Clinical, experimental and pathophysiological effects of Yaq-001: a non-absorbab le, gut-restricted adsorbent in models and patients with cirrhosis

**Clinical, Experimental and Pathophysiological effects of Yaq-001, a Non-absorbable, Gut-restricted Adsorbent in Models and Patients with Cirrhosis**

## Authorships and Affiliations


| Author | Affiliations | email | Conflict of interest |
|---|---|---|---|
| Jinxia Liu[#] | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF.<br><br>Department of Gastroenterology, Affiliated Hospital of Nantong University, Nantong, 226001, China. | liujinxia@ntu.edu.cn | |
| Jane Macnaughtan[#] | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | j.macnaughtan@ucl.ac.uk | |
| Annarein JC Kerbert | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | J.C.Kerbert@lumc.nl | |
| Javier Martinez | Hospital Ramón y Cajal, IRYCIS, CIBEREHD, Universidad de Alcalá, Madrid, Spain<br>Liver Unit, Hospital Vall d'Hebron, Universitat Autónoma, CIBERehd, Barcelona, Spain | martinez.gonzalez.javier@gmail.com | |
| Yi Jin | Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, SE1 9RT, UK. | yi.1.jin@kcl.ac.uk | |
| Frederick Clasen | Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, SE1 9RT, UK. | frederick.clasen@crick.ac.uk | |
| Abeba Habtesion | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | a.habtesion@ucl.ac.uk | |
| Huoyan Jin | Department of Gastroenterology, Affiliated Hospital of Nantong University, Nantong, 226001, China. | jihuoyan@163.com | |
| Qin Jin | Department of Gastroenterology, Affiliated Hospital of Nantong University, Nantong, 226001, China. | jinqin@sina.com | |

| | | | |
|---|---|---|---|
| Alexandra Phillips | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | alexandra.phillips@ucl.ac.uk | |
| Francesco De Chiara | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | fdechiara@ibecbarcelona.eu | |
| Ganesh Ingavle | Centre for Regenerative Medicine and Devices, School of Applied Sciences, University of Brighton, Brighton, East Sussex, BN2 4GJ, UK.<br><br>Symbiosis Centre for Stem Cell Research (SCSCR), Symbiosis School of Biological Sciences (SSBS), Symbiosis International (Deemed University), Pune 412115, India. | ganesh.ingavle@ssbs.edu.in | |
| Cesar Jimenez | Liver Unit, Hospital Vall d'Hebron, Universitat Autónoma, CIBERehd, Barcelona, Spain | cesar.jimenez@vhebron.net | |
| Giacomo Zaccherini | University of Bologna, Italy | g.zaccherini@gmail.com | |
| Katherine Husi | Gastroenterology, University Hospital Bern, CH | Kathrin.Husi@insel.ch | |
| Miguel Rodriguez-Gandia | Hospital Ramón y Cajal, IRYCIS, CIBEREHD, Universidad de Alcalá, Madrid, Spain | maldimed@gmail.com | |
| Paul Cordero-Sanchez | Symbiosis Centre for Stem Cell Research (SCSCR), Symbiosis School of Biological Sciences (SSBS), Symbiosis International (Deemed University), Pune 412115, India. | paul.sanchez@ucl.ac.uk | |
| Junpei Soeda | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | Deceased | |
| Lynda McConaghy | Yaqrit | lynda.mcconaghy@yaqrit.com | |
| Jude A Oben | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | jude.1.oben@kcl.ac.uk | |
| Karen Church | Yaqrit | karen.church@yaqrit.com | |

| | | | |
|---|---|---|---|
| Jia Li | [6]Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, Imperial College Road, South Kensington, London, SW7 2AZ. | jia.li@imperial.ac.uk | |
| Haifeng Wu | Department of Gastroenterology, Affiliated Hospital of Nantong University, Nantong, 226001, China. | wuhaifeng1986@163.com | |
| Aarti Jalan | Kings College Hospital, 125 Coldharbour Lane, London SE5 9NU, UK London | aartijalan1@gmail.com | |
| Pere Gines | Liver Unit, Hospital Clinic of Barcelona, IDIBAPS, Faculty of Medicine and Health sciences, University of Barcelona. CIBEReHD | PGINES@clinic.ub.es | |
| Elsa Sola | Liver Unit, Hospital Clinic of Barcelona, IDIBAPS, Faculty of Medicine and Health sciences, University of Barcelona. CIBEReHD | ESOLA@clinic.ub.es | |
| Simon Eaton | ICH-UCL | s.eaton@ucl.ac.uk | |
| Carrie Morgan | Yaqrit | carrie.morgan@yaqrit.com | |
| Michal Kowalski | Yaqrit | michal.kowalski@yaqrit.com | |
| Daniel Green | Yaqrit | daniel.green@yaqrit.com | |
| Amir Gander | Tissue Access for Patient Benefit: ROYAL FREE HOSPITAL | a.gander@ucl.ac.uk | |
| Lindsey Ann Edwards | Institute of Liver Studies, School of Immunology and Microbial Sciences, Faculty of Life Sciences and Medicine, King's College London, 125 Coldharbour Lane, London SE5 9NU, UK | lindsey.edwards@kcl.ac.uk | |
| I. Jane Cox | The Roger Williams Institute of Hepatology, Foundation for Liver Research, 111 Coldharbour Lane, London SE5 9NT | j.cox@researchinliver.org.uk | |
| Helena Cortez-Pinto | Clínica Universitária de Gastrenterologia, Laboratório de Nutrição, Faculdade de Medicina, Universidade de Lisboa, Portugal | hlcortezpinto@gmail.com | |
| Reiner Wiest | Gastroenterology, University Hospital Bern, CH | reiner.wiest@insel.ch | |
| Francois Durand | Hepatology, Hospital Beaujon, Clichy, France | francois.durand@aphp.fr | |
| Paolo Caraceni | University of Bologna, Italy | paolo.caraceni@unibo.it | |
| Roberto Elosua | Datarus, Barcelona | relosua@dararus.eu | |
| Joan Vila | Datarus, Barcelona | jvila@datarus.eu | |

| | | | |
|---|---|---|---|
| Marco Pavesi | European Foundation for the Study of Chronic Liver Failure (EF Clif), Barcelona | mpavesi@gmail.com | |
| Vicente Arroyo | European Foundation for the Study of Chronic Liver Failure (EF Clif), Barcelona | vicente.arroyo@efclif.com | |
| Nathan Davies | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | nathan.davies@ucl.ac.uk | |
| Rajeshwar P Mookerjee | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | r.mookerjee@ucl.ac.uk | |
| Victor Vargas | Liver Unit, Hospital Vall d'Hebron, Universitat Autónoma, CIBERehd, Barcelona, Spain | vvargas@vhebron.net | |
| Susan Sandeman | Centre for Regenerative Medicine and Devices, School of Applied Sciences, University of Brighton, Brighton, East Sussex, BN2 4GJ, UK. | s.sandeman@brighton.ac.uk | |
| Gautam Mehta | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | gautam.mehta@ucl.ac.uk | |
| Saeed Shoaie | Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, SE1 9RT, UK. | saeed.shoaie@kcl.ac.uk | |
| Julian R. Marchesi | Division of Digestive Diseases, Department of Metabolism, Digestion and Reproduction, St Mary's Hospital, Imperial College London, South Wharf Road, London, W2 1NY | j.marchesi@imperial.ac.uk | |
| Agustin Albillos | Hospital Ramón y Cajal, IRYCIS, CIBEREHD, Universidad de Alcalá, Madrid, Spain | agustin.albillos@uah.es | |
| Fausto Andreola | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. | f.andreola@ucl.ac.uk | |
| Rajiv Jalan | Liver Failure Group, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF.<br><br>European Foundation for the Study of Chronic Liver Failure (EF Clif), Barcelona | r.jalan@ucl.ac.uk | |

[#] Both authors share first authorship.

**Correspondence**: Rajiv Jalan, UCL Institute for Liver and Digestive Health, Upper third floor, Royal Free Campus, Rowland Hill Street, Hampstead, London, NW3 2PF. Email: r.jalan@ucl.ac.uk

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**Conflict of Interest**

Rajiv Jalan is the inventor of OPA, which has been patented by UCL and licensed to Mallinckrodt Pharma. He is also the founder of Yaqrit Discovery, Hepyx Limited (spin out companies from University College London), and Cyberliver. He has research collaborations with Yaqrit Discovery. Yaq-001 was licensed by Yaqrit Ltd. from UCL. JRM has received consultancy fees from EnteroBiotix and Cultech, and speaker fees from Falk Forum.

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**Authors' contributions**

RJ, FA, JL, JM, ND - contributed to the conception and design of the study. SS and GI contributed to the conception and design of the *in vitro* studies. RJ, ND, FA - provided

administrative, study supervision, obtained funding, material support. JL, JM, LE, YJ, FC, AH, AP, FD, GI, PC, JS, JO, JL, HW, JC, SS, RM - performed experiments and substantially contributed to the acquisition of data and its analysis. All authors were involved in the interpretation of data. JL and JM drafted the manuscript. All authors revised the manuscript critically for important intellectual content.

**Abstract**

**Objective:** Strategies to target bacterial translocation in cirrhosis are limited to antibiotics with risk of antimicrobial resistance. This study explored the therapeutic potential of a non-absorbable, gut-restricted, engineered carbon bead adsorbent, Yaq-001 in models of cirrhosis and acute-on-chronic liver failure (ACLF) and, its safety and tolerability in a clinical trial in cirrhosis.

**Design:** Performance of Yaq-001 was evaluated *in vitro*. Two-rat models of cirrhosis and ACLF, [4-week, bile duct ligation (BDL)], receiving Yaq-001 for 2-weeks; and two-mouse models of cirrhosis [6-week and 12-week carbon tetrachloride (CCL4)] receiving Yaq-001 for 6-weeks were studied. Organ and immune function, gut permeability, transcriptomics, microbiome composition and metabolomics were analysed. Effect of fecal water on gut permeability from animal models were evaluated on intestinal organoids. A double-blind, randomized, placebo-controlled clinical trial in 28-cirrhosis patients, administered 4-gram/day Yaq-001 for 3-months was performed.

**Results*:*** Yaq-001 exhibited rapid adsorption kinetics for endotoxin. *In vivo*, Yaq-001 reduced liver injury, progression of fibrosis, portal hypertension, renal dysfunction and mortality of ACLF animals significantly. Significant impact on severity of endotoxemia, hyperammonemia, liver cell death, systemic inflammation and organ transcriptomics with variable modulation of inflammation, cell death and senescence in the liver, kidneys, brain and colon was observed. Yaq-001 reduced gut permeability was noted in the organoids and impacted positively on the microbiome composition and metabolism. Yaq-001, regulated as a device met its primary end point of safety and tolerability in the clinical trial.

**Conclusions:** This study provides strong pre-clinical rationale and safety in patients with cirrhosis to allow clinical translation.

**Significance of this study**

**What is already known on this topic?**

Current strategies to target bacterial translocation in cirrhosis are limited to antibiotics with risk of resistance. Yaq-001 is an insoluble, non-absorbable, non-antibiotic, engineered carbon bead of tailored porosities, which works as an adsorbent in the gut and is completely excreted after oral administration.

