Scientific Report

A placebo controlled single centre double blind randomised trial to investigate the efficacy of **RIF**aximin versus placebo in improving **SYS**temic inflammation and neutrophil malfunction in patients with cirrhosis and chronic hepatic encephalopathy.

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2 SCIENTIFIC ABSTRACT

Title: A placebo controlled single centre double blind randomised trial to investigate the efficacy of **RIF**aximin versus placebo in improving **SYS**temic inflammation and neutrophil malfunction in patients with cirrhosis and chronic hepatic encephalopathy ('RifSys').

Trial design: Investigator-initiated randomised placebo-controlled single centre double blind study.

Methods:

- *Participants:* Patients with cirrhosis presenting with chronic persistent overt HE (≥ grade 1) or with ≥2 episodes of overt HE in the previous 6 months, recruited within the Institute of Liver Studies at King's College Hospital NHS Foundation Trust.
- *Intervention:* rifaximin-α 550mg (TARGAXAN) twice daily versus placebo for treatment period of 90 days.
- *Objective:* a therapeutic strategy utilising rifaximin-α to modulate gut microbiota to improve neutrophil dysfunction, reduce gut-derived systemic inflammation and metabolic profiling, and improve clinical outcomes and prolonging transplant-free survival.
- *Primary outcome:* To test if rifaximin-α reduces neutrophil spontaneous oxidative burst *ex vivo* in patients with cirrhosis and chronic HE after 30 days.
- *Secondary outcome:* To test if rifaximin-α reduces the development of systemic inflammation, organ failure and improves survival over 90 days. This will include analyses in gut microbiota profiling, alterations in faecal biomarkers, plasma and urine metabolic profiling, markers of bacterial translocation (blood bacterial DNA quantification), neutrophil dysfunction (including toll-like receptor 4 expression) and circulating plasma pro- and anti-inflammatory cytokine analyses. Clinical and mechanistic assessments undertaken at baseline (day 1) prior to initiation of medication, and then at 30 days and 90 days.
- *Randomisation***:** 50 participants to be randomised on a 1:1 basis to either rifaximin-α (n=25) or placebo (n=25)
- *Blinding:* participants, care givers, clinicians and researchers all blinded to group assignment.
- Recruitment period:

Results:

- Recruitment: 38 participants in total
- Numbers randomised: 38 participants in total – 19 to each treatment arm
- Numbers analysed: 32 participants completed to 30 days and 26 of these participants completing to 90 days.

- Outcome: clinical metadata collected and mechanistic data generated and all analysed for intended primary and secondary outcomes at baseline, day 30 and day 90.
- Adverse events: refer to relevant section of study report.

Conclusions: the trial recruited fewer participants than intended, and was therefore underpowered from the outset to demonstrate a change in the primary outcome of a 50% reduction in spontaneous neutrophil oxidative burst. Participants on rifaximin-α normalised their low grade hepatic encephalopathy whilst on therapy. Line tracing as part of PHES improved significantly on rifaximin-α.

Whilst peripheral total white cell and neutrophil count were not different between arms at baseline or during the course of the study, cytokine profiling demonstrated that TNF-α fell significantly and there was a temporary reduction in IL-10 levels whilst on therapy with rifaximin-α in comparison to placebo.

There were no differences in Quality of Life scores by either the EuroQol validated EQ-5D-3L descriptive system or the EuroQol EQ-Visual Analogue Scale across treatment groups or time-points.

Amongst the secondary outcomes which were not powered for, there were no global changes in gut microbiota compositional profiles based on 16S rRNA gene pyrosequencing when assessed by alpha and beta diversity whilst on therapy with rifaximin-α in comparison to placebo. Similarly there was no change in circulating whole blood bacterial 16S rDNA levels – as a surrogate marker of gut bacterial translocation - whilst on therapy with rifaximin-α in comparison to placebo. There were however some minor changes in the same circulating whole blood microbiota profiles as assessed by 16S rRNA gene pyrosequencing whilst on therapy with rifaximin-α in comparison to placebo as days 30 and 90. There were no changes in plasma bile acid profiles, or in the metabolic profiles of plasma, urine and faecal water undertaken by 1H NMR spectroscopy, whilst on therapy with rifaximin-α in comparison to placebo.

Ethical approval and trial registration:

- REC ref: 14/SC/0088
- Sponsor R&D ref: KCH14-183
- ClinicalTrials.gov ref: NCT 02019784
- EudraCT ref number: 2013-004708-20

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6 SCIENTIFIC BACKGROUND AND RATIONALE FOR STUDY

Patients with cirrhosis are particularly prone to infection, which is frequently a precipitant of hepatic encephalopathy, renal failure and circulatory collapse. Bacterial infections are of particular concern in patients with cirrhosis because they are poorly tolerated [1]. Sepsis and associated endotoxaemia occur in approximately 40% of hospitalized patients with cirrhosis and is a major cause of death [2].

Gut-derived and blood-borne pathogens can induce an inflammatory response within the liver and spleen, which are the major organs that remove bacteria and their endotoxin (lipopolysaccharide - LPS) from the bloodstream. Several mechanisms have been identified and proposed in this process which depends upon a balance between the barrier functions of the gut and the 'detoxifying' capacity of the liver [3, 4]. Those with established liver cirrhosis have been shown to have escape of endotoxin into the bloodstream produced by bacteria that reside in their intestines, which becomes more permeable or 'leaky' [5].

Gut dysfunction is defined by changes in the types of bacteria within the gut and in overall permeability allowing bacterial products which would otherwise be contained within the gut to travel into the bloodstream and lymphatic system with detrimental effects elsewhere in the body [6]. This passage of bacterial products is termed bacterial translocation, and their effects on the liver and general immune system can be then be measured [7, 8].

As it has now become recognised that neutrophil dysfunction predisposes to infection and may also have a more direct pathogenic role in hepatic encephalopathy it supports the neutrophil as being a novel pharmacotherapeutic target in a condition where current therapies such as lactulose are inadequate [9]. A therapeutic strategy utilising rifaximin-α, a non-absorbable antibiotic, to modulate gut ammonia bacterial producing species could potentially lower gut-derived systemic inflammation, endotoxaemia, infection and organ dysfunction in this population improving outcomes and prolonging transplant-free survival [10].

Positive results from this study would support further trials into the potential benefit of using rifaximin-α to improve immune function, as well as the recurrence of hepatic encephalopathy, in patients with cirrhosis.

7 STUDY OBJECTIVES AND ENDPOINTS

7.1 Primary objective

To assess whether rifaximin-α reduces neutrophil spontaneous oxidative burst *ex vivo* in patients with cirrhosis and chronic hepatic encephalopathy after 30 days.

7.2 Secondary objective

To assess whether rifaximin-α reduces the development of systemic inflammation, infection, organ failure and improves patient survival over 90 days. This will include analyses for changes in intestinal permeability, alterations in faecal microbiota and faecal biomarkers (e.g. calprotectin), systemic endotoxemia and immune dysfunction.

7.3 Primary Endpoint

A reduction in spontaneous neutrophil oxidative burst of 50% compared to baseline (as measured by the Burstest which measures the spontaneous production of reactive oxygen species) 30 days following the start of rifaximin-α/placebo therapy.

7.4 Secondary Endpoints

I. A reduction in systemic inflammation as measured by plasma endotoxaemia, bacterial DNA quantification and plasma pro-inflammatory cytokine profile at 90 days.

II. An improvement in neutrophil bacteriocidal capacity as measured by the Phagotest which utilises opsonised E. coli at 30 and 90 days.

III. An improvement in neutrophil phenotype and function including baseline and LPS-induced toll-like receptor 4 expression and intracellular cytokine production at 30 and 90 days.

IV. Alterations in faecal microbiota at 30 and 90 days.

V. Changes in faecal biomarkers (calprotectin) at 90 days.

VI. Changes in urinary and plasma metabonomic profile as measured by proton MR spectroscopy at 90 days.

VII. Development of recurrent overt hepatic encephalopathy, organ failure and infection during the 90 day follow up.

VIII. Improvement in Psychometric Hepatic Encephalopathy Score including Trails A and B neuropsychiatric test scores at 30 and 90 days.

8 TRIAL DESIGN

8.1 Summary

The study was designed to be performed on a total of 50 patients with cirrhosis and chronic hepatic encephalopathy aged between 18 and 75 years managed within the Liver Unit at King's College Hospital (largest tertiary Liver Transplant Centre within the United Kingdom).

The study was designed as a single centre double blind randomised placebo controlled longitudinal trial, with a 1:1 allocation ratio of rifaximin-α therapy to matching placebo, with treatment to be administered over a total of 90 days.

For the purposes of the study a patient was considered to have cirrhosis if they fulfilled two out of three diagnostic criteria of (i) biochemistry consistent with underlying cirrhosis, (ii) radiologic findings consistent with cirrhosis/portal hypertension and/or (iii) confirmatory liver histology. The diagnosis of chronic hepatic encephalopathy was based on the presence of (i) persistent overt hepatic encephalopathy (≥ grade 1) or (ii) presentation with ≥2 episodes of overt hepatic encephalopathy in the previous 6 months.

Patient demographics, clinical details (including West Haven hepatic encephalopathy grade) and blood haematology, biochemistry (including venous ammonia) and neutrophil function was assessed and collated at baseline before randomisation to one of the two treatment arms, and again at two separate time points following 30 and 90 days of Rifaximin-α therapy/placebo. Faecal and salivary (oral) microbiota analysis was performed by deep pyrosequencing techniques and plasma endotoxaemia was measured by whole blood bacterial DNA quantification as a marker of bacterial translocation, as well as plasma cytokine array. Clinically relevant outcomes including the development of recurrent overt hepatic encephalopathy, organ failure, infection and mortality was recorded for a total of 90 days.

8.2 Study Flowchart

Study procedures	Screening	Baseline	Day 30 (+/- 7 days)	Day 90 (- 7 days)	Follow-Up Period (+/- 7 days)
Signed informed consent or surrogate informed consent from Legal Representative	X				
Eligibility Criteria	X				
Participant demographics	X				
Medical and surgical histories	X		X	X	
Concomitant medication usage	X		X	X	
Vital signs	X		X	X	
Complete physical examination	X		X	X	
Haematology panel	X		X	X	
Coagulation panel	X		X	X	
Clinical biochemistry panel	X		X	X	
Child-Pugh, MELD, UKELD & CLIF-SOFA score assessments	X		X	X	
Liver screen	X				
Urinary pregnancy test (β-HCG) if applicable	X		X	X	
Liver ultrasound *(if iiver imaging not performed within the previous 3 months)*	X		X	X	
Psychometric Hepatic Encephalopathy Score		X	X	X	
EQ-5D-3L health questionnaire		X		X	
Randomisation		X			
IMP administration		X			
IMP compliance check			X	X	
Adverse events monitoring			X	X	X

Table 1: Schedule of events (1) – trial specific, clinical and monitoring procedures

Study procedures	Screening	Baseline	Day 30 (+/- 7 days)	Day 90 (- 7 days)	Follow-Up Period (+/- 7 days)
Study sampling					
Blood (heparinised) for neutrophil assays including phenotyping, phagocytosis and oxidative burst capacity		X	X	X	
Blood (whole – EDTA) for bacterial DNA quantification		X	X	X	
Blood (plasma – EDTA) for bile acid profiling		X	X	X	
Blood (serum – clotted) for cytokine analysis		X	X	X	
Blood (lithium heparinised plasma and SST serum) for metabonomic profiling		X	X	X	
Urine for metabonomic profiling		X	X	X	
Faeces for biomarker analysis		X	X	X	
Faeces for metabonomic profiling		X	X	X	
Saliva for microbiota analysis		X	X	X	

Table 2: Schedule of events (2) – trial biological sampling

9 STUDY ASSESSMENTS AND CONDUCT

9.1 Screening Visit

The following study evaluations were performed and recorded during the course of the screening visit:

- Signed informed consent or surrogate informed legal representative consent on behalf of participant deemed to be incapacitated and therefore unable to consent,
- Participant demographics,
- Review of eligibility criteria,
- Complete medical and surgical histories, including alcohol consumption, smoking and other recreational drug use,
- Record of concomitant medication usage, including use of prescription and over-the-counter medications, herbal preparations, and vitamin and/or mineral supplements,
- Vital signs (blood pressure, pulse, respiratory rate, and temperature),
- Complete physical examination, including neurological examination for encephalopathy,
- Haematology, coagulation panel, clinical biochemistry,
- Child-Pugh, MELD, UKELD and CLIF-SOFA score assessments,
- Liver screen including viral hepatitis, HIV assay (other viral screen assays as indicated), ferritin, caeruloplasmin, alpha-1-antitrypsin, auto-antibody screen, serum immunoglobulins, alpha-fetoprotein,
- Urinary pregnancy test (β-HCG) (for women of childbearing potential), and
- Liver ultrasound (if imaging not performed within the previous 3 months).

Clinically meaningful, unexpected findings in the laboratory or clinical tests that may interfere with the study conduct were all discussed with the Principal Investigator prior to entry into the study. Once the participant's eligibility was confirmed and screening was completed, the participant was seen at the baseline visit and randomised within 72 hours of eligibility confirmation.

9.2 Baseline (pre-treatment) Study Visit

The following study evaluations were performed and recorded during the course of the baseline visit:

- Review and confirmation of eligibility criteria
- Confirmation that participant wishes to continue with study participation
- Psychometric hepatic encephalopathy score testing, including Trail's A and B neurocognitive function tests
- EQ-5D-3L health questionnaire
- *Baseline study sampling – refer to next section*

9.2.1 BASELINE (PRE-TREATMENT) STUDY SAMPLING

- Blood (heparinised) for neutrophil assays including phenotyping, phagocytosis and oxidative burst capacity
- Blood (whole – EDTA) for bacterial DNA quantification
- Blood (plasma – EDTA) for bile acid profiling
- Blood (serum – clotted) for cytokine analysis
- Blood (lithium heparinised plasma and SST serum) for metabonomic profiling
- Urine for metabonomic profiling
- Faeces for biomarker analysis
- Faeces for metabonomic profiling
- Saliva for microbiota analysis

9.3 Randomisation and Post-Randomisation Double Blind Study Phase

Randomisation occurred on the day of the baseline study visit, and the IMP with medication compliance diary was dispensed to the participant by the Investigator and research team on a double blind basis, to commence study medication the next morning.

The study procedures and sampling protocol specified for the baseline visit were performed prior to commencement of the study drug, and then repeated at intervals of 30 and 90 days whilst the participant was taking the study medication, or at early termination if applicable.

9.4 Day 30 and Day 90 Study Visits after Commencement of Treatment

All participants were due to be seen at 30 days (+/- 7 days) and then 90 days (- 7 days) after start of treatment. Follow-up study procedures consisted of the following:

- Update of concomitant medications and therapies
- Vital signs (blood pressure, pulse, respiratory rate, and temperature)
- Physical examination, including neurological examination for encephalopathy and weight
- Evaluation of clinical features
- Study medication compliance check
- Haematology, coagulation panel, clinical biochemistry
- Child-Pugh, MELD and UKELD score assessments
- Urinary pregnancy test (β-HCG) (for women of childbearing potential)
- Psychometric hepatic encephalopathy score testing, including Trail's A and B neurocognitive function tests
- EQ-5D-3L health questionnaire at visit 2 (90 days) only
- Assessment and/or updates of adverse events
- On treatment study sampling – refer to next section

9.4.1 DAY 30 AND DAY 90 VISIT (ON-TREATMENT) STUDY SAMPLING

- Blood (heparinised) for neutrophil assays including phenotyping, phagocytosis and oxidative burst capacity
- Blood (whole – EDTA) for bacterial DNA quantification
- Blood (plasma – EDTA) for bile acid profiling
- Blood (serum – clotted) for cytokine analysis
- Blood (lithium heparinised plasma and SST serum) for metabonomic profiling
- Urine for metabonomic profiling
- Faeces for biomarker analysis
- Faeces for metabonomic profiling
- Saliva for microbiota analysis

9.5 Day 120 Follow-up Study Visit (30 days after Completion of Treatment)

Participants were reviewed 30 days (+/- 7 days) after completion of treatment with the IMP for clinical review and assessment of adverse events.

After clinical assessment, those participants that were assessed as potentially benefiting from treatment with standard rifaximin-α were offered treatment in the clinical setting out with of the study. Note that participants were not unblinded at the end of their trial participation unless Emergency Unblinding Procedures were required, and that standard unblinding for all participant occurred at the end of the study.

9.6 Provision for Unscheduled visits

Due to the nature of the underlying illness with advanced liver disease and related complications, it was anticipated that a significant proportion of participants might require unplanned hospital admissions for emergency treatment. This would have resulted in them being unable to attend for the 30 day (+/- 7 days) study visit or 90 day (- 7 days) study visit for study related procedures whilst being admitted elsewhere. In order to optimise the likelihood of obtaining data after the IMP has been commenced, provision for arranging unscheduled visits to equate approximately to the day 30 and day 90 visits was made possible taking into account the clinical condition and progress of the participant.

10 EFFICACY AND SAFETY ASSESSMENTS

10.1 Medical History and Physical Examinations

Medical history were taken at screening. A comprehensive physical examination was performed at the screening visit and at subsequent day 30 and day 90 study visits. Weight, assessed in ordinary indoor clothing, and height (obtained at the screening visit only) was recorded at the specified visits. Each participant's body mass index (BMI) was calculated

10.2 Vital Sign Measurements

Evaluation of the participant's respiratory rate was measured by counting the inhalations for one minute. Heart rate, systolic and diastolic blood pressure, and temperature measurements ('vital signs') were obtained at the screening visit and at subsequent day 30 and day 90 study visits. If clinically significant findings occurred, as determined by the Investigator, then that measurement was repeated at medically appropriate intervals until the value returned to within an acceptable range.

10.3 Clinical Laboratory Tests

Clinical laboratory tests were analysed by the King's College Hospital central laboratory according to standardised, validated assays. The laboratory supplied instructions and specific containers for the various clinical and screening blood, faecal and urine based investigations. Blood, urine and faecal sample volumes and quantities all met the laboratory's specification.

10.4 Liver Ultrasound (and other radiological imaging)

A liver ultrasound scan, including examination of the biliary tree and gall bladder and with major hepatic vessel Doppler studies was performed as part of the screening visit if a similar scan had not been performed in the past 3 months. If the participant had undergone imaging evaluation by either ultrasound or CT or MRI scan based modalities within 3 months of recruitment, the results of those scans were utilised in lieu of the ultrasound at screening. If the Investigator deemed it medically necessary, additional scans other than those specified in the protocol were performed during the study.

11 PROCEDURES FOR RECORDING AND REPORTING ADVERSE EVENTS

Recording and reporting of adverse events was undertaken according to definitions provided by The Medicines for Human Use (Clinical Trials) Regulations 2004 and Amended Regulations 2006, as follows:

Adverse Event (AE):

Any untoward medical occurrence in a subject to whom a medicinal product has been administered including occurrences which are not necessarily caused by or related to that product.

Adverse Reaction (AR):

Any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

Unexpected Adverse Reaction (UAR):

An adverse reaction the nature and severity of which is not consistent with the information about the medicinal product in question set out in the summary of product characteristics (SmPC) for the product.

Serious adverse Event (SAE), Serious Adverse Reaction (SAR) or Unexpected Serious Adverse Reaction (USAR):

Any adverse event, adverse reaction or unexpected adverse reaction, respectively, that:

- Results in death;
- Is life-threatening;
- Required hospitalisation or prolongation of existing hospitalisation;
- Results in persistent or significant disability or incapacity;
- Consists of a congenital anomaly or birth defect.

Important Medical Events (IME) & Pregnancy:

Events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed in the definition above should also be considered serious.

Although not a serious adverse event, any unplanned pregnancy was also be reported via the SAE reporting system.

11.1 Reporting Responsibilities

King's College Hospital NHS Foundation Trust and King's College London delegated the delivery of the Co-sponsor's responsibility for Pharmacovigilance (as defined in Regulation 5 of the Medicines for Human Use (Clinical Trials) Regulations 2004 to the King's Health Partners Clinical Trials Office (KHP-CTO).

All SAEs, SARs and SUSARs (excepting those specified in this protocol as not requiring reporting) were reported immediately (and certainly no later than 24hrs) by the Investigator to the KHP-CTO and the Investigator for Medical Review in accordance with the current Pharmacovigilance Policy.

Death as a result of disease progression and other events that are primary or secondary outcome measures were not considered to be SAEs and were consequently reported in the normal way, on the appropriate Case Report Form.

11.2 Adverse events that do not require reporting

Events or reactions listed in the SmPC did not need to be reported to KHP-CTO but were recorded in the Case Report Form. The period for AE reporting was from date of the first dose until 30 days post final IMP administration.

The development of systemic inflammation, infection, other forms of hepatic decompensation in keeping with disease progression including the development of jaundice, synthetic failure including coagulopathy or hypoalbuminaemia, new onset ascites, spontaneous bacterial peritonitis, variceal haemorrhage, and/or hepatocellular carcinoma, as well as organ failure and survival were all pre-specified outcomes of this trial and did not require expedited reporting to KHP-CTO. Deaths as a result of disease progression were not reportable in an expedited fashion.

Serious Adverse Events that were to be reported using an SAE reporting form were limited to those NOT already listed as primary or secondary outcomes, but which were deemed as reasonably occurring as a consequence of treatment with Rifaximin-α.

11.3 Participant and Study Termination

11.3.1 REASONS AND PROCEDURES FOR EARLY TERMINATION

Participants were withdrawn from the study at their own request, upon request of the Investigator, or by the Co-sponsors at any time or for any reason. Reasons for removing a participant from the study included :

- the participant does not adhere to study rules and procedures;
- the participant wishes to withdraw from the study;
- the legal representative wishes to withdraw the participant from the study;
- the participant develops an adverse event (AE) necessitating withdrawal;
- continuation of the participant is in violation of the inclusion and exclusion criteria;
- the blind being broken to the study team for the participant;
- the Investigator feels it is in the participant's best interest to terminate participation.

If a participant was lost to follow up (i.e., fails to return for study visits), reasonable efforts were made to contact the participant and complete study termination procedures. All participants who discontinue the study because of adverse events (AEs) will be followed up at suitable intervals in order to evaluate the course of the AE and to ensure the reversibility or stabilisation of the abnormality.

All participants who prematurely discontinued the study, regardless of the cause, underwent all assessments scheduled for End of Treatment on the date of discontinuation. A follow-up visit was then scheduled to occur 4 weeks following the early termination visit to ensure ongoing clinical needs of the participant are met.

11.3.2 TERMINATION OF THE STUDY

The study could have been terminated prematurely with sufficient notice in advance by the Investigator for any reason as per the terms of the study contract. The reason would have been communicated in writing to the Co-sponsors.

The trial may have been prematurely discontinued by the Co-sponsor, Principal Investigator or Regulatory Authority on the basis of new safety information or for other reasons given by the Chief Investigator and Ethics Committee concerned.

If the trial had been prematurely discontinued, active participants would be informed and no further participant data will be collected. The Competent Authority (MHRA) and Research Ethics Committee would also be informed within 15 days of the early termination of the trial.

11.4 Completion of the Study

The Investigator documented the completion or the reason for early withdrawal by a participant in the case report form. The following categories were used to describe the early withdrawal reasons in the case report form:

- Participant discretion (document reason)
- Legal representative discretion
- Investigator considers it to be in the best interest of the participant (document reason)
- Adverse event(s)
- Administrative reasons (e.g., early termination of the study)
- Participant lost to follow-up
- Major protocol violation (with approval by the Co-sponsors)
- Liver transplantation
- Death

12 ETHICS & TRIAL REGISTRATION

Full ethical approval was secured by application to and review by the NHS Health Research Authority (HRA) NRES Committee South Central - Oxford C (Bristol) and to the Medicines and Healthcare products Regulatory Agency (MHRA) for Clinical Trial Authorisation. Local study approvals were secured after review by the King's College Hospital Research and Development department, with trial oversight and monitoring undertaken by King's Health Partners Clinical Trials Office. The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of Good Clinical Practice (GCP) and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations 2004, as amended in 2006 and any subsequent amendments.

12.1 Participant Informed Consent

Potential participants were identified by clinical teams responsible for their care and approached to assess whether they were interested in participating in this research project. If agreeable, they were given the relevant study participant information sheet to read. After an appropriate time period and the opportunity to ask any questions, written informed consent was then obtained if they were in agreement with participation.

Informed consent forms were in compliance with applicable regulations and reviewed and approved by the Research Ethics Committee prior to initiation of the study. The participant information sheet contained a full explanation of the purpose and nature of the study, a description of the procedures, the possible advantages and risks, alternate treatment options, a statement of confidentiality of participant study records, an explanation of whom to contact about the research, the participant's rights, and notification that participation is voluntary and refusal will involve no penalty or loss of medical benefits. These requirements are in accordance with the most current revision of the Declaration of Helsinki.

12.2 Legal Representative Consent on behalf of Incapacitated Participants

It was recognised during study design that participants that were eligible for this study may be unable to provide informed consent due to cognitive impairment arising from hepatic encephalopathy or pharmacologic sedation. In this situation where the potential participant was unable to consent, an appropriate legal representative was sought. The legal representative was most often a close personal contact of the potential participant e.g. the patient's next of kin. They were suitable to act as the legal representative by the virtue of their relationship, availability and their willingness to do so. In the process of considering inclusion into the study, the patient's wishes and feelings were assessed and written information was provided in the form of a 'legal representative information sheet'. After an appropriate time period and the opportunity to ask any questions, the legal representative if in agreement would then sign a 'legal representative consent form'.

Participants who subsequently regained decision making capacity during either the treatment or follow-up phases of the study were notified of their participation in the study and formal re-consent for continued participation was obtained where required and appropriate to do so. They were provided with a 'Regaining Capacity Information Sheet'. This explained why and how they were recruited to the study, that they had the choice of whether to continue their involvement in the study or if they preferred to withdraw and have previous data and samples related to their prior participation destroyed.

The Investigator was responsible for obtaining written informed consent from potential participants and/or their legally authorised representatives prior to any study specific screening and entry into the study. A copy of the signed document was provided to the participant and a copy filed in their medical notes. The original was retained by the Investigator and filed in the Trial Master file with copies in the hospital records.

12.3 Documentation of Data

12.3.1 SOURCE DOCUMENTATION

Source documentation refers to the information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data is contained in source documents (original records or certified copies).

All clinical work conducted under this protocol was done so according to good medical practice rules. This included regular inspections by the Co-sponsors with regular communication with the research team. The Investigator permitted trial-related monitoring, audits, Research Ethics Committee review, and regulatory inspections by providing the Co-sponsors, Regulators and Research Ethics Committee direct access to source data and other documents (including patients' case sheets, blood test reports, X-ray reports, histology reports etc.).

12.3.2 PARTICIPANT CONFIDENTIALITY

All information obtained during the conduct of the study with respect to the participant is regarded as confidential and confidentiality of all participants will be maintained.

Monitors, auditors and inspectors that required access to a participant's medical notes for the purpose of source document verification did so on the basis that the participant's confidentiality will be maintained at all times. An agreement for disclosure of any such information would be obtained in writing and was included in the statement of informed consent. The study data will not be disclosed to a third party (with the exception of auditors and/or regulatory authorities) without the written consent of the Co-sponsors.

12.4 Data Storage

All data has been secured against unauthorised access. Study related data has been stored in an encrypted form and the Investigator holds the key. Any hard copies of data, such as investigator files, are kept in a locked dedicated research office. All data kept on NHS or University computers has been protected by password access. All data kept on laptops or portable storage devices has been encrypted and password protected. If any data has been sent outside the above mentioned areas for statistical analysis, it has first been fully anonymised and password protected.

13 PARTICIPANTS

13.1 Participant Identification

Participants (who fulfil entry criteria) were recruited following admission to the Liver and General Intensive Care Unit's at Kings College Hospital. Patients admitted to Liver wards who are eligible were also approached, as will be those that were seen in the Liver out-patient clinics.

13.2 Eligibility criteria for participants

13.2.1 INCLUSION CRITERIA

Patients with established cirrhosis complicated by hepatic encephalopathy were recruited to the study. For the purposes of the study a patient was considered to have cirrhosis if they fulfilled two out of three diagnostic criteria:

(i) biochemistry consistent with underlying cirrhosis,

(ii) radiologic findings consistent with cirrhosis/portal hypertension, and/or

(iii) confirmatory liver histology.

The diagnosis of chronic hepatic encephalopathy was based on:

(i) the presence of (i) persistent overt hepatic encephalopathy (≥ grade 1), or

(ii) presentation with ≥2 episodes of overt hepatic encephalopathy in the previous 6 months.

13.2.2 EXCLUSION CRITERIA

- Age <18 or >75 years.
- Evidence of disseminated malignancy. Note isolated hepatocellular carcinoma without evidence of secondary spread was not an exclusion criteria.
- Known coeliac or inflammatory bowel disease.
- Evidence of intestinal failure, intestinal obstruction and / or previous bowel resection.
- Pre-existing immunosuppressive states including HIV infection and chronic granulomatous diseases.
- Anti-inflammatory drug use e.g. non-steroidal medication.
- Immunomodulatory drug use e.g. prednisolone, azathioprine, mycophenalate mofetil.
- Exposure to standard rifaximin-α therapy in the previous 12 weeks.
- Already receiving concomitant oral or parenteral antibiotic therapy, including rifamycin-based antibiotics or derivatives:
  - There is no experience regarding administration of rifaximin-α to subjects who are taking another rifamycin antibacterial agent to treat a systemic bacterial infection. Accordingly, potential participants already on systemic antibiotics or who are receiving antibiotic

prophylaxis against spontaneous bacterial peritonitis will not be recruited in order to reduce the theoretical risk of developing bacterial resistance.

- Known hypersensitivity to rifaximin-α or rifamycin-derivatives.
- Infection with *clostridium difficile* or stool testing positive for *clostridium difficile toxin* in the previous 3 months.
- Pregnancy or breast feeding women.

14 INTERVENTION: STUDY MEDICINAL PRODUCT

14.1 Rifaximin-α and Placebo Supplies

The double-blind supplies of rifaximin-α (TARGAXAN®*, manufactured by Alfa-Wasserman, Bologna, Italy) were supplied in blister packs each containing 14 tablets of 550mg. Matching placebo (also manufactured by Alfa-Wasserman, Bologna, Italy) were also supplied in blister packs each containing 14 tablets with accompanying stability data of appropriate standard. In order to maintain blinding, the study drug and placebo were packaged in an identical anonymised fashion so that the medications could not be identified as active drug or placebo by either the Investigator, members of the research team (such as dispending pharmacists or nursing staff) or participants.

