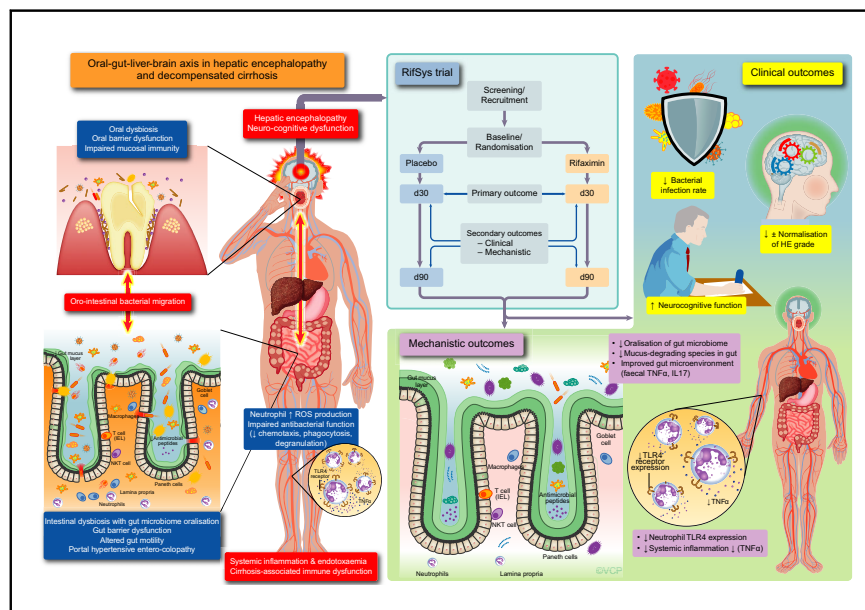


Rifaximin- α reduces gut-derived inflammation and mucin degradation in cirrhosis and encephalopathy: RIFSYS randomised controlled trial

Graphical abstract



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Lay summary

In this clinical trial, we examined the underlying mechanism of action of an antibiotic called rifaximin- α which has been shown to be an effective treatment for a complication of chronic liver disease which effects the brain (termed encephalopathy). We show that rifaximin- α suppresses gut bacteria that translocate from the mouth to the intestine and cause the intestinal wall to become leaky by breaking down the protective mucus barrier. This suppression resolves encephalopathy and reduces inflammation in the blood, preventing the development of infection.

Highlights

- Rifaximin reduced gut-derived systemic inflammation by suppressing orofixation of the gut microbiome.
- Rifaximin suppressed mucin-degrading species rich in sialidase, e.g. *Streptococcus* and *Veillonella* spp.
- Rifaximin promotes an intestinal environment augmenting responses to pathobionts and promoting gut barrier repair.
- Patients treated with rifaximin were less likely to develop infections.



Rifaximin- α reduces gut-derived inflammation and mucin degradation in cirrhosis and encephalopathy: RIFSYS randomised controlled trial

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Background & Aims: Rifaximin- α is efficacious for the prevention of recurrent hepatic encephalopathy (HE), but its mechanism of action remains unclear. We postulated that rifaximin- α reduces gut microbiota-derived endotoxemia and systemic inflammation, a known driver of HE.

Methods: In a placebo-controlled, double-blind, mechanistic study, 38 patients with cirrhosis and HE were randomised 1:1 to receive either rifaximin- α (550 mg BID) or placebo for 90 days. Primary outcome: 50% reduction in neutrophil oxidative burst (OB) at 30 days. Secondary outcomes: changes in psychometric hepatic encephalopathy score (PHES) and neurocognitive functioning, shotgun metagenomic sequencing of saliva and faeces, plasma and faecal metabolic profiling, whole blood bacterial DNA quantification, neutrophil toll-like receptor (TLR)-2/4/9 expression and plasma/faecal cytokine analysis.

Results: Patients were well-matched: median MELD (11 rifaximin- α vs. 10 placebo). Rifaximin- α did not lead to a 50% reduction in spontaneous neutrophil OB at 30 days compared to baseline ($p = 0.48$). However, HE grade normalised ($p = 0.014$) and PHES improved ($p = 0.009$) after 30 days on rifaximin- α . Rifaximin- α reduced circulating neutrophil TLR-4 expression on day 30 ($p = 0.021$) and plasma tumour necrosis factor- α (TNF- α) ($p < 0.001$). Rifaximin- α suppressed oralisation of the gut,

reducing levels of mucin-degrading sialidase-rich species, *Streptococcus* spp, *Veillonella atypica* and *parvula*, *Akkermansia* and *Hungatella*. Rifaximin- α promoted a TNF- α - and interleukin-17E-enriched intestinal microenvironment, augmenting anti-bacterial responses to invading pathobionts and promoting gut barrier repair. Those on rifaximin- α were less likely to develop infection (odds ratio 0.21; 95% CI 0.05–0.96).

Conclusion: Rifaximin- α led to resolution of overt and covert HE, reduced the likelihood of infection, reduced oralisation of the gut and attenuated systemic inflammation. Rifaximin- α plays a role in gut barrier repair, which could be the mechanism by which it ameliorates bacterial translocation and systemic endotoxemia in cirrhosis.

Clinical Trial Number: ClinicalTrials.gov NCT02019784.

Lay summary: In this clinical trial, we examined the underlying mechanism of action of an antibiotic called rifaximin- α which has been shown to be an effective treatment for a complication of chronic liver disease which effects the brain (termed encephalopathy). We show that rifaximin- α suppresses gut bacteria that translocate from the mouth to the intestine and cause the intestinal wall to become leaky by breaking down the protective mucus barrier. This suppression resolves encephalopathy and reduces inflammation in the blood, preventing the development of infection.

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Introduction

Advanced cirrhosis brings with it a plethora of complications including hepatic encephalopathy (HE), variceal bleeding, ascites

Keywords: Hepatic encephalopathy; rifaximin- α ; cirrhosis; systemic inflammation; gut microbiome; salivary microbiome.

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and a propensity to develop infections, which can lead to multi-organ failure. The development of HE in both its covert¹ and overt forms² confers a poor prognosis.

The gut microbiome has prime importance in the pathogenesis of cirrhosis, with the evolution from a healthy gut microbiome, to one characterised by dysregulated gut microbial activity or 'dysbiosis' associated with decompensation of cirrhosis.³ Dysbiosis is greater in patients with cirrhosis who develop complications correlating with plasma endotoxin levels and 30-day mortality.⁴ In cirrhosis there is an imbalance between healthy and pathogenic gut bacteria with skewed microbiota populations in favour of increased numbers of pro-inflammatory and ammonia-producing species including *Enterobacteriaceae*, *Firmicutes*, *Archaea* and *Prevotella*.⁵ Bacterial translocation (BT) is a significant driver of cirrhosis-associated immune dysfunction (CAID), although the mechanisms by which intestinal dysbiosis drives immune cell dysfunction remain unknown.^{6,7} Furthermore, there is growing evidence supporting a pivotal role of dysregulated gut microbiota in HE, as well as gut inflammation and barrier dysfunction in decompensated cirrhosis.⁸

The non-absorbable antibiotic rifaximin- α reduces the risk of recurrence of overt HE and need for hospitalisation.⁹ Treatment with rifaximin- α has been associated with significant reductions in bed days, emergency department attendances and 30-day readmissions.¹⁰ The specific mechanism of action of rifaximin- α remains to be elucidated; it has been shown to reduce circulating gut-derived endotoxins⁴ but studies of faecal microbiome composition in response to rifaximin- α have fallen short of demonstrating any distinct changes in microbial abundance utilising 16S rRNA gene sequencing.^{4,11,12}

We hypothesised that rifaximin- α reduces gut microbiota-derived systemic inflammation, a known driver of HE and CAID. A single-centre, double-blind, randomised, placebo-controlled mechanistic trial of rifaximin- α was undertaken on 38 patients with cirrhosis and HE over 90 days to delineate whether rifaximin- α ameliorates neutrophil-derived oxidative stress and systemic inflammation (as a primary objective). Secondary objectives were set to assess changes in HE grade and neurocognitive functioning, as well as to evaluate rifaximin's mechanism of action by undertaking shotgun metagenomic sequencing (MGS) of faecal and saliva samples, in conjunction with plasma and faecal metabolic profiling, whole blood bacterial DNA quantification, neutrophil toll-like receptor (TLR) expression and plasma and faecal cytokine analysis.