**What this study adds?**

1. Yaq-001 rapidly adsorbs endotoxin, ammonia and bile acids without influencing bacterial growth kinetics *in vitro*.
2. Yaq-001 reduces mortality of ACLF animals and impacts positively on markers of gut permeability, liver injury, portal pressure, brain and kidneys in animal models of cirrhosis and ACLF.
3. Yaq-001 administration was associated with positive impact on the composition of the gut microbiota, reduction in severity of endotoxemia and ammonia, severity of inflammation, liver cell death, signaling pathways and LPS sensitivity.
4. In animal models of liver fibrosis and cirrhosis, it reduces the severity of liver injury and hepatic fibrosis.
5. Enhanced permeability of intestinal organoids following incubation with fecal water from cirrhosis animals is prevented by treatment of the cirrhosis animals with Yaq-001.
6. In a Phase 2, double-blind, randomized, controlled clinical trial of Yaq-001 versus placebo in patients with cirrhosis, Yaq-001 was found to be safe and well tolerated providing evidence of clinical translatability.

**How this study might affect research, practice or policy?**

The data provide the pre-clinical rationale and clinical safety to proceed to the next phase of clinical trials in patients with cirrhosis aiming to prevent the occurrence of complications.

## INTRODUCTION

Gut dysbiosis and gut-derived bacterial ligands, in particular endotoxin, drive a dysregulated inflammatory response, which has been implicated in the development of cirrhosis and its complications such as sepsis, spontaneous bacterial peritonitis, renal dysfunction and hepatic encephalopathy[1-3]. This dysregulated inflammatory response is also central in the development of acute-on-chronic liver failure (ACLF)[4]. Markers of bacterial translocation such as endotoxin and bacterial DNA have been shown to be associated with complications of cirrhosis and diminished survival highlighting their pathogenic importance[5,6,7]. The microbiome in cirrhosis is characterized by reduced diversity and abundance of autochthonous bacteria[1]. Whilst antibiotics have been shown to impact positively on complications of cirrhosis, their use is associated with antibiotic resistance[8,9]. Furthermore, antibiotics reduce bacterial diversity rendering the microbiome less resilient.

One of the consequences of bacterial translocation in cirrhosis is that the endotoxin-sensing pathways in different organs are known to be primed resulting in heightened susceptibility to organ injury[3,10]. Adsorption of free endotoxin without exerting direct effects on bacterial growth kinetics, therefore has the potential to attenuate susceptibility to organ injury without producing the deleterious effects on the microbiome. Considering this, we developed a synthetic non-absorbable, non-antibiotic, endotoxin sequestrant and generated the hypothesis that this may be a novel therapeutic strategy to restore the microbiome, prevent bacterial translocation, systemic inflammation progression of fibrosis and cirrhosis complications. Yaq-001 is a non-absorbable, highly engineered, activated carbon of multiple porosities tailored to the micro (<2nm) and meso-macroporous (30-200 nm) range and high surface area[11-13]. These properties confer a high adsorptive capacity for larger biologically relevant molecules such as bacterial toxins in addition to smaller intraluminal targets. The most closely associated experimental oral adsorbent is AST-120, a microporous carbon bead, which has not been shown to be efficacious in patients with hepatic encephalopathy[14].

In this study, we sought to determine the adsorptive capacity of Yaq-001 and its effect on bacterial growth kinetics in *in vitro* studies. We then evaluated the *in vivo* biological effects of Yaq-001 in four animal models representing characteristics of cirrhosis and ACLF. We studied the effects of Yaq-001 on measures of multiorgan function, systemic and portal hemodynamics, immune function, multiorgan transcriptomics and microbiome composition. Finally, we performed a Phase 2 equivalent double-blind, multicenter, randomized, placebo-controlled clinical trial to assess the safety and tolerability of Yaq-001 in patients with decompensated cirrhosis.

## METHODS

Methodological details are described in **Supplementary section**.

### Functional and Structural Characteristics of Yaq-001

Adsorption of biomolecules of varying molecular weights (albumin, myoglobin, and caffeine) was evaluated. Bacterial growth was studied for *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. Scanning electron microscopy was performed to characterise the beads and pore size distribution was assessed using mercury porosimetry.

### Studies in animal models

#### *Study design*

These studies aimed to characterize the therapeutic potential of Yaq-001 in rats and mice to define its role in prevention of occurrence of cirrhosis, progression of cirrhosis and occurrence of ACLF (**Fig.S1 and Fig.S2**).

#### *Animal models*

Four-week bile-duct ligation model of advanced fibrosis

a. *Cirrhosis:* Sham (n=36); Sham-Yaq-001 (n=30); BDL (n=37); BDL-Yaq-001 (n=44).
b. *Prevention of ACLF:* Sham-LPS (n=9); Sham-LPS-Yaq-001 (n=10); BDL-LPS (n=16); BDL-LPS-Yaq-001 (n=12).

Yaq-001 (0.4 g/100g body weight per day) was administered for 2-weeks prior to sacrifice. At the time of sacrifice, mean arterial pressure (MAP) and portal pressure were measured.

Carbon tetrachloride treated model of cirrhosis

c. *Advanced fibrosis and early cirrhosis ($CCl_4$ for 6-weeks):* Control (n=6); Control-Yaq-001 (n=6); $CCl_4$ (n=12); $CCl_4$-Yaq-001 (n=12).
d. *Advanced cirrhosis ($CCl_4$ for 12-weeks)*: Control (n=6); Control-Yaq-001 (n=6); $CCl_4$ (n=12); $CCl_4$-Yaq-001 (n=12).

Yaq-001 (0.4 g/100 g body weight per day) was administered from 0-6 weeks in the 6-week model and from 6-12 weeks in the 12-week model.

***Collection and analysis of bio-samples***

Blood, stool and tissues samples were collected for later analysis. Portal venous blood was collected where possible. Peripheral blood cells and Kupffer cell reactive oxidant species (ROS) were measured. Hematoxylin-Eosin (H&E), Picrosirius Red (PSR) staining and TUNEL stains were performed in liver tissues. The mRNA in different organs was analyzed by using nSolver4.0 software (NanoString Technologies). To define effect on the microbiome, 16s microbiome study was performed. To determine the effect of Yaq-001 on modulating metabolism, urinary $^{1}$H-NMR analysis was performed.

**Assessment of gut permeability in Intestinal organoids**

Permeability of mouse intestinal organoids were detected using established protocols[15]. Fecal water generated from the stools obtained from the four groups of 6-week $CCl_4$ mice were incubated with the organoids as described previously. Permeability of the organoids were assessed.

**Clinical trial of Yaq-001 versus placebo, CARBALIVE-SAFETY study**

***Study Design***

The CARBALIVE-SAFETY clinical trial was a first in man, multicenter, double blind randomized, placebo-controlled trial of oral Yaq-001 in stable decompensated cirrhosis. Details of the study protocol is available in the Supplementary section (**Fig.S3)**. As Yaq-001 is regulated as a device, it followed both ISO standards and ICH-GCP guidance. Informed consent was obtained from each patient. The study was closely monitored and overseen by an independent data safety monitoring board (NCT03202498).

Study design is described as **Fig.S4**. The primary end point was assessed at 12-weeks. Blood and stool samples were taken at the time of randomization, 4-weeks and 12-weeks for assessment of some of the secondary and exploratory end points. Safety

assessments were performed on weeks 1, 4, 8 and 12 and comprised a physical examination, clinical laboratory tests, urinalysis, 12-lead ECG and an assessment of reported and observed adverse events. ECGs were analyzed independently. Nutritional status was assessed by the Royal Free Hospital Global Assessment tool at each safety assessment together with micronutrient analysis at baseline, week 4 and 12. Vitamin B12, A, D, E, folate, and K1 and, trace elements Copper, Zinc and Selenium were analyzed.

***Main Inclusion and Exclusion Criteria***

The main inclusion criteria were participants aged 18-years or above, clinical diagnosis of diuretic-responsive cirrhotic ascites (Child-Pugh score = 7-11 inclusive), abstinence from alcohol for at least 4-weeks prior to screening and written informed consent. The main exclusions were lack of informed consent, use of oral antibiotics, immunosuppressants or antiviral medication within 4-weeks prior to recruitment, change in dose of proton pump inhibitor therapy within 4-weeks before the start of the study treatment, hospital admission for liver-related indication for at least 4-weeks (except paracentesis), BMI > 35 or BMI < 18, presence of a transjugular intrahepatic portosystemic shunt (See protocol in supplementary for details).

***Randomization, Dosing and Compliance***

Patients were randomized 1:1 to receive 4g of oral Yaq-001 or equivalent placebo nocte for 12-weeks. Treatment compliance was assessed by the number of used or unopened sachets returned to the clinical site at each visit. Patients taking ≥70% of study medication were considered compliant.

***Endpoints and Assessments***

Primary Endpoints

The main objective of this clinical investigation is to assess the safety and tolerability of Yaq-001 throughout the three months' treatment period.

Secondary and Exploratory Endpoints

Blood and stool samples were collected for later analysis for markers of endotoxemia, systemic inflammation, bile acids, short chain fatty acids, gut permeability and the microbiome (results not reported in this paper).

## Statistical Analysis

### *Animal Studies*

Based on the *in vitro* studies, we anticipated a 50% decrease in circulating endotoxin in the treatment groups with an alpha error of 0.05 and power of 80%, resulting in a minimum sample size of 5 animals/group. As this study included several pathophysiological end points, multiple experimental groups were included. All the data accrued from these studies are described in this paper. All the rats in eight groups from three independent batches were included in the analysis as shown in **Fig.S1**. All the mice studied in eight groups were included in **Fig.S2**.

Group comparisons for continuous variables were performed using Man-Whitney U test (no-normal distribution) or unpaired t-test (normal distribution) and for categorical variables by using Chi-squared test. The data were analyzed using R package (R version 4.4.4). 16s microbiome study and circos correlation were analyzed by using Wilcoxon rank sum test and spearman correlation. Software used included Graphpad Prism 9.0 (GraphPad software, Inc., San Diego, CA).

### *CARBALIVE-SAFETY Clinical Trial*

This first-in-man clinical investigation was not powered to demonstrate statistical significance for any endpoint. All statistical analyses of study data were carried out using SAS v 9.3 or a later version. For categorical variables, summary tabulations of the number and percentage of patients within each category (with a category for missing data) of the parameter are presented. Percentage calculations are based on non-missing data unless otherwise specified. Please also see statistical analysis plan (Supplementary).

## RESULTS

### Functional and Structural Characteristics of Yaq-001

Yaq-001 beads exhibited a consistent pre-defined structure with a bead diameter within the 250-500 µm range and the prescribed porosity (**Fig.S5A**). Yaq-001 rapidly adsorbed albumin (66.5kDa), myoglobin (16.7kDa) and caffeine (0.194kDa) representing different sized biomolecules (**Fig.S5B**). Yaq-001 adsorbed LPS (18kDa) reducing the concentrations from 2.5 to 1.5 EU $mL^{-1}$ (60%) within 30 minutes. No endotoxin was detected in the control solution (0 EU $mL^{-1}$) (**Fig.S5B**). Yaq-001 also adsorbed a range of bile acids (**Fig.S5C**). Direct co-incubation of Yaq-001 with bacterial suspensions of either *E. coli* or *S. aureus* indicated that Yaq-001 did not affect bacterial growth kinetics for either species following direct contact in comparison to the antibiotic controls (**Fig.S5D**). Mercury porosimetry showed that Yaq-001 used in the clinical trial had a consistent pore size distribution plot in the meso-macroporous range from 30-200 nm (**Fig.S5E**).

Yaq-001 exhibited better performance in adsorptive capacity and effect on endotoxin kinetics than AST-120(**Fig.S5**).

