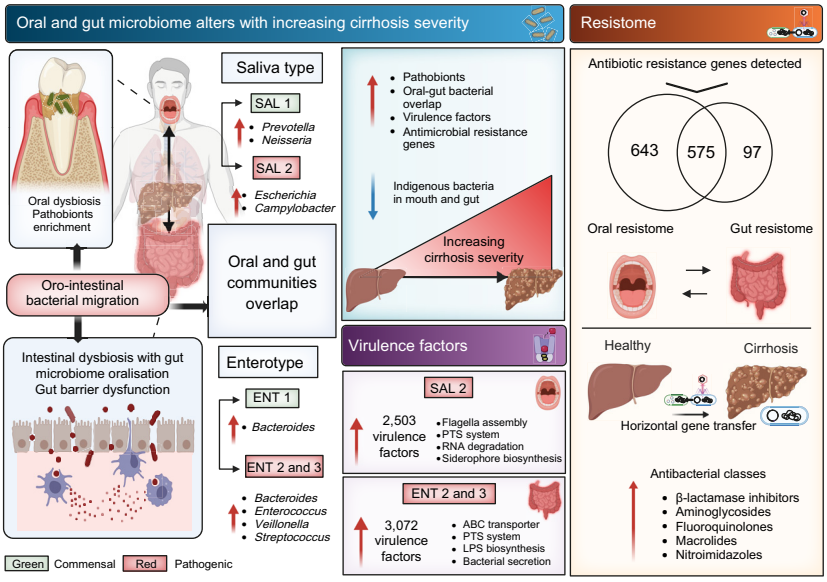
Graphical abstract



Highlights

- Increasing cirrhosis severity correlates with increased pathobionts and decreased commensals in oral and gut microbiomes.
- Significant overlap exists between oral and gut microbiomes in decompensated cirrhosis and acute-on-chronic liver failure.
- Distinct microbial clusters in the gut of advanced cirrhosis patients harbour virulence factors and antimicrobial resistance genes.
- Oral and gut microbiomes in cirrhosis show substantial antimicrobial resistance genes as well as overlap, with unique resistotypes identified.
- Reduced oral and gut microbial richness in decompensated cirrhosis, despite similar antimicrobial exposure, when compared to non-cirrhosis patients who are septic.

Impact and implications

This research underscores the crucial role of microbiome alterations in the progression of cirrhosis in an era of escalating multidrug resistant infections, highlighting the association and potential impact of increased oral-gut microbial overlap, virulence factors, and antimicrobial resistance genes on clinical outcomes. These findings are particularly significant for patients with decompensated cirrhosis and acute-on-chronic liver failure, as they reveal the intricate relationship between microbiome alterations and cirrhosis complications. This is relevant in the context of multidrug-resistant organisms and reduced oral-gut microbial diversity that exacerbate cirrhosis severity, drive hepatic decompensation and complicate treatment. For practical applications, these insights could guide the development of targeted microbiome-based therapeutics and personalised antimicrobial regimens for patients with cirrhosis to mitigate infectious complications and improve clinical outcomes.

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Oral-gut microbiome interactions in advanced cirrhosis: characterisation of pathogenic enterotypes and salivatypes, virulence factors and antimicrobial resistance

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Background & Aims: Cirrhosis complications are often triggered by bacterial infections with multidrug-resistant organisms. Alterations in the gut and oral microbiome in decompensated cirrhosis (DC) influence clinical outcomes. We interrogated: (i) gut and oral microbiome community structures, (ii) virulence factors (VFs) and antimicrobial resistance genes (ARGs) and (iii) oral-gut microbial overlap in patients with differing cirrhosis severity.

Methods: Fifteen healthy controls (HCs), as well as 26 patients with stable cirrhosis (SC), 46 with DC, 14 with acute-on-chronic liver failure (ACLF) and 14 with severe infection without cirrhosis participated. Metagenomic sequencing was undertaken on paired saliva and faecal samples. 'Salivatypes' and 'enterotypes' based on genera clustering were assessed against cirrhosis severity and clinical parameters. VFs and ARGs were evaluated in oral and gut niches, and distinct resistotypes identified.

Results: Salivatypes and enterotypes revealed a greater proportion of pathobionts with concomitant reduction in autochthonous genera with increasing cirrhosis severity and hyperammonaemia. Increasing overlap between oral and gut microbiome communities was observed in DC and ACLF *vs*. SC and HCs, independent of antimicrobial, beta-blocker and gastric acid-suppressing therapies. Two distinct gut microbiome clusters harboured genes encoding for the PTS (phosphoenolpyruvate:sugar phosphotransferase system) and other VFs in DC and ACLF. Substantial ARGs (oral: 1,218 and gut: 672) were detected (575 common to both sites). The cirrhosis resistome was distinct, with three oral and four gut resistotypes identified, respectively.

Conclusions: The degree of oral-gut microbial community overlap, frequency of VFs and ARGs all increase significantly with cirrhosis severity, with progressive dominance of pathobionts and loss of commensals. Despite similar antimicrobial exposure, patients with DC and ACLF have reduced microbial richness compared to patients with severe infection without cirrhosis, supporting the additive pathobiological effect of cirrhosis.

Introduction

One in five hospitalised patients with cirrhosis die.[1] The PREDICT study showed that almost all patients with acute decompensation with and without the development of acute-on-chronic liver failure (ACLF) had proven bacterial infections as a precipitant.[2] Infections caused by multidrug-resistant organisms (MDROs) are associated with the highest risk of developing (multi-)organ failure and account for nearly half of cases globally.[3] Bacterial infections in patients with decompensated cirrhosis (DC) typically result from breaches in innate immune barriers and inadequate immune cell clearance.[4] Antimicrobial therapy therefore forms the cornerstone of treatment in cirrhosis, both for acute infections and as prophylaxis against infection-driven complications.[5] This is however mired with challenges due to diagnostic delays and uncertainties, and an increasing frequency of MDROs.[6,7] High levels of antimicrobial resistance is increasingly concerning in Europe[8] and worldwide.[9] This is particularly worrying in patients with cirrhosis who have a heightened susceptibility to infections due to cirrhosis-associated immune dysfunction (CAID).[10] CAID is characterised by low-grade systemic inflammation and immune dysfunction; defects within the 'gut-liver axis', characterised by microbiome alterations, mucosal permeability, microbial translocation, and metabolome disarray, amplify CAID.[11,12]

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Alterations in the gut microbiome in DC and ACLF influence clinical outcomes[13] and contribute to hepatic decompensation.[14] This broadly termed gut 'dysbiosis' is causally linked to MDRO infections and associated with intestinal inflammation and defective host-microbiome compartmentalisation.[15–17] Most studies to date have employed less phylogenetically resolving 16S rRNA gene sequencing approaches instead of more advanced shotgun metagenomics (SMGS). Alterations of individual genera in the gut have been associated with cirrhosis progression, namely *Enterococcaceae* and *Enterobacterales*,[18] as well as changes in the oral microbiome.[19]