Patients and methods

The study was designed to be performed on 50 patients with cirrhosis and chronic HE recruited from King's College Hospital. 1:1 allocation of rifaximin- α (Targaxan 550 mg) to matching placebo was administered twice daily over 90 days between 15/1/2015 and 20/6/2016 with intention-to-treat analysis. A patient was considered to have cirrhosis if they fulfilled 2 of 3 diagnostic criteria: (i) biochemistry consistent with cirrhosis, (ii) radiology consistent with cirrhosis/portal hypertension and/or (iii) liver histology. The diagnosis of chronic HE was based on the presence of (i) persistent overt HE ($\geq$ grade 1) or (ii) ≥ 2 episodes of overt HE in the previous 6 months.

Exclusion criteria: age <18 or >75 years, disseminated malignancy (an isolated hepatocellular carcinoma <50 mm was not an exclusion), coeliac or inflammatory bowel disease, intestinal

failure, intestinal obstruction and/or previous bowel resection, human immunodeficiency virus infection and chronic granulomatous disease, anti-inflammatory or immunomodulatory drug use, exposure to rifaximin- α in the previous 12 weeks, patients receiving concomitant oral or parenteral antibiotic therapy, known hypersensitivity to rifaximin- α or rifamycin-derivatives, infection with *Clostridium difficile* or faecal testing positive for *Clostridium difficile* toxin in the previous 3-months, and pregnancy or breastfeeding women.

Patient demographics, clinical details (including West Haven HE grade¹³), biochemistry (including venous ammonia) and neutrophil function were assessed at baseline and after 30 and 90 days of rifaximin- α /placebo treatment. Clinically relevant outcomes including overt HE, neurocognitive function by psychometric hepatic encephalopathy score (PHES),¹⁴ health-related quality of life (HRQoL), organ failure, infection and mortality were recorded for 90 days.

Primary endpoint

A 50% reduction in spontaneous neutrophil production of reactive oxygen species (ROS) 30-days following the start of therapy.

Secondary endpoints

Clinical secondary endpoints included HE grade,¹³ PHES,¹⁴ HRQoL¹⁵ and incidence of infection and organ failure at 30 and 90 days. Mechanistic endpoints included assessment of systemic inflammation with analyses, at 30 and 90 days, of salivary/faecal microbiome, faecal calprotectin, whole blood bacterial DNA, plasma and faecal metabolome, and neutrophil phenotype and function including circulating TLR-4 expression.

Ethics and trial registration

Ethical approval was obtained from NHS Health Research Authority NRES Committee South Central-Oxford C (Bristol) [REC reference:14/SC/0088] and from the Medicines and Healthcare products Regulatory Agency for Clinical Trial Authorisation [EudraCT number: 2013-004708-20; [ClinicalTrials.gov](https://www.clinicaltrials.gov/ct2/show/study?term=NCT02019784) NCT02019784]. The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996), principles of Good Clinical Practice, Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations. Fully informed consent was obtained from all participants. Some participants eligible for this study were unable to provide informed consent due to cognitive impairment arising from HE and permission from a legal representative was sought.

Psychometric hepatic encephalopathy score

A psychometric test battery comprising 5 neurocognitive tests: trail making test A and B, digit symbol substitution test, line tracing test and serial dotting test were performed.¹⁴

HRQoL assessment

The 3-level version of EQ-5D¹⁵ consisting of the EQ-5D descriptive system and the EQ visual analogue scale were performed.

Analysis of neutrophil phenotype and function

Fluorochrome-conjugated monoclonal antibodies (anti-human CD16, CD11b, IL-8, TLR-2, TLR-4, and TLR-9; BD UK) were used for staining individual patient polymorphonuclear leucocytes from whole blood and analysed by flow cytometry using a FACS Canto II analyser and FACS Diva 6.1.2 software (BD, San Jose, CA). 50,000

granulocytes were gated on forward and side-scatter characteristics and stained with anti-CD16-phycoerythrin-IgG1κ. Fluorochrome mean fluorescence intensity was calculated to detect the receptor binding response and to measure antigen-antibody binding. Neutrophil oxidative burst was quantified using GlycoTope Biotechnology Phagoburst™ (BD Biosciences) kits measuring the percentage of phagocytic cells producing ROS at rest.¹⁶ The formation of ROS was detected using the oxidation of dihydrorhodamine-123 to rhodamine-123. Neutrophil phagocytic activity was assessed by neutrophil phagocytosis of opsonized *Escherichia coli*.¹⁶

Plasma cytokine profiling

Plasma cytokines were measured using the Meso Scale Discovery (MSD) platform. Samples were run in duplicate on U-PLEX Proinflamm Combo 1 (hu) plates, measuring interferon- γ (IFN- γ), interleukin (IL)1- β , IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12 p70, IL-13, and TNF- α .

Faecal calprotectin

Faecal calprotectin was measured using the Bühlmann EKAL2 enzyme-linked immunosorbent assay (EK-CAL, Bühlmann Laboratories, Switzerland).¹⁷ The calprotectin cut-off level representing a positive value was 60 $\mu\text{g/g}$ of faeces.

Faecal cytokines

Faecal lysates were produced from frozen faecal samples by combined chemical and mechanical homogenisation using an optimised extraction method.⁸ IL-1 β , IL-6, IL-10, IL-17A, IL-17E,

IL-17F, IL-21, IL-22, IFN- γ and TNF- α were measured in neat faecal lysates using the U-PLEX Th17 Combo 2 (hu) plates and MSD platform.

Plasma and faecal metabonomic analysis

Proton nuclear magnetic resonance (NMR) spectroscopy and reversed-phase ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry were undertaken.¹⁸ Samples were thawed and prepared for NMR using previously published protocols.^{19,20}

Whole blood 16S ribosomal DNA quantification

This was undertaken by quantitative PCR (qPCR) by Vaiomer (Labège, France). DNA was extracted from sterile whole blood. The 16S rDNA present in the samples was measured by qPCR in triplicate and normalised using a plasmid-based standard scale using the workflow described previously.²¹

Saliva and faecal 16S rDNA and metagenomic species quantification

16S analysis was undertaken by standard qPCR-based methods. Abundance of MGS, defined as clusters of >500 genes that covary in abundance among individuals, and thus belonging to the same microbial species, was estimated by mapping shotgun sequencing reads onto the genes (performed by R MetaOMiner package). Median signals of the 50 marker genes that represent a robust centroid of gene clusters of MGS were reported (supplementary materials and methods).

