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ORIGINAL ARTICLE

The impact of artificial liver support system on intestinal microbiota and serum bile acid profiles in patients with acute-on-chronic liver failure: a prospective cohort study

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Abstract

Background Acute-on-chronic liver failure (ACLF) patients exhibit an imbalance in intestinal microbiota, and bile acids (BAs) can affect the composition of intestinal microbiota. Although Artificial liver support system (ALSS) is a treatment for ACLF, the impact of ALSS on intestinal microbiota and serum BA profiles of ACLF patients remains unclear.

Methods A prospective study was conducted, which included 51 patients diagnosed with ACLF. These patients were stratified into two groups based on the utilization of an ALSS during their treatment period: a standard medical treatment group (SMT group), comprising 19 patients, and an ALSS combined with SMT group (ALSS group), comprising 32 patients. Blood and stool samples were collected from the patients on the day of admission and 14 days after treatment. Additionally, eight healthy controls were recruited, and their stool samples were also collected. The intestinal microbiota was sequenced using the 16S rRNA sequencing technique, while the serum BA profiles were determined using ultra-performance liquid chromatography/mass spectrometry.

Results ACLF patients exhibited imbalances in intestinal microbiota and abnormalities in BA profiles. Compared to SMT alone, the combined ALSS and SMT was more effective in regulating intestinal microbiota imbalance and increasing the concentrations of ursodeoxycholic acid and glycoursodeoxycholic acid. Correlation analysis revealed a significant correlation between intestinal microbiota and Bas. Furthermore, the preliminary correlation heatmap indicated that the *Faecalibaculum*, *Gemmiger*, and taurochenodeoxycholic acid were associated with clinical improvement.

Conclusions Our study identified the compositional characteristics of the intestinal microbiota and serum BA in ACLF patients, emphasizing the impact of ALSS on both intestinal microbiota and serum BA profiles.

Keywords Acute-on-chronic liver failure · Artificial liver support system · Intestinal microbiota · Bile acid

Yuyu Zeng, Dakai Gan and Kaige Zhang have contributed equally to this work.

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Abbreviations

ACLF	Acute-on-chronic liver failure
ALSS	Artificial liver support system
SMT	Standard medical treatment
BA	Bile acid
CA	Cholic acid
TCA	Taurocholic acid
GCA	Glycocholic acid
CDCA	Chenodeoxycholic acid
TCDCA	Taurochenodeoxycholic acid
GCDCA	Glycochenodeoxycholic acid
DCA	Deoxycholic acid
TDCA	Taurodeoxycholic acid
GDCA	Glycodeoxycholic acid
LCA	Lithocholic acid
TLCA	Taurolithocholic acid
GLCA	Glycolithocholic acid

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UDCA	Ursodeoxycholic acid
TUDCA	Tauroursodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
HDCA	Hyodeoxycholic acid
THDCA	Taurohyodeoxycholic acid
GHDCA	Glycohyodeoxycholic Acid
BSH	Bile salt hydrolase
PE	Plasma exchange
DPMAS	Double plasma molecular adsorb system
PDF	Plasma diafiltration
CHDF	Continuous hemodiafiltration
NAT	Nutrition support team
APASL	Asian pacific association for the study of the liver
MAFLD	Metabolic associated fatty liver disease

Introduction

Acute-on-chronic liver failure (ACLF) is defined as the acute hepatic decompensation occurring on the basis of chronic hepatitis or cirrhosis, primarily characterized by hyperbilirubinemia and severe coagulation dysfunction [1]. The hepatic injury in ACLF patients is exceptionally severe, characterized by extensive necrosis of hepatocytes within a brief timeframe. The majority of patients exhibit varying degrees of cholestasis, which is primarily caused by extensive hepatocyte necrosis, resulting in impaired bile flow into the intestine. The principal constituents of bile encompass water, bile pigments, bile acids (BAs), cholesterol, and inorganic salts.