### Studies in animal models

#### *Studies in BDL rat model of advanced fibrosis*

Effect of Yaq-001 on liver injury and portal pressure

BDL rat model was used to assess the effect of Yaq-001 in cirrhosis (**Fig.1A**). Significant reduction in 4-week body weight was observed in BDL rats ($p<0.0001$), which was prevented by administration of Yaq-001 ($p=0.045$) (**Fig.1A**). Yaq-001 was associated with a significantly lower plasma ALT ($p=0.007$). ALP, TBIL and albumin were not impacted by Yaq-001 (**Fig.S6A, B, C**). Total bile acid concentrations were not different between the BDL and Sham groups and there was no significant impact of Yaq-001 (**Fig.S6E**). MAP was lower in BDL animals and no effect of Yaq-001 was observed (**Fig.S6F**). Yaq-001 resulted in a significant reduction in portal pressure compared to untreated BDL rats [(median (IQR) 11.1 mm Hg (10.3-11.7) *vs* 12.4 mm Hg (10.8-13.3), ($p=0.025$)] (**Fig.1A**). TUNEL assay showed significantly more intense staining in the liver tissue of BDL compared to Sham rats (**Fig.1A**) ($p<0.0001$), which was significantly reduced in Yaq-001-

treated BDL rats compared to untreated-BDL rats (p=0.025). Collagen proportionate area (CPA) was significantly higher in BDL rats (p=0.0007), which was unchanged with Yaq-001 (p=0.122) (**Fig.S6D**).

Effect of Yaq-001 on ammonia, organ dysfunction, endotoxemia and bacterial translocation

*Ammonia*: Arterial and portal venous ammonia concentrations were significantly increased in BDL rats (p<0.0001), which was significantly reduced by Yaq-001 [(p=0.003) and (p=0.004) respectively] (**Fig.1A**). None of the animals showed signs of overt hepatic encephalopathy.

*Kidneys:* BDL animals had significantly higher plasma creatinine (p=0.049), which was significantly reduced with Yaq-001 (p=0.025) (**Fig.1A**). Urea was higher in BDL group (p=0.092), which was reduced with Yaq-001 treatment (p=0.095) (**Fig.1A**).

*Gut permeability, Endotoxemia, Bacterial DNA and Cytokines*: The microbial metabolite, D-lactate, a marker of gut-specific intestinal barrier damage and translocation[16] was significantly increased in BDL rats (p=0.032) and was significantly reduced by Yaq-001 (p=0.035) (**Fig.1A**). BDL rats exhibited marked endotoxemia in the portal vein and the artery (p<0.0001 for each), which was significantly reduced with Yaq-001 [(p<0.0001) (p=0.003) respectively] (**Fig.1A**). Portal venous bacterial DNA was detectable in significantly higher number of BDL rats (p<0.05), which was markedly reduced in Yaq-001 administered BDL rats (p=0.08) (**Fig.1A**). Plasma IL- β concentration were higher in the BDL rats but no significant differences were observed in TNF-a, IL-6 and IL-10. No significant changes were seen with Yaq-001 (**Table S1**).

**Studies in the BDL model of ACLF**

This experiment was performed to determine whether Yaq-001 treatment for 2-weeks prevents the occurrence of ACLF when BDL animals are administered LPS (**Fig.S1**, **Fig.1B**).

*Survival*: Animals were sacrificed either at coma stages (considered as a surrogate for mortality) or at 6-hours post LPS. Yaq-001 significantly reduced time to coma of BDL-LPS rats compared to untreated controls ($p<0.01$) (**Fig.1B**). All animals in the two Sham groups were alive at 6-hours following LPS (data not shown).

*Liver*: Yaq-001 was associated with significantly lower ALT in BDL-LPS rats compared to untreated rats ($p=0.004$) (**Fig.1B**). No significant effect of Yaq-001 was observed on ALP, TBIL and albumin (**Fig.S7 A, B, C**). The severity of fibrosis measured using CPA and the body weight were unchanged (**Fig.S7D, E**).

*Systemic and Portal hemodynamics*: No significant difference in MAP was observed between the groups treated with or without Yaq-001 (**Fig.S7F**) but Yaq-001 produced a significant reduction in portal pressure in BDL-LPS animals compared with the untreated group ($p=0.003$), (**Fig.1B**).

*Brain:* Yaq-001 significantly reduced brain water in BDL-LPS compared with the untreated group ($p=0.017$) (**Fig.1B**). Arterial and portal venous ammonia concentrations were significantly increased in BDL-LPS rats, which was significantly reduced in Yaq-001-treated animals [($p=0.007$) and ($p=0.017$) respectively] (**Fig.1B**).

*Kidneys*: Creatinine concentrations were significantly higher in BDL-LPS animals ($p=0.004$), which was significantly reduced by Yaq-001 ($p=0.03$) (**Fig.1B**).

*Cytokines*: BDL-LPS group had a significantly higher plasma IL-1β, which was significantly reduced with Yaq-001 ($p=0.003$). Plasma IL-10 was higher in BDL-LPS and was significantly reduced with Yaq-001 ($p=0.028$) (**Fig.1B**). No significant differences were observed in IL-6 or TNF-α concentrations between any of the groups (**Table S1**).

***Effect of Yaq-001 on peripheral blood cells and Kupffer cells***

Significant increase in total leucocyte, neutrophil and monocyte counts in the artery and portal vein were observed with BDL rats (**Fig.S8A, B**) ($p=0.008$ and $p=0.016$

641 respectively), which was significantly reduced with Yaq-001 in the arterial blood and
642 insignificantly reduced in the portal vein (**Fig. S8B**). To determine whether Yaq-001
643 impacts on the response of peripheral inflammatory cells and Kupffer cells to generate
644 reactive oxygen species (ROS) to LPS *ex vivo,* studies using isolated cells incubated with
645 LPS, were performed. Yaq-001 was associated with significantly lower LPS-induced ROS
646 production in $CD163^-$ Kupffer cells in BDL rats (p=0.036) and portal venous $CD43^{hi}$
647 monocyte populations of BDL rats (p=0.029) (**Fig.S8C**).
648
649 ***Transcriptomic analysis of gene expression profiles from the Liver, Colon, Brain***
650 ***and Kidneys***
651 Multiorgan transcriptomic analysis was performed to determine the possible molecular
652 mechanisms underlying the clinical effects of Yaq-001. The four groups studied were as
653 follows: Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) (**Fig.2A**).
654 All differentially expressed genes (DEGs) and related pathways in the liver, colon, kidney
655 and brain are listed in **Table S2**. The top 20 and significant DEGs are listed in **Table S3**.
656
657 Effect of Yaq-001 on gene expression profiles in the liver and gut in BDL rats
658 *Liver*: Analysis of liver tissue showed 82 DEGs at the threshold of 1.2-fold change and
659 p=0.1 in the four groups (**Fig.2B**). Compared with the Sham group, expression of 62-
660 genes was upregulated, and 15-genes were downregulated in BDL. These significantly
661 changed genes were associated with inflammation, cell death and senescence.
662 Compared to the untreated BDL group, the expression of 7-genes was upregulated and
663 12-genes were downregulated in the Yaq-001-treated BDL group, indicating the potential
664 role of Yaq-001 in reducing inflammation, cell death and cell senescence. Furthermore,
665 2-genes were upregulated, and 4-genes downregulated in Sham-Yaq-001 group in
666 comparison to Sham group (**Fig.2C**). Functional analysis demonstrated that BDL rats had
667 enriched pathways related to inflammation, cell senescence, cell death, TLR signaling
668 and other related signaling pathways in comparison with Sham (**Fig.S9A**). Yaq-001
669 treatment targeted the altered pathways compared with untreated BDL group. Additionally,
670 Yaq-001 treatment also changed the pathways in the liver when compared to Sham group,
671 demonstrating its effect in rats even without cirrhosis (**Fig.S9A**).

*Colon*: 43 DEGs were identified from the colonic tissue (**Fig.2D**). 5-genes that correlated with inflammation and cell death were upregulated and 15-genes were downregulated in BDL compared with the Sham group. Moreover, the expression of 10-genes was upregulated, and 13-genes were downregulated with Yaq-001 treatment. Only 1-gene was upregulated in the Sham-Yaq-001 group, and 16-genes were downregulated with Yaq-001 compared with the untreated Sham group (**Fig.2E**). Functional analysis indicated that inflammation, cell senescence, cell death, TLR signaling and intracellular signaling were associated with BDL in comparison with the Sham group (**Fig.S9B**). Yaq-001 targeted the altered pathways, indicating the potential mechanisms in the prevention of gut dysfunction and permeability (**Fig.S9B**).

Effect of Yaq-001 on gene expression profiles in the brain and kidney in BDL rats

*Brain*: 17 DEGs were identified from the brain tissue (**Fig.2F**). Compared with Sham group, expression of 2-genes was upregulated and 13-genes were downregulated in BDL animals. These significantly changed genes were associated with inflammation, cell death, and cell senescence. Compared to the untreated-BDL group, the expression of 5-genes was upregulated and 2-genes were downregulated in the Yaq-001-treated BDL group (**Fig.2G**). Functional analysis demonstrated that BDL rats had enriched pathways related to inflammation, cell senescence, cell death, TLR signaling and intracellular signaling (**Fig.S9C**). Yaq-001 targeted cytokine-cytokine receptor interaction, cytosolic DNA-sensing pathway, TLR signaling pathway, NOD-like receptor signaling pathway, neutrophil extracellular trap formation, TGF-beta signaling pathway and cytokine-cytokine receptor interaction pathways compared to untreated-BDL group (**Fig.S9C**).

*Kidneys*: 30 DEGs were identified from kidney tissue (**Fig.2H**). 9-genes that correlated with inflammation were downregulated in BDL. The expression of 5-genes was upregulated and 4-genes were downregulated with Yaq-001 treatment compared to untreated-BDL group. 5-genes were upregulated in Sham-Yaq-001 group, and 3-genes were downregulated with Yaq-001 compared with untreated-Sham group (**Fig.2I**). Functional analysis indicated that inflammation and TLR signaling were associated with

BDL in comparison with Sham (**Fig.S9D**). Compared with the untreated-BDL group, Yaq-001 targeted the altered pathways, indicating the potential mechanisms in the prevention of renal dysfunction (**Fig.S9D**).

***Effect of Yaq-001 on the gut microbiome profile***

The effects of Yaq-001 on the microbiome bacterial composition was assessed by metataxonomics. At the family level, an abundance of six bacteria were significantly changed at the threshold of 2-fold change and *Porphyomonadaceae* was significantly changed ($p<0.05$) comparing BDL with Sham **(Fig.3A)**. At genus level, 19 bacteria including were significantly changed in abundance. *Barnesiella* was significantly changed ($p<0.05$) comparing BDL with Sham group **(Fig.3B)**. These changes were reversed with Yaq-001 treatment compared to untreated-BDL rats (**Fig.S10A, B**, **Table S4 and Fig.S10C, D).** For between groups sample diversity, PERMANOVA analysis revealed a significant difference in beta diversity between groups ($R2 = 0.32$, $p = 0.001$). Yaq-001 appeared to moderately restore the beta diversity in the BDL group especially in PCoA2 axis (**Fig.S10E, F)**.

To further investigate the potential importance of the changes in the microbiome induced by Yaq-001, we correlated these with all significantly changed DEGs and the top 20 DEGs in the four organs. Circos plots indicated a significant correlation between them (**Fig.3C, D and Fig.S11A, B**, **C)**. *Porphyromonadaceae*, was observed to positively correlate with three DEGs - TGFB2 and CASP1 in liver tissue, and FOS in colonic tissue. Also, it correlated negatively with five DEGs- TGFB2, IL-18 and CCR5 in brain tissue, CXCL10 in colon tissue and CCL24 in kidney tissue.

***Effect of Yaq-001 on metabolomic profile***

Significant difference of acetate/creatinine, glycine/creatinine, lactate/creatinine, betaine/creatinine, trimethylamine oxide/creatinine and bile acid/creatinine ratio were observed in BDL compared to Sham. Treatment of BDL rats with Yaq-001 resulted in significant resolution of acetate/creatinine, glycine/creatinine and lactate/creatinine compared to the untreated BDL animals (**Fig.S12**).