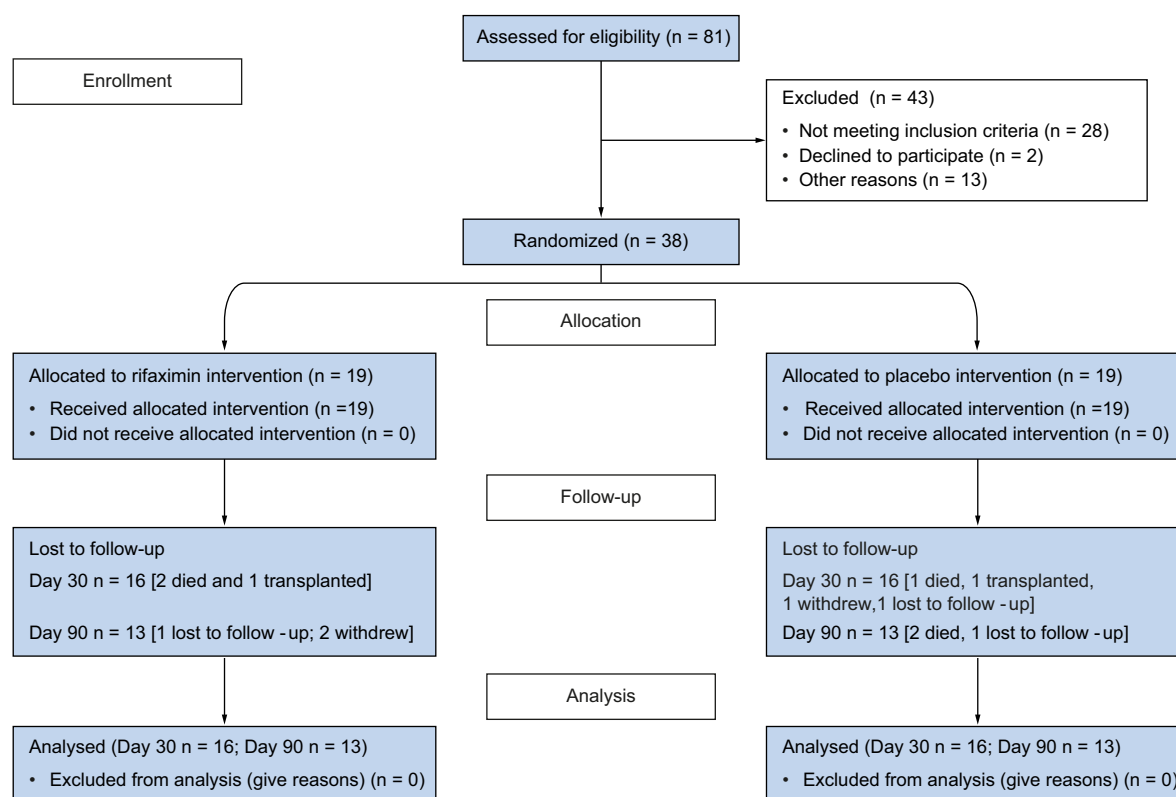


Fig. 1. Consort/patient flow diagram.

Table 1. Baseline demographic and clinical characteristics by treatment group.

	Rifaximin- α n = 19	Placebo n = 19	p value
Age	58 (52–62)	53 (49.5–60.5)	0.48
Male	16	11	0.15
Previous most severe HE grade [0–4]	3 (3–3.5)	3 (2–3)	0.029
Lactulose	7	7	1.0
Proton pump inhibitor	15	11	0.3
Beta blocker	13	7	0.10
Prior TIPS	5	1	0.18
Ascites (Yes:No)	11:8	10:9	0.75
Previous history of SBP	0	0	1.0
Smoking			0.91
Never	6	7	
Stopped	8	8	
Ongoing	5	4	
Alcohol use			0.28
Never	3	1	
Stopped	13	17	
Ongoing	3	1	
BMI (kg/m ²)	29.7 (26.3–32.7)	26.5 (23.1–29.4)	0.068
Mean arterial pressure (mmHg)	87 (78–93)	83 (75–86)	0.082
Ascites grade (1–4)	1 (1–3)	3 (1–3.5)	0.25
Glasgow coma scale (3–15)	15 (15–15)	15 (15–15)	0.29
Overt HE at day 0 (Yes)	14	10	0.31
White blood cell count [$\times 10^9$ /L]	6.34 (4.89–7.2)	5.44 (4.42–6.25)	0.4
INR	1.45 (1.26–1.78)	1.37 (1.3–1.67)	0.67
Sodium (mmol/L)	139 (137–142)	135 (132–137)	0.001
Creatinine (μ mol/L)	70 (57–87)	77 (64–84.5)	0.63
Bilirubin (μ mol/L)	39 (23–56.5)	40 (24–57)	0.66
Albumin (g/L)	36 (30–37.5)	33 (30–38)	0.59
Venous ammonia (μ mol/L)	66 (48–78)	45.5 (30–64)	0.08
Lactate (mmol/L)	1.3 (1.15–1.55)	1.7 (1.3–1.95)	0.13
MELD	11 (8–15)	10 (8–12)	0.49

HE, hepatic encephalopathy; INR, international normalised ratio; MELD, model for end-stage liver disease; SBP, spontaneous bacterial peritonitis; TIPS, transjugular intra-hepatic portosystemic shunt. Data are presented as median (range) with comparison between baseline cohorts done by Mann-Whitney *U* test. Comparison between categorical data was done by the χ^2 test. Bold text denotes statistically significant values.

Statistical analysis

Sample size was determined based on previous *in vitro* and *ex vivo* data.¹⁶ Under the assumption of a reduction in spontaneous neutrophil OB from 30% to 15% (constant 60% difference in medians -0.3) and using the Binomial proportions (Exact) method (power 80%; alpha 0.05 [2-tailed *t* test]), 22 patients were required per study arm.

Continuous data were tested for normality using the D'Agostino Pearson test. Non-normally distributed data are presented as median (range). A comparison between 2 (or more) groups was done by Student's *t* test (or Analysis of Variance) and Mann-Whitney *U* test (or Kruskal Wallis) test for normally and non-normally distributed data, respectively. Comparison between categorical data was done by χ^2 test or Fisher's exact test for small sample sizes.

For continuous data measured over 3 time points, determination of the significance of change was undertaken by repeat measures analysis of variance (RM-ANOVA) with appropriate tests for sphericity. *Post hoc* tests were used to assess statistical significance between individual time points/groups. Longitudinal ordinal data (e.g. HE grade) was analysed by ordered logistic regression.

For measures performed at set times using complex laboratory techniques, RM-ANOVA/Student's *t* test, partial least

square discriminant analysis (PLS-DA) and principal component analysis were used. Using *ropls* R package, metabolomics data were compared.

Significance was defined at a 95% level and all *p* values were 2-tailed. Analyses were undertaken utilising IBM SPSS® (version 21).

Results

Recruitment

Eighty-one patients were screened and 38 randomised to rifaximin- α or placebo (Fig. 1) using a web-based block design randomisation system.

The trial failed to complete recruitment as rifaximin- α was approved in the UK in 2014 for the prevention of recurrent overt HE. Therefore, patients that would have been candidates for participation in the trial were commenced on rifaximin- α as standard of care.

Patient characteristics

Patient demographics and baseline characteristics are summarised in Table 1. Patients were well-matched. Fourteen patients were taking lactulose (7/19 [37%] in each arm). There were no significant differences in median MELD (model for end-stage liver disease) score (11 [8–15] rifaximin- α vs. 10 [8–12] placebo), venous ammonia and severity of HE at baseline.

Table 2. Clinical parameters at baseline, 30 and 90 days post rifaximin- α or placebo.