Periodontitis and poor oral health affecting up to 68% of patients with cirrhosis and dysbiosis of the subgingival microbiota may perpetuate hepatic decompensation.[12,20,21] A proof-of-concept study demonstrated that periodontal therapy in patients with cirrhosis and hepatic encephalopathy (HE) was associated with improved oral and gut dysbiosis, systemic inflammation, cirrhosis severity scores, and cognitive function at 30 days.[22] A study reported higher levels of *Streptococcus* and *Veillonella* species, usually detected in the oral cavity, present in cirrhosis faeces.[23] Over 75,000 microbial genes differed between cirrhosis and HCs, and over 50% taxonomically assigned bacterial species were of oral origin, interpreted as an 'invasion' of the distal gut from the mouth in cirrhosis. Salivary and faecal microbiomes have been compared in patients with cirrhosis and HCs, evaluating inflammatory markers linked to the oral cavity.[24] Xerostomia is a risk factor for oral disease, and may be an iatrogenic complication of diuretic use, as these drugs are frequently required to treat ascites in decompensated cirrhosis.[25] Improving oral health awareness and access in patients with cirrhosis has been recognised as crucial to reduce systemic inflammation, enhance quality of life, and mitigate socio-economic disparities in healthcare outcomes.[11]

Antimicrobial resistance gene (ARG) profiles within a microbial community are known as the 'resistome'.[26–28] Exploration of the resistome by SMGS can provide valuable insight into mechanisms leading to the development of MDROs.[29] ARGs can also represent quorum-sensing and secretion system survival strategies independent of antibiotic exposure,[30] regulate ecological dynamics within a specific environment, and determine survival in complex microbial communities due to adaptations in phenotypic and genotypic responses to antimicrobials.[31] The gut is a reservoir for ARGs and virulence factors (VFs) with disruption of the microbiome leading to colonisation by pathogenic organisms.[32] Bacterial species can acquire ARGs through horizontal gene transfer and the high-density communities found in the gut give rise to a wide distribution of ARGs.[33] The burden of ARGs within the gut microbiome reservoir is a functional threat when dysbiosis occurs, with an over-representation of pathobionts.[34] A better understanding of the ARGs harboured by both the oral and gut microbiome in cirrhosis is critical, given the escalating rates of antimicrobial resistance, the contribution of the microbiome to heightened infection risk, and data demonstrating the strong association of MDRO infections with mortality, especially in ACLF.[3] Further explanation of ARGs and VFs is provided in the supplementary section.

In this study, we aimed to simultaneously interrogate the gut and oral microbiome utilising deep shotgun metagenomic sequencing of faecal and saliva samples, respectively, in well-phenotyped patients with cirrhosis of varying severities, in comparison with HCs and patients with sepsis and systemic inflammation but without underlying cirrhosis. Our objectives were to assess (i) the degree of overlap and alterations between oral and gut microbiome community structures, (ii) VF and ARG carriage, and (iii) crucially how these evolve with increasing cirrhosis severity, organ failure and critical illness. We provide additional novelty in exploring how these alterations relate to clinically relevant parameters, therapies and endpoints at different cirrhosis stages, and how this compares to a 'positive disease control cohort' who have sepsis with systemic inflammation but without underlying cirrhosis.

Materials and methods

Study participants and biological sampling

Patient participants were stratified and phenotyped according to clinically relevant groups based on the severity and time course of their underlying cirrhosis, degree of hepatic decompensation, and presence and extent of hepatic and extrahepatic organ failure at the time of sampling. These groups were stable cirrhosis (SC) (n = 26), DC (n = 46) and ACLF (n = 14), with a separately recruited HC cohort (n = 15). DC was defined by the acute development of one or more major complications of cirrhosis, including ascites, HE, variceal haemorrhage, and bacterial infection. ACLF was defined and graded according to the number of organ failures in concordance with criteria reported in the CANONIC study.[35,36] Further details are provided in the supplementary section around criteria and clinical data collected for non-liver-related sepsis (NLS) (n = 14) and HC.

Faecal and saliva sample acquisition

Faecal and saliva samples were obtained within 48 h of admission to hospital. Faeces and saliva samples were obtained, respectively, as follows: 81 and 66 from patients with cirrhosis of varying severities, 11 and 7 from NLS and 15 and 13 from HCs. Upon acquisition, samples were kept on ice within 2 h and stored at -80 °C within 12 h. Further details are provided in the supplementary section.

DNA extraction from faecal and saliva samples

A 2-day protocol adapted from the IHMS (International Human Microbiome Standards)[37,38] was used to extract DNA from both stored faecal and saliva pelleted samples. For faeces, a 200 mg pre-weighed and homogenised aliquot was used. For saliva, a post-centrifugation pellet was used. Please refer to the supplementary section for further details.

Library preparation

Illumina TruSeq DNA PCR-free library preparation (Illumina Cat no: 20015963, Illumina, USA) was used to generate high-quality DNA sequencing libraries, adapted for automation for the Agilent NGS Bravo workstation (Agilent Technologies, USA) in a 96-well plate format. Please refer to the supplementary section for further details.

Whole-genome shotgun metagenome sequencing

Libraries with an average size of 350 base pairs were validated, normalised, pooled and loaded onto S4 flowcells (Illumina,

USA) and sequenced on a NovaSeq S6000 (Illumina, USA). Samples were in 3 lanes S4-300, generating a minimum of 40 million reads per sample. Raw sequencing reads were filtered for high-quality (HQ) reads to a minimum of 20 million per sample, before cleaning to remove possible contaminating human and food-associated reads. This was achieved by mapping the HQ reads to the human reference genome (GRCh39), food-related genomes, *Bos taurus* (May 2014 version), and *Arabidopsis thaliana* (May 2014 version). The resulting HQ-cleaned reads were then mapped and counted using the METEOR pipeline (https://forgemia.inra.fr/metagenopolis/meteor). A minimum of 20 million HQ clean reads were generated by SMGS from faecal and saliva samples that met quality control thresholds.

Statistical analysis of clinical and metagenome sequencing data

Continuous data were tested for normality using the D'Agostino Pearson test. Comparisons between two or more groups were performed by Student's *t* test (or analysis of variance) and Mann-Whitney *U* test (or Kruskal Wallis) for normally and non-normally distributed data, respectively. Normally distributed data are presented as mean ± SD and non-normally distributed data are presented as median (IQR). Comparisons between categorical data were performed by $\chi 2$ test or Fisher's exact test for small sample sizes and data are presented as n (%). Significance was defined at a 95% level and all *p* values were 2-tailed. Analyses were undertaken utilising IBM SPSS (version 27) and GraphPad Prism (version 9.5.1).

Sequenced reads were aligned to oral[39] and gut microbiome[40] gene catalogues, and gene counts normalised after rarefying aligned reads to the same sequencing depth. Using metagenomics species pan-genomes[41] as references, the abundance of microbial species within the faecal and saliva samples was calculated. Please refer to the supplementary section for details on how sequencing data were analysed for microbiome taxonomic and functional profiling and for antimicrobial resistance gene determination.