BAs are bioactive molecules synthesized in the liver from cholesterol. BAs exhibit a unique amphipathic nature, encompassing hydrophilic hydroxyl and carboxyl groups alongside hydrophobic alkyl chains. The degree of hydrophobicity in BAs inversely correlates with the number of hydroxyl groups present in their side chains. Crucially, the toxicity of BAs is intimately linked to their hydrophobicity. As hydrophobicity increases, so does the toxicity, attributed to their enhanced insolubility at equivalent concentrations [2]. BAs exhibit diverse physiological functions. Firstly, their amphipathic nature confers them with robust interfacial activity, enabling them to reduce the surface tension between oil and water phases, thereby promoting lipid emulsification and facilitating the digestion and absorption of lipids and fat-soluble [3]. Secondly, BAs serve as signaling molecules, modulating the expression of genes associated with glucose, lipid, and energy metabolism [4]. However, when the concentrations of certain BAs within the blood or liver surpass their physiological thresholds, high concentrations of BAs can directly compromise cellular membranes, trigger sterile inflammatory responses, and ultimately induce necrosis or apoptosis of hepatocytes and cholangiocytes, leading to hepatic injury [5, 6].

The intestinal microbiota comprises a diverse assemblage of microorganisms in the human digestive system [7]. There is a close and interactive relationship between the intestinal microbiota and BAs. After primary BAs reach the intestine, approximately 95% of them are reabsorbed by intestinal mucosal epithelial cells in the terminal ileum [8]. The remaining BAs that are not reabsorbed reach the colon and are gradually converted into secondary BAs by intestinal microbiota possessing bile salt hydrolase (BSH) and 7α-dehydroxylase activity. These enzymes catalyze the conversion of cholic acid (CA) into deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) into lithocholic acid (LCA) [9]. DCA, LCA, CDCA, and glycochenodeoxycholic acid (GCDCA) are potent hydrophobic BAs exhibiting notable antibacterial properties. When some microbiome are exposed to a certain concentration of potent hydrophobic BAs, the integrity of their cell membranes is disrupted, resulting in DNA damage and oxidative stress, which ultimately causes the death of some microbiome [10]. However, when the obstruction of bile flow into the intestine, the concentration of BAs in the intestine decreases, which can result in excessive proliferation of intestinal microbiome and damage to the intestinal mucosa, ultimately leading to bacterial translocation and systemic infection.

The artificial liver support system (ALSS) is a highly effective blood purification technology that temporarily compensates for the detoxifying functions of the liver. It effectively removes toxic substances from the blood, including bilirubin, endotoxin, cytokines, and others, thereby creating conditions for hepatocyte regeneration and liver function recovery or serving as a bridge prior to liver transplantation [11]. Previous studies have demonstrated that compared to standard medical treatment (SMT) alone, ALSS can improve the prognosis of patients with ACLF and prolong survival time [12].

Previous studies have demonstrated the imbalance of intestinal microbiota in patients with ACLF [13, 14]. Nevertheless, the specific influence of ALSS on the intestinal microbiota composition in these patients remains incompletely understood. Furthermore, considering the intricate interactions among intestinal microbiota, BAs, and the liver, and with only a single study exploring the effects of ALSS on the serum BAs in ACLF patients [15]. Therefore, this study aims to clarify the unique impact of ALSS on intestinal microbiota and serum BAs in ACLF patients. Furthermore, a correlation heatmap is utilized to visualize the relationship between intestinal microbiota, serum BAs, and clinical outcomes in ACLF patients, thereby contributing to a deeper understanding of the pathophysiology and potential therapeutic targets in ACLF.