Variable	Baseline	Day 30	Day 90	Friedman test within group p value [#]	RM-ANOVA within subject effects p value [*]	RM-ANOVA between subject effects p value ^Δ
HE grade						
Rifaximin- α	1 (0–1)	0 (0–1)	0 (0–0)	0.014	0.043	0.61
Placebo	1 (0–1)	0.5 (0–1)	0.5 (0–1)	0.384		
Trails A (sec)						
Rifaximin- α	52 (46–81)	48 (36–65)	46 (37–54)	0.417	0.012	0.86
Placebo	46 (34–78)	46 (37–72)	39 (33–61)	0.293		
Trails B (sec)						
Rifaximin- α	142 (105–161)	143 (106–195)	144 (94–186)	0.88	0.98	0.84
Placebo	140 (57–234)	135 (73–205)	150 (55–194)	0.905		
Line tracing (sec)						
Rifaximin- α	205 (145–254)	185 (111–213)	167 (115–270)	0.023	0.47	0.56
Placebo	169 (154–255)	165 (131–363)	135 (120–299)	0.496		
Serial dot (sec)						
Rifaximin- α	133 (94–178)	97 (78–197)	102 (74–219)	0.218	0.94	0.54
Placebo	101 (83–154)	109 (66–189)	113 (66–173)	0.384		
Digit symbol						
Placebo	23 (20–34)	24 (19–37)	23 (17–38)	0.568	0.096	0.85
Rifaximin- α	21 (16–32)	28 (19–36)	28 (23–39)	0.026		
PHES score						
Rifaximin- α	-9 (-13 to -4)	-7 (-13 to -3)	-6 (-10 to -2)	0.045	0.009	0.617
Placebo	-7 (-13 to -2)	-6 (-11 to -2)	-7 (-12 to -1)	0.278		
MELD						
Rifaximin- α	11 (8–15)	11 (7–14)	10 (7–13)	0.27	0.97	0.99
Placebo	10 (8–12)	10 (8–13)	11 (8–13)	0.076		
White cell count $\times 10^9/L$						
Rifaximin- α	6 (3.8–7.6)	5.8 (3.3–6.9)	6.9 (2.9–6.6)	0.32	0.37	0.49
Placebo	5 (3.8–5.9)	4.3 (3.2–6.3)	4.7 (3.8–6.4)	0.075		
C-Reactive protein						
Rifaximin- α	4.6 (2.8–8.8)	5.3 (2.3–12)	4.5 (2.4–9.3)	0.28	0.64	0.96
Placebo	2 (2–9.6)	2 (2–4.7)	3.1 (2–5.2)	0.31		
Neutrophils $\times 10^9/L$						
Rifaximin- α	3 (1.8–4.4)	2.9 (1.1–3.9)	3.1 (1.4–3.9)	0.56	0.57	0.81
Placebo	2.5 (1.9–4.3)	2.5 (1.9–3.8)	2.5 (2.1–4.7)	0.58		
Creatinine (Mmol/L)						
Rifaximin- α	68 (58–78)	68 (36–81)	69 (55–81)	0.99	0.68	0.67
Placebo	78 (64–84)	86 (64–90)	79 (76–92)	0.32		
Bilirubin (Mmol/L)						
Rifaximin- α	33 (20–53)	32 (17–46)	29 (24–49)	0.55	0.37	0.7
Placebo	35 (20–46)	32 (24–47)	29 (22–47)	0.41		
INR						
Rifaximin- α	1.4 (1.2–1.8)	1.4 (1.2–1.7)	1.3 (1.2–1.5)	0.062	0.49	0.55
Placebo	1.3 (1.2–1.4)	1.4 (1.3–1.5)	1.3 (1.2–1.6)	0.58		
Venous ammonia (Mmol/L)						
Rifaximin- α	62 (49–74)	53 (34–72)	63 (41–85)	0.023	0.96	0.39
Placebo	44 (31–59)	58 (42–74)	52 (33–71)	0.024		

HE, hepatic encephalopathy; INR, international normalised ratio; MELD, model for end-stage liver disease; PHES, psychometric hepatic encephalopathy scoring; RM-ANOVA, repeated measures-ANOVA.

$p < 0.05$ represents significant difference between groups. Bold text denotes statistically significant values.

[#]Friedman test within group p value comparing change across 3 time points within group.

^{*}RM-ANOVA (log transformed for non-parametric data) reflecting within subject effect.

^ΔRM-ANOVA reflecting between subject comparison.

Primary endpoint

The trial failed to demonstrate a 50% reduction in spontaneous neutrophil OB at 30 days compared to baseline ($p = 0.48$) in patients receiving rifaximin- α .

Rifaximin- α resolved overt HE and improved cognitive function

No rifaximin- α -treated patients experienced an HE episode compared to 21% (4/19) on placebo. Patients on rifaximin- α normalised their HE grade to zero at 90 days (HE grade 0 [0–1] vs. 0.5 [0–1]; $p = 0.014$) with an improvement in PHES ($p = 0.009$)

(Table 2). Resolution of HE on rifaximin- α did not translate into an improvement in HRQoL over 90 days.

Rifaximin- α reduced systemic inflammation without changing blood ammonia concentration

Plasma TNF- α fell significantly at day 30 and 90 (all $p < 0.001$) on rifaximin- α compared to placebo ($p < 0.001$ [Fig. 2A]) with a reduction in IL-10 at day 30 ($p = 0.005$) which normalised by day 90 (Table 3). Whilst there were no changes in whole blood bacterial DNA levels, there was a significant reduction in circulating neutrophil TLR-4 expression ($p = 0.0021$) at day 30 in the

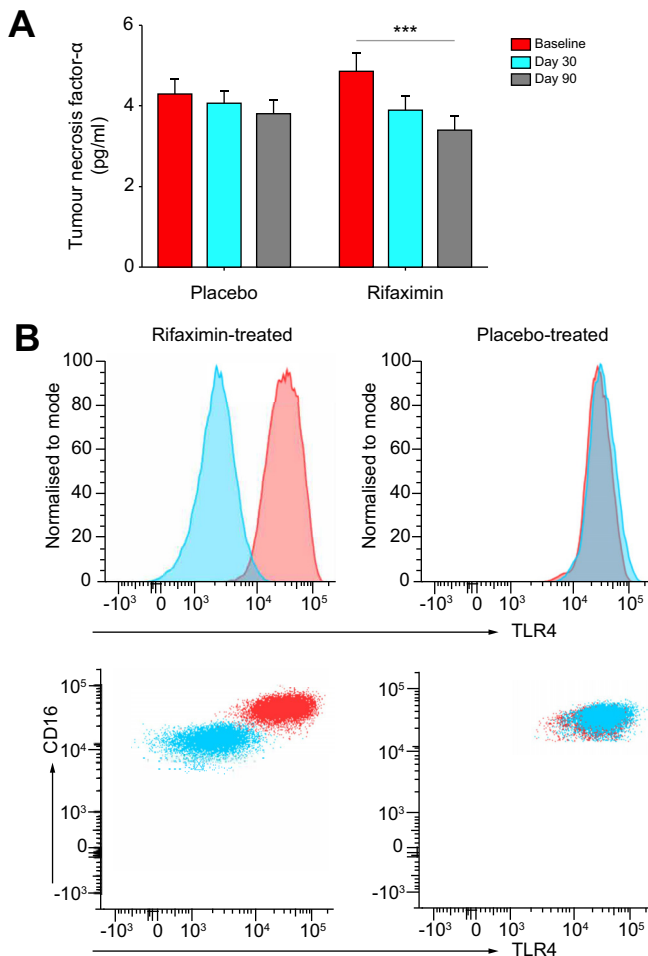


Fig. 2. Rifaximin- α reduced systemic TNF- α and neutrophil TLR-4 expression. (A) Plasma TNF- α fell on rifaximin- α at day 30 and 90 (RM-ANOVA; all $p < 0.001$) compared to placebo. (B) MFI histograms (top panels) and FACS plots (bottom panels) comparing neutrophil TLR-4 expression (MFI) observed at day 30 (blue) compared to baseline (red) in a rifaximin- α -treated compared to a placebo-treated patient. MFI, mean fluorescence intensity; RM-ANOVA, repeated measures-ANOVA; TLR-4, Toll-like receptor-4; TNF- α , tumour necrosis factor- α . (This figure appears in color on the web.)

rifaximin- α -treated patients but not in placebo-treated patients (Fig. 2B). There were no significant changes in circulating neutrophil TLR-2, TLR-9 or IL-8 expression. Those on rifaximin- α were less likely to develop an infection (3 vs. 9); odds ratio for developing an infection on rifaximin- α was 0.21 (95% CI 0.05–0.96) compared to placebo. There were no significant differences in venous ammonia levels between the treatment and placebo arms.