Total amount of study medication that was supplied included 5 extra treatment packs per treatment arm overage:

- **Rifaximin-α 550mg tablets** twice daily:
  - 25 participants + 5 spare packs x 90 days = 5,400 tablets required.
  - TOTAL = 5,460 tablets supplied (30 packs x 182 tablets per pack – including 2 spare tablets per pack).
- **Matching placebo tablets** twice daily:
  - 25 participants + 5 spare packs x 90 days = 5,400 tablets required
  - TOTAL = 5,460 tablets supplied (30 packs x 182 tablets per pack – including 2 spare tablets per pack).

14.1.1 RIFAXIMIN-α AND PLACEBO PACKAGING AND LABELLING

The packaging and labelling of study medication supplies was performed according to good manufacturing practice standards by by Norgine Limited, Hengoed, UK, the designated qualified vendor. All study drug carried a uniquely numbered label and the label affixed to the blister pack contained the drug identification for the participant and conditions for storage.

14.1.2 RIFAXIMIN-α AND PLACEBO DISPENSING AND ACCOUNTABILITY

All study drug was dispensed in the original packaging provided by the drug manufacturer in order to assure stability of the drugs. If a participant was discharged from the hospital and was then followed as an outpatient, they were instructed to return all unused tablets in order to adequately assess their compliance with dosing instructions at each visit. The number of tablets remaining was counted at each visit to assess participant compliance with study drug administration. Every effort was made to obtain all unused tablets, and dispensed packs. If such effort failed, a dated file note explaining the reason for the failure to collect the study drugs was entered on the drug accountability records.

14.2 Dosage, Participant Instructions & Compliance Diary

Participants were fully instructed on how often to take the study medication. Each participant kit contained sufficient supply to dose participants for the duration of the study. Participants were provided with a paper study medication compliance diary, and asked to complete this each day recording when the study medication was taken and if doses were taken incorrectly (e.g. duplicated or missed), how often this occurred and if they recognised an error what the reasons were for these. Participants were instructed to bring in their study medication pack and medication diary at the day 30 and day 90 study visits, including all empty blister packs, so that compliance could be assessed by the research team.

Rifaximin-α and placebo supplies were stored in a secure, lockable area. When a participant was discharged from the hospital and was being followed as an outpatient, they were be instructed to store their study medication in a safe area at room temperature.

If a participant was discharged from the hospital, rifaximin-α and placebo tablets were dispensed to participants in sufficient quantities for continuation of treatment to at least the next scheduled visit, along with instructions for the proper method of taking the study drugs.

If a participant was admitted to hospital during the study period, the research team communicated regularly with the clinical medical and nursing teams locally to ensure that the study medication was continued if deemed appropriate and safe to do so, and to ensure that standard TARGAXAN® was not prescribed in error as a substitute.

14.3 Rifaximin-α and Placebo Product Administration

Rifaximin-α or matching placebo was administered at a dose of one tablet twice daily with water at about the same time in the morning and evening. Details of dosing information (e.g., dates of dosing, missed doses and dose adjustment, if any) was captured in the study medication compliance diary completed by the participant, which was then documented in the relevant study visit-specific case report form at days 30 and 90.

14.3.1 RIFAXIMIN-α AND PLACEBO PRODUCT ADMINISTRATION VIA NASOGASTRIC TUBE

Where a participant was unable to ingest the IMP tablets, and they had a nasogastric tube (NGT) placed for their standard clinical care requirements, provision was made to allow the IMP to be crushed and administered after re-suspension in an appropriate soluent by the treating medical and nursing team at the Chief Investigator's discretion. Usual standard of care indications for the placement of NGT included establishing enteral access to allow nutritional support and for the administration of pharmacotherapy.

The double-blinding was maintained where the IMP was crushed for administration via NGT as the research team were not present at the time of nor involved in NGT administration of the IMP. Similarly, the participant was only be exposed to one IMP regimen and any associated appearances.

Consequently no changes could be detected by the participant (or administering nursing staff) if they had visualised the suspension themselves as they were receiving the same IMP consistently without any cross-over between active drug and placebo arms.

15 RANDOMISATION:

15.1 Sequence generation

The assignment to one of two possible treatment arms of the study (to either rifaximin-α 550mg BID or placebo BID) was performed randomly. Participants were distributed into one of the two treatment groups in a 1:1 ratio. The randomisation schedule was generated using a validated randomisation program and verified for accuracy using strict quality control procedures. The assignment of participant number and treatment assignment was centrally coordinated by King's Clinical Trials Unit but blinded to both investigators and participants.

Blocked randomization (also called restricted randomisation) in blocks of ten was employed to ensure that comparison groups were generated according to a predetermined ratio, to provide 1:1 treatment allocation grouping. Blocking was used to ensure a close balance of the numbers in each group at any time during the trial. For every block of ten participants as was used in the trial, five were allocated to each arm of the trial. The numbers in the two groups at any time can therefore never differ by more than half the block length.

15.2 Allocation concealment mechanism

The random allocation sequence (by way of sequentially numbered containers) was known only to the Clinical Trials Unit and IMP and placebo manufacturer and supplier. All necessary steps were taken to conceal the sequence whilst interventions were assigned to all of the patient participants, the trials team as well as all other clinical and allied healthcare professionals involved in their care. The allocation was only revealed to the trial statistician at the end of the trial once the electronic database was completed and locked.

15.3 Implementation

Random allocation sequence was generated by the King's Clinical Trials Unit which was then provided to the IMP and placebo manufacturer and supplier, Participants were enrolled to the trial after formal informed consent and screening by the trials team (Dr. Vishal Patel and RN Ane Zamalloa). Participants were assigned to the intervention based on the trial code number assigned to them on recruitment with the IMP/placebo supplied by the Clinical Trials Pharmacy team on receipt of a valid trials prescription.

16 BLINDING

The participant, their care providers, the Investigator(s), all members of the research team, clinical staff and dispensing pharmacists were all blinded after assignment to the treatment intervention. This was maintained throughout the study period, and during database data entry until the point at which the eCRF (electronic case report forms) were locked by the trial monitors. There were no known or suspected instances where blinding was compromised.

Blinding was removed only after the statistician was satisfied that all the major clinical and mechanistic analyses were undertaken, with primary outcome data locked.

16.1 Emergency Unblinding Procedures

The Investigators were able to unblind participants when it is was medically imperative to know whether a participant was receiving rifaximin-α or placebo. The emergency unblinding was only instituted by the Principal Investigator. Arrangements were made to ensure that access to the database was maintained in strict confidence to prevent a compromise of participant blinding by non-study individuals.

Only in the event of an adverse event (AE) where the Investigator decided the participant could not be adequately treated without knowing the identity of the study drug was the medication code broken for that individual participant. Every effort was made to contact the Co-sponsors before breaking the blind, and if in an emergency, as soon as possible thereafter (and no later than 24 hours after emergency unblinding).

If the blind was broken, an entry was made in the case report form (CRF) that contains the reason that the blind was broken and the name of the person contacted. The participant was then withdrawn from the trial once the study team were made aware of the treatment the participant was assigned to.

Access to randomisation codes and corresponding treatment assignment was also made available to the appropriate Co-sponsor designees and individuals responsible for reporting SAEs and suspected unexpected serious adverse reactions (SUSARs) to the regulatory authorities. This was accessed only in the event of a medical emergency. No other Co-Sponsor personnel had access to blinded participant treatment codes, and this was maintained until all study data was entered onto the study database and validated, and the database locked.

17 STATISTICAL METHODS

17.1 Sample size estimation

Sample size was determined by a power calculation based on previous *in vitro* and *ex-vivo* data based on normal neutrophils exposed to ammonia and in patients with cirrhosis and grade 1-2 HE [11]. This indicated that 22 patients were required in each study arm under the assumption of a reduction in spontaneous neutrophil oxidative burst from 30 to 15% (constant 60% difference in medians -0.3) and using the Binomial proportions (Exact) method with a power of 80% and alpha of 0.05 (2 sided t-test). It was therefore decided that 25 patients were to be recruited to each treatment arm to offset any dropouts from the study.

17.2 Statistical methods used to compare groups for primary and secondary outcomes

17.2.1 DATA ANALYSIS PLAN

Continuous data has been tested for normality using the D'Agostino Pearson test. Normally distributed data is presented as mean(standard deviation) and non-normally distributed data as median(range). Comparison between 2 (or more) groups was done by Student's t test (or Analysis of Variance) and Mann Whitney U test (or Kruskall Wallis) test for normally and non-normally distributed data respectively. Comparison between categorical data was done by the $\chi 2$ test or Fisher's exact test for small sample sizes.

For continuous data measured over three time points between the rifaximin-α and placebo group determination of significance of change was undertaken by repeat measures analysis of variance (RM ANOVA) with appropriate tests for sphericity. Log transformation was used for non-normally distributed data prior to RMANOVA if necessary. If normalisation could not be achieved then the Friedman test was used. Post hoc tests were used to assess statistical significance between individual time points/groups. Longitudinally measured ordinal data (such as HE grade) was analysed by ordered logistic regression.

Spurious data was assessed by analysis of the (log transformed) distribution and data greater than 3 SD from the mean reviewed as potential outliers. Data was only removed if deemed clinically not credible rather than on statistical grounds alone. Premature discontinuation of the study for the primary endpoint resulted in that endpoint not being reached for that participant and censoring occurred at the last available data point/study visit.

For all statistical tests significance will is defined at the 95% level and all p values will be 2-tailed.

17.2.2 STATISTICAL CONSIDERATIONS

A number of different statistical techniques have been utilised. For standard clinical outcomes over the study period suitable for Kaplan Meier analysis and Cox regression (eg death, transplantation, recurrence of HE) censoring is at the time of outcome or loss to follow up.

For measures performed at set times using complex laboratory techniques repeated measures ANOVA/Student's test or multivariate partial least squares(PLSDA)/principal components analysis(PCA) are used. Forms of multivariate PLSDA are also utilised where standard modifications to PLSDA such as orthogonalisation (OPLSDA) are insufficient to determine the change in microbiota or metabolic profile during treatment.

17.2.3 DESCRIPTION OF THERAPIES

The adherence, adverse event rate, withdrawals and outcome measures are reported using the above statistical methods for the rifaximin-α and placebo arms. The therapy consisted of rifaximin-α 550mg twice daily oral medication for 90 days or placebo.

17.2.4 RECRUITMENT AND REPRESENTATIVENESS OF RECRUITED PATIENTS

Screened but not recruited patients were compared to recruited patients in terms of captured demographic data to assess representativeness using the statistical tests described above. A CONSORT diagram is used to assess screening, randomisation and progression through the study period and to denote dropouts.

17.2.5 BASELINE COMPARABILITY OF RANDOMISED GROUPS

Groups are compared using the statistical tests described above for continuous and categorical variables.

17.2.6 ADHERENCE TO ALLOCATED TREATMENT AND TREATMENT FIDELITY

The primary analysis is intention-to-treat and per-protocol analysis is only presented for comparison if relevant and not the primary conclusive analysis of the study.

17.2.7 LOSS TO FOLLOW-UP AND OTHER MISSING DATA

Patients lost to follow up had data censored at the last study visit. Missing data was not envisaged to exceed 10% for cohort characteristic data and was subject to multiple imputation only if this threshold was exceeded else mean imputation was used.

17.2.8 INTERIM ANALYSIS

No interim analysis is envisaged in this small study with a non-clinically relevant primary outcome measure as stopping rules would be difficult to justify as this is the first study to use this novel primary outcome measure.

17.2.9 CLINICAL ENDPOINTS

Listings of clinical events (transplantation, hepatic encephalopathy, progression to organ failure, if relevant and death) are listed and summarised by treatment group. If a sufficient number of events occurred, Kaplan-Meier plots of the time to clinical worsening are presented by treatment group. Child-Pugh, MELD and UKELD scores are listed and summarised by visit and treatment group. The secondary clinical endpoints were not subject to sample size calculation.

17.3 Methods for additional analyses

17.3.1 SENSITIVITY ANALYSES

A sensitivity analysis with baselines SIRS score as a covariate and those experiencing sepsis during the first 30 days of admission will be performed.

17.3.2 PLANNED SUBGROUP ANALYSES

No planned subgroups other than the exploratory analyses were envisaged for this small study.

17.3.3 EXPLORATORY MEDIATOR AND MODERATOR ANALYSIS

This sample will likely be too small for full covariance analysis into potential mediator and moderator effects beyond the exploratory analysis envisaged above.

17.4 Statistical Software

Analyses will be performed using the validated statistical software of IBM SPSS® (version 21 or later) and any other validated software packages deemed to be of appropriate standard.

18 PARTICIPANT FLOW

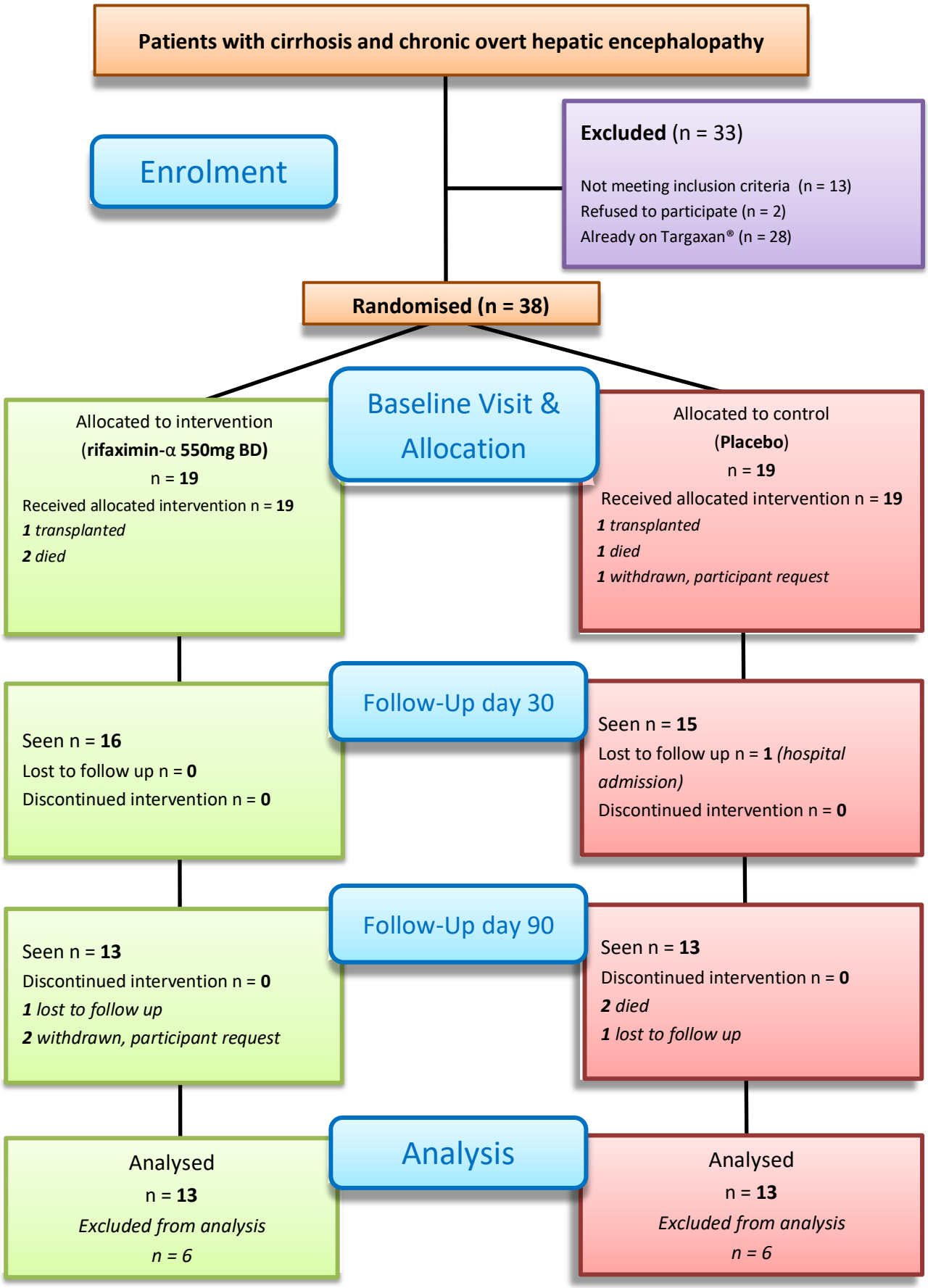


Figure 1: RifSys Trial Flow Chart

19 RECRUITMENT

The site opened to recruitment on 18th January 2015, with the first participant (RIF_01) recruited to the trial 22nd January 2015. The last participant (RIF_38) was recruited on 31st March 2016, with the final study visit completed on 20th June 2016.

Recruitment fell well below expected rates from the outset of the trial because Targaxan® (rifaximin-α) was approved by the National Institute of Clinical Excellence in mid-February 2015. The outcome of this decision is summarised in this document: NICE, Final Appraisal Determination, 'Rifaximin for preventing episodes of overt hepatic encephalopathy' (published 19 February 2014, available online at www.nice.org.uk). Thus for almost the entire duration of the trial, several patients that would have been candidates for participation in RifSys were in fact commenced on active treatment with rifaximin-α as standard of care and were therefore already on treatment at the time of screening.

The study was extended by a further 6 months from January 2016 to June 2016 to allow further recruitment to occur. This strategy enabled an additional 12 participants to be recruited and followed up scheduled for day 30 and day 90 trial visits during this time period. Despite this however, the trial team were unable to screen and recruit the 50 participants in total as planned, thus falling short by 12 participants, achieving 76% of the target number. It was decided that further extension of the study would not increase recruitment sufficiently to justify such an extension, and it was therefore closed to recruitment as above.

In summary, the original plan for the trial was to recruit 25 participants to each of the two arms equating to 50 participants in total. 38 participants were actually randomised to rifaximin-α or placebo, with 32 participants completing to 30 days and 26 of these participants completing to 90 days. Therefore 52% of original planned participants number completed the protocol schedule of visits, and 64% (n = 32) of the planned number achieving day 30 primary outcome data.

20 BASELINE PARTICIPANT CHARACTERISTICS DATA

The table below summaries the main baseline demographic and clinical characteristics for each treatment group. Overall the two groups are well matched, and where parameters do differ, these are within acceptable limits.

Table 3: Summary of baseline demographic and clinical characteristics by treatment group

	RIFAXIMIN-α N = 19	PLACEBO N = 19	P
Age	58.00 [52.00, 62.00]	53.00 [49.50, 60.50]	0.483
Male	16	11	0.151
Previous Most Severe Grade of HE	3.00 [3.00, 3.50]	3.00 [2.00, 3.00]	0.029
Receiving Lactulose	18:1	13:5	0.090
Prior TIPPS	5	1	0.180
Body Mass Index (kg/m^2)	29.65 [26.30, 32.67]	26.45 [23.14, 29.38]	0.068
Mean Arterial Blood Pressure (mmHg)	87.30 [78.35, 93.00]	83.00 [74.85, 86.00]	0.082
Fio2	0.21 [0.21, 0.21]	0.21 [0.21, 0.21]	0.317
Heart Rate (BPM)	61.00 [57.50, 72.50]	82.00 [63.50, 89.50]	**0.019**
Respiratory Rate	16.00 [16.00, 17.00]	16.00 [14.00, 17.50]	0.377
SpO_2	99.00 [97.50, 100.00]	99.00 [97.00, 100.00]	0.808
Temperature (^{0}C)	36.60 [36.50, 36.80]	36.50 [36.40, 36.70]	0.452
Grade Of Ascites (1-4)	1.00 [1.00, 3.00]	3.00 [1.00, 3.50]	0.250
Requiring haemofiltration (CVVH)	1	0	0.728
Glasgow Coma Scale	15.00 [15.00, 15.00]	15.00 [15.00, 15.00]	0.285
Overt HE (Y)	14	10	0.313
Haemoglobin	109.00 [96.50, 132.00]	112.00 [92.50, 127.00]	0.988
White blood cell count (total)	6.34 [4.89, 7.20]	5.44 [4.42, 6.25]	0.397
Neutrophils	3.60 [2.45, 4.55]	3.80 [2.35, 4.50]	0.953
INR	1.45 [1.26, 1.78]	1.37 [1.30, 1.67]	0.672
Sodium	139.00 [137.00, 142.00]	135.00 [132.00, 137.00]	**0.001**
Potassium	4.20 [3.90, 4.40]	4.20 [3.85, 4.35]	0.988
Urea	4.80 [3.90, 6.55]	4.80 [3.45, 7.05]	0.988
Creatinine	70.00 [57.00, 87.00]	77.00 [64.00, 84.50]	0.630
Bilirubin	39.00 [23.00, 56.50]	40.00 [24.00, 57.00]	0.661
Albumin	36.00 [30.00, 37.50]	33.00 [30.00, 38.00]	0.588
Ammonia	66.00 [48.00, 78.00]	45.50 [30.00, 64.00]	0.080
Lactate	1.30 [1.15, 1.55]	1.70 [1.30, 1.95]	0.128
QOL SCORE	2.2[2.2, 3.3]	2.1[2.1, 2.2]	0.543
QOL SS	55[40,68]	60[30,70]	0.929
Baseline Neutrophil Burst (MFI)	337.00 [165.00, 579.50]	218.00 [140.50, 763.00]	0.815
Baseline Neutrophil Burst (%)	8.00 [6.90, 11.97]	6.35 [3.42, 16.07]	0.474

21 NUMBERS ANALYSED

A total of nineteen participants were randomised to the active treatment group, and nineteen to the placebo group. Within the active treatment group, 3 participants were lost prior to the day 30 follow up visit; one participant underwent orthotopic liver transplantation and two participants died. Within the placebo group, 3 participants were also lost prior to the day 30 follow up visit; one participant underwent orthotopic liver transplantation, one died and one of the participants asked to be withdrawn from the study.

Consequently, sixteen participants from the active treatment arm were seen and underwent study procedures at day 30 visit. In the placebo group, an additional participant was not seen at day 30 – bringing the total seen to fifteen – as that participant was admitted to hospital elsewhere at the time. However, this participant was then seen at day 90.

At the final day 90 study visit, thirteen participants from the active treatment arm were seen and underwent study procedures. This is because 3 were lost to follow up due to non-attendance and participant request for withdrawal from the study. In the placebo group, thirteen participants were also seen at the final day 90 visit, with one lost to follow up due to non-attendance and two patients died in the interim period. Of those participants that were seen, none had discontinued the IMP.

In summary, 19 participants were randomised to active therapy and 19 to placebo, with 32 participants completing to 30 days (although one was not seen as admitted to hospital elsewhere), and 26 of these participants completing to 90 days with a near perfect 50-50 split between the active and placebo arms in terms of number of participants in each group at each time point (as seen in the study flow chart).

22 OUTCOMES AND ESTIMATION

Outcomes are described below according to primary or secondary, and by clinical and mechanistic outputs.

23 PRIMARY OUTCOME

To assess whether rifaximin-α reduces neutrophil spontaneous oxidative burst *ex vivo* in patients with cirrhosis and chronic hepatic encephalopathy after 30 days.

Neutrophil oxidative burst (OB) was quantified at all trial time points using Glycotope Biotechnology Phagoburst™ (BD Biosciences) kits which measure the percentage of phagocytic cells that produce reactive oxygen species (ROS). In brief, 100µL of heparinized whole blood was incubated for 20 minutes with 20µL of opsonized E. coli (2×10^7), or without stimulus at 37°C. Neutrophil high burst capacity was assessed by adding 5µL of phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, to 100µL of heparinized whole blood. Neutrophil low burst was assessed by adding 5µL of the chemotactic synthetic peptide formyl-Met-Leu-Phe (fMLP) for 20 minutes at 37°C. fMLP is a synthetic peptide that mimics the activity of bacterially derived peptides with formylated N-terminal methionine groups. The formation of ROS was detected using the oxidation of dihydrorhodamine-123 to rhodamine-123 which emits green fluorescence. Red blood cells were lysed and PMNs were washed with sterile PBS prior to analysis. Neutrophils were gated on forward and side-scatter characteristics and stained with anti-CD16-Phycoerythrin(PE)IgG1 κ and analyzed by FACS. OB was determined by the percentage of CD16-positive cells producing ROS, which was calculated along with the mean fluorescence intensity (MFI).

Table 4: Comparison of neutrophil oxidative burst by percentage (%) and mean fluorescence intensity (MFI) between rifaximin-α and placebo treatment groups at baseline, day 30 and day 90.

	RIFAXIMIN-α	PLACEBO	P
Baseline	**N = 19**	**N = 19**	
Neutrophil Burst (MFI)	337.00 [165.00, 579.50]	218.00 [140.50, 763.00]	0.815
Neutrophil Burst (%)	8.00 [6.90, 11.97]	6.35 [3.42, 16.07]	0.474
Day 30	**N = 16**	**N = 15**	
Neutrophil Burst (MFI)	279.50 [144.75, 380.00]	185.00 [121.75, 396.00]	0.651
Neutrophil Burst (%)	8.68 [5.09, 12.77]	3.97 [3.09, 9.16]	0.070
Day 90	**N = 13**	**N = 13**	
Neutrophil Burst (MFI)	331.00 [181.25, 512.25]	254.00 [205.50, 542.00]	0.956
Neutrophil Burst (%)	8.43 [4.48, 13.24]	7.90 [4.08, 15.15]	0.956

Table 5: Analyses of neutrophil oxidative burst by percentage (%) and mean fluorescence intensity (MFI) using median (range) and Friedman tests between rifaximin-α and placebo treatment groups at baseline, day 30 and day 90.

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE (DAY 30)	P-VALUE (ALL)	RM ANOVA
NEUTROPHIL BURST (MFI)	242 (55-3069)	222 (55-619)	319 (112-1189)	0.488	0.187	0.221
NEUTROPHIL BURST (%)	7.5 (1-30)	8.4 (2-27)	15 (1-80)	0.484	0.476	0.020
RIFAXIMIN-α NEUTROPHIL BURST (MFI)	349 (84-3069)	279 (64-557)	331 (112-952)	0.138	0.065	-
PLACEBO NEUTROPHIL BURST (MFI)	203 (55-797)	171 (55-619)	254 (123-1189)	0.632	0.461	-
RIFAXIMIN-α NEUTROPHIL BURST (%)	8 (1.9-30)	8.6 (2.5-27)	8.4 (2.1-80)	0.333	0.351	-
PLACEBO NEUTROPHIL BURST (%)	6.3 (1-28)	3.9 (2-18)	12 (1.8-37)	1	0.409	-

Primary outcome analysis using median (range) and Friedman test for non-parametric variables with pre-specified subgroup for baseline to day 30. The "all" refers to a p value for all 3 measurements. An unplanned post hoc analysis on the MFI from day 30 to day 90 for rifaximin-α patients gave a p value of 0.026 suggesting further data are required to define the long-term effects of rifaximin-α. Extensive post hoc assessment not recommended.

Using repeated measures ANOVA on both outcomes did demonstrate an effect from group membership (i.e. difference dependent on whether on active therapy or placebo) on neutrophil OB at day 90, although the number of patients who remained in the study to day 90 was low (n=26). This was on log transformed data. However, the difference was almost entirely driven by the changes in the placebo group in terms of fall in neutrophil OB and then increase to higher than baseline.

In summary, the trial failed to demonstrate a 50% reduction in spontaneous neutrophil oxidative burst compared to baseline 30 days following the start of rifaximin-α/placebo therapy. This is largely attributable to the lack of powering for the primary outcome due to the relatively low number of participants recruited, and then further fall in the number of participants followed up.

Of note multivariate analysis on multiple clinical parameters is not recommended due to the small sample size and for secondary outcomes a repeated measures analysis on all three points +/- RM ANOVA.

24 SECONDARY OUTCOMES

To assess whether rifaximin-α reduces the development of systemic inflammation, infection, organ failure and improves patient survival over 90 days. This included analyses for changes in faecal microbiota and faecal biomarkers (e.g. calprotectin), systemic endotoxemia and immune dysfunction.

24.1 Secondary Clinical Outcomes

24.1.1 GRADE OF HEPATIC ENCEPHALOPATHY

Median baseline West-Haven grade for all patients was 1 (range 0-3) and at 30 days was 0 (0-2), p value 0.014. Patients on rifaximin-α normalised their low grade hepatic encephalopathy whilst on therapy. Line tracing appeared to improve significantly on rifaximin-α.

24.1.2 PLASMA AMMONIA

There was a non-statistically significantly higher level in plasma ammonia at baseline for those that then went on to receive rifaximin-α. While patients receiving rifaximin-α had an initial fall in ammonia this had returned to baseline by day 90 and those on placebo had an increase in ammonia concentrations which had begun to fall again by day 90. These changes in both treatment groups appeared statistically significant.

24.1.3 CLINICAL INDICES OF INFECTION AND SYSTEMIC INFLAMMATION

Peripheral total white cell and neutrophil count were not different between arms at baseline or during the course of the study. Cytokine profiling demonstrated that TNF-α fell significantly on therapy with rifaximin-α and IL-10 to have a temporary reduction on therapy.

24.1.4 QUALITY OF LIFE SCORES

There were no differences in Quality of Life scores by either the EuroQol validated EQ-5D-3L descriptive system or the EuroQol EQ-Visual Analogue Scale across treatment groups or time-points.

Table 6: Comparison of main clinical parameters including HE grade and assessment tests, MELD and laboratory markers of inflammation between rifaximin-α and placebo treatment groups, at baseline, day 30 and day 90 **(bold text indicates statistically significant)**.