Materials and methods

Study participants

We prospectively recruited all patients with ACLF hospitalized in the Affiliated Infectious Diseases Hospital of Nanchang University from May 2023 to December 2023. These patients were diagnosed in accordance with the diagnostic criteria recommended by the Asian Pacific Association for the Study of the Liver (APASL) [1]: patients exhibiting acute hepatic insult, characterized by jaundice (serum bilirubin ≥ 5 mg/dL and coagulopathy (INR ≥ 1.5 or prothrombin activity < 40%) complicated within 4 weeks by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis, and is associated with a high 28 day mortality. Patients were excluded if they met any of the following criteria: (1) patients who received antibiotic, probiotic, or ursodeoxycholic acid (UDCA) treatment within 2 weeks before sample collection; (2) patients with biliary obstruction; (3) patients with severe diseases of vital organs such as heart, brain, lung, and kidney; (4) patients with circulatory failure. Additionally, eight healthy volunteers who underwent physical examination in our hospital during the same period were included as the healthy control group. Referring to the "Clinical guidelines on nutrition in end-stage liver disease in China (2019 Edition)", the general dietary principle for patients with end-stage liver disease is "low-fat, low-protein, and high-carbohydrate." In this study, the dietary management of ACLF patients was conducted by a nutrition support team (NST) composed of hepatologists, nurses, nutritionists, and pharmacists from our hospital. The NST developed an individualized full-course nutrition intervention plan for each patient. There was no difference in the dietary structure between the two groups of patients. This study was conducted at the Affiliated Infectious Diseases Hospital of Nanchang University. Informed consent was obtained from all individual participants included in the study, and the study was supervised by the Ethics Committee of the Affiliated Infectious Diseases Hospital of Nanchang University with an ethics approval number of [2023] LJSZ (19).

Samples collection

Blood and stool samples were collected from patients on the day of admission and at 14 days after treatment. Blood samples were taken from patients after an 8 h fasting period, and were immediately centrifuged at 3500 revolutions per minute for 5 min. Ten milliliters of supernatant were extracted and stored at – 80 °C in a constant temperature freezer. Similarly, the collected stool samples were immediately stored at a – 80 °C constant temperature freezer for cryopreservation. All patients underwent serum BA profiling and stool microbiota analysis, and stool samples from eight healthy controls were also collected for microbiota analysis.

Grouping method

ACLF patients were divided into two groups based on whether they received ALSS treatment during the period from admission to the second sample collection. Specifically, patients who underwent ALSS in conjunction with SMT were assigned to the ALSS group, whereas those who underwent only SMT were assigned to the SMT group. SMT encompassed bed rest, treatment of underlying causes, administration of liver-protecting, jaundice-reducing, and enzyme-lowering drugs, maintenance of electrolyte and acid–base balance, and active prevention and treatment of complications. The treatment modalities of ALSS encompassed plasma exchange (PE), double plasma molecular adsorption system (DPMAS), hemofiltration, plasma diafiltration (PDF), and continuous hemodiafiltration (CHDF), among others. For ACLF patients with normal renal function, significant hyperbilirubinemia, and low PTA, the primary treatment choice was PE combined with DPMAS; for patients with abnormal renal function, the choice was PE combined with PDF or CHDF. In summary, there are many ALSS modalities, each with its own advantages and disadvantages, depending on the patient's condition, auxiliary examinations, personal wishes and economic situation. Each patient received ranging from one to three times, with an interval of 3–7 days between each treatment. The assessment of therapeutic outcomes adhered strictly to the criteria established by the APASL [1]: clinical improvement was defined as a remission of symptoms at the time of discharge, while clinical deterioration was defined as poor disease control or even death at the time of discharge.

High throughput sequencing

The collected stool samples were transported under low temperature conditions to Personal Biotechnology, Co., Ltd (Shanghai, China) for sequencing. Initially, a DNA extraction kit was utilized to extract the colony DNA. Subsequently, the V4(a) region of the 16S rRNA gene was amplified using the primer sets 520F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′). Double-ended sequencing of the colony DNA fragments was performed using the Illumina platform. ASVs/OTU sequences were obtained through the DADA2 method, and Quantitative Insights into Microbial Ecology 2 (QIIME2) was employed for

analysis. Categorization was achieved by comparison with the Greengenes database 13.8. The fecal samples collected were subjected to α-diversity, β-diversity, and species composition difference analyses. High-throughput sequencing data of the study have been uploaded to the NCBI database (https://www.ncbi.nlm.nih.gov/), under accession number PRJNA1096945.