Results

Participant characteristics

Table S1 summarises demographic, clinical and biochemical characteristics of the recruited patients and controls (supplementary section). Patients with cirrhosis and NLS were older than HCs. Predominant aetiologies of cirrhosis included alcohol-related liver disease (SC/DC/ACLF: 50%/63%/71.4%) and metabolic dysfunction-associated steatotic liver disease (SC/DC/ACLF: 7.7%/17.4%/7.1%), respectively. Patients with DC and ACLF presented with ascites (76.1%/71.4%) and HE (8.7%/42.9%) as the predominant manifestation of hepatic decompensation, respectively. None of the patients with DC and ACLF had experienced a variceal haemorrhage in the 7 days prior to recruitment nor had developed prior spontaneous bacterial peritonitis during that admission. Patients with DC, ACLF and NLS were more frequently receiving antibiotics (71.7%/100%/100%, respectively) compared to those with SC (26.9%). There was no difference in use of rifaximin-α across the cirrhosis cohorts, nor in proton pump inhibitor (PPI) and H2 receptor antagonist use, including in the NLS group. Patients with DC and ACLF were more likely to be treated with lactulose and non-selective beta-blockers than the SC and NLS groups. Haematological, biochemical and disease severity and prognostic composite scores followed expected patterns. Almost one-fifth and approaching one-third of patients with DC and ACLF died, respectively, whilst approximately one-quarter of patients with SC and ACLF and over one-third of patients with DC were transplanted over the 12-month follow-up period.

Compositional oral and gut microbiome alterations and distinct entero- and salivatypes in cirrhosis

As seen in Fig. 1A,B (and Fig. S1), both gut and oral microbiome communities showed significant reductions in alpha-diversity with three different diversity metrics: gene richness, Shannon and Inverse Simpson indices, with increased cirrhosis severity and hepatic decompensation. Taxa (*i.e.* at a family phylogenetic level) at gut and oral sites were compared between the different cirrhosis and control cohorts. Here, particular patterns emerged, in particular between the various cirrhosis groups, differentiated by increasing disease severity. Pathobionts, including *Enterococcaceae* and some classified under the order *Enterobacterales*, and bacterial families strongly associated with cirrhosis, including *Veillonellaceae* and *Streptococcaceae*, were significantly increased in both niches as cirrhosis severity increased (Fig. 1C,D). Conversely, a decreasing relative abundance in taxa conventionally classified as autochthonous was observed. These gut bacterial families, including *Oscillospiraceae* and *Ruminococcaceae*, and oral commensals, including *Neisseriaceae* and *Prevotellaceae*, decreased in relative abundance as cirrhosis severity increased. Thus, an increasing proportion of pathobionts with a relative reduction in commensal bacteria drove a significant alteration in overall microbial community structures affecting both the gut and oral niches simultaneously, as cirrhosis severity increased. Higher taxonomic resolution of species and genus-level comparisons for both gut and oral microbiome communities are available in the supplementary section (Table S2; Figs S2 and S3).

Next, we identified the hidden community structures of the gut and oral microbiome by Dirichlet multinomial mixture modelling. This approach enabled the identification of three distinct clusters within the gut microbiome (ENT1/2/3; Fig. 1E) and two distinct clusters within the oral microbiome (SAL1/2; Fig. 1G), denoted as 'enterotypes' and 'salivatypes', respectively. Fig. S4 shows how the minimal model fit evaluation determined the number of optimal clusters as three and two for enterotypes and salivatypes, respectively.

There was enrichment of known genera for enterotypes, such as Bacteroides in ENT1 and ENT2. However, we also found that pathobionts such as *Enterococcus* were dominant in ENT2 and ENT3 (Fig. 1F). Notably, bacteria which are usually commensal within the oral niche, such as *Veillonella* and *Streptococcus*, were also enriched in ENT2 and ENT3, in keeping with the transfer of these bacteria from the oral cavity into the lower gut, as previously observed.[42] The relative proportion of these oral commensals detected in the gut also increased as cirrhosis severity and hepatic decompensation progressed. Among salivatypes, SAL1 was enriched with *Prevotella* and *Neisseria* which are known oral commensal