Rifaximin- α led to significant changes in faecal and salivary microbiome whilst preserving beta diversity

Rifaximin- α reduced species richness compared to placebo in both faeces (Fig. 3A) and saliva (Fig. 3B). Global beta diversity was preserved in the rifaximin- α -treated cohort, but significantly reduced in both faeces ($p < 0.05$ day 90) and saliva ($p < 0.05$ at day 30 and 90) in the placebo-treated cohort (Fig. S1). At the phylum level, rifaximin- α increased faecal Tenericutes and decreased Verrucomicrobia ($p < 0.05$) (Table S1). At the genus level, significant reductions in mucin-degrading genera, such as *Veillonella*,

Akkermansia and *Hungatella*, were observed in the faecal samples ($p < 0.05$) (Fig. S3 and Table S2). In the saliva, rifaximin- α reduced opportunistic pathogenic genera including *Filifactor* and *Abiotrophia* (Table S3). Three distinct genus-based microbial clusters were identified in the faeces (enterotypes): *Prevotella*, *Bacteroides* and *Firmicutes* (Fig. S4). Rifaximin- α enriched the firmicutes enterotype (Fig. 3C). Similarly, 3 distinct microbial clusters were identified in the saliva (oraltype): *Prevotella*, *Neisseria* and *Lactobacillus* (Fig. S4) with rifaximin- α enriching *Lactobacillus* (Fig. 3D).

Rifaximin- α suppressed growth of orally originating species in the gut with mucin-degrading capacities

Rifaximin- α suppressed the growth of orally originating species in the faeces with mucin-degrading capacities and virulence at day 30 and 90, including *Veillonella* spp and *Streptococcus* spp as well as *Akkermansia* and *Hungatella* (Fig. 4A–C; Fig. S2; Tables S2,4,6). In the saliva, rifaximin- α decreased the opportunistic pathogens *Abiotrophia defectiva*, *Olsenella uli* and *Filifactor alocis*, and significantly increased oral commensal species such as *Streptococcus* spp (Fig. 4B,D; Tables S5 and S7). We determined the mucin-degrading capacity of those significantly contrasted species based upon the carbohydrate-active enzyme (CAZyme) annotations of the given species such as sialidase (GH33). The CAZyme families that degrade O-glycans of human mucins are shown in Fig. 4E. Most gut and oral species associated with increased plasma TNF- α and neutrophil TLR-4 expression were enriched with sialidase (GH33) and other mucin-degrading CAZymes (GH2/GH20/GH92/GH130/GH18/GH29 and CBM50). For example, 94% and 81% of the co-abundant gut and oral microbes were enriched with mucin-degrading CAZymes and 19% and 27% were enriched with sialidases, respectively [Fig. S7].

Rifaximin- α enhanced faecal cytokines suppressing pathobionts associated with reduced plasma lactate

PLS-DA revealed differing enrichments of plasma metabolites over time between the rifaximin- α - and placebo-treated cohorts, such as decreased lactate with no substantial changes in acetoacetate, phosphocholine and trimethylamine-N-oxide, which increased over time in the placebo cohort (variable importance of projection > 1 and fold change $> 5\%$) (Fig. 5A; Fig. S5; Tables S8–11). No changes in plasma bile acids were seen (data not shown).

Rifaximin- α enhanced day-30 faecal TNF- α ($p = 0.0058$) and IL-17E ($p = 0.011$) concentrations and suppressed faecal *Veillonella* and *Streptococcus* spp.

Increased faecal IL-17A ($p < 0.05$), which is important for neutrophil recruitment and augmentation of antibacterial responses to pathogenic bacteria,²² was observed on rifaximin- α (Fig. 5B; Table S12).

Whilst baseline faecal calprotectin was elevated ($> 60 \mu\text{g/g}$) in the majority of patients, consistent with chronic intestinal inflammation, levels did not change on rifaximin- α . No changes were seen in faecal water metabolites on rifaximin- α .

Adverse events

Recorded AEs were almost twice as likely in the placebo-treated group ($n = 33$ placebo vs. $n = 17$ rifaximin- α). Infection-related AEs were more frequent on placebo. Only 1 serious AE was recorded; small bowel perforation in 1 participant treated with rifaximin- α . This was assessed clinically as a spontaneous event unrelated to the study medication.

Table 3. Inflammatory indices at baseline, 30 and 90 days post rifaximin- α or placebo.

Variable	Baseline	Day 30	Day 90	Friedman test within group p value [#]	RM-ANOVA within subjects effect p value [*]	RM-ANOVA between subjects effect p value ^Δ
TNF- α (pg/ml)						
Rifaximin- α	4 (3.1–5.3)	3.4 (2.8–4.1)	3.3 (2.5–3.8)	<0.001	<0.001	0.717
Placebo	4.3 (3–5.9)	3.5 (3–5.1)	3.7 (3–4.5)	0.578		
IL-8 (pg/ml)						
Rifaximin- α	34 (28–50)	27 (18–68)	29 (20–47)	0.409	0.547	0.811
Placebo	38 (23–84)	30 (20–114)	25 (21–107)	0.733		
IL-6 (pg/ml)						
Rifaximin- α	4.1 (1.8–10.8)	3.7 (2.7–4.7)	3.8 (2–5.4)	0.935	0.412	0.239
Placebo	9.1 (2.8–19.1)	7.1 (2.9–8.3)	6.4 (2.3–9.7)	0.384		
IL-10 (pg/ml)						
Rifaximin- α	0.42 (0.23–0.57)	0.23 (0.17–0.19)	0.4 (0.19–0.47)	0.005	0.216	0.076
Placebo	0.61 (0.3–1)	0.48 (0.21–0.77)	0.44 (0.22–0.98)	0.274		
IFN- γ (pg/ml)						
Rifaximin- α	18 (15–37)	19 (12–35)	16 (10–37)	0.935	0.206	0.911
Placebo	23 (16–36)	24 (16–39)	17 (9–104)	0.039		
Bacterial DNA (x10 ³)						
Rifaximin- α	3.2 (1.7–4.6)	3.5 (1.1–4.5)	3.3 (1.2–4.3)	0.181	0.447	0.717
Placebo	2.2 (1.6–2.7)	2.5 (1.8–3.7)	2.9 (2.1–3.9)	0.076		
Faecal calprotectin (μ g/g of faeces)						
Rifaximin- α	105 (55–155)	44 (14–129)	71 (14–166)	0.176	0.658	0.58
Placebo	116 (48–211)	40 (24–149)	122 (24–149)	0.032		

IFN- γ , interferon- γ ; IL-, interleukin-; RM-ANOVA, repeated measures-ANOVA; TNF- α , tumour necrosis factor- α .

p < 0.05 represents significant difference between groups. Bold text denotes statistically significant values.