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE	RM ANOVA
HE GRADE (WESTHAVEN)					
- *Rifaximin*	1(0-1)	0(0-1)	0(0-0)	0.014	0.606; **0.043**
- *Placebo*	1(0-1)	0.5(0-1)	0.5(0-1)	0.384	
TRAILS A TEST					
- *Rifaximin*	52(46-81)	48(36-65)	46(37-54)	0.417	0.859; **0.012**
- *Placebo*	46(34-78)	46(37-72)	39(33-61)	0.293	
TRAILS B TEST					
- *Rifaximin*	142(105-161)	143(106-195)	144(94-186)	0.880	0.843; 0.975
- *Placebo*	140(57-234)	135(73-205)	150(55-194)	0.905	
LINE TRACING TEST					
- *Rifaximin*	205(145-254)	185(111-213)	167(115-270)	**0.023**	0.555;0.473
- *Placebo*	169(154-255)	165(131-363)	135(120-299)	0.496	
LINE TRACING ERRORS					
- *Rifaximin*	18(5-26)	11(6-18)	11(2-14)	0.285	0.650; 0.096
- *Placebo*	11(3-31)	11(3-37)	12(5-22)	0.367	
SERIAL DOT TEST					
- *Rifaximin*	133(94-178)	97(78-197)	102(74-219)	0.218	0.543;0.943
- *Placebo*	101(83-154)	109(66-189)	113(66-173)	0.384	
PLASMA AMMONIA					
- *Rifaximin*	62(49-74)	53(34-72)	63(41-85)	0.023	0.394; 0.958
- *Placebo*	44(31-59)	58(42-74)	52(33-71)	0.024	
MELD					
- *Rifaximin*	11(8-15)	11(7-14)	10(7-13)	0.266	0.990; 0.974
- *Placebo*	10(8-12)	10(8-13)	11(8-13)	0.076	
SODIUM					
- *Rifaximin*	140(138-142)	138(136-139)	138(136-140)	0.006	0.005;0.112
- *Placebo*	136(133-138)	134(132-138)	136(133-139)	0.832	
CREATININE					
- *Rifaximin*	68(58-78)	68(36-81)	69(55-81)	0.986	0.666;0.679
- *Placebo*	78(64-84)	86(64-90)	79(76-92)	0.323	
BILIRUBIN					
- *Rifaximin*	33(20-53)	32(17-46)	29(24-49)	0.546	0.698; 0.367
- *Placebo*	35(20-46)	32(24-47)	29(22-47)	0.409	
INR					
- *Rifaximin*	1.4(1.2-1.8)	1.4(1.2-1.7)	1.3(1.2-1.5)	0.062	0.545; 0.493
- *Placebo*	1.3(1.2-1.4)	1.4(1.3-1.5)	1.3(1.2-1.6)	0.579	
WHITE BLOOD CELL					
- *Rifaximin*	6.0(3.8-7.6)	5.8(3.3-6.9)	6.0(2.9-6.6)	0.316	0.494; 0.373
- *Placebo*	5.0(3.8-5.9)	4.3(3.2-6.3)	4.7(3.8-6.4)	0.075	
NEUTROPHILS					
- *Rifaximin*	3.0(1.8-4.4)	2.9(1.1-3.9)	3.1(1.4-3.9)	0.558	0.808;0.572
- *Placebo*	2.5(1.9-4.3)	2.5(1.9-3.8)	2.5(2.1-4.7)	0.578	
QUALITY OF LIFE SCORE (EQ-5D-3L) / 10^5					
- *Rifaximin*	2.1(1.1-3.3)	2.2(2.1-3.3)	2.1(1.1-2.3)	0.358	0.525;0.244
- *Placebo*	2.1(1.6-2.3)	2.1(1.1-2.3)	2.1(1.1-2.2)	0.929	
QUALITY OF LIFE SCORE (EQ-VAS) / 100					
- *Rifaximin*	60(47-90)	70(53-90)	75(38-90)	0.210	0.430; 0.581
- *Placebo*	64(40-77)	67(35-80)	63(35-70)	0.976	

24.2 Baseline neutrophil toll-like receptor (TLR) expression

Neutrophils are key innate immune effector cells that are rapidly recruited to sites of infection and inflammation to provide early defense against invading microorganisms. This function is facilitated by the expression of Toll-like receptor (TLR) family members by neutrophils, allowing the recognition of an extensive array of pathogen-associated molecular patterns (PAMPs) and thus triggering the response to invading pathogens. TLR activation leads to important cellular processes including reactive oxygen species (ROS) generation, cytokine production and increased survival, all of which can contribute to the pathogenesis of chronic inflammation when signaling becomes dysregulated. In turn, inflammation and tissue injury results in the release of endogenous TLR ligands, known as damage-associated molecular patterns (DAMPs), which are a rapidly growing class of potent inflammatory stimuli. DAMPs act in an autocrine manner, alerting the host of damage, but can also amplify inflammation leading to further tissue damage. [12]

TLR2 and TLR4 are amongst the most studied of the neutrophil TLRs, mediating responses to Gram-positive and Gram-negative bacteria respectively. TLR2 heterodimerises with TLR1 to detect triacylated peptides or TLR6 to detect diacylated peptides, whilst TLR4 recognises the lipid A component of lipopolysaccharide (LPS) [13]. Neutrophils also express TLR co-receptors, including CD14 and CD11b/CD18 [14], which cooperate with TLR4 or TLR2 on the plasma membrane [15].

Table 7: Baseline neutrophil TLR-2 and TLR-9 expression analyses between rifaximin-α and placebo treatment groups, at baseline, day 30 and day 90.

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE (ALL)	RM ANOVA
TLR-2					
- *Rifaximin*	7.3(6.8-8.3)	8.1(7.3-9.5)	7.4(6.7-8.7)	0.605	0.645; 0.086
- *Placebo*	7.4(6.6-8.3)	7.9(7.1-9.2)	8.5(8.1-9.5)	0.080	
Expressed as 10^3					
TLR-4					
- *Rifaximin*	1.3(0.8-1.9)	1.0(0.6-1.4)	1.6(0.9-3.4)	0.112	0.154; 0.745
- *Placebo*	1.4(1.0-2.3)	2.3(0.8-3.3)	1.6(0.7-6.2)	0.871	
Expressed as 10^4					

24.2.1 BASELINE TLR-2 EXPRESSION

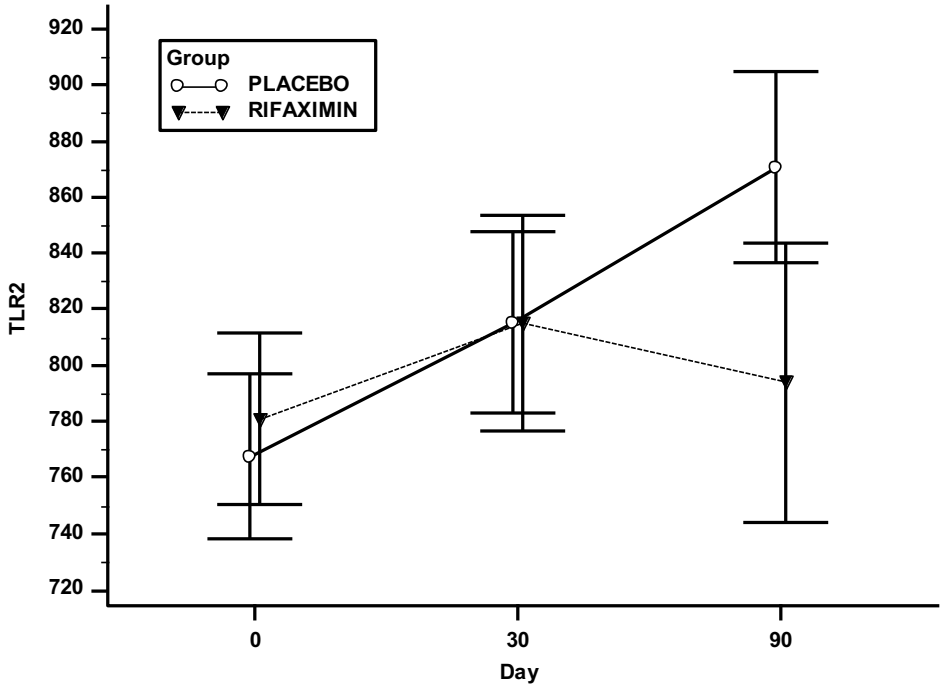


Figure 3: TLR2 expression in patients treated with rifaximin-α or placebo. RM ANOVA and day specific ANOVA p values are all non-significant.

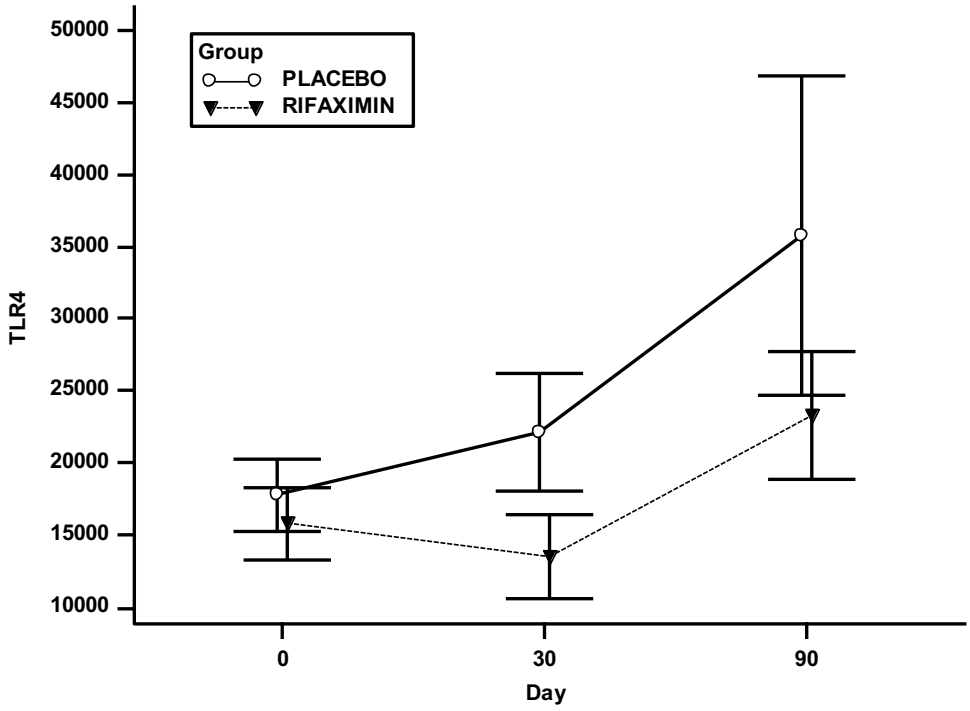


Figure 2: TLR4 expression in patients treated with rifaximin-α or placebo. RM ANOVA and day specific ANOVA p values are all non-significant, with p=0.09 for day 30.

24.2.2 BASELINE TLR-4 EXPRESSION

24.3 Plasma cytokine profiling

Cytokine levels were measured in participant plasma (EDTA) via the Meso scale Discovery (MSD) platform. Assays using this multiplexing platform are highly sensitivity with excellent precision and can be up to 100-fold better than ELISA with a large linear dynamic range of 3-4 logs. In addition, MSD assay formats minimise both matrix effects improving performance; often a problem with Luminex based assays for example.

In brief, U-PLEX assay plates consist of biotinylated capture reagents coupled to U-PLEX Linkers. The U-PLEX Linkers then self-assemble onto unique spots on the U-PLEX plate. After analytes in the sample bind to the capture reagents, detection antibodies conjugated with electrochemiluminescent labels (MSD GOLD SULFO-TAG) bind to the analytes to complete the sandwich immunoassay. Once the sandwich immunoassay is complete, the plate is placed into an MSD instrument where the amount of analyte present in the sample is measured.

U-PLEX assays were read on an MSD instrument using no complicated fluidics or calibration procedures. Plasma samples were run – in duplicate - on U-PLEX Proinflam Combo 1 (hu) plates, which measure a combination of human cytokine assays that are involved in inflammation and immune system regulation, including IFN-gamma, IL-1β, IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12 p70, IL-13, and TNF-α.

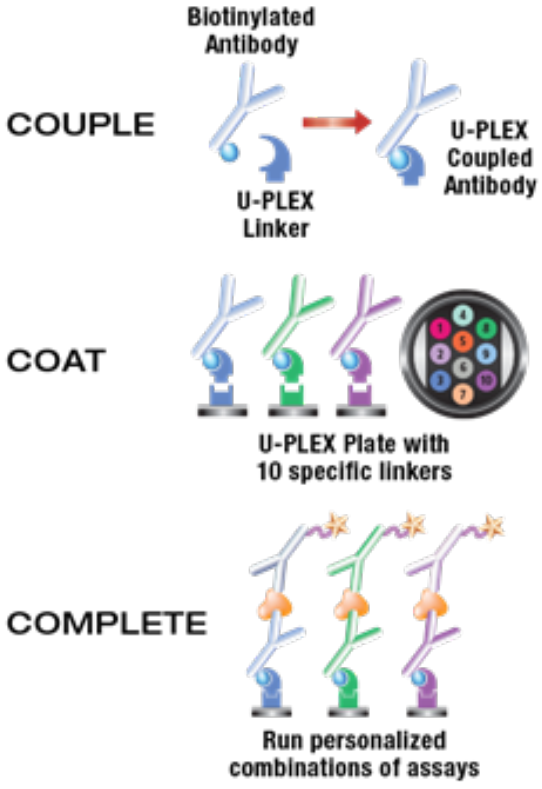


Figure 4: Meso scale Discovery (MSD) U-Plex assay schematic

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE (ALL)	RM ANOVA
IFN-γ					
- *Rifaximin*	18(15-37)	19(12-35)	16(10-37)	0.935	0.911;0.206
- *Placebo*	23 (16-36)	24(16-39)	17(9-104)	0.039	
IL-10					
- *Rifaximin*	0.42(0.23-0.57)	0.23(0.17-0.19)	0.40(0.19-0.47)	**0.005**	0.076;0.216
- *Placebo*	0.61(0.30-1.00)	0.48(0.21-0.77)	0.44(0.22-0.98)	0.274	
IL-6					
- *Rifaximin*	4.1(1.8-10.8)	3.7(2.7-4.7)	3.8(2.0-5.4)	0.935	0.239;0.412
- *Placebo*	9.1(2.8-19.1)	7.1(2.9-8.3)	6.4(2.3-9.7)	0.384	
IL-8					
- *Rifaximin*	34(28-50)	27(18-68)	29(20-47)	0.409	0.811;0.547
- *Placebo*	38(23-84)	30(20-114)	25(21-107)	0.733	
TNF-α					
- *Rifaximin*	4.0(3.1-5.3)	3.4(2.8-4.1)	3.3(2.5-3.8)	**<0.001**	0.717; **<0.001**
- *Placebo*	4.3(3.0-5.9)	3.5(3.0-5.1)	3.7(3.0-4.5)	0.578	
NOTE: IL-1, IL-2 IL-4, IL-12, IL-13 WERE MOSTLY BELOW THE DETECTION THRESHOLD (LLOD PG/ML)) AND THEREFORE NOT PURSUED FURTHER.					

Table 8: Summary and analyses of plasma cytokine levels by treatment group.

In summary, there was a significant fall in pro-inflammatory TNF-α levels in the actively treated group which was maintained at each time point whilst on therapy (p <0.001). This is a highly significant finding, suggesting that rifaximin-α has an anti-inflammatory effect by ameliorating the release of this particular cell signaling protein which Is involved on systemic inflammation often part of the acute phase response. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, natural killer cells, and neutrophils.

There was also a reduction in IL-10 which is an anti-inflammatory cytokine in in the actively treated group at day 30, but this was not sustained at day 90. L-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway. The JAK-STAT signaling pathway transmits information from extracellular chemical signals to the nucleus resulting in DNA transcription and expression of genes involved in immunity, proliferation, differentiation, apoptosis and oncogenesis.

There were no significant changes in IL-6 and IL-10 levels (both pro-inflammatory), nor in interferon gamma which is an important immunoregulatory cytokine critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IL-1, IL-2 IL-4, IL-12, IL-13 were mostly below the detection threshold (Lower Limit of Detection 'LLOD') and therefore not pursued further statistically.

Table 9: Meso scale Discovery (MSD) U-Plex assay Lower Limit of Detection 'LLLOD'

	LLOD (pg/mL)									
Assays	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNF-α
U-PLEX	1.7	0.15	0.7	0.08	0.33	0.15	0.14	0.69	3.1	0.51

	LLOD (pg/mL)							
Assays	Eotaxin	IP-10	MCP-1	MCP-4	MDC	MIP-1α	MIP-1β	TARC
U-PLEX	3.2	0.49	0.74	7.5	8.4	7.7	1.5	0.51

24.4 Whole blood 16S rDNA quantification

Whole blood 16S rDNA quantification was undertaken by quantitative polymerase chain reaction, as a surrogate marker of gut bacterial translocation from the intestinal compartment into the systemic circulation, as a measure of gut permeability and bacterial DNA as a potential pro-inflammatory pathogen associated molecular pattern ('PAMP').

DNA was extracted from sterile processed and batch stored whole blood samples using an optimised plasma-specific technique. The 16S rDNA present in the samples was measured by qPCR in triplicate and normalised using a plasmid-based standard scale. The amount of bacterial DNA was assessed using the "Universal 16S Real Time qPCR" workflow established by Vaiomer (Vaiomer SAS, Labège, France), summarised in brief below.

24.4.1 GENOMIC DNA EXTRACTION

DNA was extracted from samples using an optimised blood specific technique. Total genomic DNA is collected in a final 50µl extraction volume. Total DNA concentrations were determined by UV spectroscopy (Nanodrop®, Thermo Scientific).

24.4.2 qPCR METHOD

Real-time PCR amplification was performed using 16S universal primers targeting the V3-V4 region of the bacterial 16S ribosomal gene (Vaiomer universal 16S primers). The qPCR step is performed on a VIIA 7® PCR system (Life Technologies) using Sybr Green technology and the following amplification cycles: hold stage of 10 min at 95 °C, then 40 cycles of 15 sec at 95 °C, 1 min at 63°C and 1 min 72 °C. The absolute number of copies of 16S rDNA was determined by comparison with a quantitative standard curve of 16S rDNA plasmids generated by serial dilution of plasmid standards (Vaiomer Universal standard plasmids).

24.4.3 STANDARD CURVE

The total 16S rDNA present in the samples was measured by qPCR in triplicate and normalised using a plasmid-based standard scale. The construction of standard curves allows for a proper quantification of 16S rDNA gene copy in the sample, but also enables the determination of the

efficiency, linear dynamic range, and reproducibility of the qPCR assay. In these experiments, the efficiency calculated from the standard curve was required to be between 80-120%, and the R2 of the standard curve greater than 0.980.

Samples	Regression	R	Efficiency
All (N= 93)	y = -3.259x + 36.63	0.996	102.69%

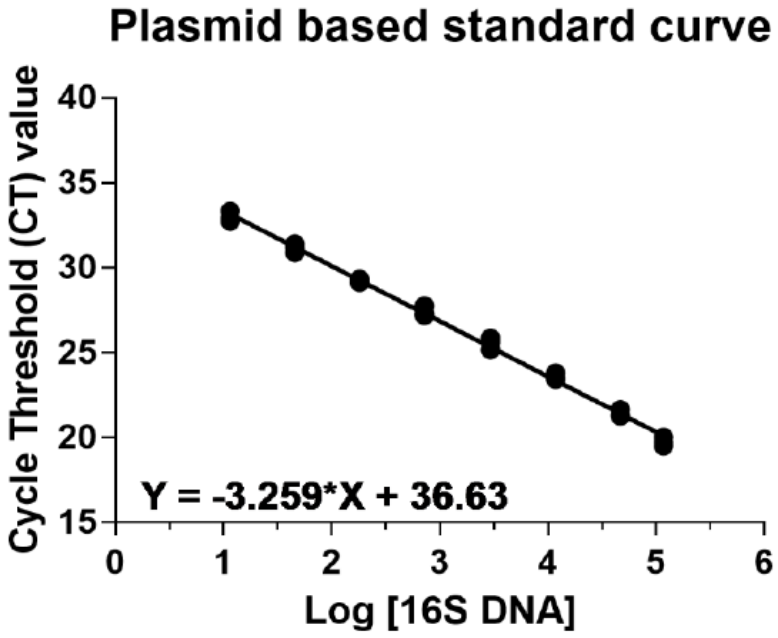


Figure 5: 16S rDNA qPCR plasmid based standard curve.

24.4.4 QUALITY CONTROL

Melting curve analysis is the assessment of heat induced dissociation-characteristics for double-stranded DNA. The specificity of all qPCR products was assessed by systematic analysis of the post-PCR dissociation curve performed between 60°C to 95°C. Melting curve analysis helps to ensure the specificity of the target PCR amplicons, including identification of the presence of non-specific products and primer-dimers. This property is valuable because the presence of secondary non-specific products and primer-dimers can affect the accuracy of the qPCR assay.

24.4.5 DATA

The results have been reported as number of copies of 16S rDNA per µl of blood (and therefore as a concentration of bacterial DNA per volume of whole blood) and summarised.

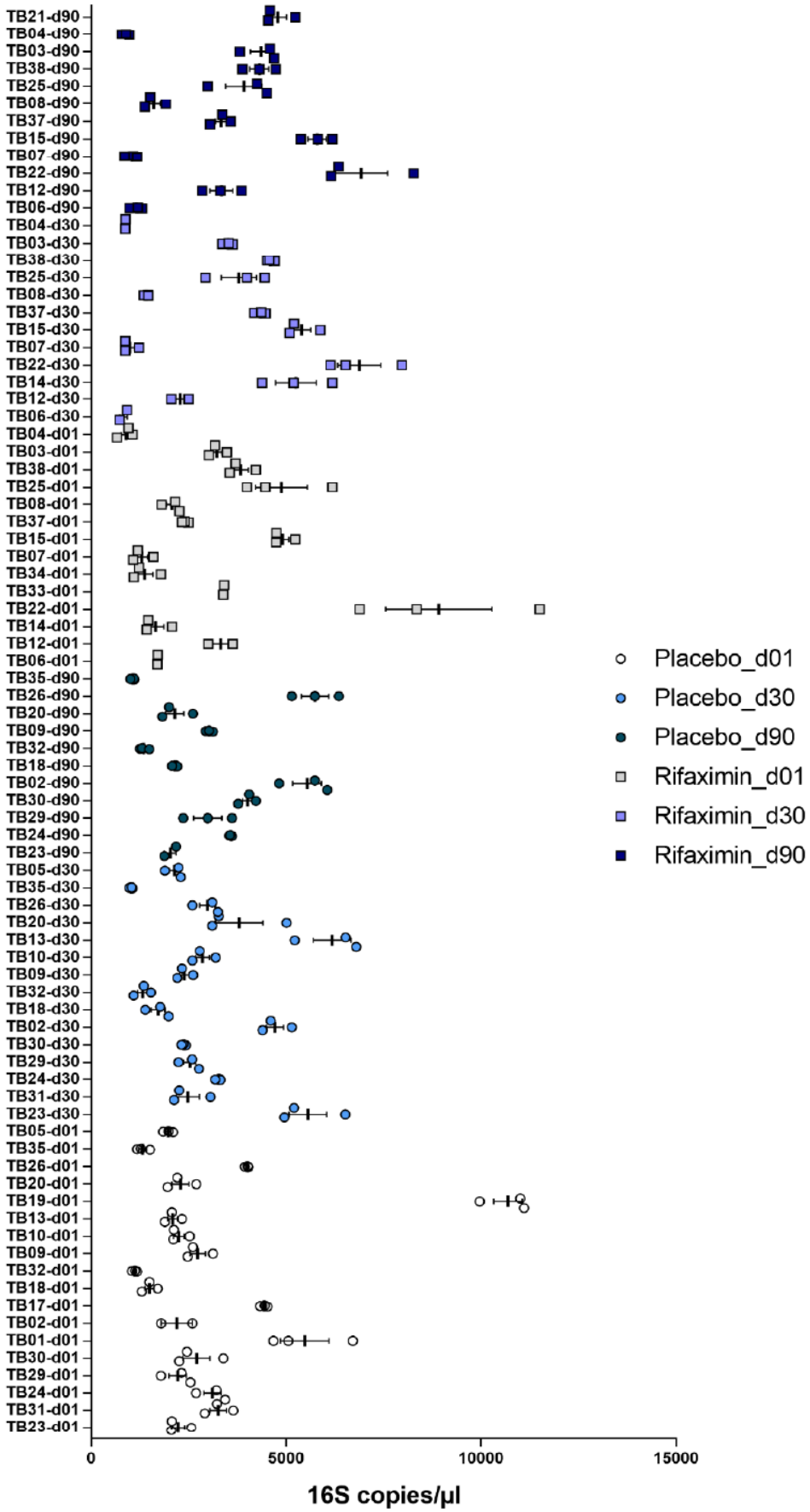


Figure 6: 16S rDNA quantity per total volume of whole blood (16S copies / µL)

Table 10: Summary and analyses of whole blood 16S rDNA concentration (16S copies/µL) by treatment group.

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE (ALL)	RM ANOVA
BACT DNA ($x10^3$)					
- *Rifaximin*	3.2(1.7-4.6)	3.5(1.1-4.5)	3.3(1.2-4.3)	0.181	0.717;0.447
- *Placebo*	2.2(1.6-2.7)	2.5(1.8-3.7)	2.9(2.1-3.9)	0.076	

The table above summaries the raw median values (with interquartile ranges) of whole blood 16S rDNA concentration (expressed as number of 16S copies/µL) by treatment group and time point, with the overall analyses showing no difference in circulating bacterial DNA concentration within and between treatment groups at the various time points. The figures below demonstrate the whole blood 16S rDNA concentration by treatment group and time points, with confirmation that there is no statistically significant change within the groups comparing on-treatment data to baseline samples, as well as between groups at the different time points.

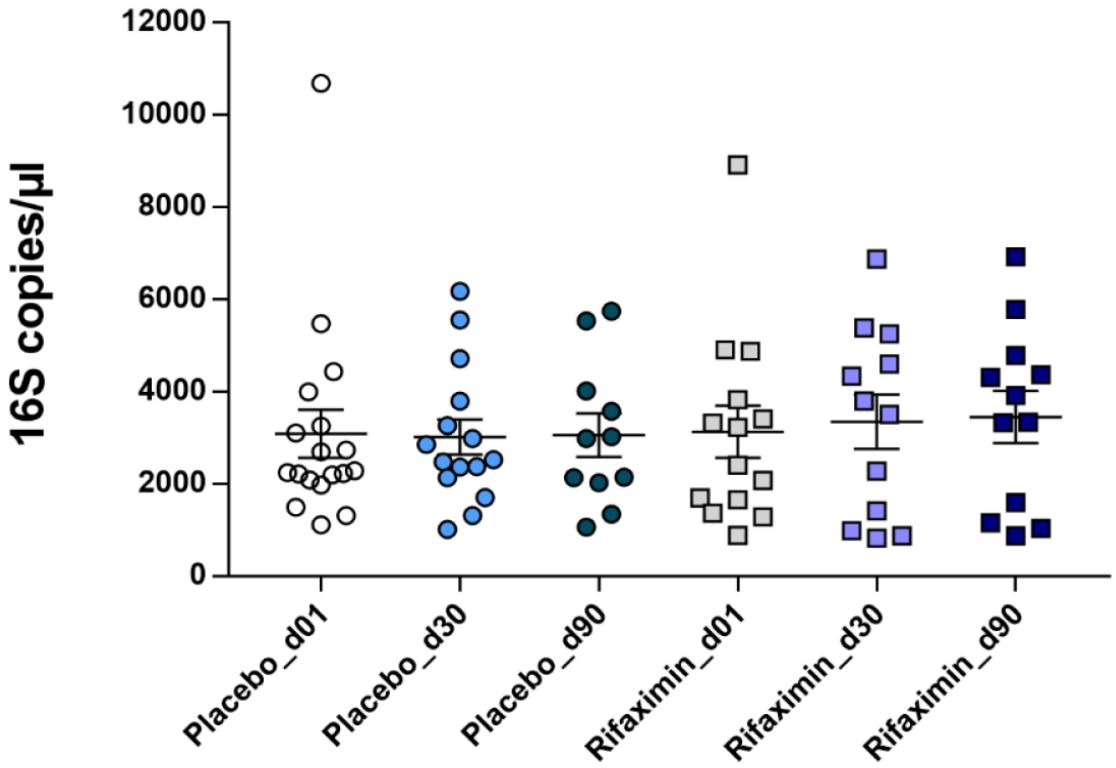


Figure 7: Whole blood 16S rDNA concentration (16S copies/µL) by treatment group and time points

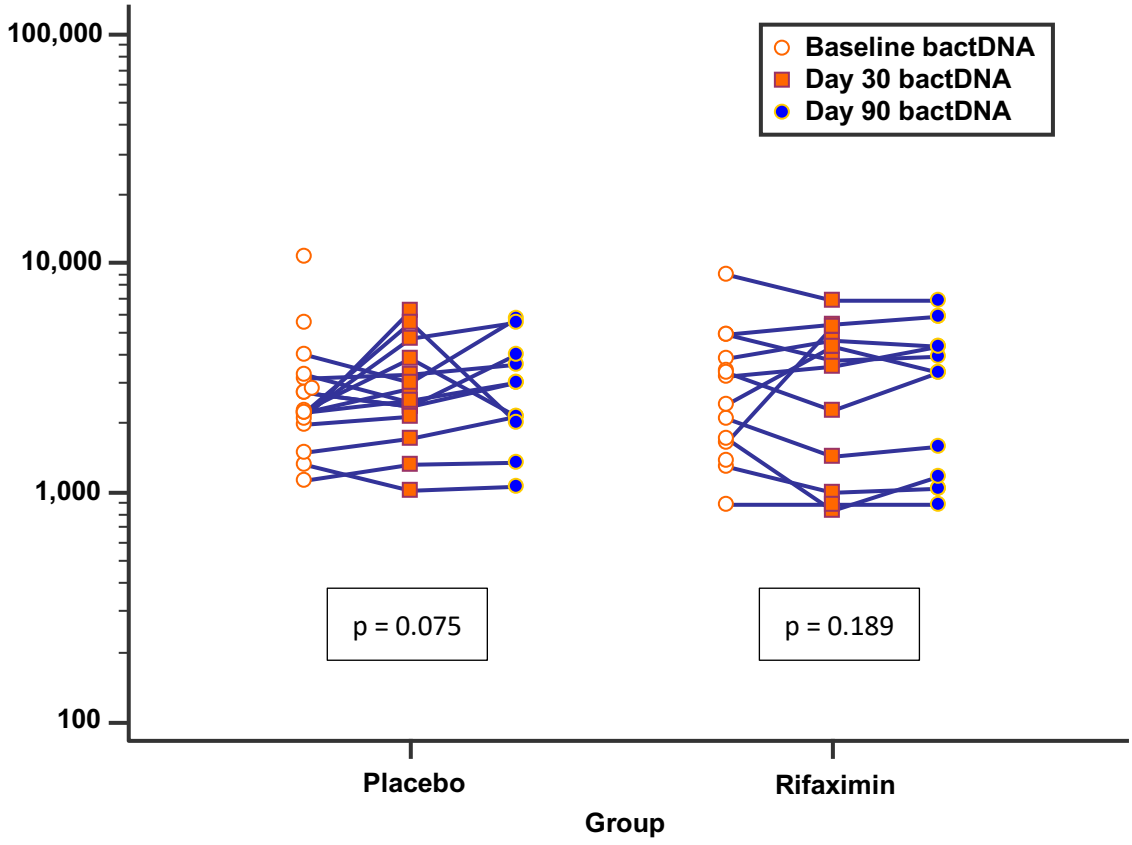


Figure 8: Analyses of whole blood 16S rDNA concentration (16S copies/µL – logarithmic scale) by treatment group and time points

24.5 Whole blood 16S rDNA metagenomics

Bacterial populations contained in circulating whole blood samples were determined using next generation high throughput sequencing of variable regions (V3-V4) of the 16S rDNA bacterial gene. The metagenomics workflow used was to identify organisms from a sample by amplifying specific regions in the 16S ribosomal DNA gene. This metagenomics workflow was exclusive to bacteria. The main output was a classification of reads at several taxonomic levels: phylum, class, order, family, genus, and species, using the workflow established by Vaiomer (Vaiomer SAS, Labège, France), summarised in the steps below.