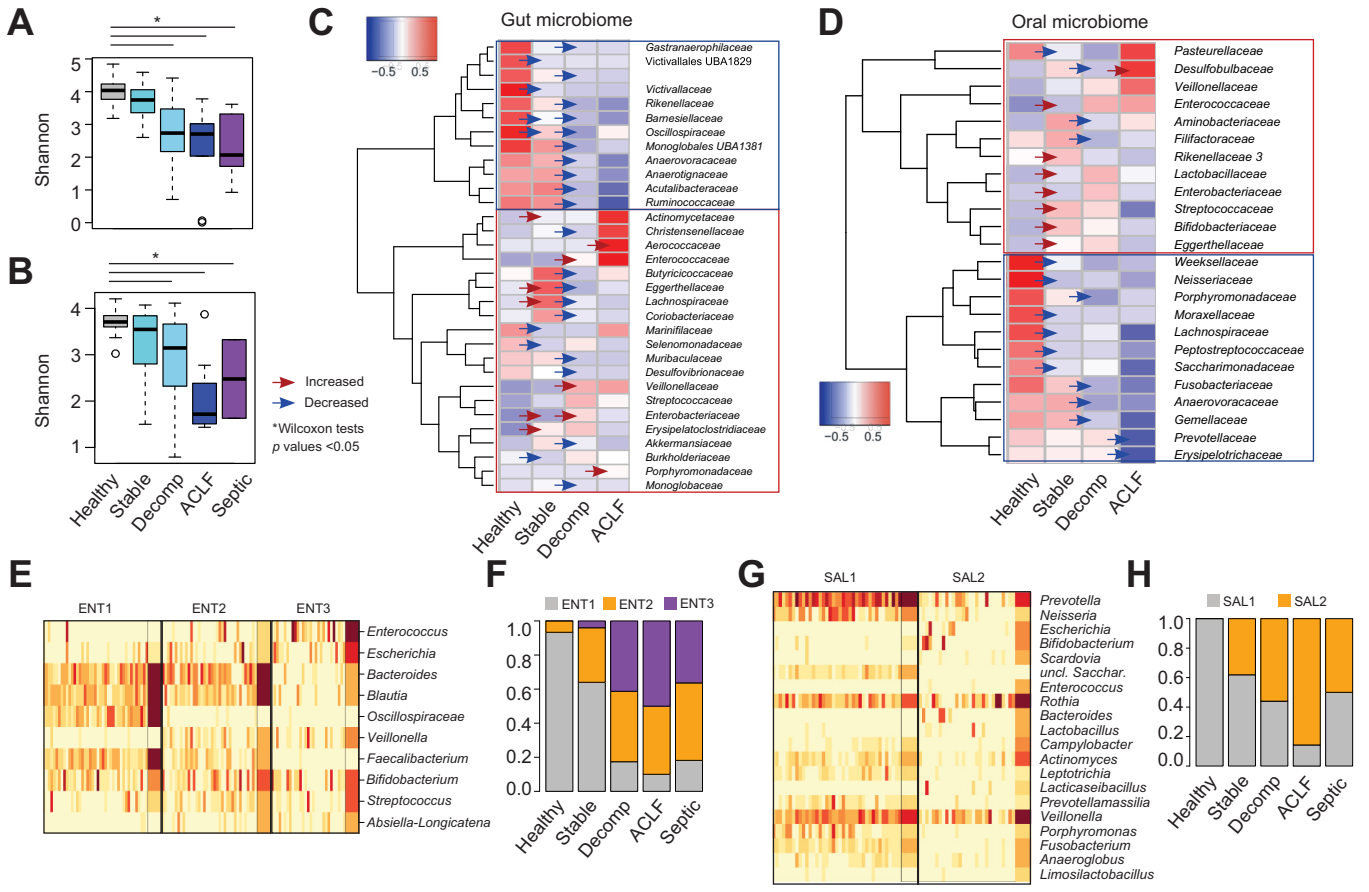

Fig. 1. Microbial diversity and community structure alterations by cirrhosis severity. (A,B) Alpha-diversity of (A) gut and (B) oral microbiome based on alterations in cirrhosis severity, in comparison to HC and NLS groups. A significant reduction of alpha-diversity of both oral and gut microbiomes was observed in patients with decompensated cirrhosis, ACLF and NLS compared to HCs (Wilcoxon rank sum tests *p* values <0.05). (C,D) Gut and oral microbiome alterations at the family phylogenetic level, based on cirrhosis severity, compared to HCs. Families with significant changes by stage (Wilcoxon rank sum tests *p* values <0.05) are denoted by red (increase) and blue (decrease) arrows. (E,G) Microbial community structures by unsupervised clustering method, denoting enterotype (gut) and salivatype (oral). Three microbial clusters from the gut microbiome – ENT1, ENT2 and ENT3 (E), and two microbial clusters from the oral microbiome – SAL1 and SAL2 (G), identified, with varying degrees of commensal bacteria and pathobionts. (F,H) Overall proportion of different enterotypes (F) and salivatypes (H) in cirrhosis, in comparison to HC and NLS. The proportion of ENT2/ENT3 and SAL2 (which represent a greater proportion of pathobionts) increased based on cirrhosis severity, whilst ENT1 and SAL1 (which represents mainly commensals), which are most prevalent in HC, decreased. ACLF, acute-on-chronic liver failure; HCs, healthy controls; NLS, non-liver-related sepsis.

bacteria dominant in HCs. SAL2 conversely was enriched with pathobionts, including *Escherichia* and *Campylobacter*, commensals in the lower intestine which are not usually present in the oral cavity. The relative proportion of the more pathogenic SAL2 salivatype – like ENT2 and ENT3 in the gut – also increased with cirrhosis severity and hepatic decompensation (Fig. 1H). When assessing clinical parameters relative to cirrhosis enterotypes (Fig. S5), the pathogenic enterotypes (ENT2/3) but not salivatypes (SAL2) (Fig. S6), were significantly associated with the presence of ascites, higher model for end-stage liver disease (MELD) and Child-Pugh scores, higher plasma ammonia levels, as well as active antimicrobial and laxative therapy. In summary, the fractions of both enterotypes and salivatypes enriched with pathobionts (ENT2, ENT3 and SAL2) increased significantly with cirrhosis severity. The degree of pathobiont enrichment in the NLS cohort for ENT3 and SAL2 more closely resembled the DC cohort, with the ACLF cohort having an overall higher relative abundance of pathobionts than in NLS.

To validate these findings and to evolve from the use of unsupervised clustering alone, we applied Dirichlet multinomial mixture models and constructed enterotypes for a publicly available and comparable cirrhosis gut metagenome dataset that was generated from a Chinese population published by Qin *et al.* in 2014, with the disease cohort of 123 patients having a combination of stable cirrhosis and hospitalised decompensated cirrhosis phenotypes whilst the healthy control group consisted of 114 individuals from whom faecal samples were shotgun metagenomically sequenced.[23] Further details are available in Figs S7 and S8.

Overlap between oral and gut microbiome community structures is associated with increasing cirrhosis severity

We showed that microbes increasingly co-exist and 'overlap' in both the oral and gut niches as cirrhosis severity worsens, whilst the degree of overlap in the relative proportions of these different types of bacteria increases, mirroring disease

progression (Fig. 2A). Based on co-existing metagenomics species pan-genomes in both gut and oral sites, including *Streptococcus spp.*, *Veillonella spp.*, *Escherichia spp.*, *Enterococcus spp.*, and *Lactobacillus spp.*, we identified overlap of oral and gut metagenomes (Fig. 1C,D). Notably, we found that oral and gut microbiome community structures increasingly merged from SC to DC, with the greatest similarity in ACLF.

We then classified individuals based on the degree of similarity of their oral and gut microbiome into two binary groups: "close" and "distant", with close describing a higher degree of overlap in bacterial genera between oral and gut niches, and distant being the converse and as observed in health. Based on this classification, we explored a variety of relevant clinical parameters that might impact upon and/or be affected by the degree of oral and gut microbiome community overlap. These included aetiology, disease severity scores (MELD and Child-Pugh), decompensating symptoms (ascites, HE), ammonia levels, antimicrobial and laxative treatments, gastric acid-suppressing treatments (PPI and H2 receptor antagonists), non-selective beta-blockers that can affect gut motility, and 1-year mortality (Fig. 2). Fractions of enterotypes and salivatypes compared by 'close' and 'distant' groupings showed that those classed as 'close' consisted of >70% pathogenic enterotypes (ENT2/3) and salivatype (SAL2) (Fig. S9).