***Studies in $CCl_4$ mice***

Effect of Yaq-001 on liver injury and fibrosis

6-week and 12-week $CCl_4$ mice models were used to further confirm the effect of Yaq-001 in cirrhosis (**Fig.4A，B**). Yaq-001 was associated with a significantly lower plasma ALT ($p<0.0001$, $p<0.0001$) in both 6-week and 12-week $CCl_4$ models. ALP and TBIL were reduced by Yaq-001 in 6-week $CCl_4$ mice ($p=0.040$, $p=0.001$) (**Fig.4A，B**). CPA was significantly higher in both $CCl_4$ mice compared with control animals ($p=0.0001$, $p=0.0001$), which was significantly reduced with Yaq-001 ($p=0.024$, $p=0.012$) (**Fig.4A, B**). TUNEL assay showed significantly more intense staining in the liver tissue of $CCl_4$ compared to Control mice ($p<0.001$, $p<0.001$), which was significantly reduced in Yaq-001-treated $CCl_4$ mice compared to untreated-$CCl_4$ mice ($p=0.021$, $p=0.017$) (**Fig.4A, B**).

Effect of Yaq-001 on ammonia, organ dysfunction and endotoxemia

*Ammonia*: Ammonia concentrations were significantly increased in the 6-week and 12-week $CCl_4$ mice compared with controls ($p=0.002$, $p=0.001$), which was significantly reduced by Yaq-001($p=0.025$, $p=0.035$) (**Fig.4A, B**). None of the animals showed signs of hepatic encephalopathy.

*Kidneys:* Higher plasma creatinine was significantly reduced by Yaq-001 treatment ($p=0.005$, $p=0.003$) in 6-week and 12-week $CCl_4$ animals (**Fig.4A, B**).

*Endotoxemia*: Both 6-week and 12-week $CCl_4$ mice exhibited marked endotoxemia compared with controls ($p=0.007$, $p=0.007$), which was significantly reduced with Yaq-001($p=0.007$, $p=0.043$) (**Fig.4A, B**).

***In vitro* studies in intestinal organoids to assess gut permeability.**

Intestinal organoids were successfully derived and cultured from small intestine of C57BL/6 mice. Intestinal organoids underwent eversion into apical-out polarity in the first 12h of suspension culture and collected for identification and subsequent experiments (**Fig.5A**). Immunostaining of the microvilli (mv; F-actin) demonstrated that intestinal

765 organoids in suspension had reversed polarity such that the apical surface faces outward
766 (**Fig.5A**). Apical-out intestinal organoids possessed goblet cells, which were identified
767 with MUC2 staining (**Fig.5B**). Gut permeability of apical-out intestinal organoids was
768 significantly increased by coculturing with fecal water from $CCl_4$ group compared with the
769 control group (p=0.003) (**Fig.5C,D**). The gut permeability was significantly decreased with
770 fecal water from Yaq-001 treated $CCl_4$ animals compared with the $CCl_4$ group (p=0.001)
771 (**Fig.5C,D**).
772
773 **CARBALIVE-SAFETY Clinical Trial**
774 The data regarding safety and tolerability are reported here. Other secondary and
775 exploratory end points will be described elsewhere.
776
777 ***Patient Characteristics***
778 Thirty-four-patients were screened for this study at 8-European centers. 28-patients met
779 the study entry criteria and were randomized to either active or placebo groups. Six-
780 patients screened did not meet the study entry criteria. Dosing was not initiated in 2-
781 patients randomized to placebo due to withdrawal of consent (**Fig.S3**, CONSORT). 3-
782 patients were included for the second dosing cohort of 8 g. This part of the study was
783 terminated prematurely due to the coronavirus pandemic with none of the patients
784 completing the study duration (data not included).
785
786 In accordance with study entry criteria, all patients had cirrhosis with diuretic-responsive
787 ascites and Child-Pugh score of 7-8. The baseline demographics were similar across
788 treatment groups. The ratio of male patients to female patients was reflective of the
789 disease state. Compliance in the active and placebo groups was 92.9% and 66.7%
790 respectively (**Table 1**).
791
792 ***Safety and Tolerability***
793 Of the 14-patients enrolled in the Yaq-001 treatment group, 13 (93%) completed 12-
794 weeks of therapy. The median duration of exposure was 83 (6-94) days. Ten of the 12
795 (83%) patients who received placebo completed the treatment. The median duration of

exposure was 83 (14-86) days. No deaths or serious adverse events were reported in the study. The difference in treatment-emergent adverse events (TEAEs) in patients treated with Yaq-001 and those treated with placebo is presented in **Table 2**. The most frequently reported TEAEs were gastrointestinal in nature in both the active and placebo groups. Of these, only constipation and diarrhea were evaluated by the clinical investigator as possibly related to the investigational product. One placebo-treated patient withdrew from the study due to diarrhea.

Across both treatment groups, 40/51(78%) of the reported TEAEs were evaluated by the clinical investigator as not related or unlikely related (32/38; 84% for the active treatment group; 8/13; 62% for the placebo group). The incidence of adverse events reported is reflective of the targeted subject population for this clinical investigation. The majority of the TEAEs reported were not considered by the clinical investigator to be related to treatment and were mild in intensity. Systemic antibiotics were administrated for the following TEAEs in the active arm: Amoxicillin – acute bronchitis; Clarithromycin – acute bronchitis; Phosphomycin – urinary tract infection. None of these infections were related to the administration of the investigational product. Drugs received by the patients at the time of randomization and during follow up are listed in Supplementary Tables. Treatment-emergent, clinically significant laboratory abnormalities are listed in **Table 3**. None were deemed treatment-related by the investigator.

***Clinical, hematological and biochemical variables***

The data are summarized in **Table 3**. No significant changes in any of the clinical parameters were observed in any of the groups. Although there was a trend towards a reduction in the white cell count and C-reactive protein in the Yaq-001 group, the differences were not significant.

***Nutritional status***

No significant differences were observed in either treatment group with regards to global nutritional status, vitamin B12 and folate, Vitamin A or E, or copper zinc, and selenium. Median vitamin A, zinc and baseline vitamin D concentrations were below the limit of

normal range but no differences between treatment groups were observed. No changes were observed in any of the micronutrient parameters with treatment and these were evenly matched between groups. Any baseline abnormalities were attributable to the underlying natural history of cirrhosis.

## Discussion

The results of the study showed that Yaq-001 prevented progression of liver injury and fibrosis in animal models of cirrhosis and significantly reduced the mortality of ACLF animals. This was associated with positive impact on markers of gut permeability, liver injury, portal pressure, brain and kidneys. These pleiotropic effects of Yaq-001 were associated with partial restoration of the composition of the microbiome bacterial community, reduction in the severity of endotoxemia, ammonia, inflammation, cell death, signaling pathways and LPS sensitivity. A Phase 2 equivalent, double-blind, multicenter, placebo-controlled, randomized clinical trial in patients with cirrhosis confirmed regulatory compliance and, safety and tolerability of Yaq-001, thereby, providing evidence of clinical translatability. The data provide the rationale to proceed to further clinical trials.

Translocation of bacteria, its products and metabolites are critically important in the progression of hepatic fibrosis and pathogenesis of complications of cirrhosis[1,17-20]. Indeed, selective gut decontamination using norfloxacin or rifaximin are the current standard of care for secondary prophylaxis of patients with spontaneous bacterial peritonitis and hepatic encephalopathy respectively[21-22]. However, the use of these antibiotic strategies is limited to patients with advanced cirrhosis and induces the risk of antibiotic resistance.[23] The data presented here provide a safe, gut-restricted, non-antibiotic strategy, Yaq-001, which has the potential to diminish translocation and prevent the progression of hepatic injury, fibrosis and, prevent extrahepatic organ injury in models of cirrhosis. The *in vitro* studies demonstrate that Yaq-001 has the optimal pore size distribution to bind intraluminal factors such as free endotoxin. We also tested *in vitro* bacterial growth kinetics of two species, which were not affected by Yaq-001, an observation that was subsequently confirmed with studies in the BDL animal model where no change diversity were observed.

Endotoxemia has also been implicated in immune dysfunction resulting in a dysregulated systemic inflammatory response, which is strongly associated with the progression of fibrosis, cirrhosis and occurrence of ACLF[24,25]. Yaq-001 reduced the severity of endotoxemia and bacterial DNA positivity, which was associated with attenuated systemic

inflammation. Significant improvements in LPS-induced ROS production were observed in trafficking portal venous monocytes suggesting that Yaq-001 had attenuated the primed state of monocyte/macrophage populations within the gut-liver axis. This observed reduction in LPS-induced ROS production may be important in explaining the reduction in plasma IL-1β in LPS-treated BDL rats.

Plasma D-lactate, a marker of increased gut permeability was reduced in the Yaq-001 treated BDL rats[26]. Elevated plasma D-lactate levels in cirrhosis is associated with decompensation[22]. Transcriptomic analysis of colonic tissue demonstrated upregulation of genes associated with necroptosis, apoptosis and inflammation in BDL animals. Functional analyses pointed to modulation of colonic inflammation by Yaq-001, IL-17 signaling, which is known to have diverse biological functions, promoting protective immunity against many pathogens, neutrophil recruitment, antimicrobial peptide production and enhanced barrier function[27, 28]. To further validate the potential effect of Yaq-001 in modulating gut permeability, we performed experiments in intestinal organoids that were incubated with fecal water[29]. The data confirmed that even in *in vitro* settings, fecal water obtained from the feces of CCL4-induced cirrhosis animals enhanced permeability of the organoids, which was prevented in the fecal water obtained from animals that were treated with Yaq-001. The data support the hypothesis that Yaq-001 impacts on the factors in the gut responsible for increasing gut permeability in cirrhosis.

Yaq-001 significantly reduced the severity of liver injury and portal hypertension in both the BDL and BDL-LPS models of cirrhosis and ACLF. The lack of significant differences in CPA between untreated and Yaq-001-treated BDL groups suggests that the reduction in portal pressure is possibly due to modulation of the dynamic component of portal hypertension, in which inflammation is known to play a role[30,31] and proposes Yaq-001 as a potential treatment for portal hypertension. Reduction in ALT levels and TUNEL staining confirmed a reduction in liver injury in the Yaq-001 treated animals. The reduction in liver injury in the LPS treated BDL animals suggests that Yaq-001 has a particular effect on endotoxin sensitivity *in vivo.* This hypothesis was tested in isolated Kupffer cells, which

confirmed that LPS-induced ROS production was significantly impacted by Yaq-001 treatment.

Transcriptomic analysis of liver tissue demonstrated that the upregulated genes, CXCL16, CASP1 and TGFB2 in BDL rats was prevented by Yaq-001 administration. Silencing of CXCL16 alleviates hepatic ischemia reperfusion injury and CXCL16 variant is also associated with Hepatitis B virus related acute liver failure[32]. CASP1 mediates pro-inflammatory cytokine release and pyroptotic cell death in cirrhosis and its inhibition has been shown to prevent ACLF [33]. TGFB2 is an important mediator of cellular senescence[34, 35]. Of note, Yaq-001 also modified necroptosis and cytosolic DNA-sensing pathways representing cell death, which are known to be activated by LPS that can trigger systemic inflammation[36]. These effects of Yaq-001 potentially explains the effect of Yaq-001 in reducing liver injury [37, 38].