24.5.1 LIBRARY CONSTRUCTION AND SEQUENCING

PCR amplification was performed using 16S universal primers targeting the V3-V4 region of the bacterial 16S ribosomal gene (Vaiomer universal 16S primers). The joint pair length was set to encompass 467 base pairs amplicon thanks to 2 x 300 paired-end MiSeq kit V3. For each sample, a sequencing library was generated by addition of sequencing adapters. The detection of the sequencing fragments was performed using MiSeq Illumina® technology.

24.5.2 BIOINFORMATICS PIPELINE & DATA ANALYSES

The targeted metagenomic sequences from whole blood microbiota were analysed using the bioinformatics pipeline established by Vaiomer from the FROGS guidelines. Briefly, after demultiplexing of the bar-coded Illumina paired reads, single read sequences are cleaned and paired for each sample independently into longer fragments. Operational taxonomic units (OTUs) are produced with via single-linkage clustering and taxonomic assignment is performed in order to determine community profiles.

The following specific filters have been applied for this analysis in order to obtain the best results:

- The last 10 bases of reads R1 were removed (lower quality preventing good read pairing)
- The last 60 bases of reads R2 were removed (lower quality preventing good read pairing)
- Amplicons with a length < 350 nt or a length > 500 nt are removed.
- OTUs with abundance lower than 0.005% of the whole dataset abundance are removed.

Reads obtained from the MiSeq sequencing system were processed using Vaiomer bioinformatics pipeline. The steps include quality-filtering, clustering into OTUs with the Swarm algorithm and taxonomic affiliation.

24.5.3 QUALITY CONTROLS (1) - NUMBER OF READ PAIRS CLASSIFIED IN OTUS

Vaiomer bioinformatics pipeline uses a quality control pipeline that is intended for evaluating abundance, fragment length and sample quality of DNA libraries. The red line illustrates the targeted 37,500 raw read pairs per sample, which was experimentally determined to be the number of reads to have exhaustive coverage of the community profiles present in high diversity samples. The bar plots below (Figure 9) indicate the number of raw read pairs (brown) and read pairs that were classified into OTU (blue) per sample.

In this study, the number of raw read pairs is approximately 35,000. The number of read pairs classified in OTU is around 20,000.

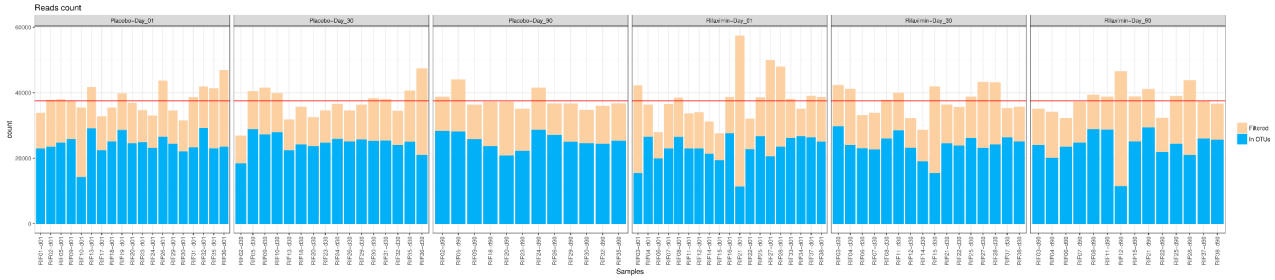


Figure 9: Reads count – red line illustrates targeted 37,500 raw read pairs per sample

24.5.4 QUALITY CONTROLS (2) - RAREFACTION ANALYSIS

Rarefaction analysis is used to assess richness of bacterial taxa from the sequencing results. Rarefaction curves are created by randomly re-sampling the pool of N sequences multiple times and then plotting the average number of operational taxonomic unit found in each sample. Rarefaction curves generally grow rapidly at first, as the most common bacterial taxa are found, but the curves plateau as the rarest taxa remain to be sampled. If the curve becomes flatter to the right, a reasonable number of sequences have been produced and more intensive sequencing is likely to yield only few additional taxa. Rarefaction analysis curves were plotted for each group independently (Figure 10 below).

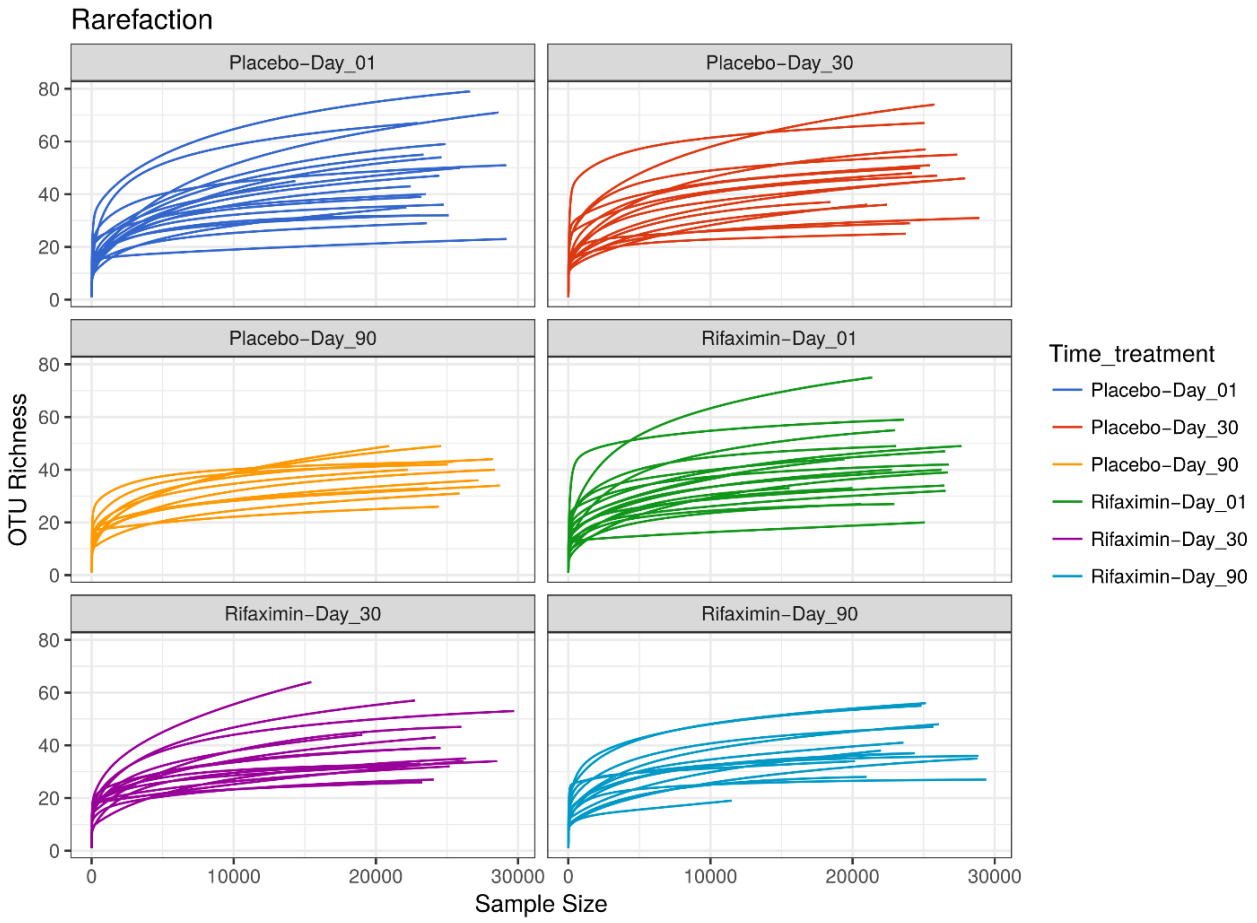


Figure 10: Rarefaction curves per group

The rarefaction analysis curves suggest that the sample diversity was captured as expected given the average sequence pairs classified into operational taxonomic units (OTU).

24.5.5 ALPHA DIVERSITY

Alpha diversity (α-diversity) represents the taxa diversity within each sample. Alpha diversity analyzed with different methods is represented in Figure 11 (median + interquartile), 1) Observed, 2) Chao1, 3) Shannon, 4) Simpson, and 5) Inverse Simpson. Observed and Chao1 indexes calculate the alpha diversity in term of richness (number of taxa that are present in the samples). Shannon, Simpson and inversed Simpson indexes calculate the alpha diversity regarding the evenness of taxa in the samples. In this study, at the OTU level and for both richness and evenness, the groups were not significantly different between each other.

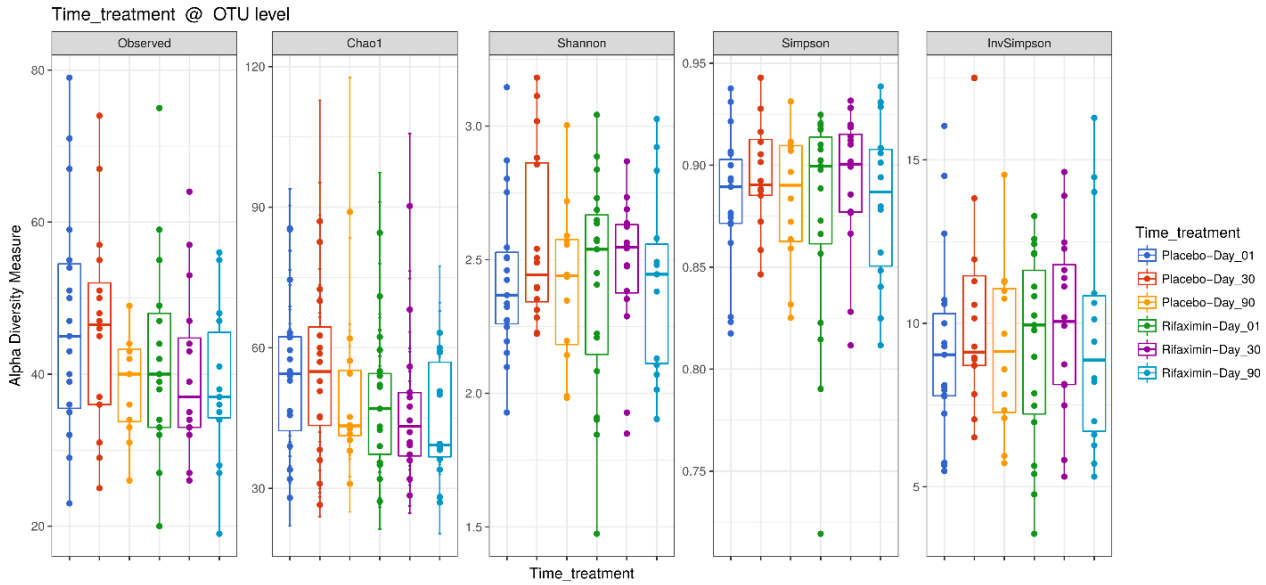


Figure 11: Alpha diversity measures of circulating whole blood 16S rDNA sequencing at OTU level.

24.5.6 MULTIDIMENSIONAL SCALING ON BETA DIVERSITY

Multidimensional scaling (MDS) ordination allows visualisation at the global level the level of similarity between individual bacterial profiles. Multidimensional Scaling (MDS) ordination was performed for comparison of sample groups/class based on four methodologies for β-diversity: A) Bray-Curtis, B) Jaccard, C) Unifrac, and D) Weighted Unifrac (Figures 12, 13, 14 and 15). These figures represent the distance between samples (calculated by the 4 different methods) using the OTU distribution of each sample. The distance is represented on 2 axes summarising the entire distribution of all the OTU present in the samples.

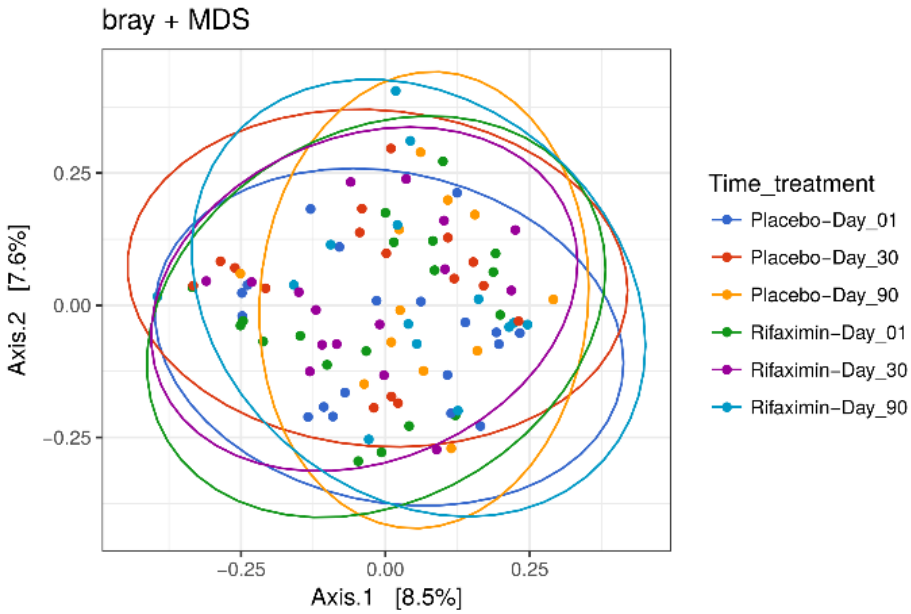


Figure 12: Multidimensional scaling on beta diversity measures of circulating whole blood 16S rDNA sequencing at OTU level – Bray Curtis methodology.

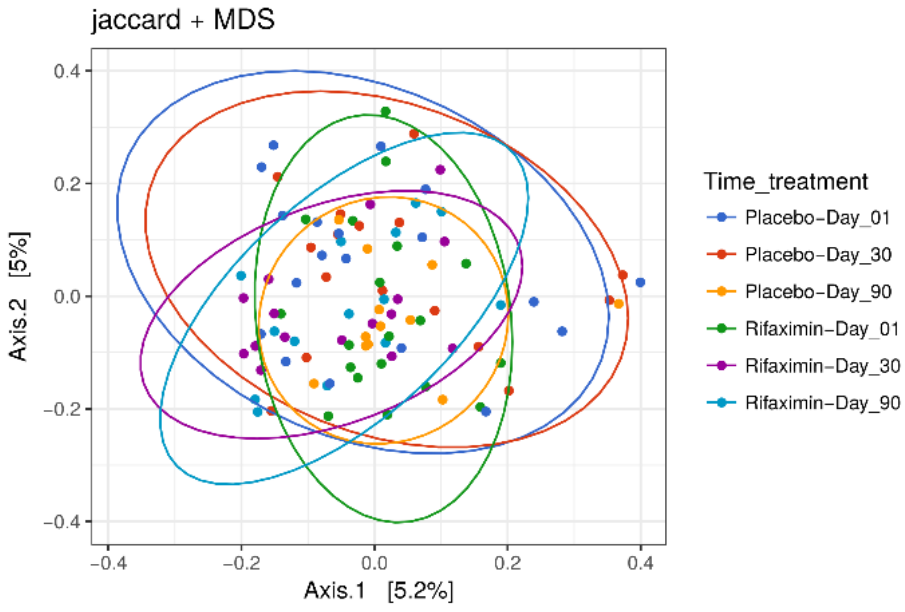


Figure 13: Multidimensional scaling on beta diversity measures of circulating whole blood 16S rDNA sequencing at OTU level – Jaccard methodology.

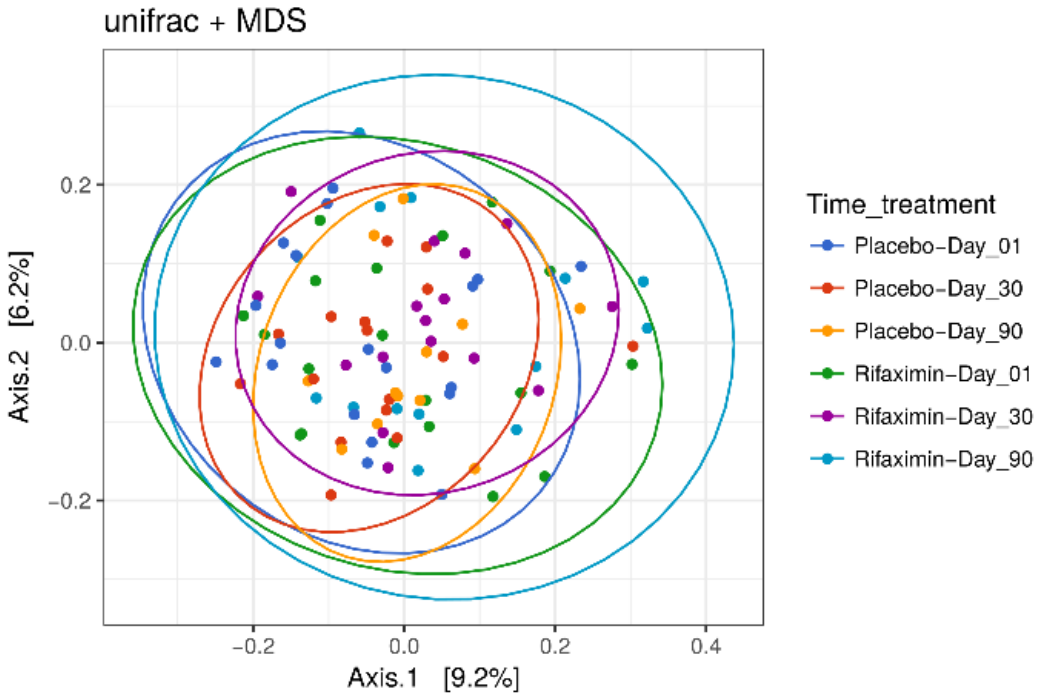


Figure 14: Multidimensional scaling on beta diversity measures of circulating whole blood 16S rDNA sequencing at OTU level – Unifrac methodology.

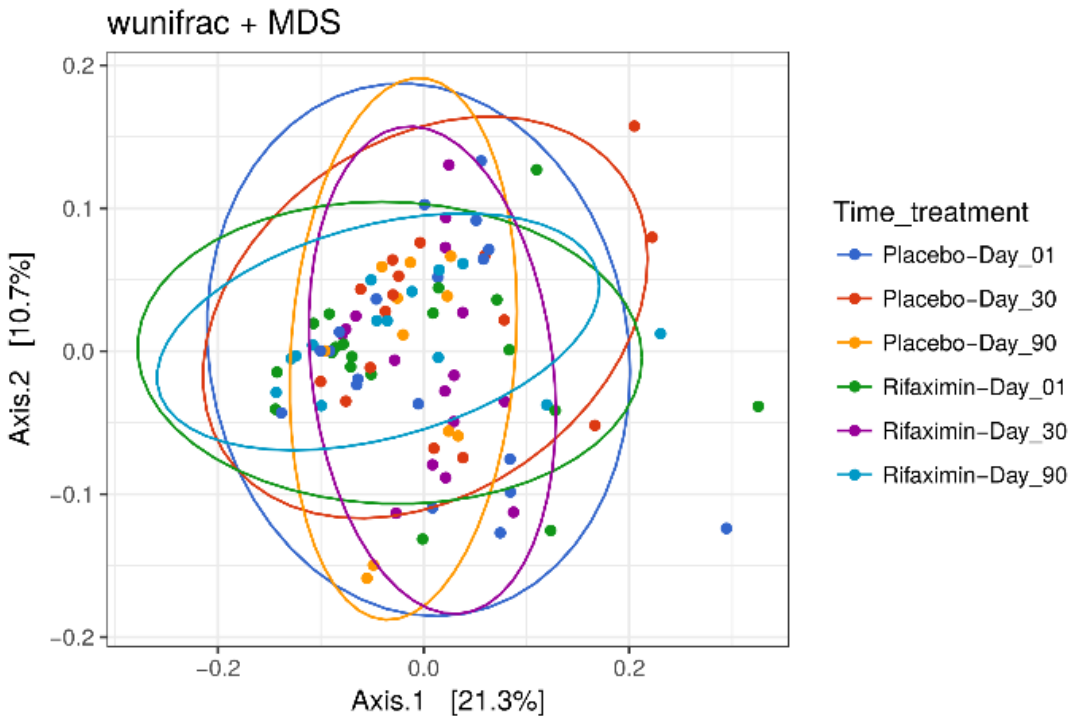


Figure 15: Multidimensional scaling on beta diversity measures of circulating whole blood 16S rDNA sequencing at OTU level – Weighted Unifrac methodology.

24.5.7 HIERARCHICAL CLUSTERING ON BETA DIVERSITY

Hierarchical clustering allows visualisation at the global level the degree of similarity between individual bacterial profiles using a dendogram. Ward's method criterion was applied to generate the hierarchical clustering with the A) Bray-Curtis, B) Jaccard, C) Unifrac, and D) Weighted Unifrac beta diversity measures (Figures 16-19).

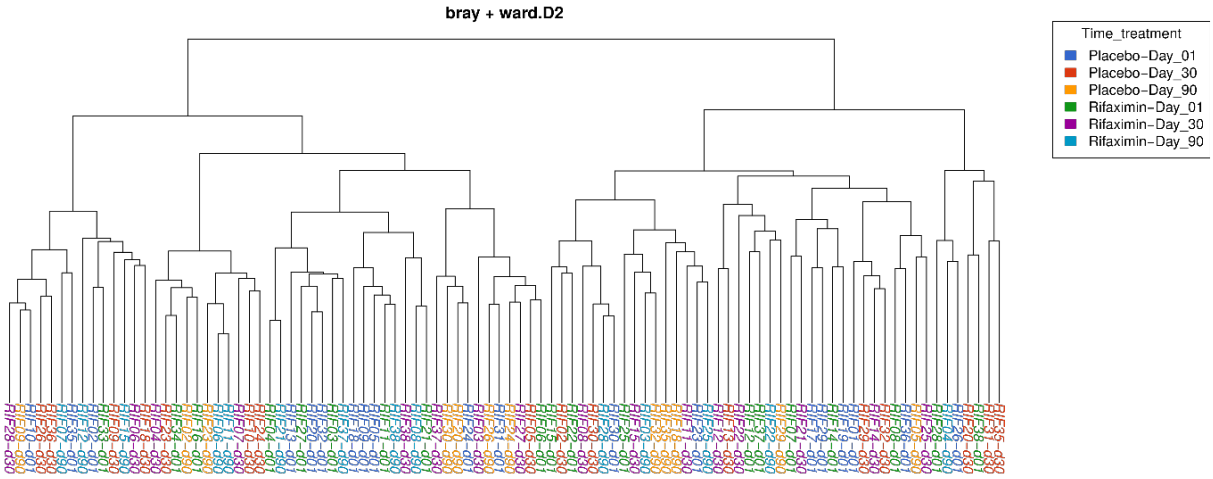


Figure 16: Hierarchical clustering on beta diversity – Bray Curtis.

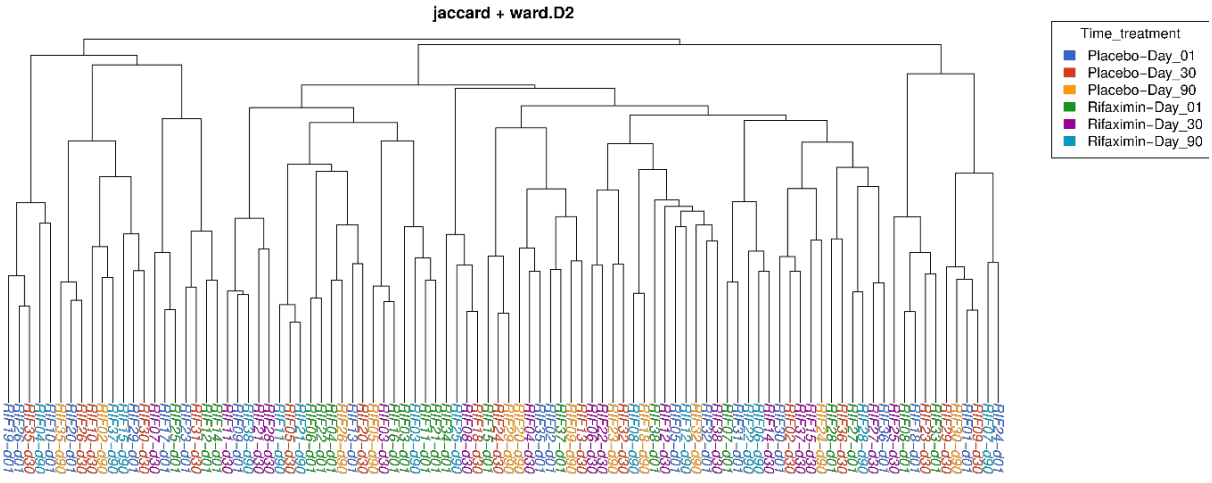


Figure 17: Hierarchical clustering on beta diversity – Jaccard

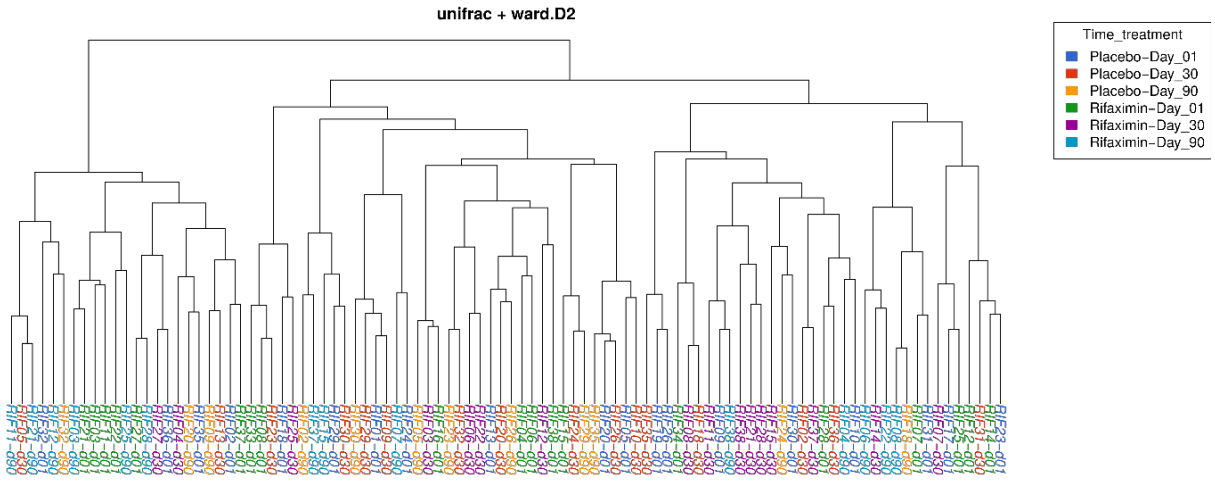


Figure 18: Hierarchical clustering on beta diversity – Unifrac.

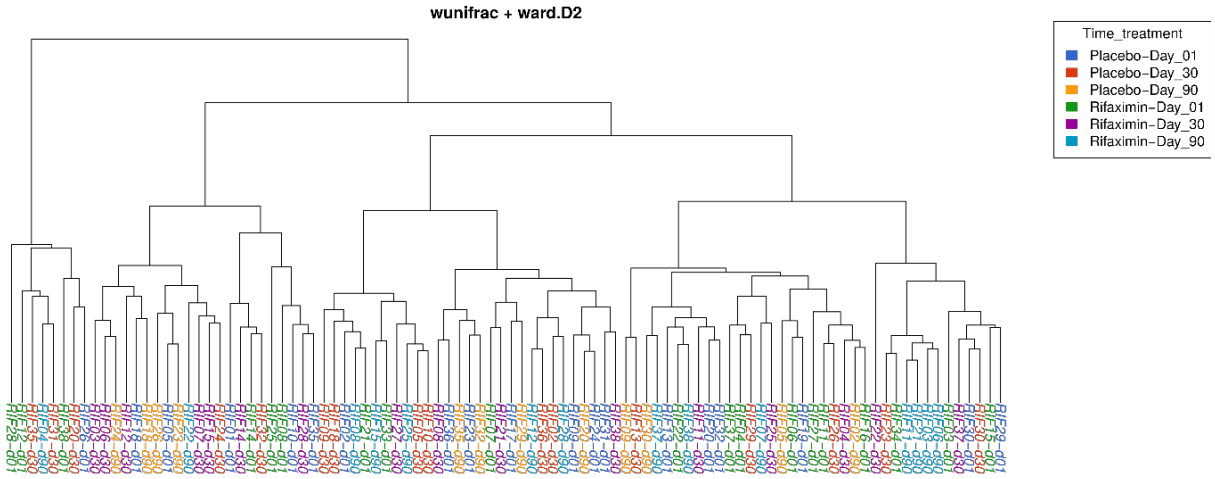


Figure 19: Hierarchical clustering on beta diversity – weighted Unifrac.

24.5.8 RELATIVE ABUNDANCE AT EACH TAXONOMIC LEVEL

Based on these results, graphical representations are made of the relative proportion of taxa for each taxonomic level (phylum, class, order, family, and genus– Figures 20-24) present in individual study samples. Taxa are identified by name in the plot for the most abundant taxa. Taxa are merged into the "Other" category if less abundant. Taxa are merged into the "Multi-affiliation" category when they can correspond to two or more different affiliations.

These figures (next pages) show the taxa relative abundance per sample at each taxonomic level. The visualization of the top 15 bacteria for each sample helps to identify outliers and differences between groups.