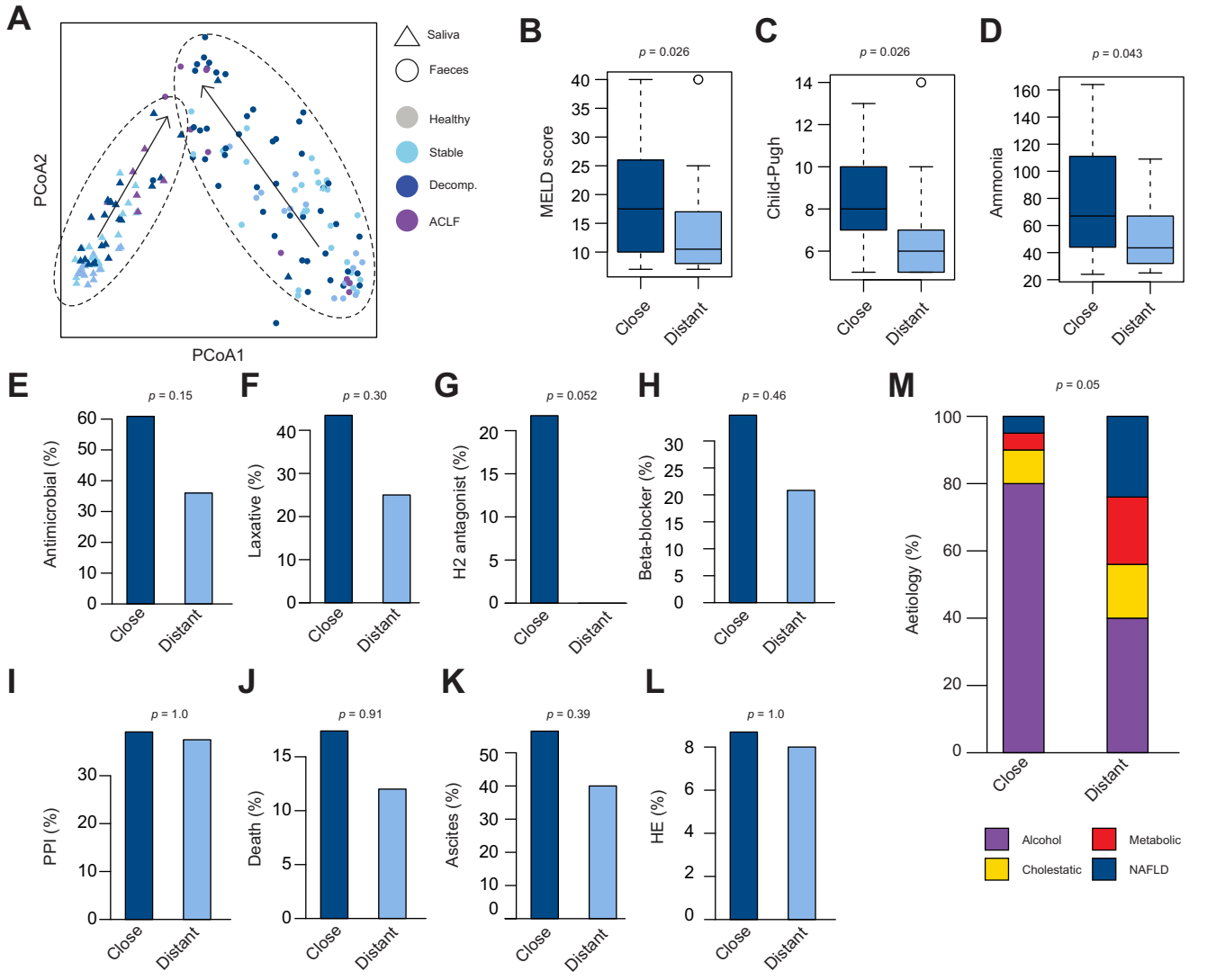

Fig. 2. Oral and gut microbiome bacterial community overlap by cirrhosis severity and compared against cirrhosis composite severity scores, specific clinical parameters and aetiology. (A) Bray-Curtis dissimilarity ordination plots of gut and oral microbiome based on species between anatomical sites. Gut and oral microbiome communities increase in similarity as cirrhosis severity increases (see arrows). (B-M) Patients with cirrhosis were then stratified based on the degree of similarity of their oral and gut microbiome community structures. "Close" denotes a greater degree of microbial overlap between oral and gut communities, whilst "distant" denotes greater differences and separation in community structures. The clinical parameters that exhibited the highest degree of oral and gut microbiome bacterial community overlap (*i.e.* 'close') were (B,C) MELD and Child-Pugh scores, (D) plasma ammonia levels, and aetiology of cirrhosis (M), alcohol-related cirrhosis *vs.* other causes of cirrhosis. Other clinical parameters including pharmacotherapies (E) antimicrobials, (F) laxatives (G) H2 receptor antagonists, (H) non-selective beta-blockers; (I) proton pump inhibitors; (J) mortality at 1 year; and manifestations of hepatic decompensation (presence/absence of (K) ascites and (L) HE) did not associate with a higher degree of oral and gut microbiome bacterial community overlap. Wilcoxon rank sum tests and chi-square tests were performed for continuous and categorical variables, respectively.

Worsening disease severity characterised by MELD score and Child-Pugh grade (Fig. 2B,C, respectively), and higher plasma ammonia levels (Fig. 2D), correlated with the degree of oral-gut microbiome overlap. Alcohol-related liver disease was associated with greater overlap whilst those with MASLD were observed to have more distinct oral and gut microbiomes (Fig. 2M). Notably, drug therapies thought to impact on microbiome composition such as antimicrobials, laxatives, gastric acid suppressants and non-selective beta-blockers were not associated with alterations in the degree of oral and gut microbiome overlap (Figs 2E-I).

Pathogenic entero- and salivatypes are enriched with virulence factors

Putative functional profiles of the enterotypes and salivatypes were explored. By aligning the sample-specific gene count profiles with KEGG orthology (KO) annotations, we generated functional profiles summarising all gene counts per KO detected. A total of 10,007 and 15,464 KOs were annotated in all the faecal and saliva samples that were sequenced, respectively.

KO profiles between pathogenic enterotypes, ENT2 and ENT3, and commensal enterotype, ENT1, were compared, identifying 3,072 enriched and 4,429 depleted KOs. We performed enrichment analysis based on hypergeometric tests and found enriched pathways and modules amongst KOs in ENT2/ENT3 (Fig. 3A and Fig. S10). ENT2/ENT3 harboured genes encoding for the phosphoenolpyruvate:sugar phosphotransferase (PTS) system, further explained in the supplementary section.

Bacterial genera within ENT3 harboured more virulence factors, including biofilm formation,[43] lipopolysaccharide biosynthesis,[44] bacterial secretion systems,[45] and ascorbate degradation, which can initiate inflammation, transfer virulence factors and hijack host nutrients. Dissimilatory nitrate reduction modules that generate ammonia – central to the pathogenesis of HE – were additionally found to be enriched in patients with advanced cirrhosis harbouring ENT2 and ENT3 in the gut. Biofilms promote horizontal gene transfer through the exchange of bacterial genome fragments and/or mobile genetic elements, which contributes to the spread of antibiotic-resistance genes.[46]

KO profiles between SAL2, pathogenic salivatype, and SAL1, commensal salivatype, were also compared. Among 2,503 enriched and 5,980 depleted KOs in SAL2 samples, we identified enriched pathways and modules based on hypergeometric tests (Fig. 3B and Fig. S11). Several virulence factors that can contribute to pathogenic properties of those bacteria in SAL2, including flagella assembly, siderophore biosynthesis, autoinducer (AI)-2 transport, and type IV secretion system,[47] were also identified. Here we found the upregulation of carbohydrate transport and metabolism in both the oral (SAL2) and gut (ENT2, ENT3) microbiome of patients with DC and ACLF, including significant enrichment of the PTS system hijacking host nutrients with enrichment of galactose metabolism, of which excess metabolites, such as galacitol, can lead to oxidative stress or act as metabotoxins.[48]

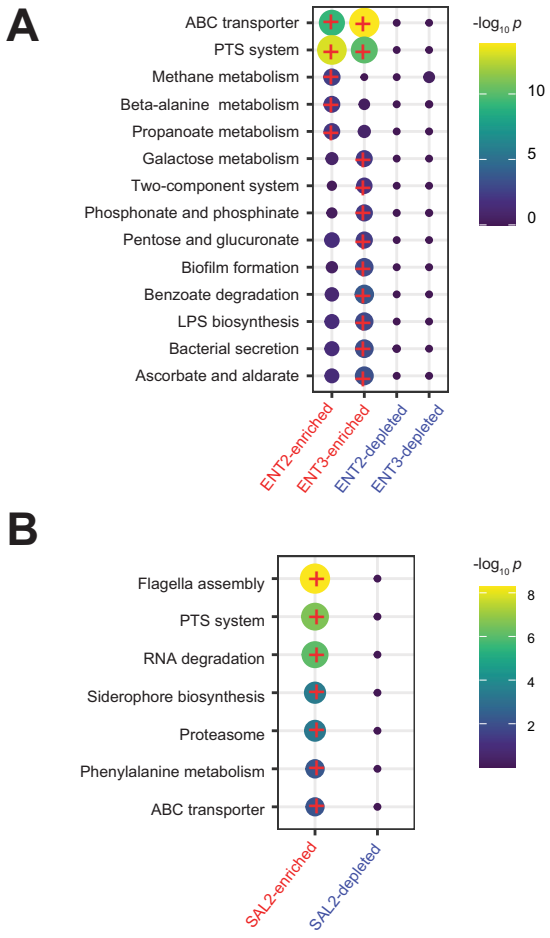

Fig. 3. Pathogenic enterotypes and salivatypes harbouring virulence factors. Enriched KEGG pathways from enriched/depleted KEGG ortholog groups, contrasting (A) pathogenic enterotypes, ENT2 and ENT3 to ENT1, and (B) pathogenic salivatype SAL2 to SAL1 (hypergeometric tests p values <0.01). LPS, lipopolysaccharide; PTS, phosphoenolpyruvate:sugar phosphotransferase.