Yaq-001 administration had a significant impact on time to coma of ACLF rats, which is considered as a surrogate for mortality compared to untreated controls. Yaq-001 also significantly lowered portal venous and arterial ammonia levels, which was associated with reduced brain water. Transcriptomic analysis of brain tissue showed that IL-18, TGFB2, CCR5 and IL-23A were dysregulated in BDL rats and these were corrected by Yaq-001. IL-18 is released during pyroptosis by activation of the inflammasome complex in neuroinflammatory and neurodegenerative diseases[39]. The effect of Yaq-001 on TGFB2 may mean that it has an impact on senescence, which is known to be associated with hepatic encephalopathy. CCR5 has been implicated in neuroprotection and is novel therapeutic target in stroke[40]. The impact of Yaq-001 on IL-23A indicates possible reduction in neuroinflammation.

In both cirrhosis and ACLF models, Yaq-001 reduced renal dysfunction. Transcriptomic analysis of kidney tissue showed that CCL24 was downregulated in BDL rats, which was prevented in the Yaq-001-treated animals. CCL24 protects renal function in the development of early diabetic nephropathy by exerting an anti-inflammatory effect[41]. Yaq-

001 impacted, on the cytokine-cytokine receptor interactions and chemokine and toll-like signaling pathways, which were abnormal in the BDL rats.

BDL animals become sarcopenic and lose weight[42], which was significantly abrogated by Yaq-001. The possible mechanisms underlying this effect are likely multifactorial[43]. Yaq-001 reduced ammonia significantly, which has been shown to induce sarcopenia[44]. Weight loss in cirrhosis is also attributed to an increased catabolic state in the context of systemic inflammatory response and thus the observed improvement in body weight may reflect the diminished catabolic state with reduced inflammation[43].

The clinical effects of Yaq-001 observed in the BDL models was validated in the CCL4 induced liver injury animal models. Two models were studied. In the first (6-week model), Yaq-001 was administered in a preventative mode starting its administration with the onset of liver injury during parenteral administration of CCL4. The results showed significant reduction in the severity of liver injury, fibrosis and progression to cirrhosis, endotoxemia, creatinine and ammonia levels. In the second (12-week model), Yaq-001 was administered starting at 6-weeks when the animal already had advanced fibrosis/ cirrhosis. Again, significant reduction in markers of liver injury, fibrosis, endotoxemia, creatinine and ammonia were observed. Extrapolating these observations to the humans, the results from the 6-week model suggests that Yaq-001 may well be useful to prevent the progression of fibrosis in patients without cirrhosis and, from the 12-week model, the possibility of prevention of progression of liver disease in those with well compensated cirrhosis.

Gut microbiota are important in modulating intestinal health, permeability, bacterial translocation, systemic inflammation and complications of cirrhosis[45-47]. BDL was associated with marked changes in the abundance of microbiota, which were reversed by Yaq-001. In particular, the abundance of *Porphyromonadaceae* and *Barnesiella* were significantly elevated in BDL rats and significantly decreased with Yaq-001. This change is potentially important as *Porphyromonadaceae* is a pro-inflammatory bacterium that has been positively correlated with hepatic encephalopathy[48] and, *Barnesiella* and

*Porphyromonadaceae* have been associated with liver cancer [49,50]. Urinary NMR analysis reflects the combined metabolic status of both host and microbiota. Yaq-001 was associated with a distinct shift of acetate, glycine and lactate in metabolomic profile in BDL rats. These metabolites are generated by mixed acid fermentation (MAF), typically by bacteria such as Enterobacter. MAF is not the preferred metabolic pathway for facultative anaerobes and may be indicative that Enterobacter populations are under conditions of metabolic stress in Yaq-001 treated BDL animals. As these species are often pathogenic in cirrhosis, this may represent a beneficial change. However, the exact mechanisms by which the change in the microbiome results in improvement in distant organ function and gene expression cannot be directly inferred from the data derived from this study. One possibility is that alongside LPS adsorption and modulation of other unmeasured toxins, the *milieu* of the gut is changed allowing proliferation of more autochthonous bacteria[47], which impacts on gut inflammation that reduces gut permeability. This hypothesis is supported by the organoid experiments. Reduction in permeability would result in a reduction in endotoxemia, systemic inflammation, improvement of organ function and LPS-sensitivity. In this study, most of these changes have been described individually but whether this is happening in sequence has not been investigated.

As Yaq-001 is completely excreted unchanged in the stool, it is regulated in Europe as a device but as a drug in the US, the clinical trial was performed both according to ISO standards and ICH-GCP guidance. The results of this first-in-man randomized, placebo-controlled trial suggested that oral Yaq-001 at a dose of 4g nocte was well tolerated with a favorable safety profile. Despite the rapid adsorption kinetics for bacterial toxins and metabolites, Yaq-001 treatment had no negative impact on micronutrient levels or impact on nutritional profile as assessed by the gold standard Royal Free Global Assessment tool. This data must be interpreted keeping in mind that Yaq-001 was administered post prandially, separated from meals by 4-hours and from drugs by about 6-hours as necessitated by the protocol. It is important to note that the studies were performed in stable cirrhosis patients, many of whom had minimal evidence of systemic inflammation and therefore, any clinical effect of this intervention was difficult to gauge. However, future

analysis of the available samples from the blood and stool will provide answers as to whether Yaq-001 modulates the gut microbiome, inflammation and endotoxemia.

These results must be considered in view of some limitations. First, the rodent microbiome is not directly analogous to the human and further clinical studies will be required to verify the effects on the gut microbiome's bacterial composition. Second, although Yaq-001 was effective in adsorbing a variety of bile acids *in vitro* and reduced bile acids significantly in Sham animals, no impact on bile acids was seen in BDL animals. This possibly reflects the effect of the BDL model, where no increase in bile acids was observed. Also, no changes in bile acids were observed in CCL4 animals but these animals did not have elevated bile acid either. Third, although, Yaq-001 was observed to impact positively on the gene expression profiles of multiple pathways, their exact relevance at the protein or cellular level has not been explored. Fourth, as only one dose of Yaq-001 was tested in the clinical trial, further dose-ranging studies will be needed to define optimal dosing for safety and efficacy. However, the animal toxicity studies that were performed by an independent laboratory for regulatory purposes, showed evidence of safety in much larger doses than that administered in the present studies (summary in Supplementary).

In conclusion, the data provides compelling evidence for the potential of Yaq-001 as a novel therapy targeting the gut microbiome, bacterial translocation and gut permeability that impacts on systemic inflammation, liver injury and fibrosis and, organ function in models of cirrhosis and improves survival in ACLF. The placebo-controlled clinical trial of Yaq-001 in cirrhosis patients provides evidence of safety and tolerability allowing translation to next phase of clinical studies to define its potential as a novel therapeutic for patients with cirrhosis.

**Abbreviations**

ACLF, acute-on-chronic liver failure; LPS, lipopolysaccharides; BDL, bile duct ligation; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; MAP, mean arterial pressure; CPA, collagen proportionate area; PSR, picrosirus red ; PP, portal pressure; ROS, reactive oxidant species; DEGs, differential expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; TLR, toll-like receptor; TNF-a, tumor necrosis factor-a.

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**Figure legends**

**Fig.1. Effect of Yaq-001 on organ dysfunction, endotoxemia and bacterial translocation in BDL and ACLF rats.**

(A) Rats underwent bile duct ligation for 4-weeks as a model of cirrhosis (n=23-37/group). Treatment groups received Yaq-001 for 2 weeks before sacrifice.

*4-week body weight in four groups*: Sham (n=31), Sham-Yaq-001 (n=24), BDL (n=31) and BDL-Yaq-001 (n=38). Significantly lower final body weights were observed in BDL compared to Sham controls ($p<0.001$). Yaq-001-treated BDL rats had a significantly higher body weights compared to untreated-BDL rats ($p<0.05$).

*Plasma alanine transaminase (ALT) concentrations* in Sham (n=17), Sham-Yaq-001 (n=14), BDL (n=17) and BDL-Yaq-001 (n=26) groups and *Portal pressure (PP) measurements in Sham (n=17), Sham-Yaq-001 (n=19), BDL (n=14) and BDL-Yaq-001 (n=26) groups*. Significantly higher ALT and PP were observed in BDL compared to Sham controls ($p<0.0001$). Yaq-001-treated BDL rats had a significantly lower ALT and PP compared to untreated-BDL rats ($p<0.01$, $p<0.05$).

*TUNEL assay of liver tissue with quantification of staining by digital image analysis*. Significantly higher TUNEL staining was observed in BDL compared to Sham controls ($p<0.0001$). Yaq-001-treated BDL rats had a significantly lower TUNEL staining compared to untreated-BDL rats ($p<0.05$) indicative of a reduction in liver cell death with Yaq-001 treatment.

*Arterial ammonia concentrations i*n Sham (n=7), Sham-Yaq-001 (n=5), BDL (n=19), BDL-Yaq-001 (n=21) groups and *Portal venous ammonia concentrations* in Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=13), BDL-Yaq-001 (n=18) groups. Significantly increased arterial ammonia concentrations and portal venous ammonia concentrations were observed in BDL compared to Sham controls ($p<0.0001$, $p=0.0001$). Yaq-001 significantly decreased arterial and portal venous ammonia concentrations in BDL rats ($p<0.01$ for both).

*Serum creatinine* in Sham (n=19), Sham-Yaq-001 (n=17), BDL (n=20), BDL-Yaq-001 (n=17) and *serum urea* in Sham (n=28), Sham-Yaq-001 (n=23), BDL (n=30), BDL-Yaq-001 (n=34) groups. Yaq-001 markedly decreased serum creatinine levels in BDL rats ($p<0.05$).

*Plasma D-lactate* in Sham (n=7), Sham-Yaq-001 (n=8), BDL (n=6), BDL-Yaq-001 (n=7). Plasma D-lactate was significantly increased in the BDL group compared with Sham animals ($p<0.05$). Yaq-001 resulted in a significant reduction in plasma D-lactate in BDL rats ($p<0.05$).

*Portal venous endotoxin* [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=7)] and *arterial endotoxin* [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=7)].

*Portal venous bacterial DNA positivity* [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=13)] and *arterial plasma bacterial DNA positivity* [Sham (n=6), Sham-Yaq-001 (n=6), BDL (n=12) and BDL-Yaq-001 (n=7)]. Significantly higher portal venous endotoxin and arterial endotoxin were observed in BDL rats compared to Sham rats ($p<0.0001$). Significantly higher portal venous plasma bacterial DNA positivity was observed in BDL rats compared to Sham rats ($p<0.05$). Yaq-001 administration was associated with a significant reduction of portal venous and arterial endotoxin compared to untreated-BDL rats ($p<0.0001$, $p<0.01$). Yaq-001 administration reduced bacterial DNA positivity, which was not statistically different ($p>0.05$).

(B) Rats underwent sham biliary surgery or BDL for 4-weeks. The treated group received Yaq-001 for 2 weeks prior to LPS injection. Animals were sacrificed either at coma stages or 6 hours after LPS injection (n=9-16/group).

*Kaplan-Meier analysis* of BDL-LPS rats with (n=16) or without (n=12) Yaq-001 treatment. Yaq-001 treatment significantly improved the survival of BDL-LPS rats compared to untreated-BDL-LPS rats (log rank test, $p=0.003$).

*Plasma ALT concentrations* in Sham-LPS (n=7), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=10) and BDL-LPS-Yaq-001 (n=9) groups and *PP measurements* in Sham-LPS (n=8), Sham-LPS-Yaq-001 (n=10), BDL-LPS (n=9) and BDL-LPS-Yaq-001 (n=9) groups. Yaq-001-treated BDL-LPS rats had a significantly lower ALT and PP compared to untreated-BDL-LPS rats ($p<0.005$).