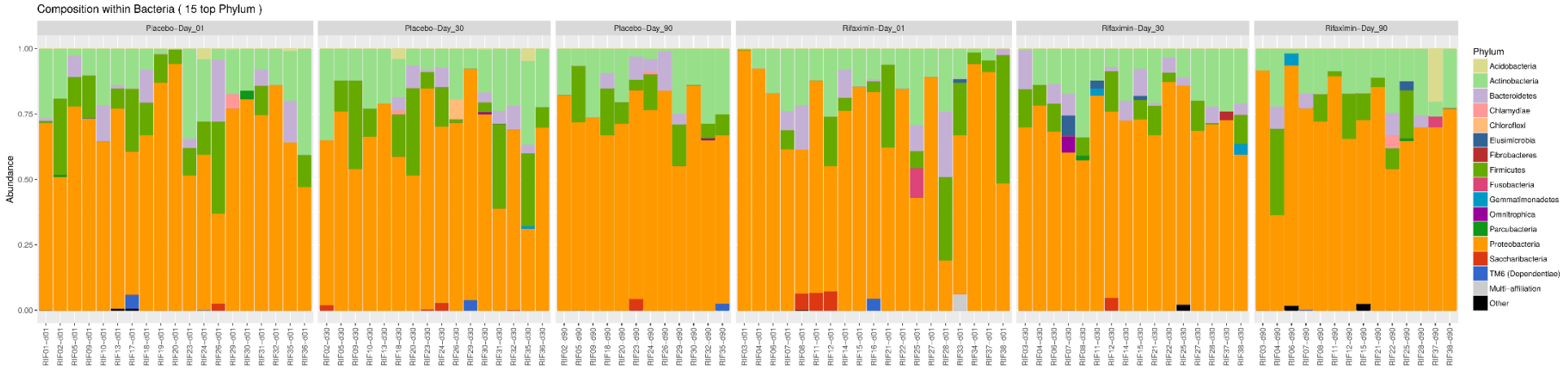


Figure 20: Relative proportion taxa for each taxonomic level: Phylum level.

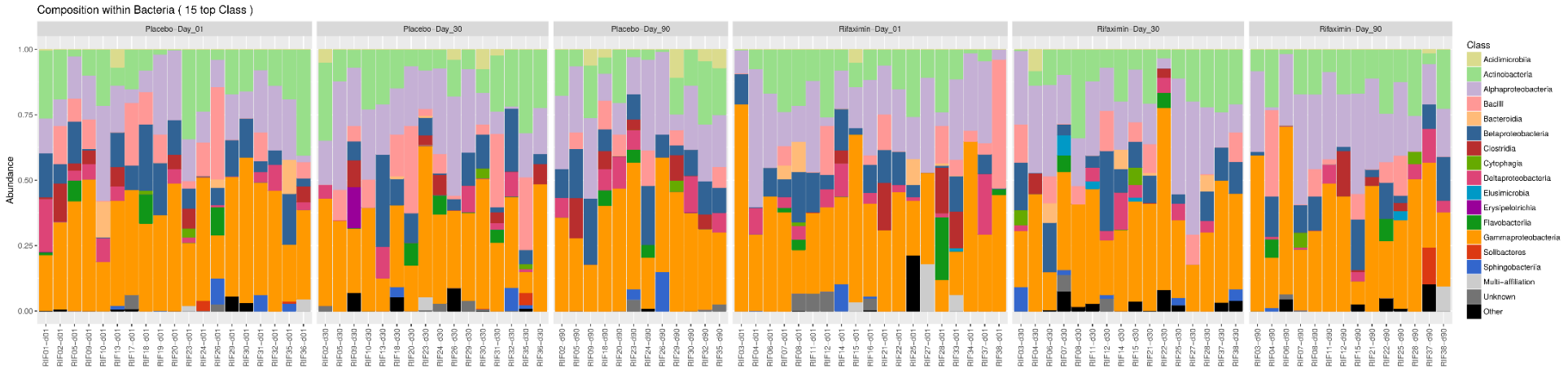


Figure 21: Relative proportion taxa for each taxonomic level: Class level.

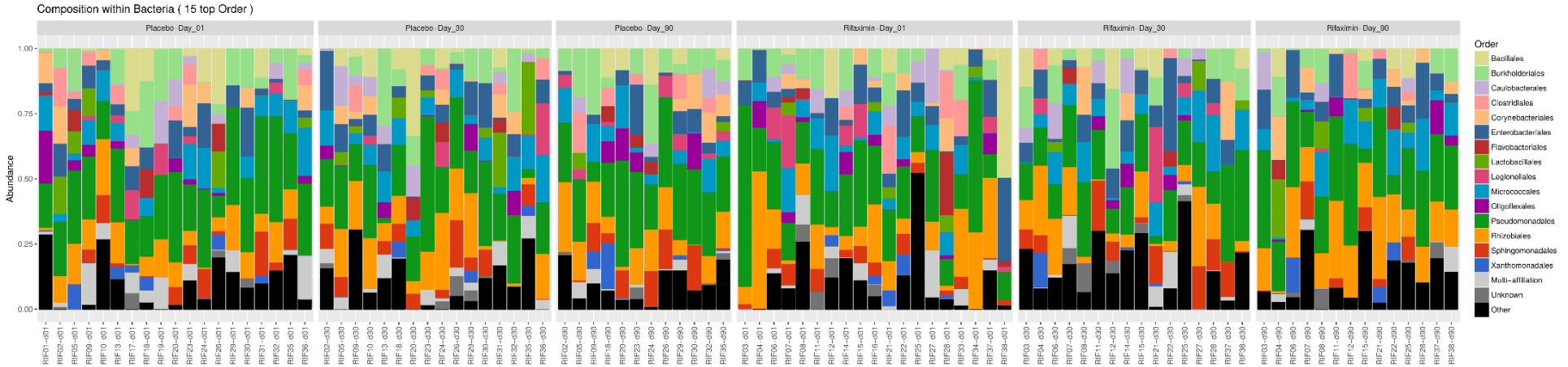


Figure 22: Relative proportion taxa for each taxonomic level: Order level

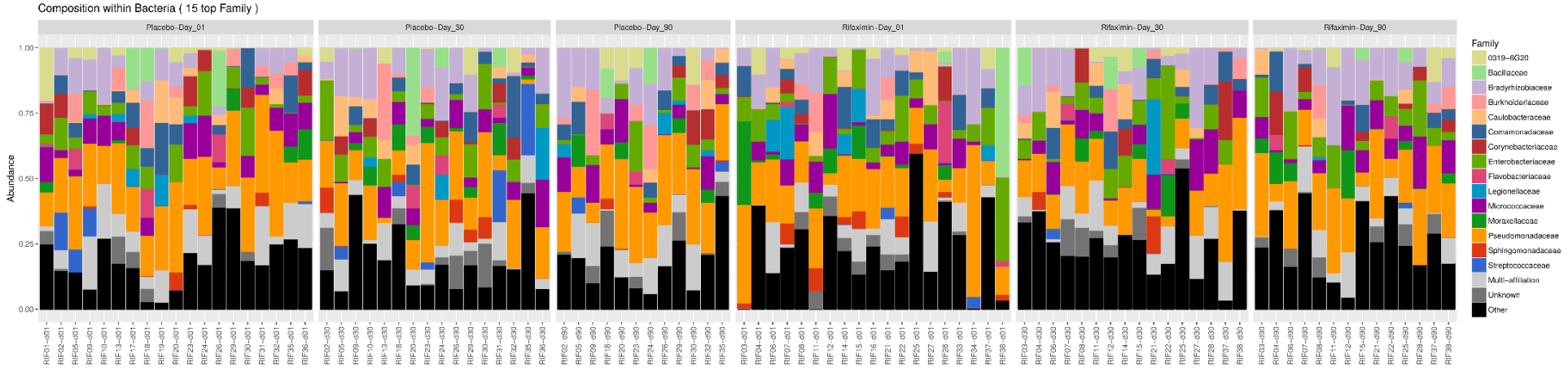


Figure 23: Relative proportion taxa for each taxonomic level: Family level.

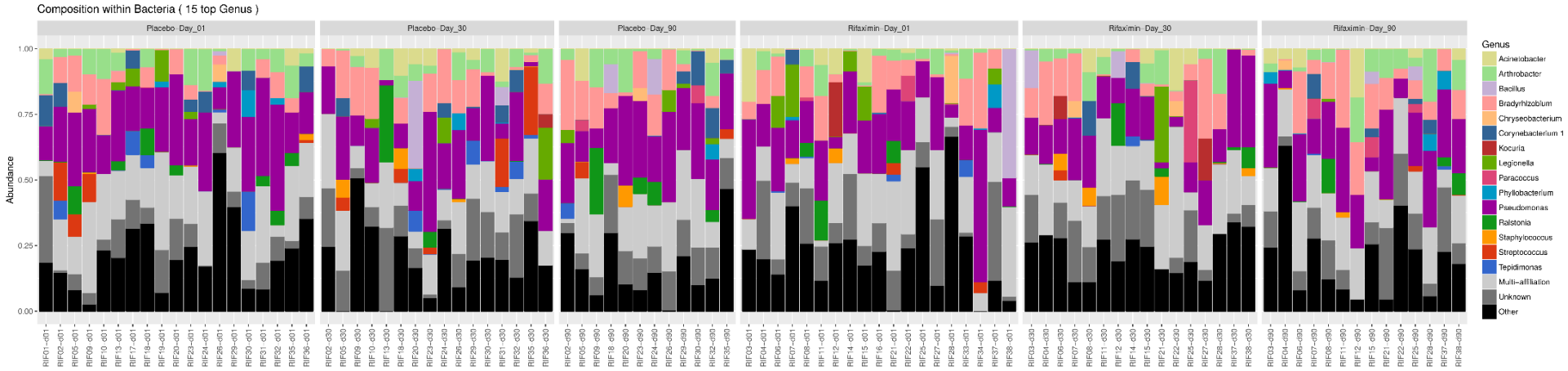


Figure 24: Relative proportion taxa for each taxonomic level: Genus level.

24.5.9 LINEAR DISCRIMINANT ANALYSIS EFFECT SIZE

Linear discriminant analysis effect size (LEfSe) is an algorithm for high-dimensional biomarker discovery and explanation that can identify taxonomic groups characterising the differences between two or more biological conditions, or in this case, interventions. It emphasises both statistical significance and biological relevance to identify differentially abundant features that are also consistent with biologically meaningful categories (subclasses). LEfSe first robustly identifies features that are statistically different among biological classes.

LEfSe analysis was performed on the complete sequence data (no OTU threshold) for the following groupings:

- Placebo vs rifaximin-α (all patients at all time points – composite comparison) (Figure 26).
- Rifaximin-α-Day_01_vs_Placebo-Day_01 (Figure 27).
- Rifaximin-α-Day_30_vs_Placebo-Day_30 (Figure 28).
- Rifaximin-α-Day_90_vs_Placebo-Day_90 (Figure 29).
- Placebo-Day_01_vs_Placebo-Day_30 (Figure 30).
- Placebo-Day_01_vs_Placebo-Day_90 (Figure 31).
- Rifaximin-α-Day_01_vs_ Rifaximin-α-Day_30 (Figure 32).
- Rifaximin-α-Day_01_vs_ Rifaximin-α-Day_90 (Figure 33).

The LEfSe result cladograms (genus and above levels) are shown for each analysis below. The cladograms represented here (next pages) indicate the bacterial taxa that are significantly different between the 2 groups being compared. This analysis helps to identify a first selection of differential bacterial taxa.

For the all the cladograms below:

Taxa more abundant in "red" group
Taxa more abundant in "green" group

24.5.10 EXAMPLE CLADOGRAM AND TAXONOMIC STRUCTURE

The following example cladogram has been modified from the original output so that all taxonomic names can be read. The biomarkers found by LEfSe are highlighted (red and green for pairwise analysis) with the class (group) having the highest median. Please note that the remaining data cladograms are not retouched from the originals (to separate overlapping taxonomic names).

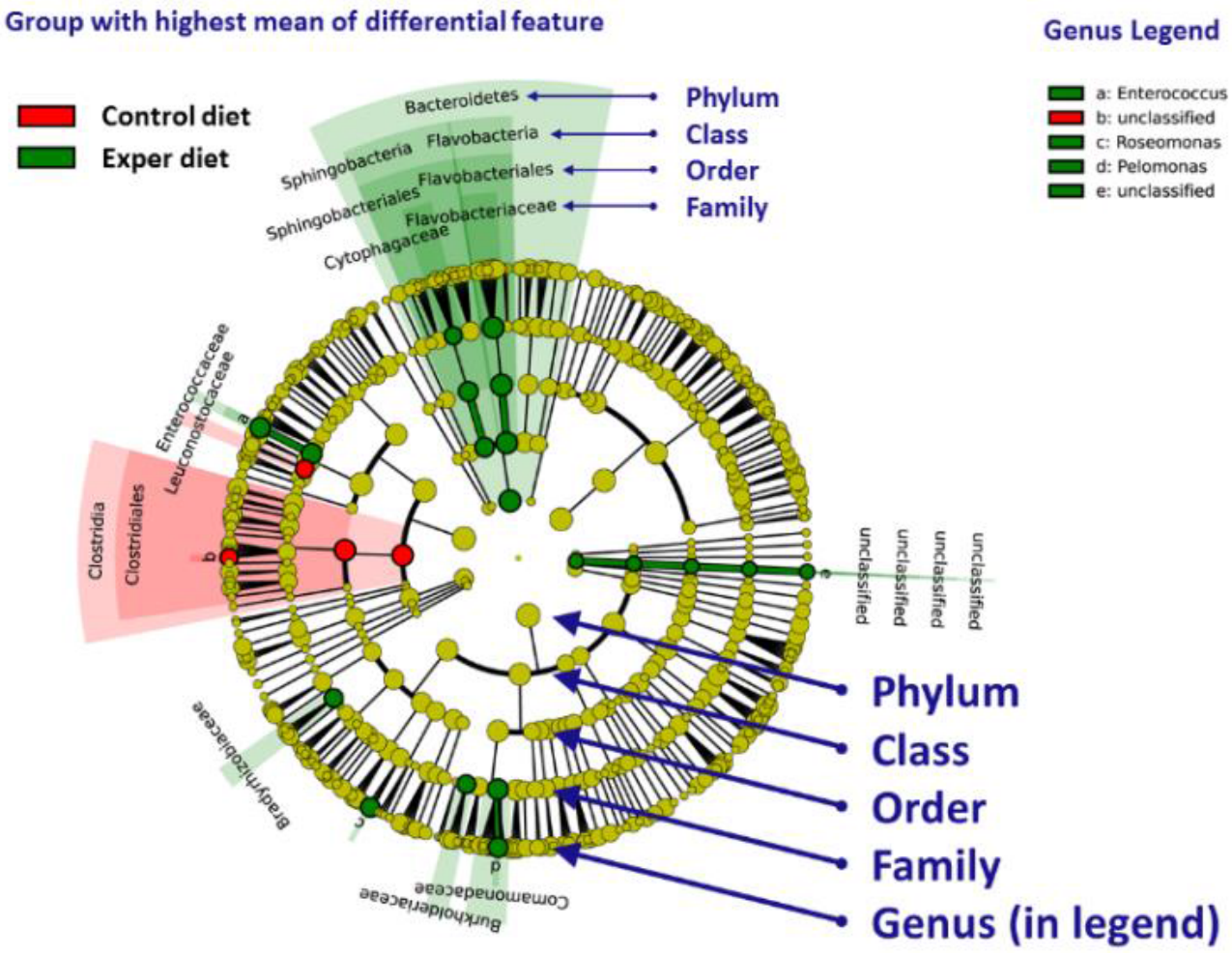


Figure 25: LEfSe Cladogram schematic example/key

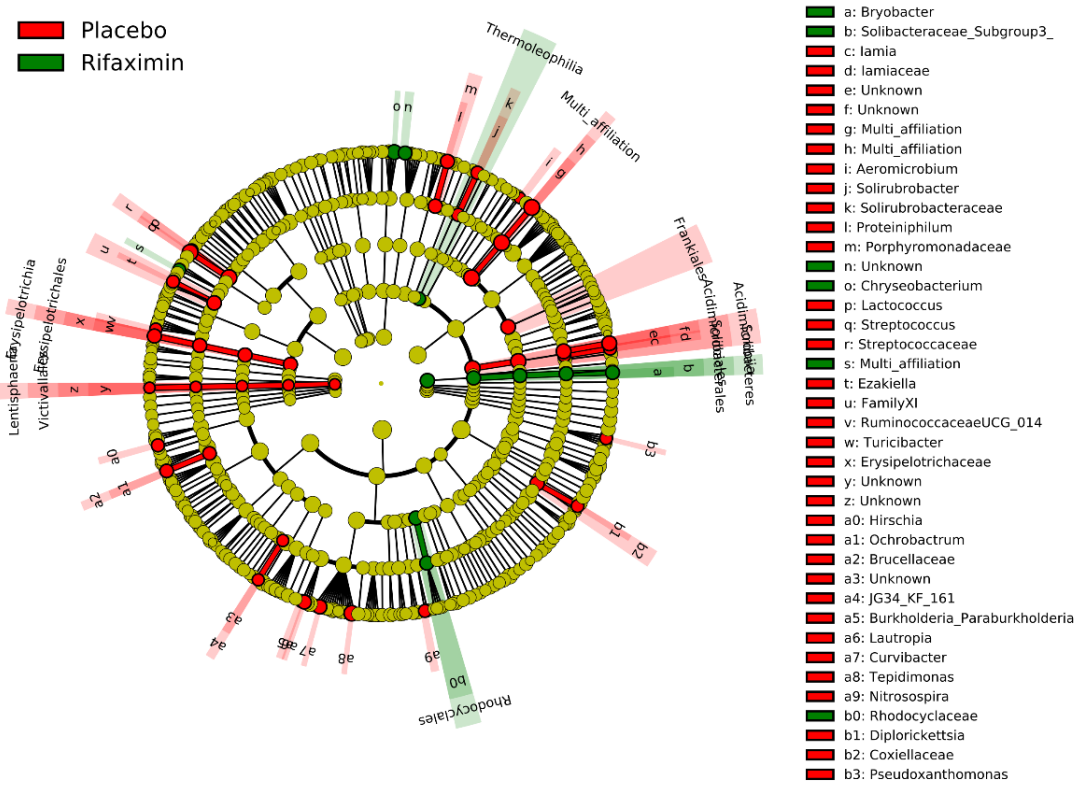


Figure 26: Cladogram of pairwise analysis: Placebo vs Rifaximin (all participants at all time points)

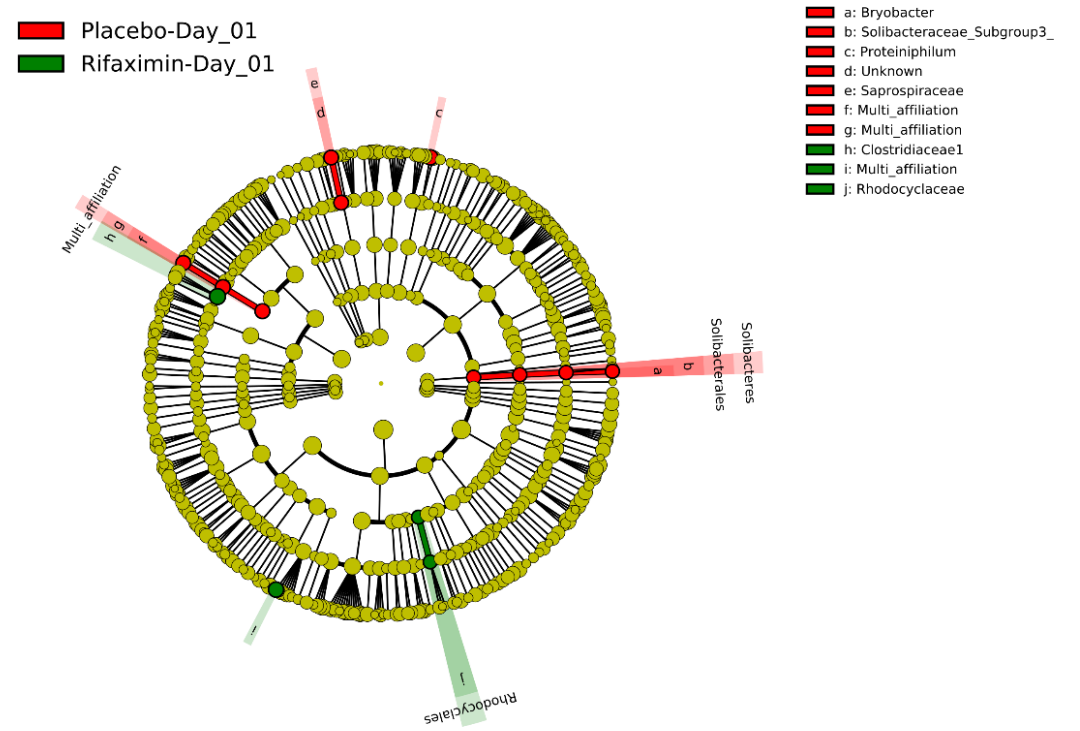


Figure 27: Cladogram of pairwise analysis: Rifaximin-Day_01_vs_Placebo-Day_01 (baseline comparator)

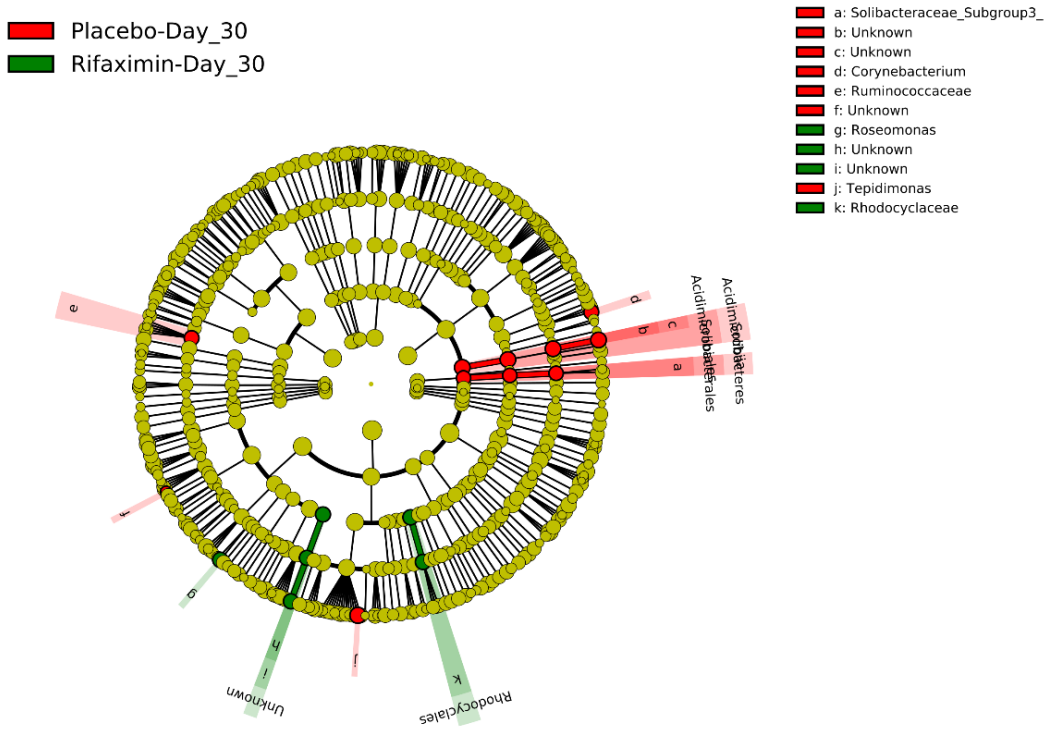


Figure 28: Cladogram of pairwise analysis: Rifaximin-Day_30_vs_Placebo-Day_30

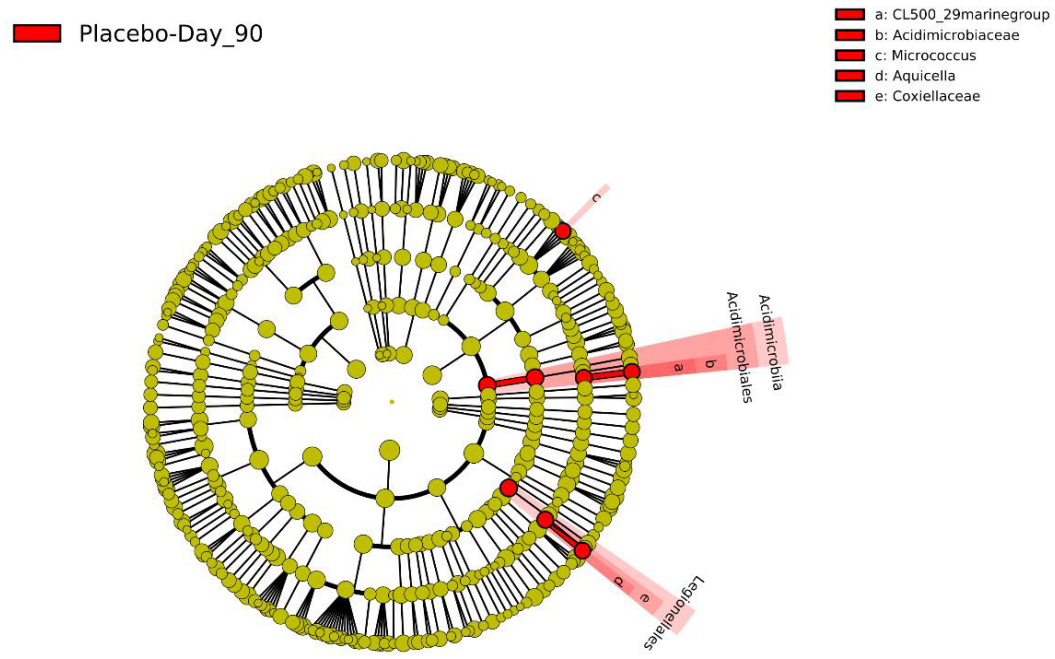


Figure 29: Cladogram of pairwise analysis: Rifaximin-Day_90_vs_Placebo-Day_90

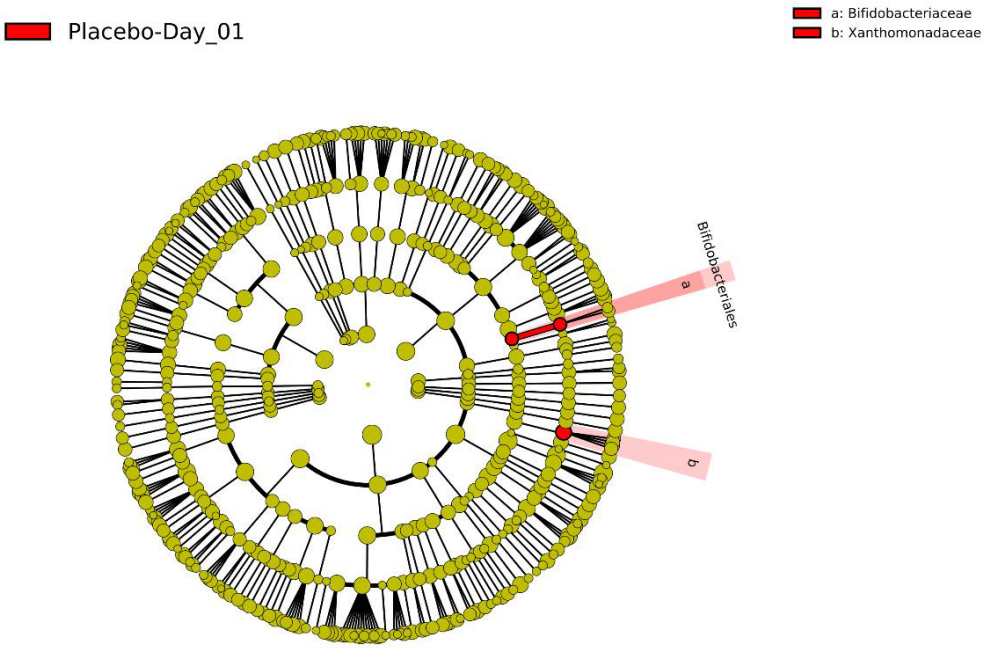


Figure 30: Cladogram of pairwise analysis: Placebo-Day_01_vs_Placebo-Day_30

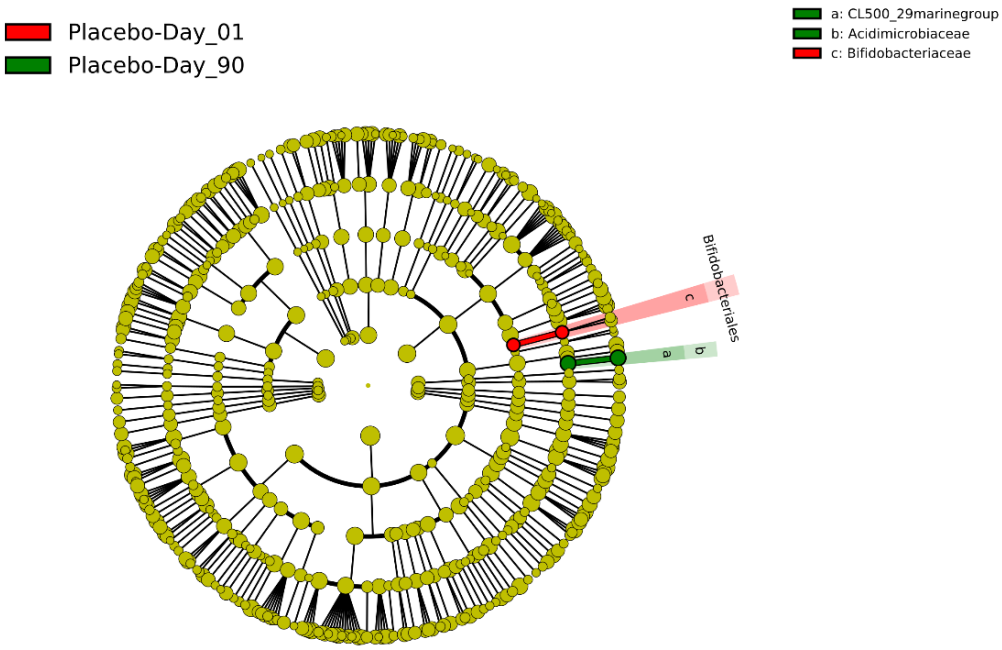


Figure 31: Cladogram of pairwise analysis: Placebo-Day_01_vs_Placebo-Day_90

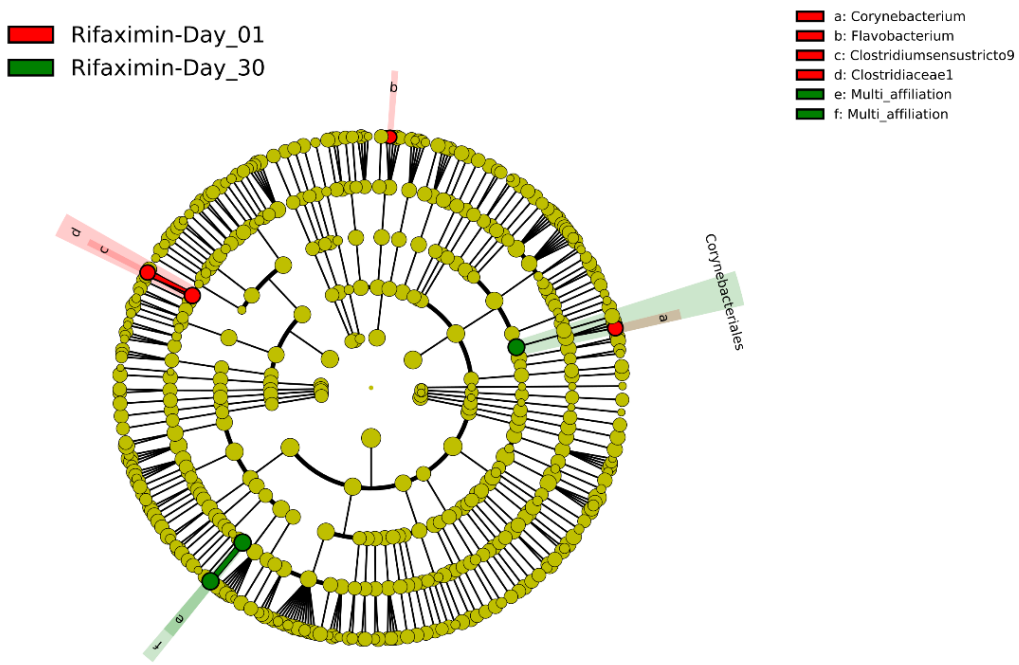


Figure 32: Cladogram of pairwise analysis: Rifaximin-Day_01_vs_Rifaximin-Day_30

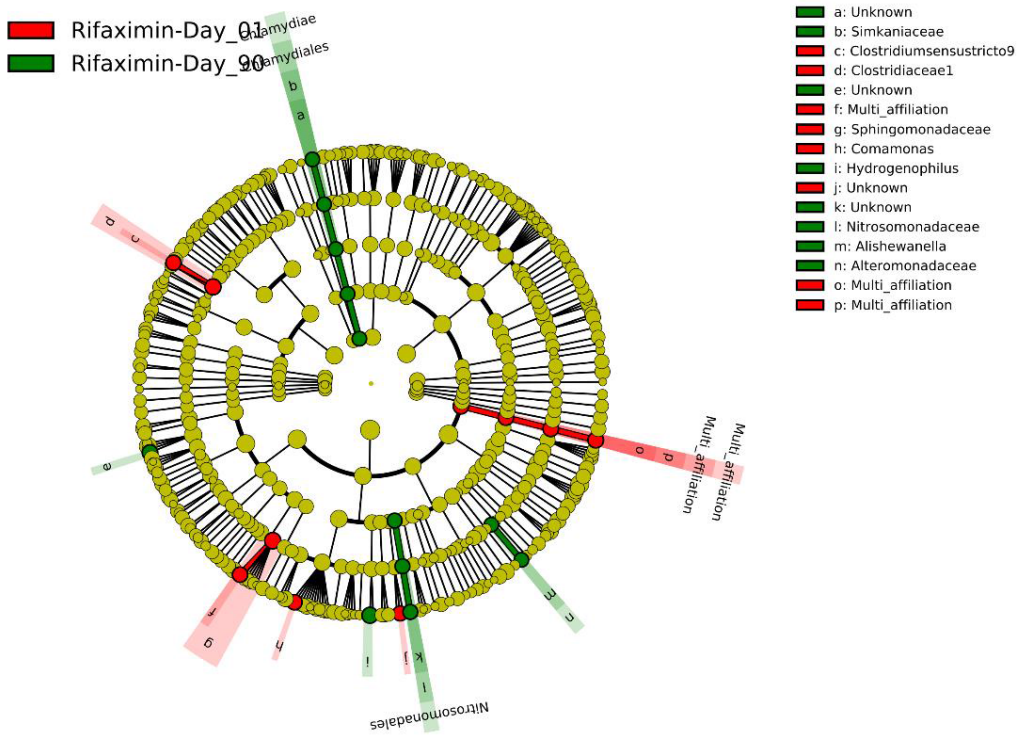


Figure 33: Cladogram of pairwise analysis: Rifaximin-Day_01_vs_Rifaximin-Day_90

The following is a summary of observations from the whole blood bacterial 16S ribosomal DNA gene sequencing data presented above.