[#]Friedman test within group p value comparing change across 3 time points within group.

^{*}RM-ANOVA (log transformed for non-parametric data) reflecting within subject effect.

^ΔRM-ANOVA reflecting between subject comparison.

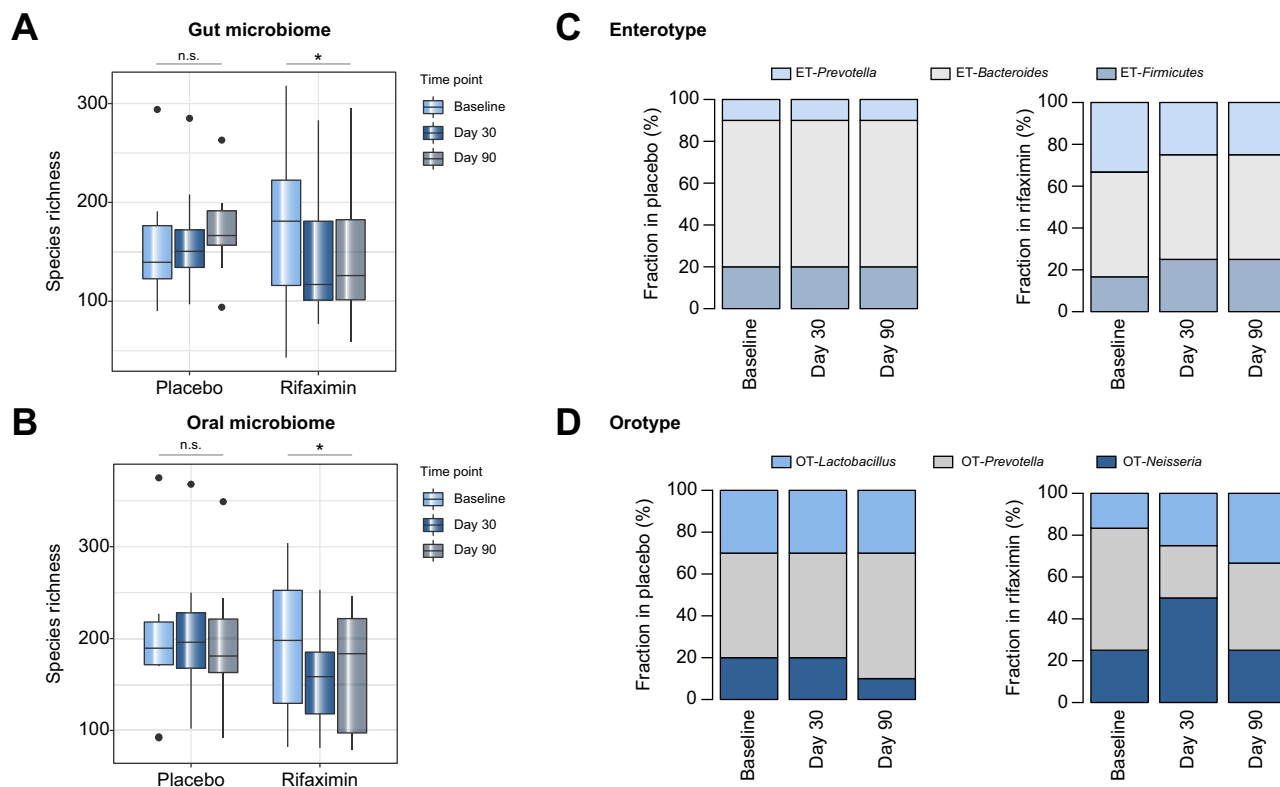


Fig. 3. Rifaximin- α led to significant changes in the microbial community. Rifaximin- α reduced species richness in both (A) faecal and (B) salivary microbiome not observed with placebo (Wilcoxon signed rank one-sided tests; *p value < 0.05). Three distinct microbial clusters in the faecal microbiome were identified enriched with *Prevotella*, *Bacteroides* and *Firmicutes* genera, named enterotype (C). Likewise, 3 distinct microbial clusters were identified in the salivary microbiome enriched with *Prevotella*, *Neisseria* and *Lactobacillus*, named orotype (D). Fractions of different enterotypes/orotypes were changed by rifaximin- α , e.g. increasing *Firmicutes*-type in the faecal and *Lactobacillus*-type in the salivary microbiome. ET, enterotype; OT, orotype. * Wilcoxon signed rank one-sided test p value < 0.05