*Brain water* percentage in Sham-LPS (n=4), Sham-LPS-Yaq-001 (n=4), BDL-LPS (n=7), BDL-LPS-Yaq-001 (n=13) groups. *Arterial ammonia* concentrations in Sham-LPS (n=5), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=7), BDL-LPS-Yaq-001 (n=7) groups. *Portal*

*venous* ammonia concentrations in Sham-LPS (n=5), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=6), BDL-LPS-Yaq-001 (n=5) groups. Yaq-001 decreased brain water percentage and arterial/portal venous ammonia concentrations in BDL-LPS rats compared to untreated rats ($p<0.05$, $p<0.01$, $p<0.05$).

*Serum creatinine* in Sham-LPS (n=4), Sham-LPS-Yaq-001 (n=3), BDL-LPS (n=12) and BDL-LPS-Yaq-001 (n=6) groups. Serum urea in Sham-LPS (n=8), Sham-LPS-Yaq-001 (n=4), BDL-LPS (n=12) and BDL-LPS-Yaq-001 (n=8) groups. Yaq-001 significantly decreased creatinine levels in BDL-LPS rats ($p<0.05$).

*Plasma cytokines* in Sham-LPS (n=6), Sham-LPS-Yaq-001 (n=9), BDL-LPS (n=8) and BDL-LPS-Yaq-001 (n=8) groups. Yaq-001 significantly decreased plasma IL-1β and IL-10 concentrations in BDL-LPS groups ($p<0.01$, $p<0.05$).

**Fig.2.Effect of Yaq-001 on gene expression profiles in the multiorgans in BDL rats.** (A) Rats underwent BDL for 4-weeks as a model of cirrhosis (n=3-4/group) and the treatment groups received Yaq-001 for 2-weeks before sacrifice. Liver, colon, brain and kidney were collected for transcriptomic analysis. (B, D, F, H) Heatmap of differentially expressed genes (DEGs) in different organs between Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) groups. DEGs were identified at 1.2-fold change and p=0.1 threshold in three pairwise groups (BDL versus Sham, BDL-Yaq-001 versus BDL, Sham-Yaq-001 versus Sham). (C, E, G, I) Volcano plot of pairwise DEGs in four organs among Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) groups. The vertical dashed lines indicated the threshold for 1.2-fold change. The horizontal dashed line indicated the adjusted p=0.05 and p=0.1 threshold. The right part indicates up-regulation of gene expression, and the left part indicates down-regulation of gene expression. The top 20 genes are indicated by gene names.

**Fig.3. Effect of Yaq-001 treatment on the microbiome composition.** (A) Heatmap of gut microbiome associated with the effect of Yaq-001 as determined by 16S PCR at the family level. The Family *Porphyromonadaveae* with asterisk was statistically differently abundant between BDL (n=7) vs Sham (n=6), and between BDL-Yaq-001 (n=7) vs BDL groups (n=7) (Wilcoxon rank sum test, $p<0.05$). The abundance of this family was

statistically higher in BDL group than in Sham group, and its abundance statistically decreased in the BDL-Yaq-001 group than in the BDL group. The other six families in the heatmap were with marked fold changes between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (|log2FC|>2). Of these, five were more abundant in the BDL group than in the Sham group. The abundance largely decreased in the Yaq-001-treated group. In addition, of these, one family was less abundant in the BDL group than in the Sham group. The abundance increased in the Yaq-001-treated group. (B) Heatmap of gut microbiome at the Genus level. The Genus *Barnesiella* with asterisk was statistically differently abundant between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (Wilcoxon rank sum test, $p<0.05$). The abundance of this genus was statistically higher in BDL group than in the Sham group, and its abundance statistically decreased in the BDL-Yaq-001 group. The other 19 genera in the heatmap represent those with significant fold change values between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (|log2FC|>2). Of these, 14 were more abundant in the BDL group compared with the Sham group. The abundance decreased in the Yaq-001-reated BDL group. In addition, 5 genera were less abundant in the BDL group than in the Sham group. Their abundance increased in the Yaq-001-treated BDL animals. (C, D) Correlation plots between markedly changed genes and gut microbiome at family/genus. The genes were from amongst the top 20 changed genes in BDL animals with Yaq-001 treatment. Nodes represent either genes (lower semi-circular part) or bacteria (upper semi-circular part) at the family and genus level. The nodes are colored based on the log-fold change for the differential gene expression and differences in the bacterial abundance. The red nodes indicate an increase and blue nodes indicate a decrease. Edges represent the correlation coefficient calculated between genes and microbial genus or family with red indicating a positive correlation and blue a negative correlation. Correlation coefficients greater or equal to 0.4 were plotted in plot C (Spearman's coefficient >= 0.4), and D shows all correlations.

**Fig.4. Effect of Yaq-001 on organ dysfunction, ammonia and endotoxemia in $CCl_4$ mice.**

(A) Mice underwent $CCl_4$ injection for 6-weeks as a model of cirrhosis (n=6-12/group) and the treatment groups received Yaq-001 for 6 weeks before sacrifice.

*Plasma ALT, ALP and TBIL concentrations* in Control (n=6), Control-Yaq-001 (n=6), 6-week $CCl_4$ (n=12) and 6-week $CCl_4$-Yaq-001 (n=12) groups. Significantly higher ALT, ALP and TBIL were observed in $CCl_4$ compared to controls (p=0.0001, p=0.0007, p=0.012). Yaq-001-treated $CCl_4$ mice had a significantly lower ALT, ALP and TBIL compared to untreated-$CCl_4$ mice (p<0.0001, p=0.040, p=0.001).

*H&E and PSR staining of liver tissue*. $CCl_4$ mice were associated with a significant increase in CPA compared to Controls (p=0.0001). Yaq-001 had significant effect on CPA in $CCl_4$-Yaq-001 compared to $CCl_4$ mice(p=0.024).

*TUNEL staining liver tissues.* Significantly greater staining was observed in $CCl_4$ compared to Controls (p=0.0001). Yaq-001-treated $CCl_4$ mice had a significantly lower TUNEL staining compared to untreated-$CCl_4$ (p=0.021) with Yaq-001 treatment.

*Venous ammonia concentrations and serum creatinine* levels in Control (n=6), Control-Yaq-001 (n=6), 6-week $CCl_4$ (n=12) and 6-week $CCl_4$-Yaq-001 groups(n=12). Significantly increased ammonia concentrations were observed in $CCl_4$ compared to Controls (p=0.0020). Yaq-001 significantly decreased venous ammonia concentrations and serum creatinine levels in $CCl_4$ mice (p=0.025, p=0.005).

*Venous endotoxin concentrations* in Control (n=3), Control-Yaq-001 (n=3), 6-week $CCl_4$ (n=10) and 6-week $CCl_4$-Yaq-001 groups(n=10). Significantly higher venous endotoxin was observed in $CCl_4$ mice compared to Control mice (p=0.007). Yaq-001 administration was associated with a significant reduction of venous endotoxin compared to untreated-$CCl_4$ mice (p=0.007).

(B) Mice underwent $CCl_4$ injection for 12-weeks as a model of cirrhosis (n=6-12/group) and the treatment groups received Yaq-001 for 6-weeks before sacrifice.

*Plasma ALT, ALP and TBIL* concentrations in Control (n=6), Control-Yaq-001 (n=6), 12-week $CCl_4$ (n=12) and 12-week $CCl_4$-Yaq-001 (n=12) groups. Significantly higher ALT, ALP and TBIL were observed in $CCl_4$ compared to controls (p=0.0001, p=0.0008, p=0.007). Yaq-001-treated $CCl_4$ mice had a significantly lower ALT compared to untreated-$CCl_4$ mice (p<0.0001).

*H&E and PSR staining of liver tissue* in $CCl_4$ mice. $CCl_4$ mice were associated with a significant increase in CPA compared to Controls (p=0.0001). Yaq-001 had significant effect on CPA in $CCl_4$-Yaq-001 compared to $CCl_4$ mice(p=0.012).

*TUNEL staining of liver tissues*. Significantly higher TUNEL staining was observed in $CCl_4$ compared to Controls (p=0.0001). Yaq-001-treated $CCl_4$ mice had a significantly lower TUNEL staining compared to untreated-$CCl_4$ (p=0.017) indicative of a reduction in liver cell death with Yaq-001 treatment.

*Venous ammonia*. Significantly increased ammonia concentrations were observed in $CCl_4$ compared to Controls (p=0.001). Yaq-001 significantly decreased venous ammonia concentrations in $CCl_4$ mice (p=0.035).

*Serum creatinine*: Yaq-001 significantly decreased serum creatinine levels in $CCl_4$ mice (p=0.003).

*Venous endotoxin concentrations* in Control (n=3), Control-Yaq-001 (n=3), 12-week $CCl_4$ (n=10) and 12-week $CCl_4$-Yaq-001 groups(n=10). Significantly higher venous endotoxin was observed in $CCl_4$ mice compared to Control mice (p=0.007). Yaq-001 administration was associated with a significant reduction of venous endotoxin compared to untreated-$CCl_4$ mice (p=0.043).

**Fig.5. Effect of Yaq-001 on gut permeability in intestinal organoids.**

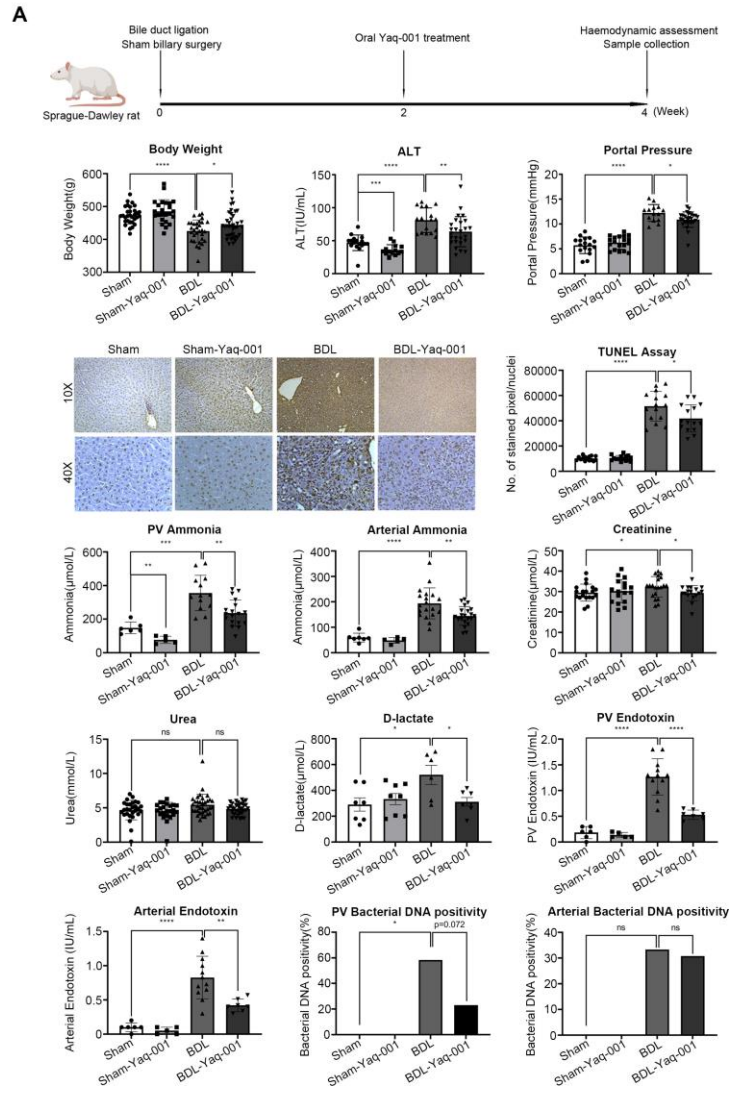
(A) Intestinal organoids derived and cultured from small intestine of C57BL/6 mice underwent eversion into apical-out polarity in the first 12 h of suspension culture. Immunostaining of the microvilli (mv; F-actin) demonstrated that intestinal organoids in suspension have reversed polarity from basolateral-out to apical-out.