- The sequence quality was very good. The average number of reads assigned to OTU per sample was around 20,000.
- Curve plots for rarefaction analysis suggest that the sequence depth was sufficient to capture the diversity in all samples.
- Alpha diversity analyses do not show significant differences between groups.
- Beta diversity analyses do not show a major separation between groups; however, significant differences exist between groups as shown for example in the LEfSe analysis.
- Visual comparison of the relative abundance barplots indicates differences between the groups as seen also in the pairwise group LEfSe analyses (log(LDA Score)>2.0).

24.6 Faecal 16S rRNA sequencing microbiota analysis

24.6.1 FAECAL SPECIMEN HANDLING

Faecal samples were obtained as close to the time of planned sampling as possible, and often within 12hrs of the participant having a bowel motion. Fresh faecal samples were obtained and stored in standard clinical collection specimen containers, and following delivery were placed in a cooler containing ice (4^{o}C). Within 12hrs, all faecal samples were divided into aliquots stored within 2ml microtubes without any additional storage medium or preservative and stored for future DNA isolation and molecular microbiological analysis.

24.6.2 ISOLATION OF FAECAL BACTERIAL DNA

Bacterial DNA was isolated from a total of 91 faecal samples. This was performed utilising an ISOLATE Fecal DNA Kit (Bioline, UK; BIO-52082). The steps are summarised in Figure X. In summary, faecal samples were thawed from -80^{o}C at room temperature for up to 30 minutes. Up to 150mg of faecal material per sample was added directly to a Bashing Beads Lysis Tube and rapidly lysed by bead beating in a vortex, without the use of organic denaturants or proteinases. The DNA was then bound, isolated and purified using spin columns. To optimise the DNA binding step, β-mercaptoethanol was added to eliminate deoxyribonucleases released during cell lysis, by reducing the disulfide bonds within the deoxyribonuclease enzymes. This prevents enzymatic digestion of the DNA during its extraction procedure, increasing the overall yield. The eluted DNA, free of contaminants and enzyme inhibitors, was then used for downstream molecular biology applications including sequencing. Sequencing and bioinformatic processing was undertaken by collaborators at the South Australian Health and Medical Research Institute, Adelaide, South Australia.

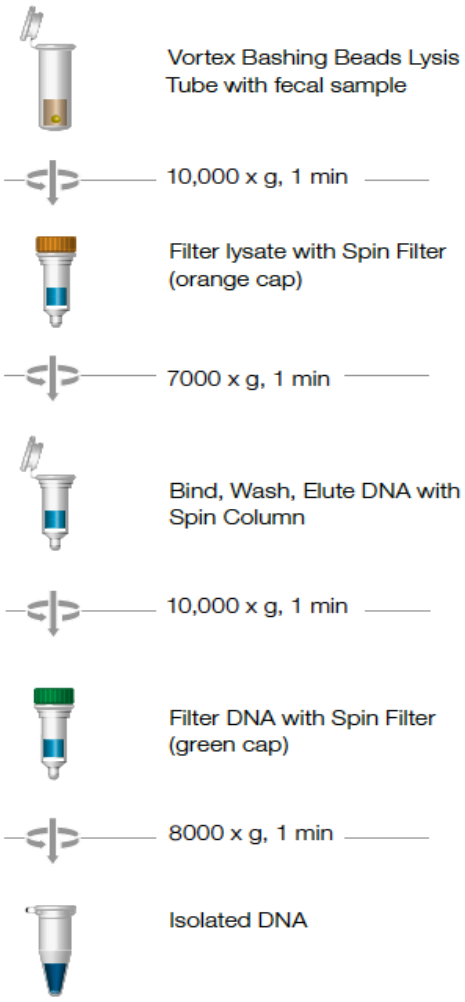


Figure 34: Isolation of faecal DNA using ISOLATE Fecal DNA Kit (Bioline, UK)

24.6.3 16S rRNA Gene Amplicon Library Preparation and Sequencing

Amplicons were generated using the fusion degenerate forward and reverse primers targeting the V4 hypervariable region of the bacterial 16S rRNA gene. The primers had ligated overhang Illumina adapter consensus sequences and allowed barcoded amplicon libraries for bacterial community analysis to be prepared on an Illumina MiSeq sequencing platform using Illumina Nextera XT index kit (Illumina, Inc., Victoria, Australia). In brief, the PCR reactions were performed on a Veriti 96-well Thermal Cycler (Life Technologies, Australia) with library preparation based on a standard protocol of 25 cycles for amplicon generation and 8 cycles for barcoding. The PCR reactions were performed in the following programme, with paired sequence reads for samples:

- initiation enzyme activation at 95°C for 3 min,
- followed by 25 cycles consisting of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec.
- after 25 cycles, the reaction was completed with a final extension of 7 min at 72 °C.

The Illumina Nextera XT Index kit (Illumina Inc., San Diego. CA, USA) with dual 8-base indices were used to allow for multiplexing. Two unique indices located on either end of the amplicon were chosen based on the Nextera dual-indexing strategy. To incorporate the indices to the 16S amplicons, PCR reactions were performed on a Veriti 96-well Thermal Cycler (Life Technologies, Australia). Cycling conditions consisted of one cycle of 95°C for 3 min, followed by eight cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, followed by a final extension cycle of 72°C for 5 min.

Library preparation included the use of Agencourt AMPure XP PCR purification system (Beckman Coulter, Inc., Indianapolis) to clean up post PCR allowing removal of unincorporated deoxyribose containing nucleotide triphosphates (dNTPs), primers, primer dimers, salts and other contaminants. The Nextera XT Index Kit (Illumina) was used to allow PCR produced DNA to be simultaneously fragmented and tagged with sequencing adapters in a single tube enzymatic reaction, for barcode addition and PCR amplicon preparation. The AMPure XP kit was used to clean up the barcoded PCR products prior to normalisation and library pooling. Following library preparation, samples with low amplicon concentration (<2ng/µl) were excluded from sequencing.

Prior to library pooling, the barcoded libraries were quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Results from this quantification step (amplicon concentration) were used in downstream processing to eliminate contamination. The libraries were sequenced by 2 x 300 base pair paired-end sequencing on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina) at the Flinders Genomics Facility, Adelaide, Australia.

24.6.4 NUMBER OF READS FOR FAECAL SAMPLES

All samples had a sequence read range from 9,581 to 41,910, except for one which had a very low number: 44 sequence reads (RIF07_d01). Various control samples were sequenced as follows:

- Extraction control (# 92): 77 reads
- Water used for dilution (# 93): 319 reads
- Elution buffer 1X before dilution (# 94): 52 reads
- Sequencing buffer, 5uL elution buffer + 15uL dH_2O (# 95): 72 reads

24.6.5 BIOINFORMATIC PROCESSING

24.6.5.1 Demultiplexing and chimera removal

Samples were demultiplexed using QIIME v.1.8.0 with individual sequences assigned to their original samples. The demultiplex step contained further quality filtering steps as follows: truncation following three consecutive low quality base calls, removal of reads with <75% high quality base calls and removal of sequences with an unclear base call (N).

Chimeras are DNA sequences composed of DNA from two or more parents and are artifacts produced during the PCR process. On occasion, an amplicon may terminate prematurely before it is completely finished. When PCR picks up again in the next cycle another DNA strand may attach where the first left off if the starting region is similar enough, and complete the amplicon from this second parent. If this sequence were to go straight to classification without being checked for chimeric status it would likely be returned as a 'novel' sequence. This would create the false impression of a previously unknown organism being discovered when in fact it is merely a combination of two previously classified microbes.

24.6.5.2 OTU picking and taxonomic assignment

For OTU picking, SILVA 128 16S rRNA reference database was used as the current definitive open-source bioinformatics data analysis pipeline for performing 16S microbiome analysis from raw DNA sequencing data.

24.6.5.3 Rarefaction curve generation

Rarefaction curves were generated for all contaminant-filtered and non-filtered samples. Appropriate subsample depth was established by visual inspection of rarefaction curves to ensure adequate sample depth while retaining low read samples. It was confirmed that reducing the sequence number in this way did not result in a significant reduction in profile diversity, as determined using the Simpson' s Index of Diversity (1-D). Accordingly, all samples were subsampled to 9,581 reads. Subsampling eliminated 1% of all samples (1 out of 91 samples) where less than 9,581 reads were obtained.

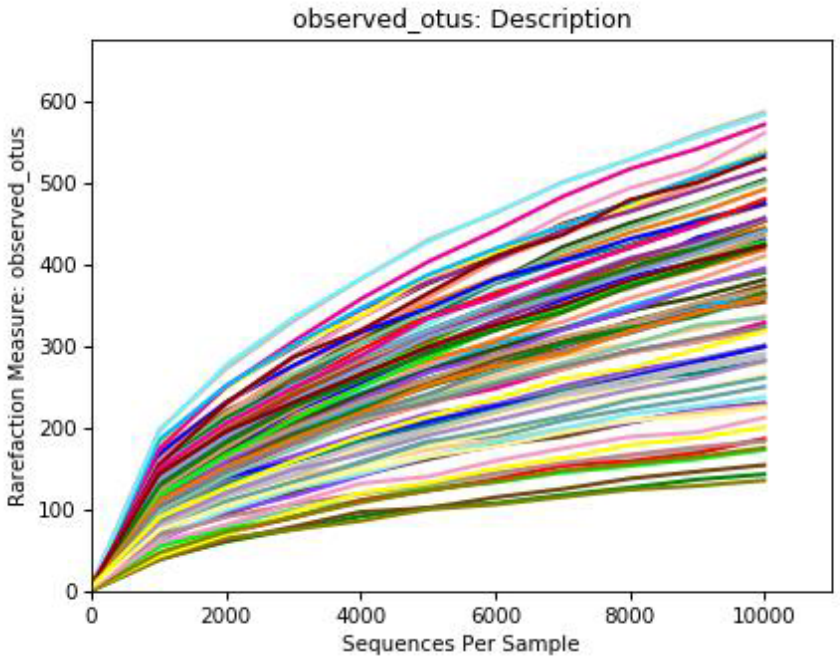


Figure 35: Alpha rarefaction curve at 10,000 reads

24.6.5.4 Biostatistical analysis

The subsampled cohort was at a threshold of 9,581 reads. This resulted in a dataset without the control samples and RIF07_d01.

Alpha and beta-diversity measures were calculated as measures of 'within-sample' and 'between sample species' diversity, respectively, combining richness metrics with a measure of the evenness of abundance of the different species present. Bray-Curtis (BC) similarity matrices were created using QIIME for principle coordinates analysis (PCoA), and are a measure of compositional (dis)similarity between different samples. PRIMER v.6 (PRIMER-E Ltd, Plymouth, UK) was used to calculate SIMilarity of PERcentages (SIMPER) and Analysis of similarity (ANOSIM) analyses. SIMPER was used to determine the contribution made by specific OTUs to the observed similarity between sample types. To identify discriminating features, SIMPER calculates the average Bray-Curtis dissimilarity between all pairs of inter-group samples. Because the Bray-Curtis dissimilarity measure incorporates the contribution of each feature (e.g. each taxa), the average dissimilarity between sites can be expressed in terms of the average contribution from each species.

Two-factors nested permutational multivariate analysis of variance (PERMANOVA) was used to determine the significance and influence of variables on the two treatment cohorts.

24.6.6 FAECAL MICROBIOTA RELATIVE ABUNDANCE OF BACTERIAL TAXA

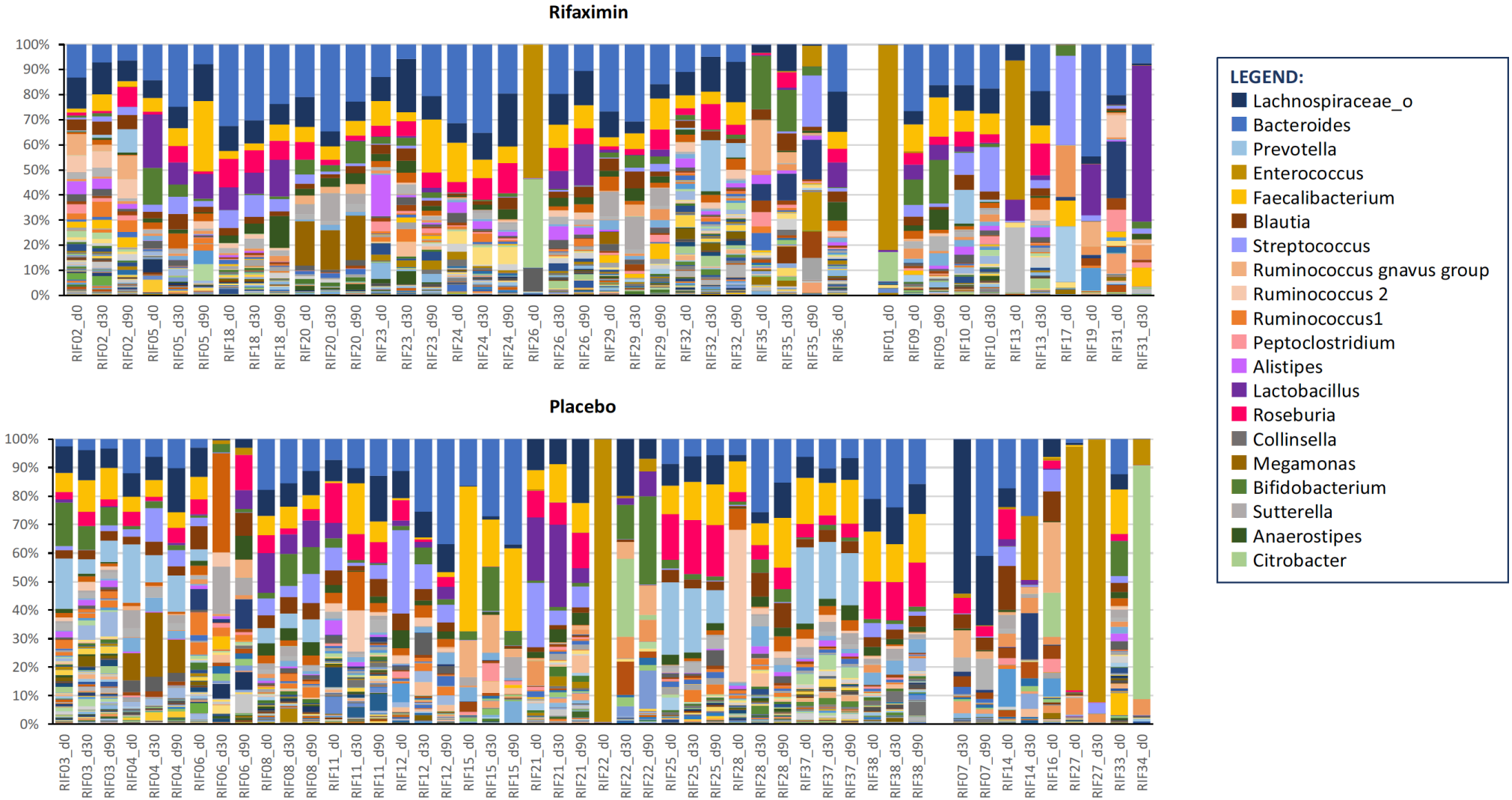


Figure 36: Faecal microbiota relative abundance of bacterial taxa

24.6.7 BASELINE CORE FAECAL MICROBIOTA IN BOTH TREATMENT COHORTS

The presence of bacterial taxa in faecal samples were based on a threshold of > 0.001 relative abundance (at least 10 sequence reads).

Bacterial taxa including *Bacteroides, Faecalibacterium, Roseburia, Blautia, Streptococcus, Bifidobacterium, Lachnoclostridium, Anaerostipes* and those from the family Lachnospiraceae were present in more than 70% of the individuals in the study.

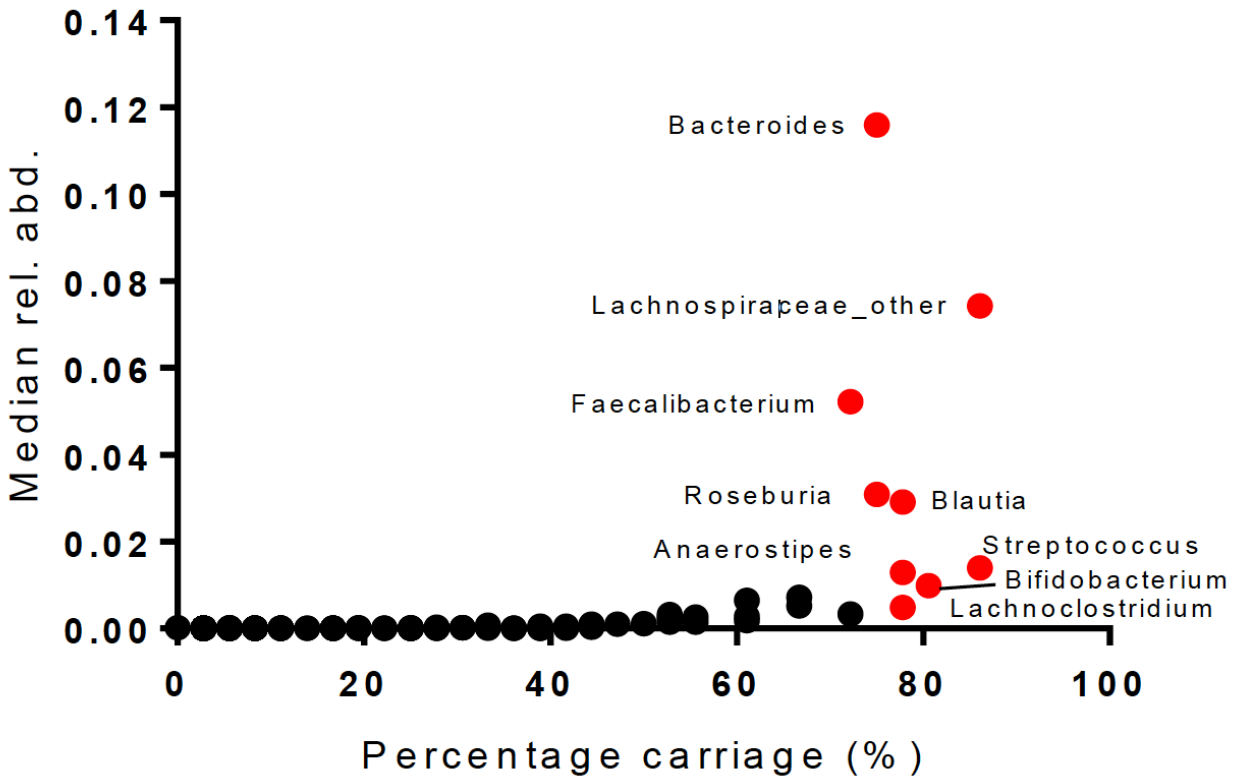


Figure 37: Overview of Core faecal microbiota

24.6.8 ALPHA DIVERSITY ANALYSIS

Alpha diversity measures for microbial richness (observed species) and microbial phylogenetic diversity (Faith's phylogenetic diversity) were performed using a paired t-test. Changes in microbial evenness (Simpson's index (1-D) was assessed using the Wilcoxon test (non-parametric, paired t-test) to assess for within group changes for the placebo or rifaximin-α treatment groups.

No significant changes in alpha diversity measures of richness and diversity was observed within the baseline and d30 or d90 time points for each group (Paired t-test, $p > 0.05$). Microbial evenness were significantly altered in the rifaximin-α group between baseline and the d90 timepoint, but not to d30 (Wilcoxon test, $p<0.006$). No significant changes were observed for these comparisons in the placebo group.

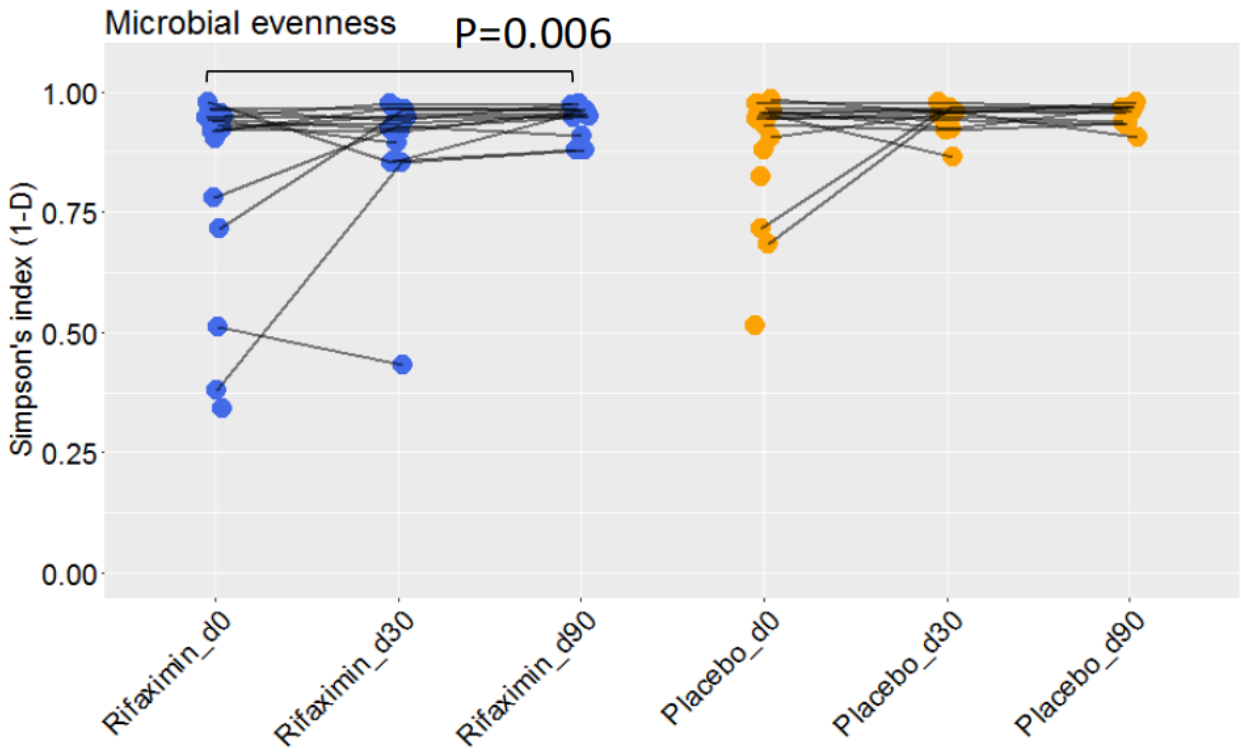


Figure 38: Alpha diversity analysis of faecal microbiota: microbial evenness

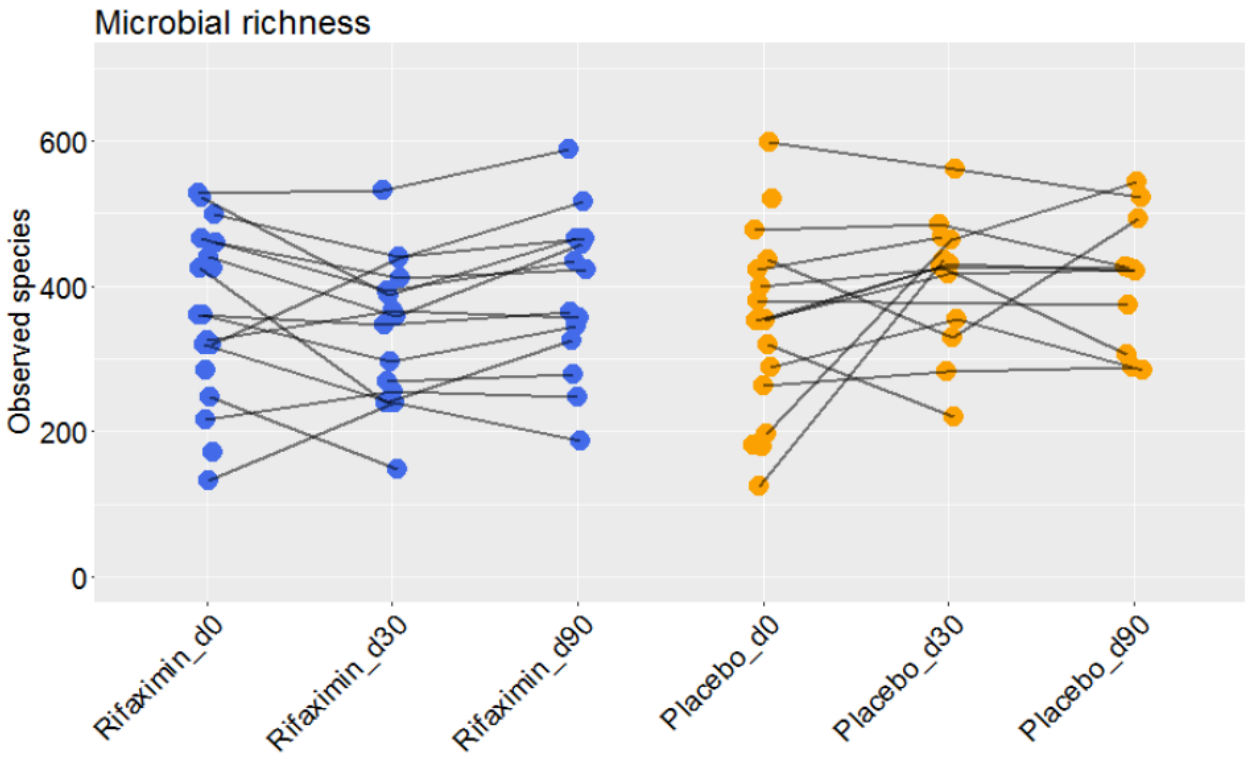


Figure 39: Alpha diversity analysis of faecal microbiota: microbial richness

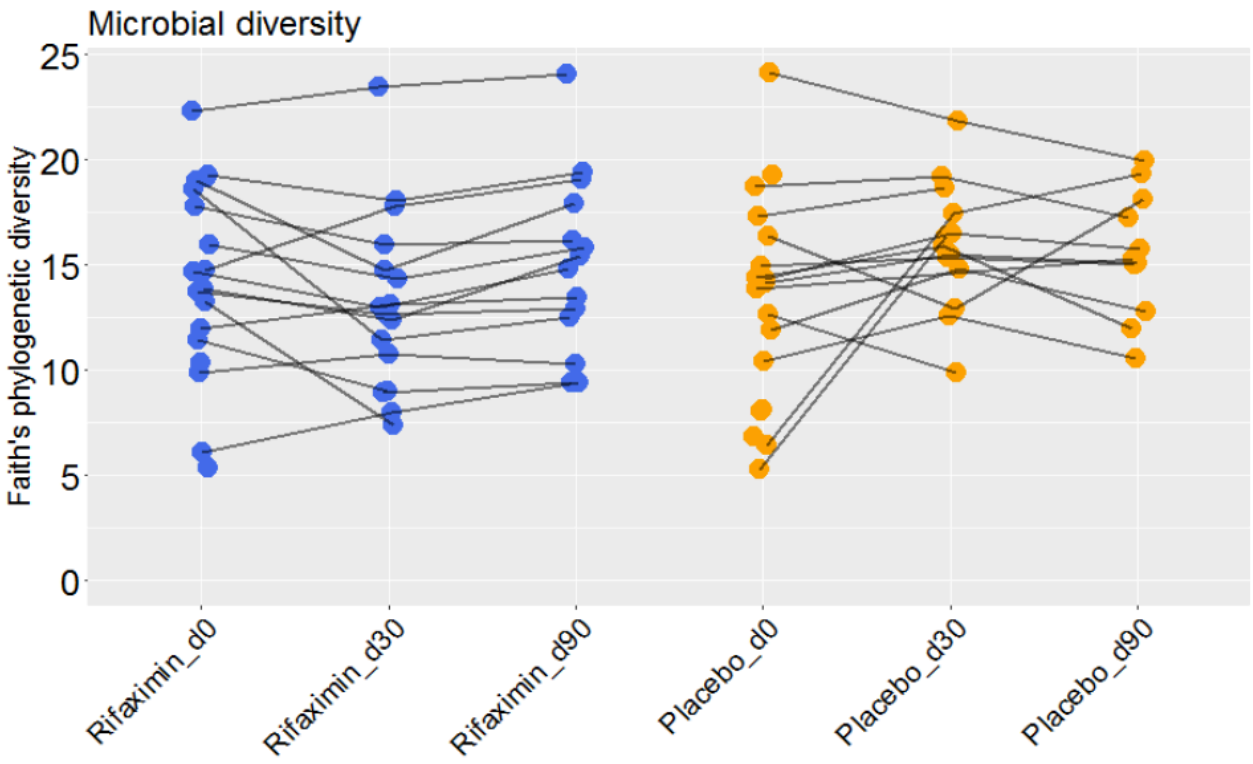


Figure 40: Alpha diversity analysis of faecal microbiota: microbial diversity

24.6.9 BETA DIVERSITY ANALYSIS

No significant compositional differences were observed between the placebo and Rif groups across all time points analysed.