Alterations in oral and gut microbiome antimicrobial resistance genetic profiles

To investigate the frequency and potential for harbouring ARGs, we profiled ARGs within the oral and gut microbial datasets utilising the CARD (Comprehensive Antimicrobial Resistance Gene Database) (Fig. 4A). We found that in most patients with cirrhosis, the oral and gut microbiome harboured substantial numbers of ARGs (1,218 and 672 genes for the oral and gut microbiome, respectively). Many of these ARGs were common to both sites (575 genes), although a greater proportion of these shared ARGs were detected in the gut (>85%) compared to the oral niche (47%). ARG abundances in both the oral and gut microbiome were greater with increasing cirrhosis severity and as hepatic decompensation worsened (Fig. 4B,C). For both niches, the NLS group had the highest total ARG abundances, although there were no statistically significant differences when compared to the cirrhosis cohorts.

To further explore the ARG profiles (resistome) of oral and gut samples across the different cirrhosis severities, we performed principal coordinate analysis of ARG profiles for both niches (Fig. 4D,E). As previously described, we found that HCs harboured unique oral and gut microbiome resistomes. However, the resistome of patients with cirrhosis was significantly different to that of the HCs. By performing unsupervised clustering, three and four resistotypes[49,50] of the oral and gut

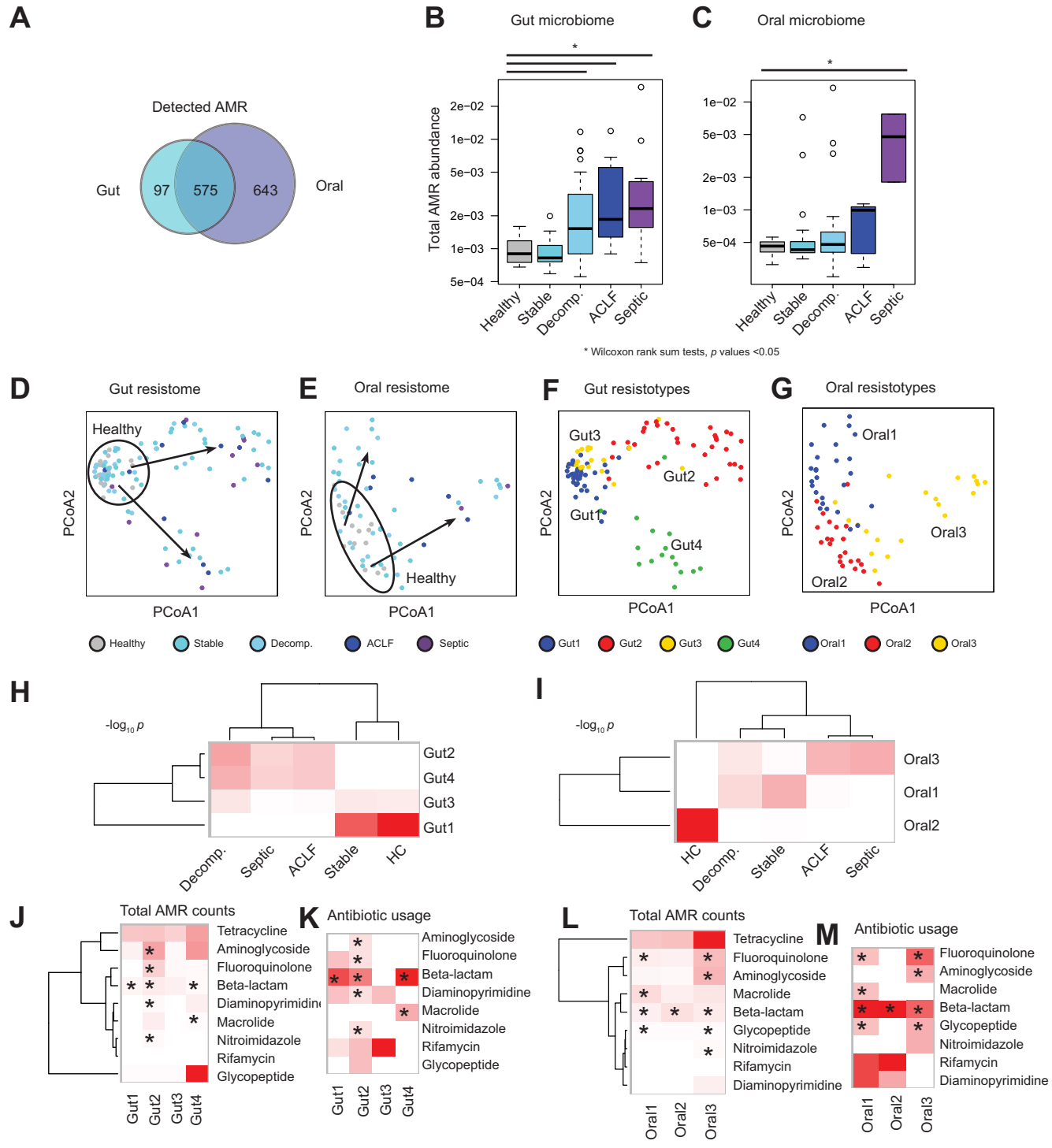

Fig. 4. Oral and faecal ARG carriage and overlap by cirrhosis severity, with evaluation of resistomes and resistotypes, relating to antimicrobial drug classes and concomitant administered antimicrobial therapy. (A) ARGs in cirrhosis faecal and saliva samples show that the majority of ARGs are shared between the gut and oral microbiome, with the latter harbouring a greater number of ARGs overall. (B,C) Total ARG abundance by cirrhosis severity in (B) the gut and (C) oral microbiome, compared with HC and NLS (Wilcoxon rank sum tests *p* values <0.05). (D,E) Principal coordinate analysis of ARG profiles of (D) gut and (E) oral resistomes of all study cohorts, demonstrating that HC and SC cluster separately from DC, ACLF and NLS groups. (F,G) Gut and oral resistotypes, respectively, by Dirichlet multinomial mixture modelling – Gut1/2/3/4 and Oral1/2/3. (H) Gut1/3 are enriched in HC and SC, whereas Gut2/4 are enriched in DC, ACLF and NLS. (I) Oral1/3 are enriched in SC and DC, Oral3 in ACLF and NLS, whilst Oral2 is enriched in HC. (J) ARG abundances by antibiotic classification of gut resistotypes and (K) proportions of patients with cirrhosis concomitantly treated with antimicrobial therapy, based on class of antibiotic administered. (L) ARG abundances by antibiotic classification of oral resistotypes and (M) proportions of patients with cirrhosis concomitantly treated with antimicrobial therapy, based on class of antibiotic administered. ARGs for beta-lactams were prevalent in the majority of gut and all oral resistotypes. Asterisks denote simultaneous resistotypes by antibiotic class detected in gut and oral microbiomes in cirrhosis, against the class of antibiotic treatment being administered at the time of sampling. ACLF, acute-on-chronic liver failure; AMR, antimicrobial resistance; ARG, antimicrobial resistance gene; DC, decompensated cirrhosis; HCs, healthy controls; NLS, non-liver-related sepsis.

microbiome, respectively, were determined (Fig. 4F,G). Of the gut resistotypes, Gut1 and to a lesser extent Gut3 were both enriched amongst the HC and SC cohorts, in contrast to Gut2 and Gut4 which were enriched in patients with DC and ACLF and to a lesser extent in NLS (Fig. 4H). For the oral resistotypes, Oral2 was specific to HCs whilst Oral1 was most enriched in SC and then DC, whilst Oral3 was most enriched in ACLF and NLS and then in DC.