(B) Apical-out intestinal organoids in suspension culture generate goblet cells (MUC2).

(C)Gut permeability of apical-out intestinal organoids was significantly increased by coculturing with fecal water from $CCl_4$ group than control group (p=0.003). Gut permeability was notably decreased in fecal water from $CCl_4$-Yaq-001 group compared to CCl4 group (p=0.001).

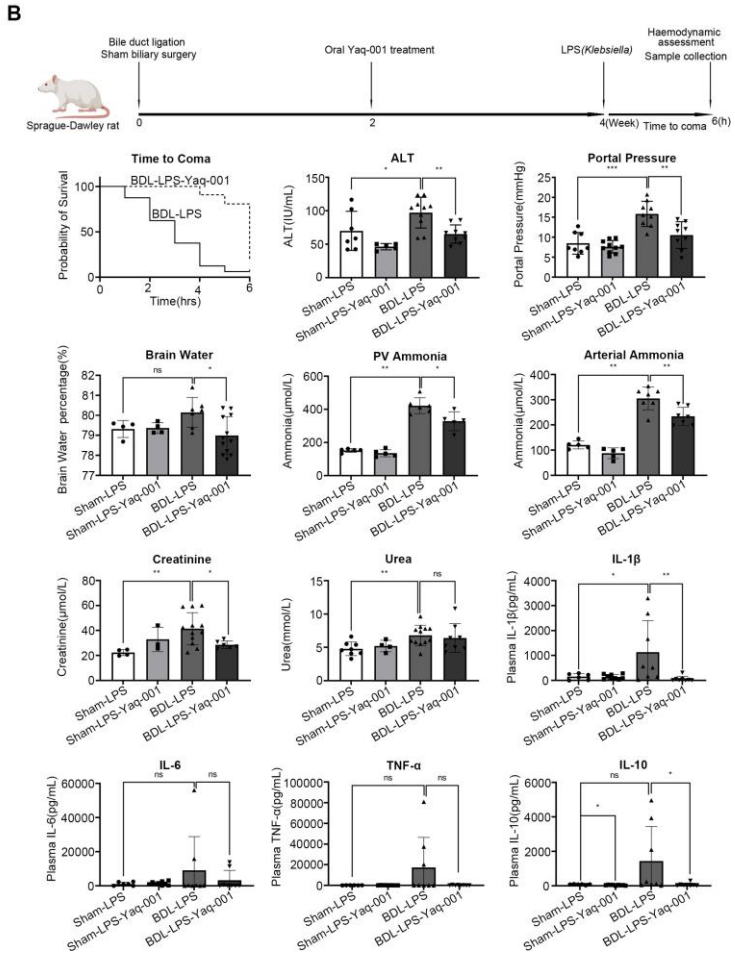
(D)Quantification of the integrated density/area of each group.

## Figure 1

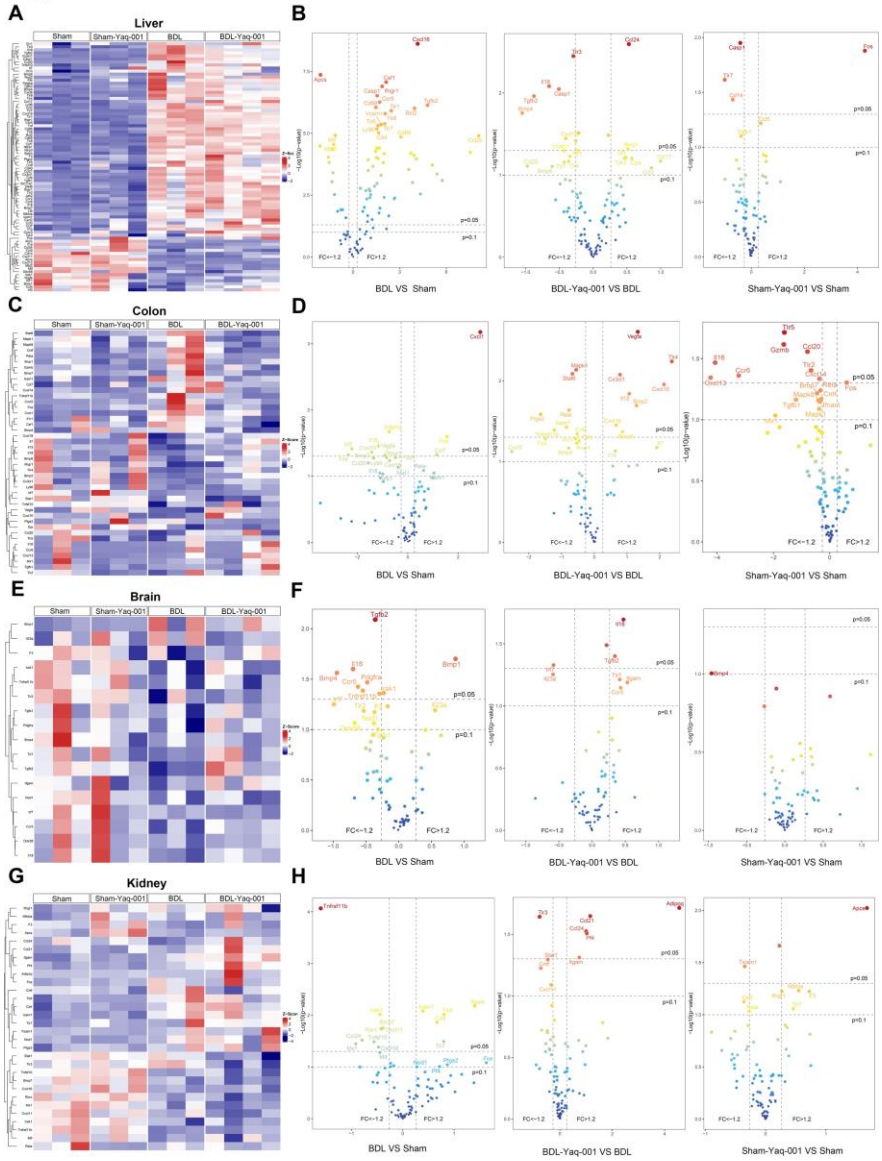



1359

## Figure 1

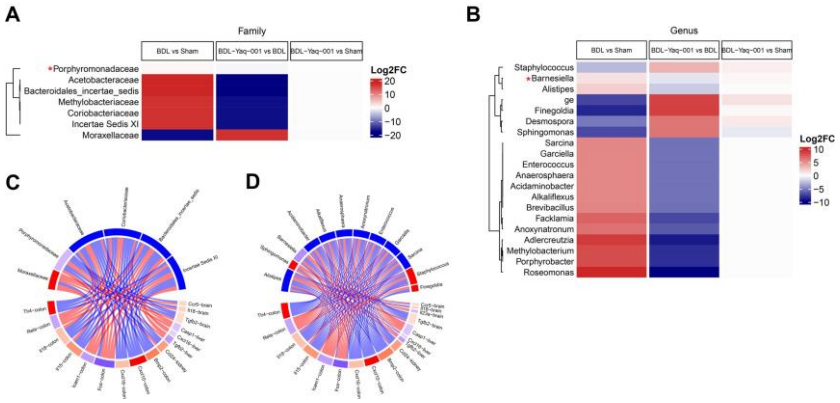

B
Bile duct ligation
Sham biliary surgery
Oral Yaq-001 treatment
LPS(Klebsiella)
Haemodynamic assessment
Sample collection
Sprague-Dawley rat
0
2
4(Week)
Time to coma
6(h)
Time to Coma
BDL-LPS-Yaq-001
BDL-LPS
Probability of Surival
Time(hrs)
ALT
ALT(IU/mL)
Portal Pressure
Portal Pressure(mmHg)
Brain Water
Brain Water percentage(%)
PV Ammonia
Ammonia(μmol/L)
Arterial Ammonia
Creatinine
Creatinine(μmol/L)
Urea
Urea(mmol/L)
IL-1β
Plasma IL-1β(pg/mL)
IL-6
Plasma IL-6(pg/mL)
TNF-α
Plasma TNF-α(pg/mL)
IL-10
Plasma IL-10(pg/mL)
Sham-LPS
Sham-LPS-Yaq-001
BDL-LPS
BDL-LPS-Yaq-001

## Figure 2




1361

## Figure 3


A
Family
BDL vs Sham
BDL−Yaq−001 vs BDL
BDL−Yaq−001 vs Sham
*Porphyromonadaceae
Acetobacteraceae
Bacteroidales_incertae_sedis
Methylobacteriaceae
Coriobacteriaceae
Incertae Sedis XI
Moraxellaceae
Log2FC
20
10
0
−10
−20
B
Genus
BDL vs Sham
BDL−Yaq−001 vs BDL
BDL−Yaq−001 vs Sham
Staphylococcus
*Barnesiella
Alistipes
ge
Finegoldia
Desmospora
Sphingomonas
Sarcina
Garciella
Enterococcus
Anaerosphaera
Acidaminobacter
Alkaliflexus
Brevibacillus
Facklamia
Anoxynatronum
Adlercreutzia
Methylobacterium
Porphyrobacter
Roseomonas
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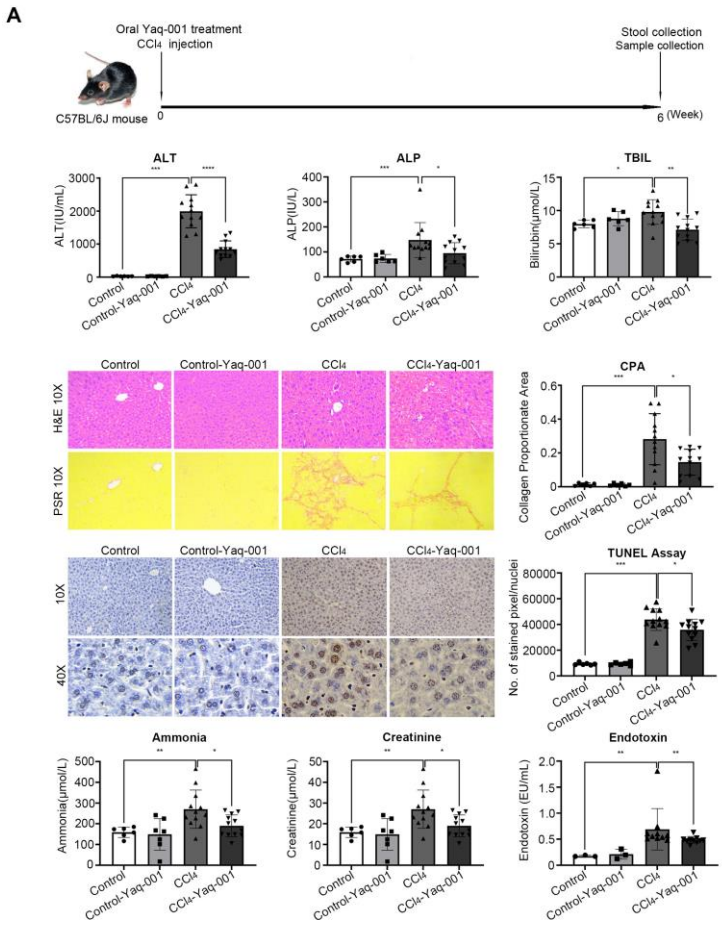
10
5
0
−5
−10
C
D