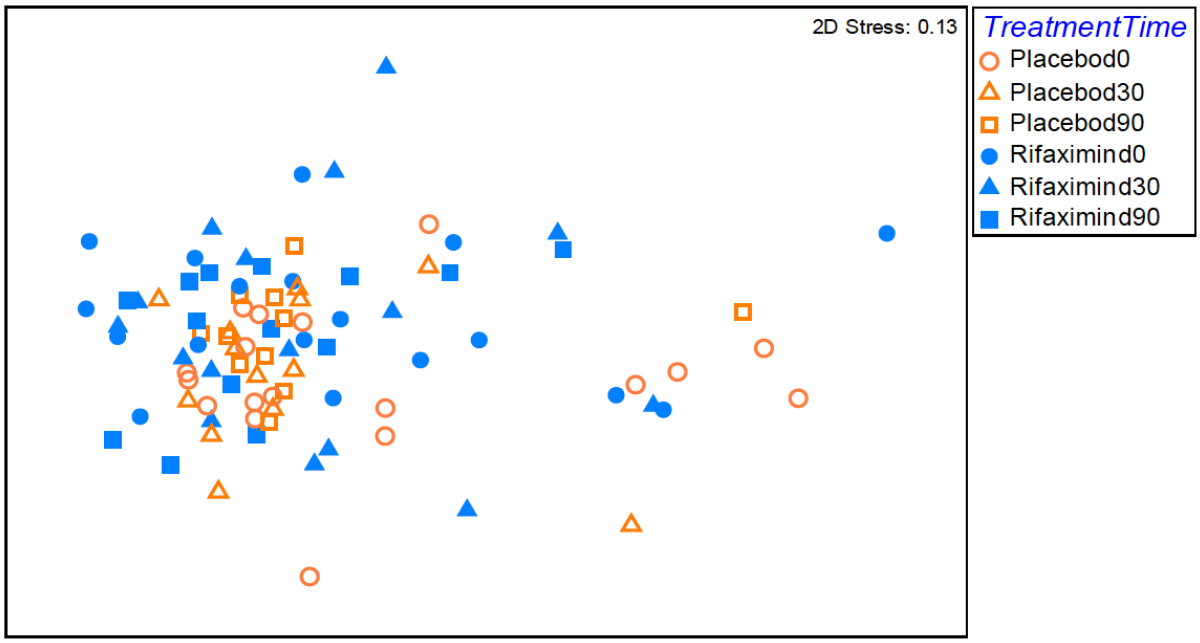


Figure 41: Non-metric multidimensional scaling (NMDS) plot identification based on treatment groups & timepoints

Table 11: Permutational multivariate analysis of variance (PERMANOVA) analysis

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Tr	1	8.4442E-2	8.4442E-2	0.97416	0.3799	9922	0.3974
Ti	2	4.5449E-2	2.2725E-2	0.90937	0.5261	9920	0.5204
Su(Tr)	35	3.6906	0.10545	4.2196	0.0001	9786	0.0001
TrxTi	2	7.5156E-2	3.7578E-2	1.5038	0.114	9921	0.1196
Res	49	1.2245	2.4989E-2				
Total	89	5.2272					

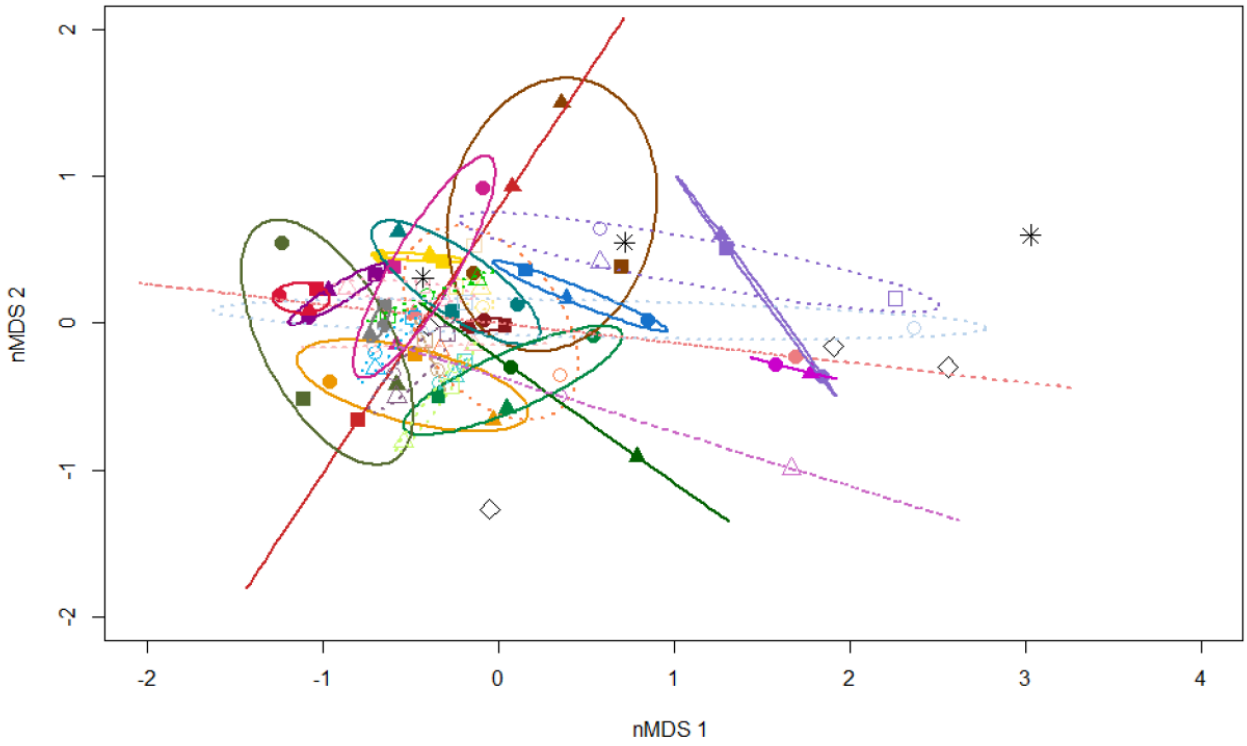
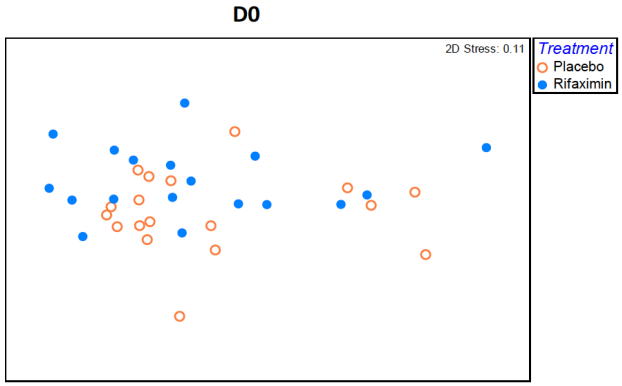


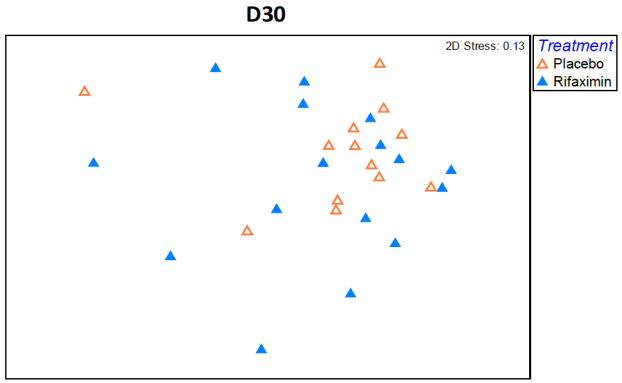
Figure 42: Non-metric multidimensional scaling (NDMS) identification based on subjects, with a 95% confidence interval ellipse across subject time-points.

24.6.9.1 Pairwise Comparison between Treatment Groups by Time Point

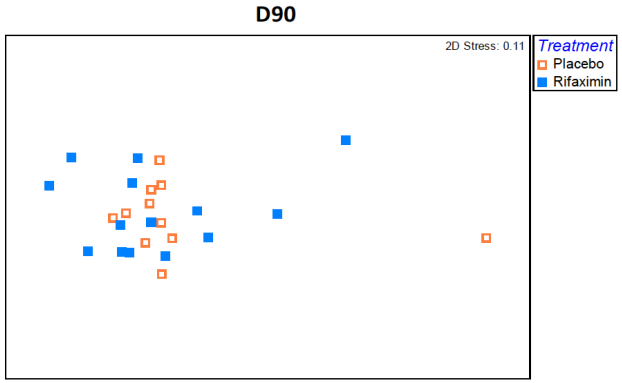
Beta diversity analysis describes the similarity between samples from one individual at different time points, or between samples from different subjects. In addition to describing the characteristics of the microbiota in an individual sample, it can be useful to compare the characteristics of multiple different samples. Inter-sample measures of similarity or dissimilarity are referred to as β-diversity and, again, can be based on many different facets of microbiota composition.



D0			
Groups	t	Unique perms	P(perm)
Rif, Placebo	1.049	9938	0.299



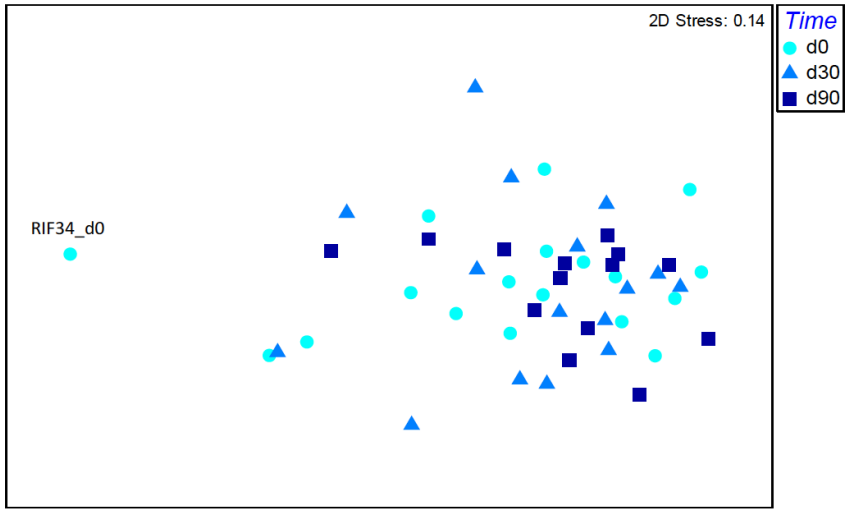
D30			
Groups	t	Unique perms	P(perm)
Rif, Placebo	1.022	9927	0.377

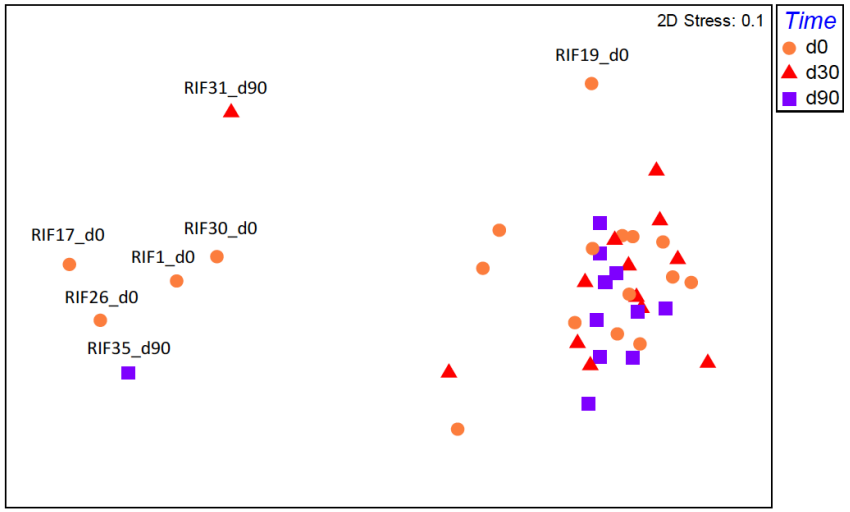


D90			
Groups	t	Unique perms	P(perm)
Rif, Placebo	0.821	9912	0.752

In this analysis of pairwise comparison between the two treatment group by each time point, there were no significant compositional differences between the rifaximin-α or placebo treatment groups at each time point analysed (PERMANOVA p> 0.05). Whilst this would be expected at baseline pre-treatment (d01), the lack of microbiota compositional difference was also measured at day 30 and day 90.

24.6.9.2 Pairwise Comparison between Time Points within Treatment Group





Rifaximin			
Groups	t	Unique perms	P(perm)
d0, d30	1.211	9931	0.173
d0, d90	1.138	9940	0.253
d30, d90	0.780	9952	0.703

Placebo			
Groups	t	Unique perms	P(perm)
d0, d30	1.060	9938	0.326
d0, d90	0.976	9946	0.410
d30, d90	1.458	9938	0.061

Similarly, when comparing compositional changes in faecal microbiota between time points within both the rifaximin-α and placebo treatment groups, no significant compositional differences were measured in beta diversity (PERMANOVA p> 0.05).

24.6.10 COMPARISON OF BACTERIAL TAXA RELATIVE ABUNDANCES

The Friedman's test was performed for non-parametric analysis of paired samples across the d01, d30 and d90 time points, for the rifaximin-α and placebo treated groups. Thirteen sets of faecal samples were available at all time points from the Rifaximin treatment group, and ten sets from the placebo treatment group. The relative abundances of 17 bacterial taxa in the rifaximin-α group and 5 bacterial taxa in the placebo group were found to significantly differ during the study time points. However, none of these changes were significant when false discovery rate correction (Benjamini-Hochberg) was applied for multiple comparison ($p > 0.05$).

Table 12: Comparison of bacterial taxa relative abundances (non-parametric analysis)

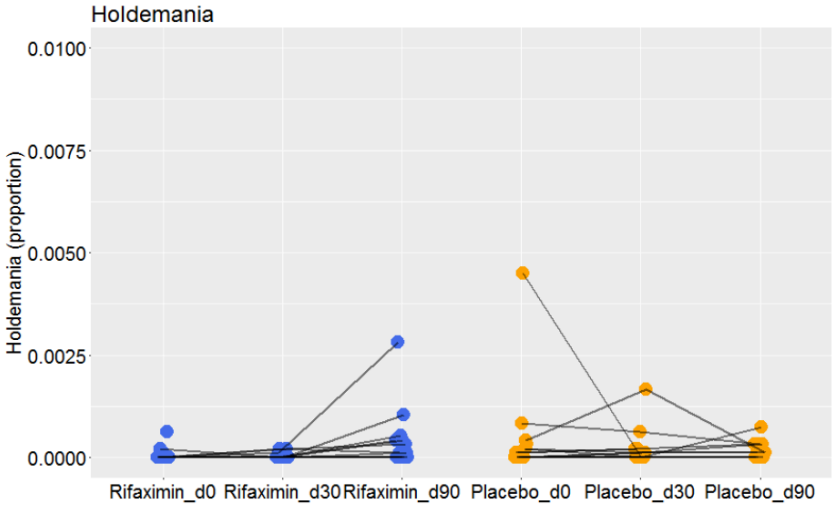
Rifaximin

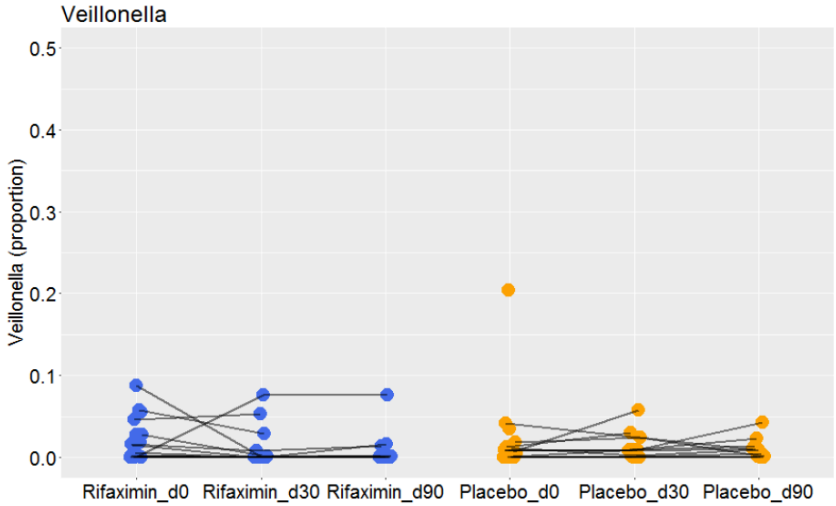
Test	df	W	p	fdr
D_0__Bacteria.D_1__Firmicutes.D_2__Erysipelotrichia.D_3__Erysipelotrichales.D_4__Erysipelotrichaceae.D_5__Holdemania	2	11.14286	0.003805	0.556172
D_0__Bacteria.D_1__Firmicutes.D_2__Negativicutes.D_3__Selenomonadales.D_4__Veillonellaceae.D_5__Veillonella	2	10.57143	0.005063	0.556172
D_0__Bacteria.D_1__Firmicutes.D_2__Erysipelotrichia.D_3__Erysipelotrichales.D_4__Erysipelotrichaceae.Other	2	9.172414	0.010191	0.556172
D_0__Bacteria.D_1__Proteobacteria.D_2__Gammaproteobacteria.D_3__Pasteurellales.D_4__Pasteurellaceae.D_5__Haemophilus	2	9.172414	0.010191	0.556172
D_0__Bacteria.D_1__Firmicutes.D_2__Erysipelotrichia.D_3__Erysipelotrichales.D_4__Erysipelotrichaceae.D_5__.Clostridium..innocuum.group	2	9.052632	0.01082	0.556172
D_0__Bacteria.D_1__Bacteroidetes.D_2__Bacteroidia.D_3__Bacteroidales.D_4__Prevotellaceae.D_5__Prevotella.9	2	8.060606	0.017769	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Lachnospiraceae.D_5__Lachnospiraceae.NC2004.group	2	7.913043	0.01913	0.573
D_0__Bacteria.D_1__Actinobacteria.D_2__Actinobacteria.D_3__Actinomycetales.D_4__Actinomycetaceae.D_5__Actinomyces	2	7.74359	0.020821	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Ruminococcaceae.D_5__Ruminococcus.2	2	7.736842	0.020891	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Lactobacillales.D_4__Lactobacillaceae.D_5__Lactobacillus	2	6.76	0.034047	0.573
D_0__Bacteria.D_1__Verrucomicrobia.D_2__Verrucomicrobiae.D_3__Verrucomicrobiales.D_4__Verrucomicrobiaceae.D_5__Akkermansia	2	6.727273	0.034609	0.573
D_0__Bacteria.D_1__Proteobacteria.D_2__Gammaproteobacteria.D_3__Enterobacteriales.D_4__Enterobacteriaceae.Ambiguous_taxa	2	6.5	0.038774	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Ruminococcaceae.D_5__Ruminiclostridium.5	2	6.408163	0.040596	0.573
D_0__Bacteria.D_1__Actinobacteria.D_2__Actinobacteria.D_3__Bifidobacteriales.D_4__Bifidobacteriaceae.D_5__Bifidobacterium	2	6.156863	0.046031	0.573
D_0__Bacteria.D_1__Bacteroidetes.D_2__Bacteroidia.D_3__Bacteroidales.Other.Other	2	6.117647	0.046943	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Ruminococcaceae.D_5__Oscillibacter	2	6.076923	0.047909	0.573
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Lachnospiraceae.D_5__Lachnospiraceae.UCG.010	2	6	0.049787	0.573

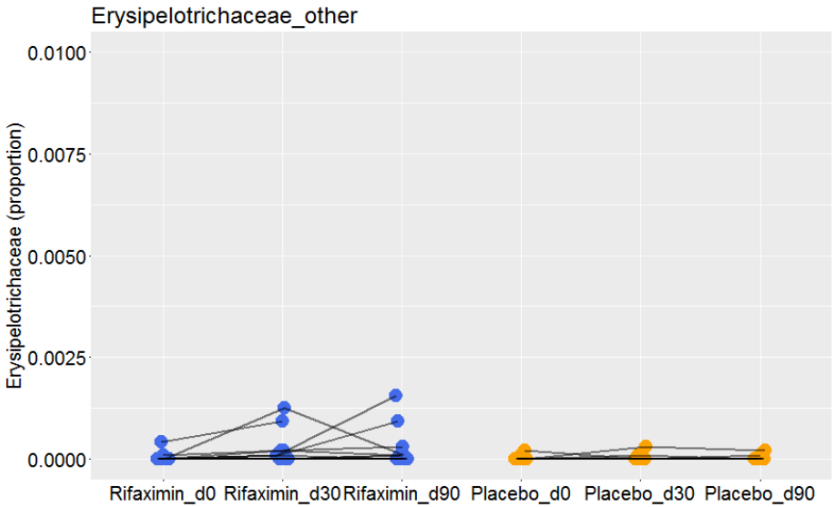
Placebo

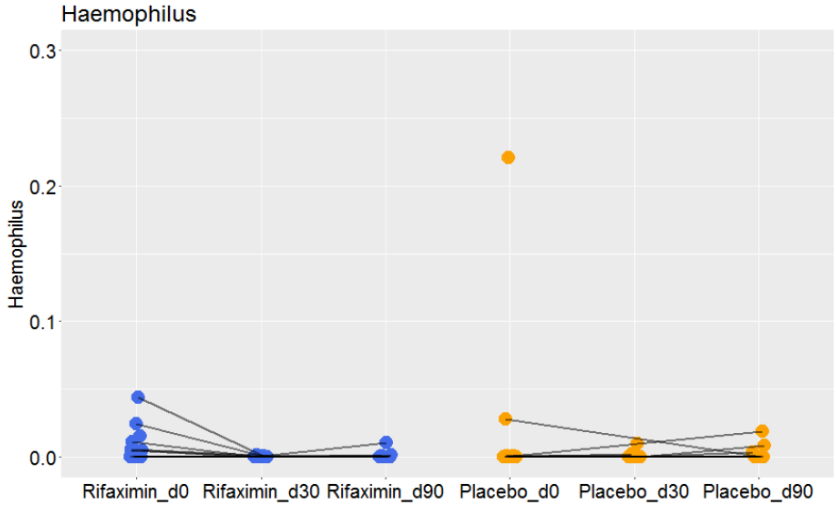
Test	df	W	p	fdr
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Lachnospiraceae.D_5__.Eubacterium..fissicatena.group	2	9.294118	0.00959	0.637834
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Lachnospiraceae.D_5__Lachnospiraceae.NK4A136.group	2	8.358974	0.015306	0.637834
D_0__Bacteria.D_1__Proteobacteria.D_2__Deltaproteobacteria.D_3__Desulfovibrionales.D_4__Desulfovibrionaceae.D_5__Bilophila	2	7	0.030197	0.637834
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Lachnospiraceae.D_5__Marvinbryantia	2	6.125	0.046771	0.637834
D_0__Bacteria.D_1__Bacteroidetes.D_2__Bacteroidia.D_3__Bacteroidales.D_4__Rikenellaceae.D_5__Alistipes	2	6	0.049787	0.637834

The relative abundance changes of the top 4 bacterial taxa that were identified from the rifaximin-α treatment group (from the table on previous page) are plotted below. Many of these taxa were low relative abundance bacterial taxa.

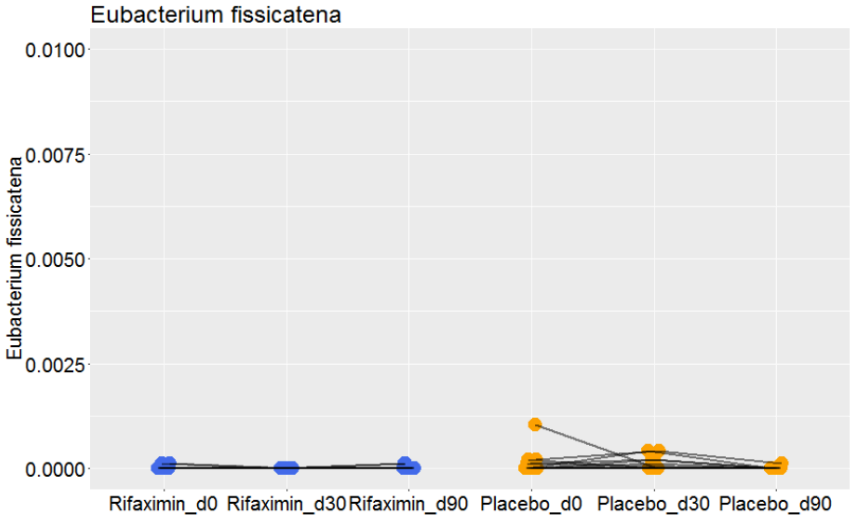


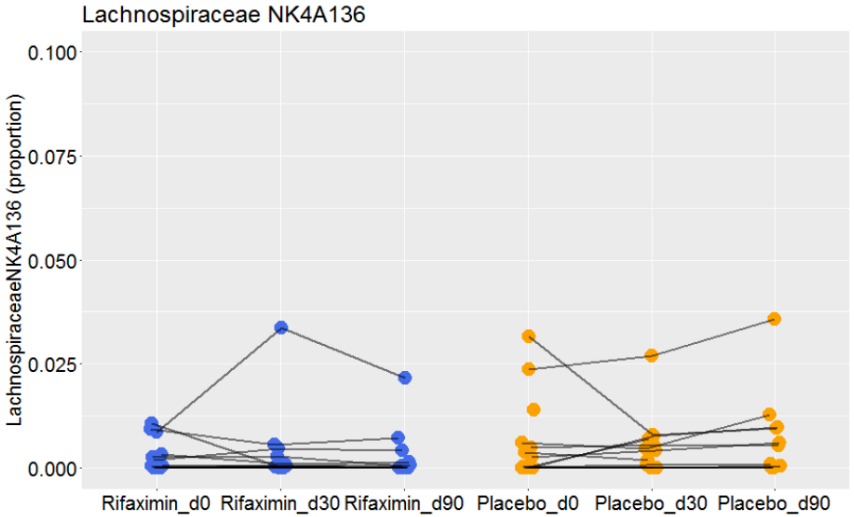


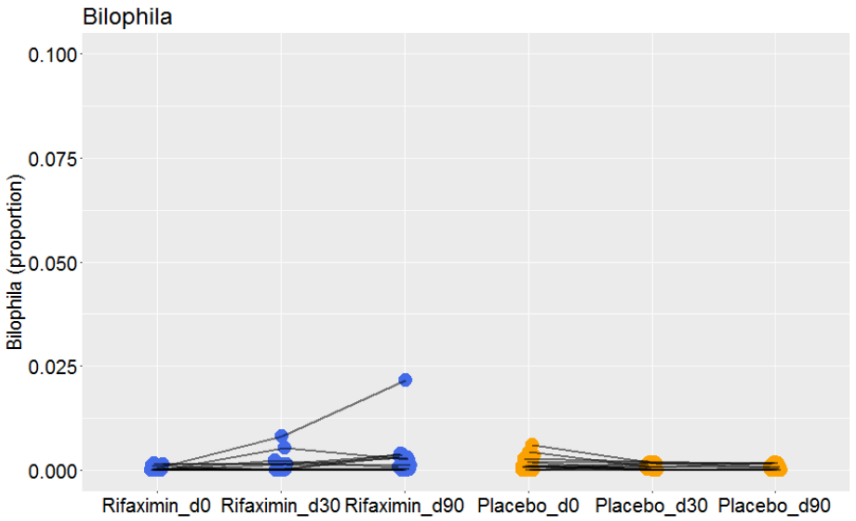




The relative abundance changes of the top 3 bacterial taxa that were identified from the placebo treatment group are plotted below. Similar to those observed for the Rifaximin group, many of the taxa identified in the placebo group were low relative abundance bacterial taxa.







24.7 Plasma bile acid profiling

Bile acids are synthesised in the liver as a breakdown product of cholesterol and are secreted into the gall bladder. The primary bile acids synthesized in the human liver are cholic acid and chenodeoxycholic acid. They are released into the small intestine where they solubilise dietary lipids such as cholesterol aiding their absorption. Bile acids are reabsorbed from the portal blood by hepatocyte extraction and re-excreted into bile, passing through the enterohepatic circulation several times before final excretion. As liver function declines due to cirrhosis for example, the efficiency of extraction of bile acids from the blood declines, concentrations within blood therefore increase. Bile acid levels also rise when bile flow is reduced or blocked (cholestasis) and additional bile acids escape into the bloodstream.