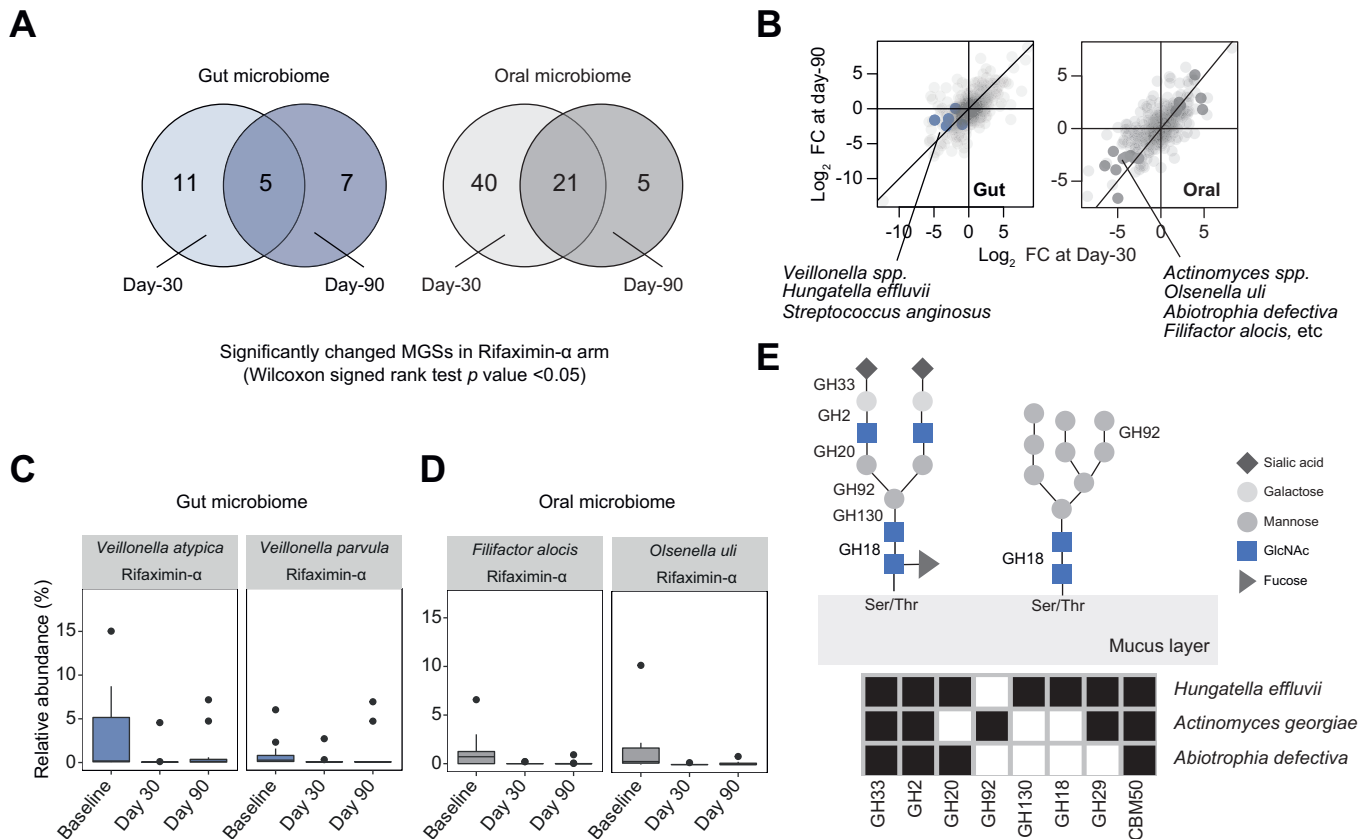


Fig. 4. Rifaximin- α suppressed growth and mucin-degrading capacity of orally originating species in faeces. (A) The number of significantly contrasted species in the faeces/saliva microbiome following rifaximin- α between baseline and days 30/90 are indicated in Venn diagrams. Significantly contrasted species were identified by comparing MGS abundance between baseline and day 30 or 90 by Wilcoxon signed rank tests ($p < 0.05$). Five species in faeces and 21 species in saliva were significantly contrasted on both day 30 and 90. (B) Log₂ FC of species abundance following rifaximin- α treatment between baseline and day 30 and 90 (Wilcoxon signed rank test; $p < 0.05$). Significantly contrasted species at both day 30 and 90 are coloured blue (faeces) and orange (saliva). (C) Boxplots for relative abundances (%) of the orally originating species in the faeces i.e. *Veillonella atypica* and *Veillonella parvula* in those treated with rifaximin- α vs. placebo. (D) Boxplots for relative abundances (%) of opportunistic pathogens in the saliva i.e. *Filifactor alocis* and *Olsenella uli*. (E) The mucin-degrading capacity of significantly contrasted species in faeces and saliva based on CAZyme annotations of given MGS. The CAZyme families that degrade O-glycans of human mucins (black cells of heatmap) represent microbes with mucin-degrading CAZyme classification. CAZyme, carbohydrate-active enzyme; FC, fold change; MGS, metagenomic sequencing. (This figure appears in color on the web.)

Discussion

In this double-blind, randomised, placebo-controlled mechanistic trial of rifaximin- α vs. placebo in patients with cirrhosis and HE, rifaximin- α improved HE at 30 days in association with a reduction in biomarkers of gut-derived systemic inflammation, including plasma TNF- α and neutrophil TLR-4 expression. Rifaximin- α suppressed growth of opportunistic orally originating pathogens that were identified in cirrhotic faeces including *Veillonella atypica*, *Veillonella parvula* and *Streptococcus* spp., as well as *Akkermansia* and *Hungatella*, all of which are rich in sialidase that degrades O-glycans in the gut mucin barrier. In the saliva, rifaximin- α decreased opportunistic pathogens including *Abiotrophia defectiva*, *Olsenella uli* and *Filifactor alocis*, and led to a significant increase in *Lactobacillus* and *Streptococcus* spp. associated with oral health. Furthermore, rifaximin- α changed the intestinal microenvironment, resulting in an increase in faecal TNF- α and IL-17E; increased faecal IL-17A being associated with reduced faecal *Veillonella* and *Streptococcus* spp.

Whilst the efficacy of rifaximin- α in reducing the risk of recurrent overt HE is well-established,⁹ its mechanism of action

remains to be elucidated. Rifaximin- α reduces circulating endotoxin levels⁴ but previous studies have fallen short of demonstrating any distinct changes in microbial abundance by 16S rRNA faecal microbiota profiling.^{4,11} One study demonstrated a significant increase in potentially beneficial serum fatty acids and intermediates of carbohydrate metabolism with rifaximin- α .⁴

This is the first study utilising shotgun metagenomic sequencing to explicitly identify changes in the salivary and faecal microbiome in response to rifaximin- α . This was associated with reduced systemic inflammation as evidenced by a reduction in plasma TNF- α and neutrophil TLR-4 expression, surrogate markers for a reduction in circulating endotoxin. Our *a priori* hypothesis had been that rifaximin- α would reduce neutrophil ROS, as circulating neutrophil dysfunction in cirrhosis has been shown to determine 90-day and 1-year mortality.¹⁶ Whilst not proven, the study was underpowered to reach this endpoint, after rifaximin- α was introduced into national clinical guidelines, making completion of recruitment difficult. However, the improvement in systemic inflammation and neutrophil TLR-4 expression in response to rifaximin- α was evident.

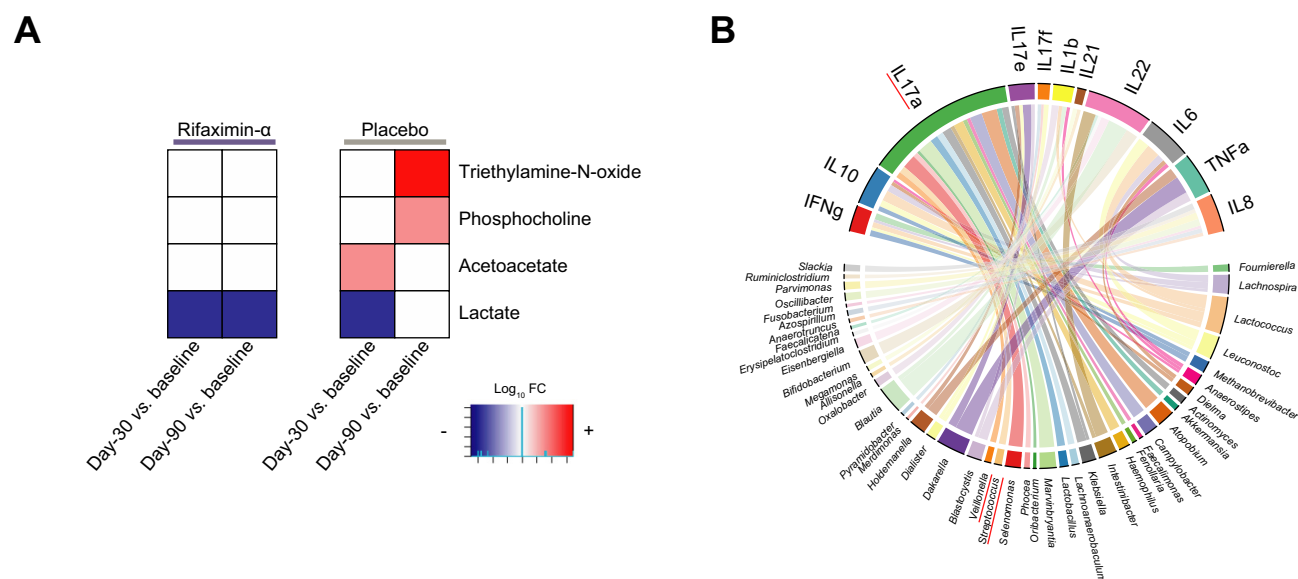


Fig. 5. Plasma metabolome and gut microbiome associations with faecal cytokines. (A) Enriched or depleted plasma metabolites at day 30 or 90, compared to baseline. Based on PLS-DA, enriched or depleted metabolites were identified from the 2 study arms. D-lactate, a marker of intestinal barrier damage⁸ was consistently depleted with rifaximin-α, whereas acetoacetate, phosphocholine, and trimethylamine-N-oxide were only enriched with placebo. Log₁₀ FC of significantly altered metabolites (VIP > 1) between baseline and day 30 or 90 were coloured on the heatmap from blue (negative value) to red (positive value). (B) Circos plot demonstrating association ($R_m^2 > 10\%$) of species abundances in faeces with faecal cytokines. Weights of the edges from explained variances of fixed effect variables were calculated with linear-mixed effects models by *lme4* R package and visualized by the *circosize* R package. FC, fold-change; IFN-γ, interferon-γ; IL-, interleukin-; PLS-DA, partial least square discriminant analysis; TNF-α, tumour necrosis factor-α; VIP, variable importance of projection. (This figure appears in color on the web.)