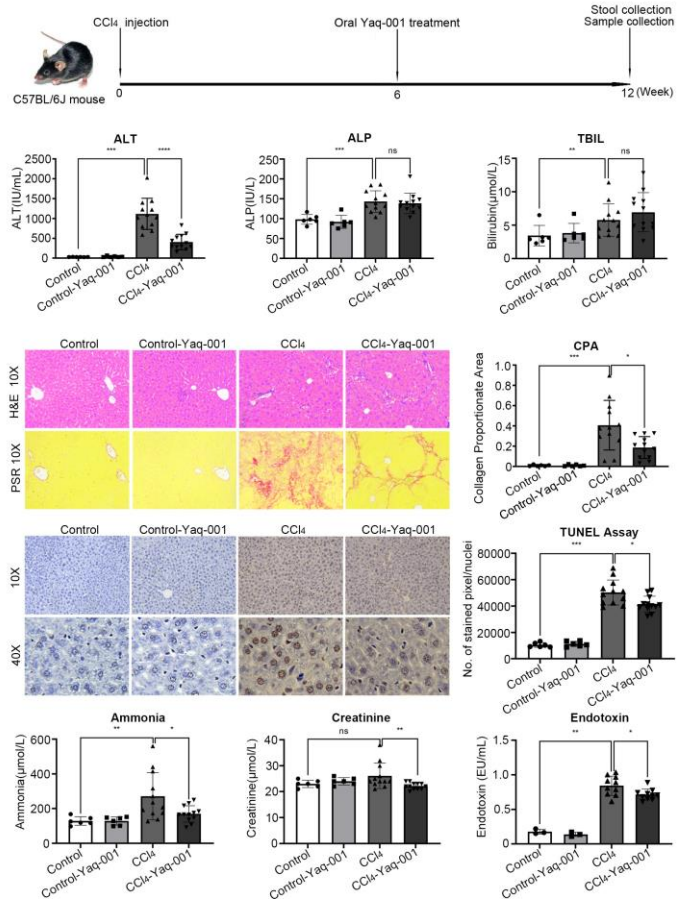
## Figure 4




1363

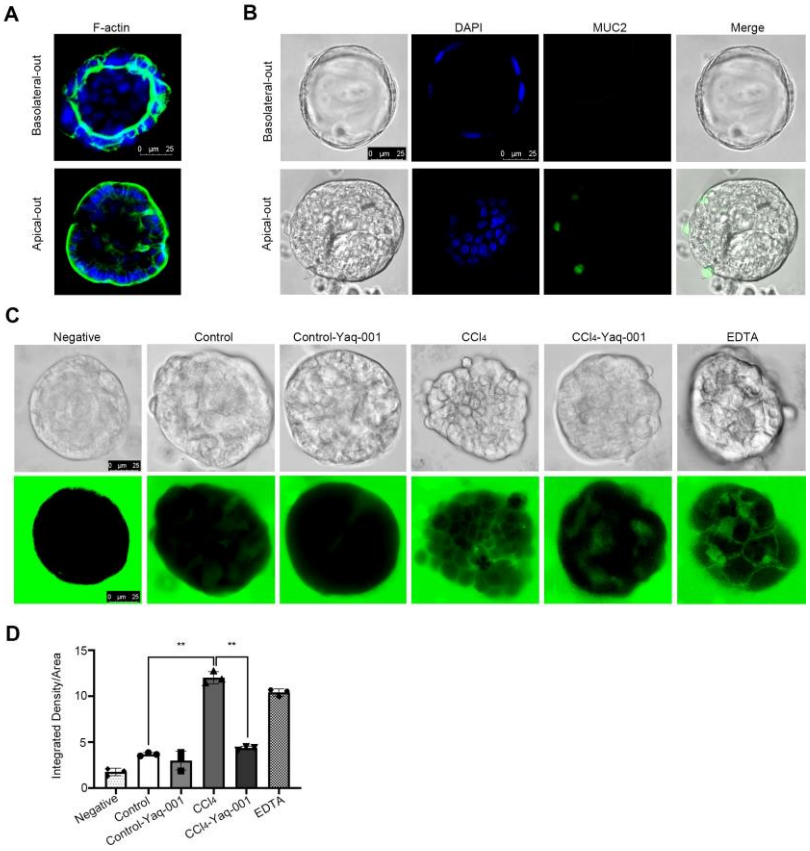
## Figure 4

**B**




1364

## Figure 5




1365

**Table 1.** Patient Characteristics

| | **Placebo** | **Active** |
|---|---|---|
| Number | 12 | 14 |
| Age(years) | 58.5(35-75) | 58.5(47-68) |
| Male sex | 9(75%) | 10(71.4%) |
| Race/ethnicity | | |
| Black | 0(0%) | 0(0%) |
| Other | 12(100%) | 14(100%) |
| BMI (kg/m$^2$) (SD) | 27.8(22.9-32.8) | 26.3(20.4-32.8) |
| Child Pugh Score | 7(7-8) | 7(7-8) |
| MELD score | 13.2(10.2-16.1) | 12.6(9.7-13.5) |
| Decompensation history | | |
| Alcoholic hepatitis | 2(16.7%) | 0(0.0%) |
| Ascites | 8(66.7%) | 11(78.6%) |
| Hepatic encephalopathy | 5(41.6%) | 2(14.3%) |
| Varices | 2(16.7%) | 6(42.9%) |
| Laboratory values | | |
| Bilirubin( μ mol/L) | 31(19-40) | 41(17-68) |
| Albumin(g/L) | 34(30-38) | 34(30-39) |
| Creatinine ( μ mol/L) | 65(53-83) | 70(60-81) |
| Sodium(mmol/L) | 136.5(134-137) | 137.2(134-140) |

**Table 2.** Adverse and Serious Events

| **Adverse Event** | **Placebo n (%)** | **Active n (%)** |
|---|---|---|
| Constipation | 3(25%) | 2(14%) |
| Epigastric pain | 0(0%) | 1(7%) |
| Nausea | 0(0%) | 1(7%) |
| Meteorism | 0(0%) | 1(7%) |
| Osophageal reflux | 0(0%) | 1(7%) |
| Diarrhoea | 2(17%) | 0(0%) |
| Diuresis | 0(0%) | 1(7%) |
| **Serious Event** | | |
| Death | 0(0%) | 0(0%) |
| 50% increase in MELD | 0(0%) | 0(0%) |
| 100% increase in creatinine | 0(0%) | 0(0%) |
| 50% reduction in BMI | 0(0%) | 0(0%) |
| Acute decompensation | 0(0%) | 0(0%) |
| Episode of ACLF | 0(0%) | 0(0%) |

**Table 3. Safety Parameters: Clinical laboratory assessments, Royal Free Global Assessment and Micronutrient concentrations**

| Parameters | Placebo | | | Active | | |
|---|---|---|---|---|---|---|
| | Baseline | 4 Weeks | 12 Weeks | Baseline | 4 Weeks | 12 Weeks |
| **Laboratory Parameters** | **Median(range)** | | | **Median(range)** | | |
| Haemoglobin(g/dL) | 122(108-143) | 119(107-145) | 119(105-139) | 122(113-134) | 123(113-133) | 121(110-137) |
| Leucocyte count($10^9$/L) | 4.22(3.47-4.97) | 4.05(3.43-4.84) | 3.94(3.20-4.90) | 5.22(4.20-6.99) | 5.12(4.21-6.09) | 4.70(4.26-5.52) |
| Platelets ($10^9$/L) | 81(75-113) | 93(83-104) | 93(85-100) | 95(81-127) | 84(75-107) | 93(75-105) |
| Bilirubin(µmol/L) | 41(17-68) | 31(16-79) | 47(20-71) | 31(19-40) | 42(21-52) | 31(17-47) |
| ALT(IU/L) | 25.9(19.88-8.55) | 22.89(18.07-31.33) | 24.1(18.07-31.33) | 25.9(24.1-34.94) | 27.11(24.1-33.13) | 28.31(19.88-34.94) |
| ALP(IU/L) | 49.2(37.8-52.8) | 37.2(24-54) | 40.8(27-56.4) | 46.8(31.2-51) | 46.8(30-69) | 45(30-61.2) |
| Albumin(g/L) | 34(30-39) | 35(31-40) | 32(31-38) | 34(30-38) | 35(30-38) | 32(30-38) |
| Sodium(mmol/L) | 137(135-140) | 138(132-140) | 138(135-140) | 137(134-137) | 136(132-137) | 137(136-138) |
| Creatinine (µmol/L) | 70(60-81) | 77(63-83) | 65(63-90) | 65(53-83) | 64(53-75) | 70(57-72) |
| INR | 1.4(1.3-1.6) | 1.3(1.2-1.5) | 1.4(1.3-1.5) | 1.3(1.2-1.4) | 1.4(1.2-1.4) | 1.3(1.2-1.4) |
| Child Pugh Score | 7(7-8) | 7(6-8) | 8(6-8) | 7(7-8) | 8(6.7-9) | 8(7-9) |

| | | | | | | |
|---|---|---|---|---|---|---|
| MELD score | 12(10-17) | 12(10-17) | 13(12-16) | 13(11-14) | 14(11-14) | 13(9-15) |
| MELD Na score | 15(10-21) | 13(9-19) | 16(12-21) | 15(13-17) | 16(15-17) | 14(13-19) |
| **Nutritional Status** | | **n (%)** | | | **n (%)** | |
| Adequate | 10(83%) | 8(73%) | 10(91%) | 13(93%) | 13(100%) | 10(77%) |
| Moderate malnourishment | 2(17%) | 3(27%) | 1(9%) | 1(7%) | 0(0%) | 3(23%) |
| Severe malnourishment | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) |
| **Micronutrient Concentrations** | | **Median(range)** | | | **Median(range)** | |
| Vitamin B12(ng/L) | 533(290-765) | 530(384-970) | 524(275-815) | 503(337-790) | 483(365-694) | 462(326-749) |
| Folate(µg/L) | 16(9-22) | 17(13-19) | 23(21-26) | 22(20-30) | 20(18-24) | 21(17-27) |
| Vitamin D(nmol/L) | 28(20-51) | 45(25-57) | 43(26-61) | 32(25-72) | 57(22-72) | 70(37-72) |
| Vitamin A(µmol/L) | 0.48(0.13-2.18) | 0.47(0.12-1.90) | 0.39(0.09-1.80) | 0.46(0.15-1.84) | 0.39(0.16-1.86) | 0.62(0.17-1.82) |
| Vitamin E(µmol/L) | 28.4(17.7-42.0) | 32.5(17.6-36.5) | 29.9(20.8-36.1) | 30.0(19.5-45.6) | 30.3(18.3-46.2) | 29.4(17.7-37.9) |
| Vitamin K(µg/L) | 0.58(0.16-3.28) | 0.33(0.12-2.5) | 0.35(0.17-4.2) | 0.58(0.14-4.3) | 0.41(0.12-5.7) | 0.60(0.14-2.8) |
| Copper(µmol/L) | 13.6(8.30-22.6) | 13.6(7.10-23.7) | 13.5(11.2-24.1) | 13.7(10.0-30.1) | 15.5(9.10-26.7) | 15.1(7.80-37.6) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Zinc(μmol/L) | 9.10(5.60-15.3) | 8.85(5.00-13.7) | 8.40(5.70-14.3) | 8.20(5,60-12.9) | 8.60(4.70-15.0) | 9.00(5.00-15.8) |
| Selenium(μmol/L) | 0.78(0.56-1.10) | 0.86(0.47-1.12) | 0.90(0.65-1.04) | 0.77(0.55-1.14) | 0.93(0.48-1.23) | 0.91(0.49-1.46) |