Primary bile acids are those synthesised by the liver. Secondary bile acids result from bacterial actions in the colon. In humans, taurocholic acid and glycocholic acid (derivatives of cholic acid) and taurochenodeoxycholic acid and glycochenodeoxycholic acid (derivatives of chenodeoxycholic acid) are the major bile salts in bile and are roughly equal in concentration. The conjugated salts of their 7-alpha-dehydroxylated derivatives, deoxycholic acid and lithocholic acid, are also found, with derivatives of cholic, chenodeoxycholic and deoxycholic acids accounting for over 90% of human biliary bile acids.

24.7.1 PLASMA BILE ACID FRACTIONATION

Analysis was performed according to a method published by the local laboratory [16], summarised below.

Standard preparation: A 4 mM stock solution of all bile acids was prepared in methanol and stored at 4oC. Calibration standards at 20, 10, 5, 1, 0.1 and 0.05 µM of all bile acids were prepared by serial dilution in phosphate buffered saline (PBS). The protein precipitating solution of acetonitrile was prepared containing d4-DC, d4-GDC and d4-TDC at approximately 1 µM and stored at 4oC.

Sample extraction: 250 µL of plasma sample was mixed with 800 µL of protein precipitating solution and vortex-mixed for 1 min. Samples were centrifuged at 13000 RPM for 10 min. 900 µL of the supernatant was transferred to a microtube and blown to dryness with compressed air in a 60oC heated block. The residue was dissolved in 250 µL of 50:50 methanol and water (v/v) containing 5mM ammonium acetate and 0.012% formic acid (v/v). 10 µL of the final solution was injected corresponding to 8.57 µL of original plasma.

24.7.2 LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

Separation was achieved using an Ascentis Express fused core C18 analytical column (150 x 4.6 mm, 2.7 µm, Sigma-Aldrich Co.) at 40oC. Mobile phases were A: methanol and B: water, both containing 5 mM ammonium acetate and 0.012% formic acid (v/v). A gradient mobile phase as detailed in Table 1 was delivered at 0.6 mL/min. A 100 µL wash injection of acetonitrile/iso-propylalcohol/acetone (7:2:1 v:v:v), was placed between each sample while the column re-

equilibrated to prevent carry-over. This step ran for 5 minutes with 70% Solvent A and 30% Solvent B.

Table 13: Gradient mobile phase for LC-MS/MS bile acid profiling method

Time (min)	Solvent A (%)	Solvent B (%)
0	70	**30**
10	95	**5**
14	**95**	**5**

Table X: LC gradient used. Solvent A = methanol and solvent B = deionised water, both containing 5 mM ammonium acetate and 0.012% (v/v) formic acid

Negative ion mass spectra of the eluates were recorded in MRM mode. The transitions are shown in Table 2. The operating conditions were: curtain gas, 30psi; ion spray voltage -3500 V; temperature, 750oC; nebuliser gas (zero air), 40psi; heater gas (zero air), 22psi; collision gas (nitrogen) 3mTorr. Data were acquired using AnalystTM Software version 1.4.2 (Applied Biosystems) and quantitated using peak area analysis corrected by comparison to the respective internal standard.

Table 14: Transitions used for each bile acid as part of LC-MS/MS bile acid profiling method

Bile Acid(s)	m/z transition
DC, CDC, UDC	391.2 / 391.2
CA	407.1 / 407.1
LC	375.2 / 375.2
GDC, GCDC, GUDC	448.2 / 74.1
GCA	464.2 / 74.1
GLC	432.1 / 74.1
TDC, TCDC, TUDC	498.2 / 80.0
TCA	514.0 / 80.0
TLC	482.2 / 80.0
d4-DC	395.2 / 395.2
d4-GDC	452.1 / 74.1
d4-TDC	502.2 / 80.0

As can be seen from Table X, there were no significant changes in either primary or secondary bile acid levels between the rifaximin-α and placebo treatment groups and any time point.

Table 15: Summary and analyses of plasma bile acid profiles by treatment group and time point.

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE	RM ANOVA
CHOLIC ACID					
- Rifaximin	0.14(0.04-0.29)	0.06(0.04-0.14)	0.04(0.04-0.08)	0.075	0.047;0.337
- Placebo	0.51(0.12-1.16)	0.61(0.15-0.95)	0.41(0.18-0.98)	0.878	
CHENODEOXYCHOLIC ACID					
- Rifaximin	0.60(0.18-0.98)	0.45(0.34-1.55)	0.53(0.34-1.40)	0.767	0.229;0.333
- Placebo	1.12(0.26-1.85)	1.16(0.86-1.51)	1.08(0.57-2.38)	0.926	
DEOXYCHOLIC ACID					
- Rifaximin	0.15(0.04-0.81)	0.04(0.04-0.32)	0.12(0.04-0.69)	0.468	0.949; 0.261
- Placebo	0.22(0.06-0.39)	0.47(0.11-0.57)	0.28(0.10-0.41)	0.003	
GLYCODEOXYCHOLIC ACID					
- Rifaximin	0.04(0.04-3.82)	0.14(0.04-3.88)	1.09(0.04-2.14)	0.731	0.646; 0.927
- Placebo	2.18(0.12-4.43)	1.43(0.81-4.61)	1.75(0.59-2.80)	0.824	
TAURODEOXYCHOLIC ACID					
- Rifaximin	0.15(0.40-1.10)	0.30(0.03-1.67)	0.51(0.03-1.36)	0.875	0.816;0.982
- Placebo	0.83(0.11-1.76)	0.82(0.24-1.81)	0.67(0.27-1.11)	0.775	
GLYCOCHENODEOXYCHOLIC ACID	21(13.1-29.7)	24.3(16-33.8)	23.6(13-32.3)	0.888	0.999;0.501
- Rifaximin	23(19.7-28.3)	25.3(18.4-30.2)	26.5(12.3-28.4)	0.578	
- Placebo					
TAUROCHENODEOXYCHOLIC ACID	18.5(2.5-29.2)	18.1(8.01-23.2)	18.2(7.3-30.9)	0.073	0.089;0.729
- Rifaximin	9.3(7.4-12.3)	10.6(6.7-14.5)	9.9(5.0-15.8)	0.273	
- Placebo					
URSODEOXYCHOLIC ACID					
- Rifaximin	0.035(0.035-0.135)	0.035(0.035-0.35)	0.035(0.035-0.47)	0.195	0.987;0.013
- Placebo	0.035(0.035-0.303)	0.183(0.035-0.498)	0.120(0.035-0.328)	0.200	
GLYCOURSODEOXYCHOLIC ACID					
- Rifaximin	0.6(0.3-2.1)	0.4(0.2-1.2)	1.0(0.1-2.2)	0.623	0.366;0.380
- Placebo	1.8(1.0-2.3)	1.6(0.95-3.4)	2.1(0.3-2.9)	0.578	
TAUROURSODEOXYCHOLIC ACID					
- Rifaximin	0.11(0.03-0.29)	0.10(0.03-0.51)	0.17(0.02-0.64)	0.274	0.400;0.109
- Placebo	0.23(0.16-0.43)	0.31(0.16-0.43)	0.23(0.12-0.44)	0.505	
GLYCOCHOLIC ACID					
- Rifaximin	9.33(4.3-13.4)	9.77(3.8-13.9)	11(3.4-13.4)	0.888	0.329;0.654
- Placebo	11.5(7.6-15.8)	10.1(8.2-13.4)	9.5(5.0-15.7)	0.353	
TAUROCHOLIC ACID					
- Rifaximin	5.1(1.5-13.0)	4.3(2.9-11.2)	8.2(3.1-12.1)	0.137	0.230;0.737
- Placebo	5.3(3.7-6.8)	4.4(2.9-7.7)	3.2(2.1-6.6)	0.353	
LITHOCHOLIC ACID					
- Rifaximin	0.08(0.08-0.19)	0.08(0.08-0.08)	0.08(0.08-0.08)	0.812	0.782;0.665
- Placebo	0.08(0.08-0.14)	0.08(0.08-0.13)	0.08(0.08-0.08)	0.646	
GLYCOLITHOCHOLIC ACID					
- Rifaximin	0.08(0.08-0.62)	0.08(0.08-0.32)	0.08(0.08-0.55)	0.322	0.365;0.936
- Placebo	0.14(0.08-0.32)	0.18(0.08-0.27)	0.18(0.08-0.26)	0.700	
TAUROLITHOCHOLIC ACID					
- Rifaximin	0.08(0.08-0.08)	0.08(0.08-0.19)	0.08(0.08-0.33)	0.254	0.401; 0.941
- Placebo	0.08(0.08-0.18)	0.08(0.08-0.14)	0.08(0.08-0.13)	0.505	

24.8 Faecal biomarkers: calprotectin

Faecal calprotectin was measured using a commercially available enzyme-linked immunosorbent assay (Bühlmann Laboratories AG, Schönenbuch, Switzerland) that measures quantitative calprotectin. To aliquots of 50-100 mg faeces was added extraction buffer at a weight/volume ratio of 1:50 and homogenized by vortexing for 30 minutes. 2 mL of the homogenate was then centrifuged in a microcentrifuge for 5 min at 3000 g. Following centrifugation, the calprotectin ELISA was performed using an automated Triturus platform. The ELISA plate is coated with a monoclonal capture antibody highly specific to the calprotectin heterodimeric and polymeric complexes. After incubation, washing, a second incubation with a specific detection antibody, and a further washing step, tetramethylbenzidine (blue color formation) followed by a stop solution (change to yellow color) are added by the Triturus. The absorption was determined at an optical density of 450 nm. The linear range of the test was 10-600µg calprotectin/g faeces with concentration-dependent intra- and inter-assay coefficients of between 2-5% and 4-8%, respectively. The calprotectin cut-off level representing a positive value was equal or greater than 60 µg/g.

As can be seen in Table 17, there was no significant difference in faecal calprotectin levels between the with rifaximin-α or placebo treated groups at any of the time points compared. There did however appear to be some perturbation of faecal calprotectin levels in the placebo group longitudinally which was significant when comparing the baseline, day 30 and day 90 values. This is also visualised in Figure 43.

Table 16: Faecal calprotectin levels by treatment group and follow up intervals

FAECAL CALPROTECTIN	PLACEBO	RIFAXIMIN	P-VALUE
	116 [48-211]	105[55-155]	0.939

VARIABLE	BASELINE	DAY 30	DAY 90	P-VALUE	RM ANOVA
- Rifaximin	105 (55-155)	44(14-129)	71(14-166)	0.176	0.580; 0.658
- Placebo	116(48-211)	40(16-81)	122(24-149)	**0.032**	

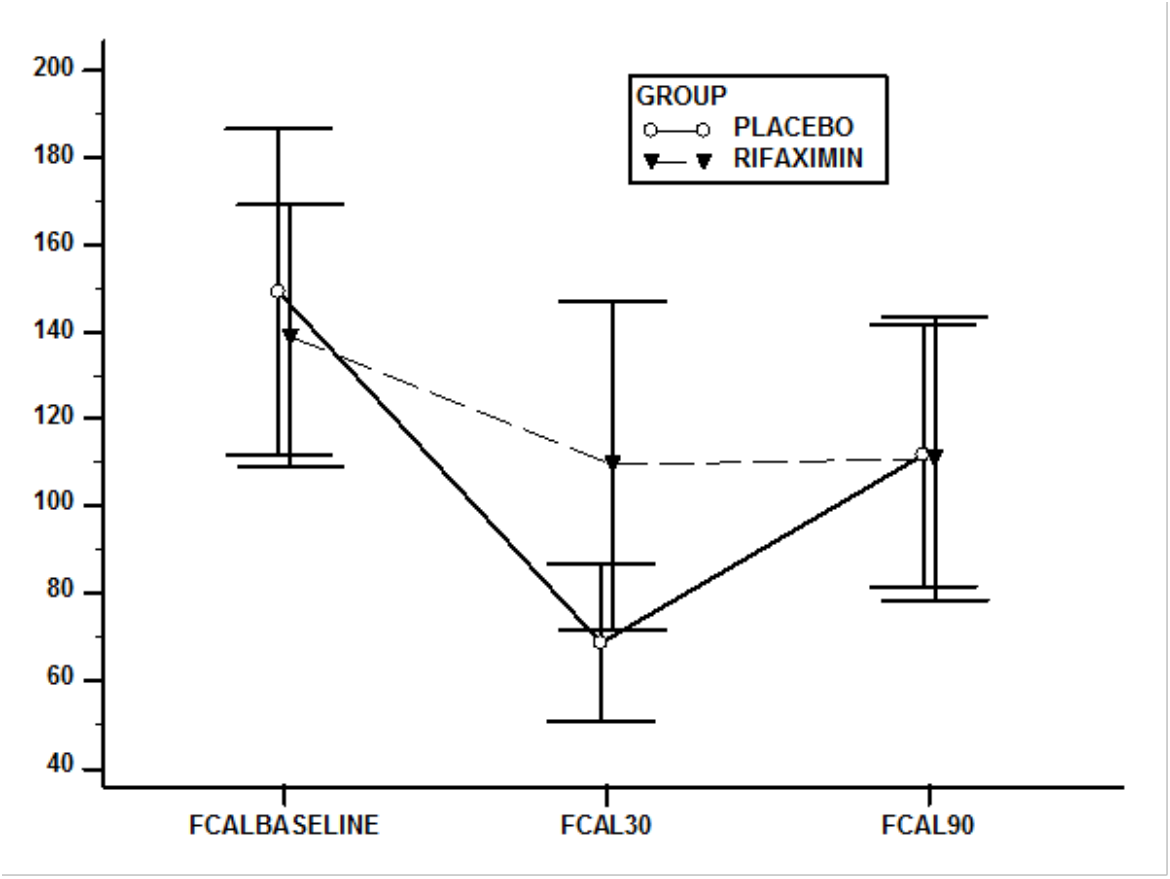


Figure 43: Faecal calprotectin levels comparing patients treated with rifaximin-α or placebo demonstrating no discernible difference in profiles at baseline and follow up

24.9 Metabonomic analysis - plasma, urine and faeces

Plasma, urine and faecal water were prepared for 1H NMR spectroscopy by standard method [17]. Post-acquisition spectra were phased, baseline corrected, log transformed and aligned using in house MATLAB scripts at Imperial College London and imported into SIMCA for multivariate analysis. The results are summarised on the following three pages.

The interpretation of these data is that there is no discernible difference in urinary, plasma or faecal water profile seen throughout the study, based on treatment interventions and time-points. In the urinary analysis the PLSDA model was invalid but with positive Q2. This is more likely to indicate under-powering as the CV ANOVA of 0.11 is lower than that for the plasma and faecal water analysis.

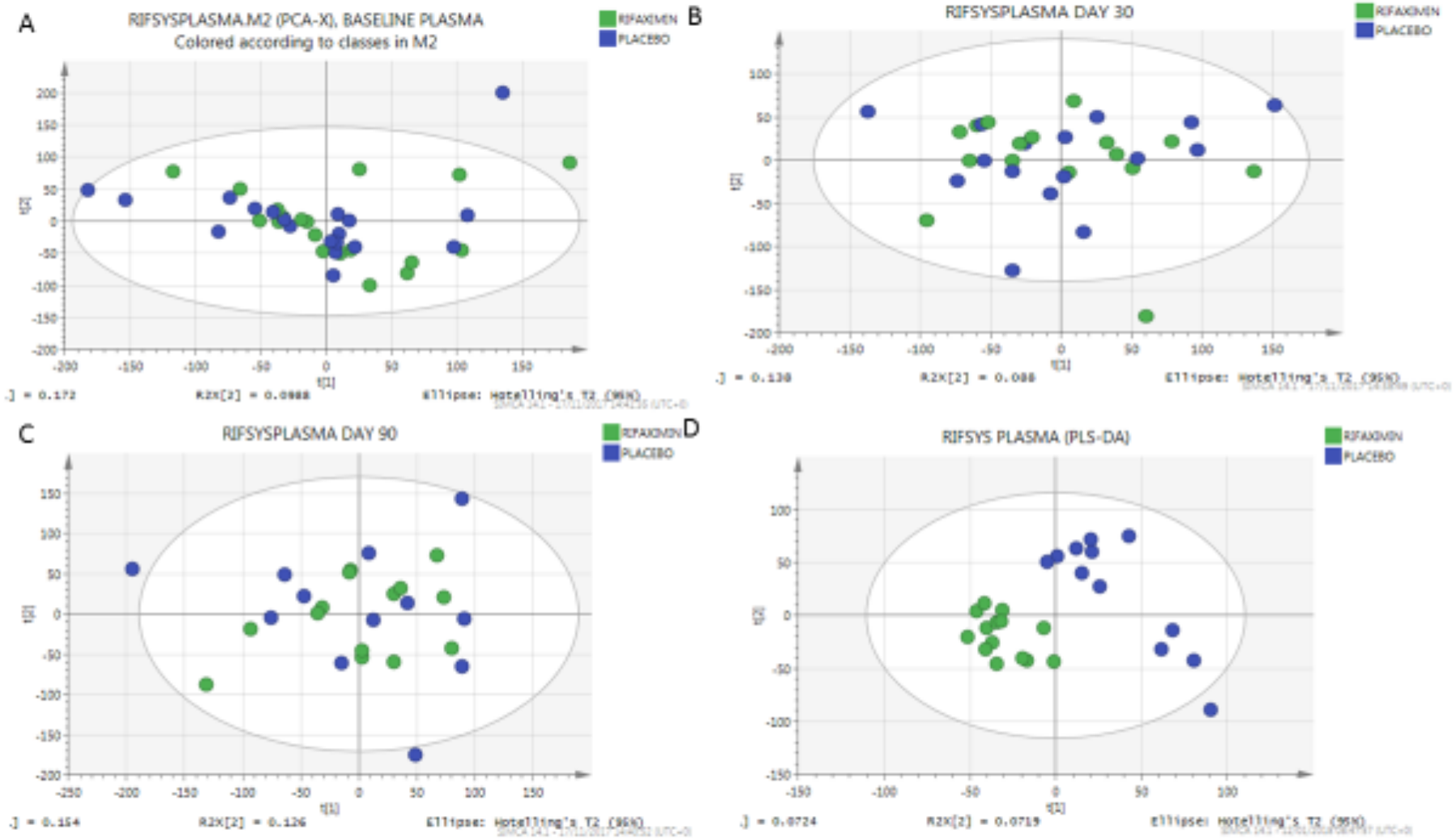


Figure 44: Plasma 1H NMR multivariate analysis comparing patients treated with rifaximin-α or placebo demonstrating no discernible difference in profiles at baseline and follow up A) Principal Components analysis of 1H NMR plasma profiles at baseline (R2 0.27 Q2 0.1 at B) day 30 R2 0.26 Q2 0.01 and C) day 90 R2 0.28 Q2 0.01. A PLSDA model was produced at day 90 but this was invalid (R2 0.14 Q2 0.11 CV ANOVA p=1).

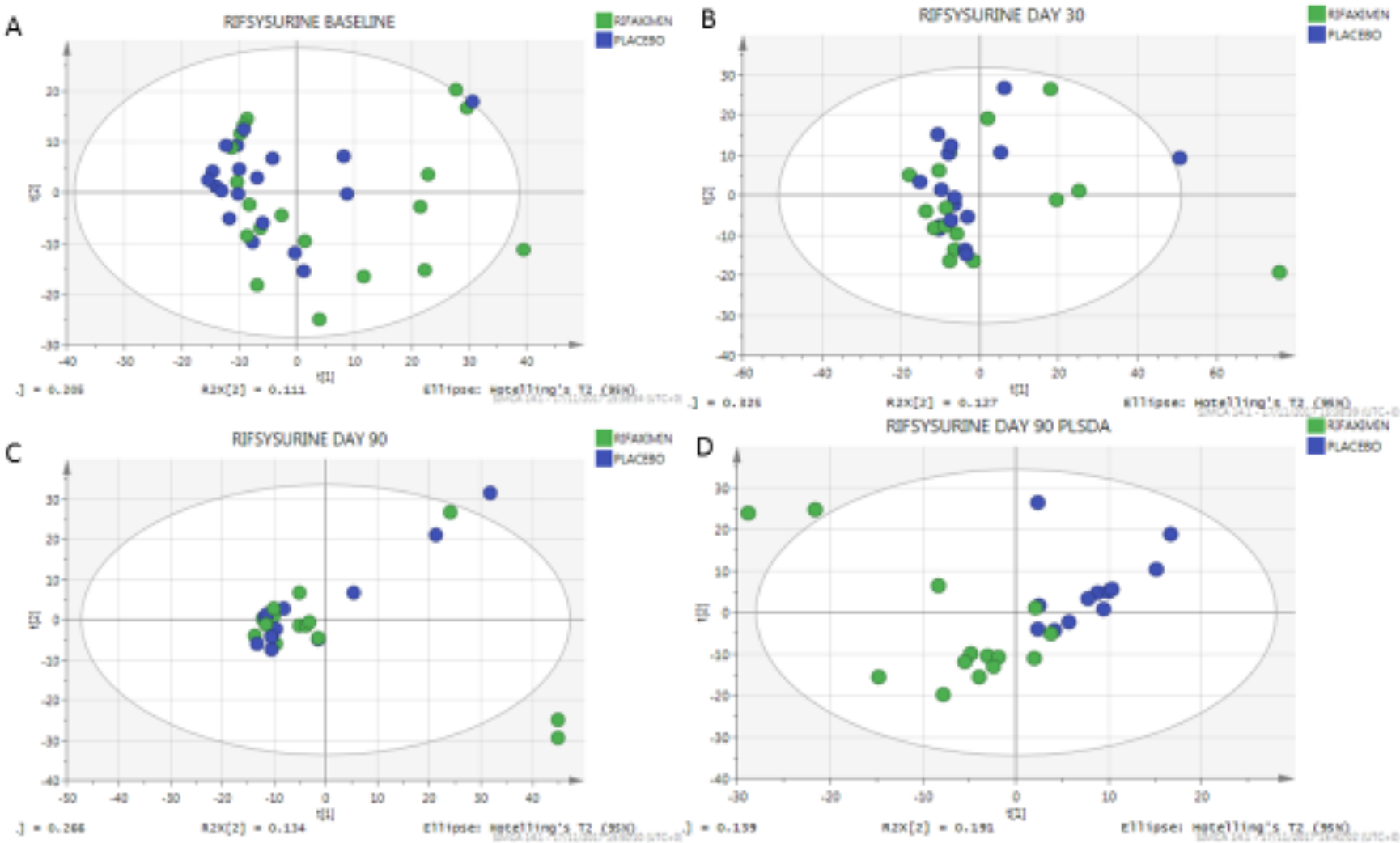


Figure 45: Urinary 1H NMR multivariate analysis comparing patients treated with rifaximin-α or placebo demonstrating no discernible difference in profiles at baseline and follow up A) Principal Components analysis of 1H NMR urinary profiles at baseline (R2 0.31 Q2 0.17 at B) day 30 R2 0.45 Q2 0.21 and C) day 90 R2 0.43 Q2 0.19. A PLSDA model was produced at day 90 but this was invalid (R2 0.33 Q2 0.26 CV ANOVA p=0.11) indicating underpowering.

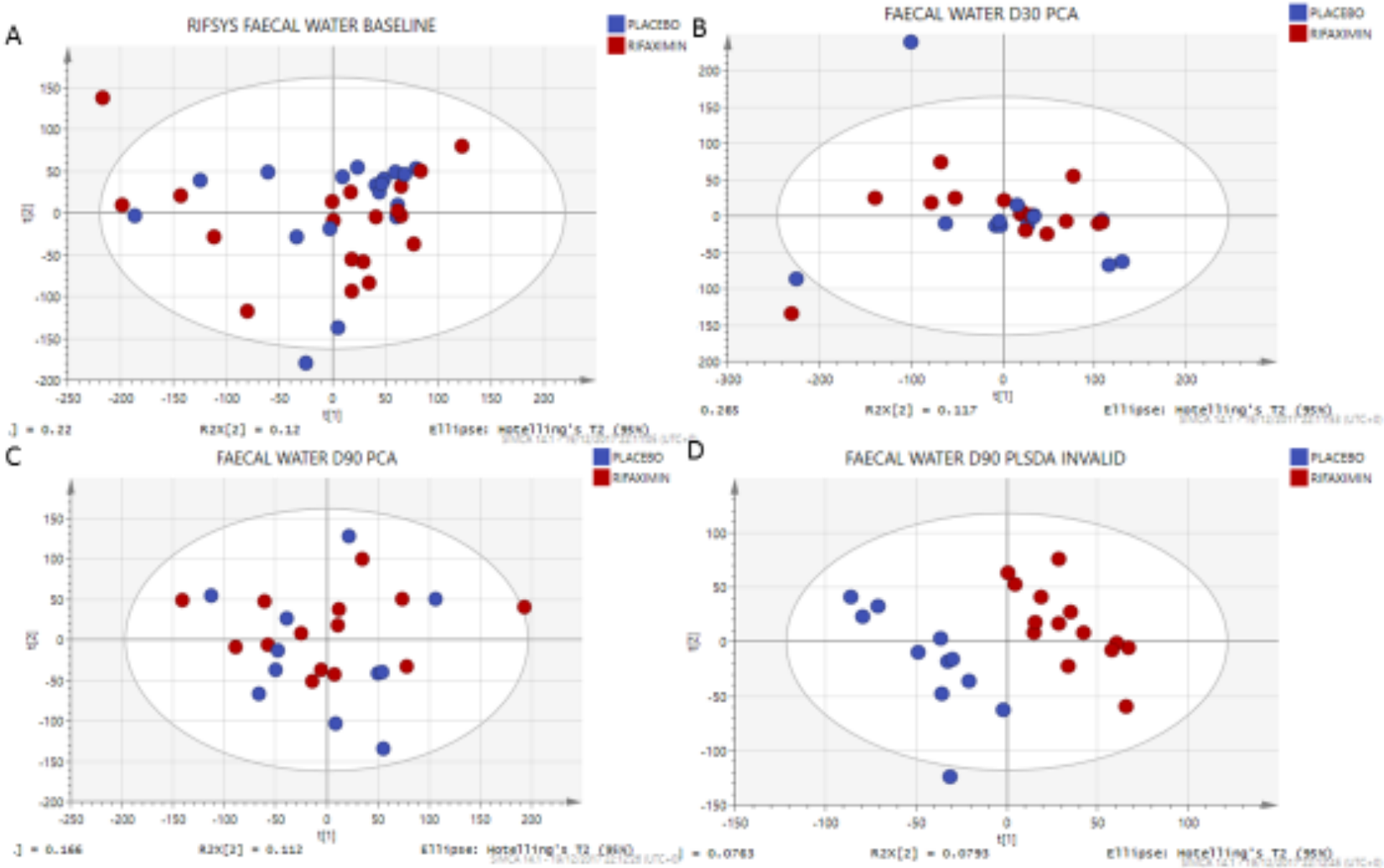


Figure 46: Faecal water 1H NMR multivariate analysis comparing patients treated with rifaximin-α or placebo demonstrating no discernible difference in profiles at baseline and follow up A) Principal Components analysis of 1H NMR faecal water profiles at baseline (R2 0.41 Q2 0.22 at B) day 30 R2 0.49 Q2 0.19 and C) day 90 R2 0.34 Q2 0.39. A PLSDA model was produced at day 90 but this was invalid (R2 0.21 Q2 -0.02).

25 ADVERSE EVENTS

A summary of the adverse events recorded by treatment group is provided in Table X with a . As can be seen, the total number of adverse events (AEs) recorded was almost twice that in the placebo treated group (33) when compared to the rifaximin-α group (17). The number of moderate to severe AEs was also much more frequent in the placebo treated group (23) when compared to the rifaximin-α group (5). The number of AEs that did not resolve was much more frequent in the placebo treated group (20) when compared to the rifaximin-α group (9). The number of infection related AEs was also much more frequent in the placebo treated group (9) when compared to the rifaximin-α group (3). Of note, none of the rifaximin-α group experienced hepatic encephalopathy as a recorded AE whereas there were four episodes of HE recorded as an AE in the placebo treated group.

None of the AEs were assessed as being related to the trial medication by the trials team during the double blinded trial phase.

Table 17: Summary of all adverse and serious adverse events

	Rifaximin-α	Placebo
Total number of AEs recorded	**17**	**33**
AE intensity: mild	12	10
AE intensity: moderate	3	22
AE intensity: severe	2	1
Number of AEs related to study drug	0	0
Number of AE's resolved	8	13
Number of AE's not resolved	9	20
AE episodes infection related	3	9
AE episodes HE	0	4
SAEs	**1**	**0**

Table 18: Summary of all adverse and serious adverse events by body system category

Category	*Adverse event*	Rifaximin-α	Placebo
Eye Disorder	Blurred vision	0	0
Cardiovascular	Chest pain	0	0
	Palpitations	0	0
	Peripheral oedema	**2**	0
Respiratory	Dyspnoea	**1**	**1**
	Cough	0	0
	Pneumonia	0	**1**
Hepatic	Ascites	**9**	**17**
	Spontaneous bacterial peritonitis	0	**1**
	Jaundice	0	0
	Variceal haemorrhage	**1**	**2**
	Hepatocellular encephalopathy	0	**6**
	Hepatocellular carcinoma	0	**1**
Gastrointestinal	Abdominal pain	0	0
	Nausea +/- vomiting	0	0
	Diarrhoea	0	0
	Constipation	0	0
	Bowel perforation	**1***	0
Renal	Acute kidney injury	**1**	**2**
Genito-urinary	Urinary tract infection	0	**1**
Endocrine		0	0
Haematological	Anaemia	0	0
Musculo-skeletal	Back pain	0	0
	Arthralgia	0	0
	Muscle spasms	0	0
Neurological	Dizziness	0	0
	Headache	0	0
Psychological	Insomnia	0	0
	Depression	0	0
Immunological	Pyrexia	0	0
Infection	Sepsis	**1**	**1**
Dermatological	Pruritus	0	0
	Rash	0	0
	Cellulitis	**1**	0
Allergies		0	0
Ears, Nose & Throat		0	0
Neoplasia		0	0
Other	Other	0	0
TOTAL		**17**	**33**

* deemed to be a Serious Adverse Event

26 SERIOUS ADVERSE EVENTS

Only one serious adverse event was recorded and this was an episode of small bowel perforation in on participant in the rifaximin-α treated group. This was assessed clinically as a spontaneous event and not assessed as related to the IMP. This SAE was reported immediately by the Investigator to the KHP-CTO and the Investigator for Medical Review in accordance with current Pharmacovigilance Policy.

27 OTHER INFORMATION

27.1 Registration

The study was registered with ClinicalTrials.gov and on the European Union Clinical Trials Register as part of the ethics application and approval process:

- ClinicalTrials.gov reference number: **NCT 02019784**,
- European Union Clinical Trials Register EudraCT Number: **2013-004708-20**.

27.2 Protocol

The full trial protocol can be accessed by written request to the Chief Investigator.

27.3 Funding

Norgine UK Ltd.

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