ARG classes were assessed for oral and gut resistomes, based on their drug classifications as determined by CARD. Enrichment of ARGs for β-lactamase classes were detected in all oral and all but one of the gut resistotypes. ARGs encoding for resistance against aminoglycoside, fluoroquinolone, macrolide and nitroimidazole drug classes were specifically enriched in oral and gut resistotypes specific to patients with DC and ACLF, including Oral1/Oral3 and Gut2/Gut4 (Fig. 4J,L). There was a high amount of antibiotic use in patients with cirrhosis, where their oral and gut resistotypes indicated resistance against those specific types of antibiotic classes that patients were treated with (starred in Fig. 4K,M) at the time of sampling. A high degree of ARGs to β-lactamase inhibitors (*e.g.* piperacillin-tazobactam) and carbapenems (*e.g.* meropenem) were detected in all but the Gut3 resistotype in patients with DC and ACLF, with 87.9% of all these patients receiving some form of β-lactamase antibiotic; 71.4% and 16.5% were simultaneously being treated with either a β-lactamase inhibitor or a carbapenem, respectively.

ARGs for rifamycin from which rifaximin-α, a prophylactic therapy used in HE, is derived, were not significantly increased in abundance in most oral and gut samples from patients with cirrhosis. This is despite up to 25% of patients with SC, DC and ACLF being treated, either concomitantly or up to hospital admission, with rifaximin-α for secondary prophylaxis of HE.

Discussion

There is increased recognition of the critical role of the oral-gut-liver axis in cirrhosis in driving systemic complications, and the impact on clinical outcomes including decompensation, survival and quality of life.[11] In this study, we showed an overlap in the oral and gut microbiomes and, crucially, interrogated specific functional alterations based on distinct cirrhosis severities, by harnessing SMGS analysis and applying bioinformatic approaches. This was achieved by the simultaneous assessment of the salivary and faecal microbiome, as surrogates for oral and gut microenvironments, respectively, in robustly phenotyped cirrhosis cohorts. These findings were contrasted with HCs and uniquely to a positive disease control cohort of NLS that represents patients who are acutely unwell with systemic inflammation but without underlying chronic liver disease, in effect controlling for the contribution of cirrhosis. After interrogating community structures and assessing them against specific clinical parameters, we provide additional novelty by evaluating VFs that offer insight into putative oral and gut microbiome functions, as well as assessing ARG abundance based on oral and gut 'resistotypes', and how the so-called 'resistome' alters as cirrhosis severity progresses irrespective of concomitant antimicrobial exposure.

We identified simultaneous, substantial bacterial alterations in the gut and oral microbiome, beginning with a significant reduction in (alpha-)diversity affecting both communities as cirrhosis progresses. This is consistent with previous reports where one or other gut/oral microbial community has been studied, in relation to decompensation with HE, pharmacotherapies such as PPI, and/or hospitalisation.[19,51] Recent large cohort studies have reported on the utility of simultaneous evaluation of the salivary and faecal microbiome in cirrhosis[52] and when comparing cirrhosis cohorts from the USA and Mexico, where greater linkages between the faecal microbiome with plasma metabolites, compared to saliva, were reported.[53] However, these studies were taxonomically limited by employing lower resolution V1-V2 16S rRNA gene analysis instead of V3-V4 analysis or deep SMGS used in this study, which enables species and potentially even strain level interrogation,[54] recognising that different strains in the same microbial species can be substantially different phenotypically.

Family-level alterations affecting both the oral and gut microbiome as cirrhosis severity worsened showed that pathobionts (*Enterococcaceae, Enterobacterales* and *Veillonellaceae*) were over-represented in both anatomical niches. In contrast, the reduction in relative abundance of indigenous bacteria in the mouth (*Neisseriaceae* and *Prevotellaceae*) and the gut (*Oscillospiraceae* and *Ruminococcaceae*) with worsening cirrhosis has implications for host-requiring metabolic activities including nitrate reduction and butyrate production, respectively, that impact upon gut barrier integrity. Species level changes are important when considering biological properties and whether individual bacterial types have particular pathological or commensal activities. Whilst previous cirrhosis studies have focused on gut alterations, our findings are also consistent with oral microbiome studies whereby *Veillonella* is associated with cirrhosis and *Neisseria* associated with HCs.[55] Functional predictions in this study showed a significantly higher proportion of genes associated with carbohydrate transport and metabolism, defence mechanisms and membrane transport, all indicative of enhanced pathogenicity, mirroring our data. Increasing cirrhosis severity associated with the greatest degree of oral-gut microbiome overlap, as did higher plasma ammonia levels and alcohol-related aetiology, all providing new insights into key clinical factors involved in this phenomenon.

In addition to the evolving concepts of 'invasion' and 'oralisation' of the intestinal microbiome in cirrhosis,[56] there is now considerable focus on the role of the oral microbiome as a distinct entity, which is increasingly recognised as predisposing to hepatic decompensation.[19,57] The hypothesis that oral microbes can extend into and/or invade the lower intestine may be propagated by impaired gastric acid, changes in intestinal pH and/or bile acid dysregulation[58,59] that occur in advanced cirrhosis. However, we did not observe an association between gastric acid-suppressing therapy and degree of oral and gut microbiome overlap. A recent study using mouse models of gut dysbiosis (and without hepatic fibrosis) employing 16S rRNA sequencing of oral swab and faecal samples reported a relative, but not absolute, increased abundance of oral bacteria, reflecting the 'marker' hypothesis.[60] The same study went on to analyse human microbiome datasets of paired oral and gut samples from patients after allogeneic hematopoietic cell transplantation and patients with inflammatory bowel disease, and determined that there was a relative, but not absolute, abundance increase of oral bacteria within the gut, consistent with depleted gut microbiota, also supporting the marker

hypothesis. It remains to be seen in advanced cirrhosis in humans to what extent there is true invasion of oral microbes into the gut where they 'expand', or whether their relative increase 'marks' the depletion of commensal gut bacteria.

As cirrhosis severity increased, the commensal enterotype (ENT1) and salivatype (SAL1) were found to be significantly reduced, whereas pathogenic enterotypes (ENT2 and ENT3) and salivatype (SAL2) were significantly increased. The NLS cohort more closely resembled the DC cohort, whilst the ACLF cohort had greater relative abundance of pathobionts, suggesting that there is an additive pathobiological impact of cirrhosis that is associated with higher pathobiont enrichment affecting both the oral and gut microbiome in those who develop organ failure. Substantial overlap of gut and oral microbiome communities, such as *Enterococcaceae*, *Streptococcaceae*, and *Veillonellaceae*, for both pathogenic enterotype and salivatype, may imply bi-directional colonisation from not only the oral to more distal intestinal niches, but also from the intestine to the more proximal oral niche. The relocation of bacteria from the oral to intestinal niche has been reported in clinical trials of patients with DC and HE. Rifaximin-α was reported to suppress the growth of orally originating species – commonly found in dental plaque and associated with periodontal disease – in cirrhosis faeces in the setting of a randomised-controlled trial.[42] These oral species have putative functions related to intestinal mucus degradation, such that a reduction in these pathobionts seeding into the gut promotes gut barrier repair, further emphasising the intimate relationship of the oral-gut-liver axis.