A change in gut microbiome composition and function impacts on a multitude of vital homeostatic functions including immunomodulation.²³ Patients with cirrhosis have gut dysbiosis with small bowel bacterial overgrowth and translocation of bacteria and their products (such as lipopolysaccharide and bacterial DNA) across a more permeable gut epithelial barrier, exacerbated by underlying portal hypertension and endothelial dysfunction.⁷ This culminates in systemic inflammation and endotoxemia, inducing CAID through TLR signalling, predisposing to infection, and the development of hepatic decompensation. In this study, patients treated with rifaximin-α experienced fewer infections during the 90-day follow-up period than patients on placebo. This is in keeping with published experience that highlights a potential role for rifaximin-α beyond the prevention of overt HE by augmenting intestinal barrier function and reducing BT and CAID.^{24,25} Furthermore, plasma lactate, a metabolic biomarker of poor prognosis associated with non-survival in decompensated cirrhosis¹⁸ was reduced by rifaximin-α.

Gut microbiome perturbations are linked to the pathogenesis of cirrhosis and progression to advanced liver disease.²⁶ Over 75,000 microbial genes differ between patients with cirrhosis and healthy individuals, with over 50% taxonomically assigned bacterial species of oral origin, suggesting an invasion of the distal gut from the mouth in cirrhosis.²⁷ Salivary dysbiosis is also observed in patients with cirrhosis.²⁸ Our data confirm salivary dysbiosis – bacteria that are normally resident in the oral cavity translocating to the gut and associated with the generation of a systemic inflammatory milieu. Rifaximin-α suppressed the growth of these orally originating species in the faeces including *Veillonella atypica*, *Veillonella parvula* and *Streptococcus spp* rich in sialidases, enzymes that degrade O-glycans of human mucins.

These species are commonly found in dental plaque²⁹ being associated with periodontal disease³⁰ and cystic fibrosis.³¹ Many bacteria that colonise the mouth express sialidases that degrade sialoglycoprotein substrates and can use sialic acid and/or underlying sugars as carbon sources, improving their survival while facilitating access to the epithelium. Sialidases are produced by oral *Streptococci viridans* including most strains of *Streptococcus oralis*, *intermedius* and *mitis*.^{32,33} Bacterial sialidases unmask underlying ligands to which bacteria or their toxins adhere.³⁴ In the saliva, rifaximin-α decreased opportunistic pathogens including *Abiotrophia defectiva*, *Olsenella uli* and *Filifactor alocis*, with significant increases in *Lactobacillus* and oral commensal species, such as *Streptococcus spp*, associated with oral health. Previous studies have also shown that rifaximin-α promotes the growth of beneficial strains in ulcerative colitis³⁵; additionally, in a mouse model of visceral hyperalgesia, *Lactobacilli* grew in the ileum in response to rifaximin-α.³⁶ Rifaximin-α reduced adhesion, invasion and motility of *E. coli* independent of its antimicrobial effect³⁷ reducing the virulence of resident microbiota.³⁸ A recent metagenomic study evaluating patients with cirrhosis before and after rifaximin-α demonstrated collapse of bacterial-phage interactions, especially phages directed against pathobionts associated with cirrhosis, such as *Streptococcus*, *Pseudomonas* and *Enterobacteriaceae spp*.³⁹

Rifaximin-α increased faecal TNF-α and IL-17E concentrations whilst the suppression of *Veillonella spp* and *Streptococcus spp* was associated with increased faecal IL-17A. IL-17A is a mucosal-associated cytokine involved in local immune modulation. IL-17A and IL-17E are secreted by TH17 cells and play a critical role in establishing local host antimicrobial immunity and promote gut barrier repair.⁴⁰ IL-17 and TNF-α induce antimicrobial peptides in mucosal organs with IL-17A serving as a potent neutrophil

recruiter.⁴¹ They also promote epithelial cell proliferation and replacement of cells lost through homeostatic shedding.⁴² In addition, TNF- α is an important regulator of intestinal microbiota populations.⁴³ IL-17E, a barrier surface cytokine, promotes epithelial cell division and increases mucus secretion.⁴⁴ Therefore it can be postulated that rifaximin- α promotes an intestinal microenvironment conducive to increased mucus production and gut barrier repair. This may be a mechanism by which rifaximin- α reduces BT of enteropathogens and endotoxaemia although the design of this study did not allow direct interrogation of the mucus layer. Furthermore, the gut barrier is complex with a multitude of factors contributing to barrier function and immune-competence. *In vitro/in vivo* studies will be needed to further investigate the impact of rifaximin- α on gut barrier function which are beyond the scope of this trial.

In summary, the mechanism of action of rifaximin- α in patients with cirrhosis has been further elucidated. Rifaximin- α improved overt HE and neurocognitive function and ameliorated systemic inflammation by suppressing oralisation of the gut microbiome *via* suppression of *Veillonella spp* and *Streptococcus spp* as well as *Akkermansia* and *Hungatella*; all species rich in mucin-degrading enzymes and known to induce gut barrier damage. Rifaximin- α promoted a TNF- α and IL-17E-enriched intestinal microenvironment conducive to improved antimicrobial function and gut barrier repair.

Abbreviations

BT, bacterial translocation; CAID, cirrhosis-associated immune dysfunction; CAZyme, carbohydrate-active enzyme; HE, hepatic encephalopathy; HRQoL, health-related quality of life; LPS, lipopolysaccharide; MGS, metagenomic species; OB, oxidative burst; PHES, psychometric hepatic encephalopathy score; PLS-DA, partial least square discriminant analysis; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF- α , tumour necrosis factor- α .

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Conflict of interest

Dr V Patel and Ms A Zamalloa have delivered paid lectures for Norgine Pharmaceuticals Ltd. Professor Shawcross has participated in advisory boards for Norgine Pharmaceuticals Ltd, EnteroBiotix, Kaleido Biosciences, Mallinckrodt and Shionogi and has delivered paid lectures for Norgine Pharmaceuticals Ltd, Falk Pharma and Alfa Sigma.

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

Authors' contributions

DLS conceived the study and served as Chief Investigator. VCP co-designed the study with DLS. VCP served as the lead investigator, recruiting the patients, undertaking data collection and coordinating the analyses with the support of AZ who recruited patients, undertook data collection and patient follow-up. MJWM performed the statistical analyses. SL and EW performed the saliva microbiome analyses. KDS and SG performed both faecal and saliva microbiome analyses. SL and SS^{3,8} performed the downstream analyses on the faeces and saliva microbiome data, integrative and functional analyses. SS⁴, GKMV, XH, SG, MC and LAE undertook the remaining laboratory analyses. ELC and NP supervised the microbiome analyses. NG generated microbiome sequence data and SDE conceived and coordinated the microbiome data generation and analyses serving as a co-investigator. JW and KB were involved throughout the trial as co-investigators. The manuscript was written by DLS and critically reviewed by all co-authors who approved the final submitted manuscript.

Data availability statement

All the metagenomic data are publicly available in the European Nucleotide Archive: <https://www.ebi.ac.uk/ena/browser/home>; Identifier number: PRJEB38481. All remaining data including the metabonomic data will be made available on request. All requests should be sent to the corresponding author: debbie.shawcross@kcl.ac.uk.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2021.09.010>.

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Author names in bold designate shared co-first authorship

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