Alterations in functional capacity of pathogenic enterotypes and salivatypes implicate changes in the homeostatic metabolism of the gut and oral microbiome. This not only provides new opportunities for pathobionts to disrupt homeostatic mechanisms by several processes, but also the loss of host-benefitting functions due to the progressive loss of commensals, which are reported here. These functional alterations include host nutrient hijacking by the PTS system, ABC transporters, siderophore biosynthesis, and ascorbate degradation;[61,62] (2) host tissue invasion due to enhanced bacterial secretion systems and flagella assembly;[63] (3) promoting host inflammation by lipopolysaccharide biosynthesis,[64] and (4) promoting dysfunctional metabolic pathways that generate greater oxidative stress via pentose and glucuronate metabolism[65] and galactose metabolism.[66] Specific to ammonia metabolism, another novel finding was that dissimilatory nitrate reduction modules that generate ammonia were enriched in advanced cirrhosis, implicating increased ammonia production in patients with DC and ACLF. Ammonia is central to the pathogenesis of HE[67] and these findings show a causal link to changes in the gut microbiome.[68]

Key differences in the overall resistome profile between the gut and oral cavity were identified, with different 'resistotypes' in each site, in common with previous work.[69] In addition, the lack of a significant rise in total ARG abundance in the oral microbiome in contrast to the gut microbiome supports the concept that the oral resistome is inherently more stable, as is proposed here and previously by others.[69,70] As part of the resistome analysis, striking alterations in the ARG repertoire were identified in both gut and oral microbiomes based on cirrhosis severity. However, it was notable that resistance to the most commonly used β-lactam antibiotic class did not show a similar level of increase. Whilst this is potentially due to the increased resistance of piperacillin to Gram-negative β-lactamases, it is more likely due to the use of combination drugs, such as piperacillin/tazobactam.

These ARG alterations described in the oral and gut resistome reflect disease type and cirrhosis severity. Another study comparing faecal ARG burden between patients with SC and DC (with HE and ascites), reported high gut ARG counts in cirrhosis[52] that was notably distinct from the resistome in chronic kidney disease and diabetes mellitus. Faecal ARG burden worsened with cirrhosis progression regardless of ascites and HE, and was associated with risk of hospitalisation and death, independent of cirrhosis severity, prior antibiotic exposure, hospitalisations, or concomitant medications. This study is the first to describe and contrast in detail the cirrhotic oral and gut resistome simultaneously in relation to cirrhosis severity. These data point to cirrhosis-driven selection pressures which select for ARGs, beyond direct antibiotic-induced effects.

We have previously shown that biomarkers of intestinal inflammation and gut barrier dysfunction rise with increasing cirrhosis severity.[71] Increased ARG carriage in cirrhosis may be exacerbated by intestinal inflammation and gut barrier damage.[72] Previous studies have linked gut inflammation with enhanced ARG transfer, potentially through a rise in horizontal gene transfer.[15] It is possible that these ARGs are co-selected with other genes, such as those involved in virulence primarily through conjugative plasmids, or that they are carried at higher levels in pathobionts identified in the context of loss of bacterial diversity as cirrhosis progresses. It is important to note, however, that cause-and-effect cannot be determined here in patients with advanced cirrhosis more frequently treated with antibiotics, which would require longitudinal assessment.

In view of the single centre, largely Westernised cohort of patients with cirrhosis and controls employing single time point biological sampling, future work to expand on these findings will require a multicentre approach that involves patients from a wider spectrum of ethnicities and geographies. Whilst there are advantages to a cross-sectional approach to determine differences in microbial communities between different anatomical niches and patient cohorts, longitudinal studies are now required to determine how the microbiome alters as cirrhosis progresses and complications occur, to begin to better evaluate causality, and provide a more comprehensive perspective on microbiota diversity (within-patient and between-patient diversities),[73] as well as develop more sophisticated, precision therapies. In addition, integrating these high-resolution metagenomic datasets with more detailed oral health assessment, nutritional, and other environmental and lifestyle factors including smoking, will allow for a better understanding of the effect of these potential confounders,[74] in addition to the impact of disease severity and phenotype.[75] Large scale studies such as MICROB-PREDICT (MICROBiome-based biomarkers to PREDICT decompensation of liver cirrhosis and treatment response[76]), a multi-centre pan-European study, should begin to address some of these issues.

Therapeutically targeting the gut microbiome is increasingly garnering attention as a potential approach in cirrhosis[77–79] as well as for specific complications such as HE,[80,81] as knowledge expands around the putative role of these microbial alterations, to which this study further contributes. This is

especially relevant in an era of increasing MDRO infections in cirrhosis, where non-antibiotic-dependent approaches are urgently required.[7] In addition to the gut, the oral microbiome in cirrhosis remains a therapeutic target, including via nutrition-based approaches.[11,12,22,82] Enhancing our fundamental understanding of functional alterations in the oral and gut microbiome remains crucial to fully and safely exploit these novel pathways for individuals with cirrhosis.

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Abbreviations

ACLF, acute-on-chronic liver failure; ARG, antimicrobial resistance gene; DC, decompensated cirrhosis; HCs, healthy controls; HE, hepatic encephalopathy; HQ, high-quality; KO, KEGG orthology; MELD, model for end-stage liver disease; MDRO, multidrug-resistant organisms; NLS, non-liver-related sepsis; PPI, proton pump inhibitor; SC, stable cirrhosis; VF, virulence factors.

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Conflict of interest

VCP has delivered paid lectures for Norgine Pharmaceuticals Ltd and Menarini Diagnostics Ltd. DLS has undertaken consultancy for Norgine Pharmaceuticals Ltd, EnteroBiotix, Mallinckrodt Pharmaceuticals and ONO Pharma UK Ltd and has delivered paid lectures for Norgine Pharmaceuticals Ltd, Falk Pharma and Aska Pharmaceutical Co. Ltd. SSH is co-founder of Bash Biotech Inc and Gigabiome Ltd. AH is now an employee of AstraZeneca and may or may not own stock options.

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

Authors' contributions

SL performed both faecal and saliva microbiome analyses. AZ recruited patients, assisted with biological sampling and undertook data collection. BA undertook sample processing, saliva and faecal DNA extractions and raw data handling. EW assisted with saliva DNA extraction optimisation. MaS undertook data curation with MeM, and statistical analysis of clinical metadata. MeM with VP generated the graphical abstract. NB and DM performed mycobiome data analyses and interpretation. AH and HC contributed to sequencing data curation. RW, SC, MJM, AM, MC, GP and DLS provided project oversight and mentorship. SS designed, supervised and coordinated the bioinformatic analysis and with AM and MU secured funding for metagenomic sequencing. VP conceptualised, designed, supervised and project managed the study and served as the study Principal Investigator, coordinated and secured funding and resources for study participant recruitment, data acquisition and bench-based experimental work including DNA extractions, and wrote and edited the original manuscript with SL, LAE and SSH, and addressed reviewers' comments. SSH led and secured funding for the generation and analysis of the microbiome data, and bioinformatics work. All authors provided editorial input and approved the final version of the manuscript.

Data availability statement

The shotgun metagenome raw data sequenced as part of this study can be found from EBI ENA repository under the project accession PRJEB52891.

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Supplementary data

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Author names in bold designate shared co-first authorship

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