Bile acid profiling

The collected serum samples were transported under low temperature conditions to Personal Biotechnology, Co., Ltd (Shanghai, China) for detecting. Firstly, metabolites were extracted through sample collection and pretreatment, and then detected by the ultra-performance liquid chromatography/mass spectrometry (UPLC-MS) (AB SCIEX QTRAP 6500+) machine. A database was constructed based on standard samples for qualitative analysis of the mass spectrometry data. The scheduled multiple reaction monitoring (MRM) mode of the triple quadrupole mass spectrometry was used to obtain the mass spectrometry analysis data of different samples. Chromatographic peaks of all targets were integrated, and quantitative analysis was performed through standard curves. Liquid chromatography/mass spectrometry profiling data of the study have been uploaded to the MetaboLights database (https://www.ebi.ac.uk/metabolights), under accession number MTBLS9898.

Statistical analysis

Data analysis and graphing were conducted using SPSS (v26.0) and GraphPad Prism (v8.0). For normal quantitative data, they were expressed as mean ± standard deviation (x ± s), and the *t*-test was used for comparison between groups. For skewed quantitative data, they were represented by the median with 25th and 75th percentiles [M(P25 ~ P75)], and the Mann-Whitney test was used for comparison between groups. For qualitative data, the chi-square test was used for comparison between groups. For multiple group comparisons, analysis of variance (ANOVA) was used. Regarding 16S sequencing, R software (v4.3.2) was used to calculate α-diversity indices (including Chao_1, Shannon, and Pielou_e) and β-diversity. Spearman correlation analysis was used to investigate the association between serum BAs and the intestinal microbiota. A significance level of $p < 0.05$ was set for all statistical tests.

Results

Enrollment of patients

A total of 51 patients with ACLF were enrolled in our study, with 19 patients in the SMT group and 32 patients in the ALSS group. Blood and stool samples were collected from all 51 patients on the day of hospitalization. However, during the subsequent 14 days after hospitalization, several events occurred that precluded the complete collection of second samples from all patients. Specifically, due to infections treated with antibiotics in 17 patients, transfer to another hospital for liver transplantation in 3 patients, and death in 5 patients. Finally, the second blood and stool samples were collected from 12 patients in the SMT group to 14 patients in the ALSS group, respectively (Fig. 1).

Baseline clinical characteristics

Compared with the SMT group, patients in the ALSS group exhibited higher levels of liver inflammation indicators, specifically alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ($p < 0.05$) (Table 1). However, there were no statistically significant differences in other indicators such as age, total bilirubin, and MELD scores (Table 1).

Clinical outcome of patients with ACLF

As shown in Table 2, a total of 24 patients (75.0%) in the ALSS group showed clinical improvement at the time of discharge, while 8 patients (42.1%) in the SMT group showed clinical improvement. The difference in the clinical improvement rates between the two groups was statistically significant ($p < 0.05$).

The impact of ALSS on the intestinal microbiota of ACLF patients

We successfully collected fecal samples from all 51 enrolled ACLF patients at the time of hospital admission (ACLF = 51). After 14 days of treatment, we collected second fecal samples from 12 patients in the SMT group (SMT = 12) to 14 patients in the ALSS group (ALSS = 14). The baseline clinical characteristics of the 26 ACLF patients whose second samples were collected after fourteen days of treatment are summarized in Table 3.

In the α-diversity analysis, the Chao 1 ($p < 0.05$) (Fig. 2a), Shannon ($p < 0.01$) (Fig. 2b), and Pielou_e ($p = 0.11$) (Fig. 2c) indexes in the ACLF group were significantly lower compared to the HC group. Compared to the SMT group, the above indexes in the ALSS group increased slightly, but there was no statistically significant distinction. Additionally, the β-diversity analysis based on the Bray-curtis distance algorithm revealed that there was a clear separation between the ACLF group and the HC group on both the PCo1 and PCo2 axes (Fig. 2d). There was no significant separation between the SMT group and the ALSS group on the PCo1 axis, but there

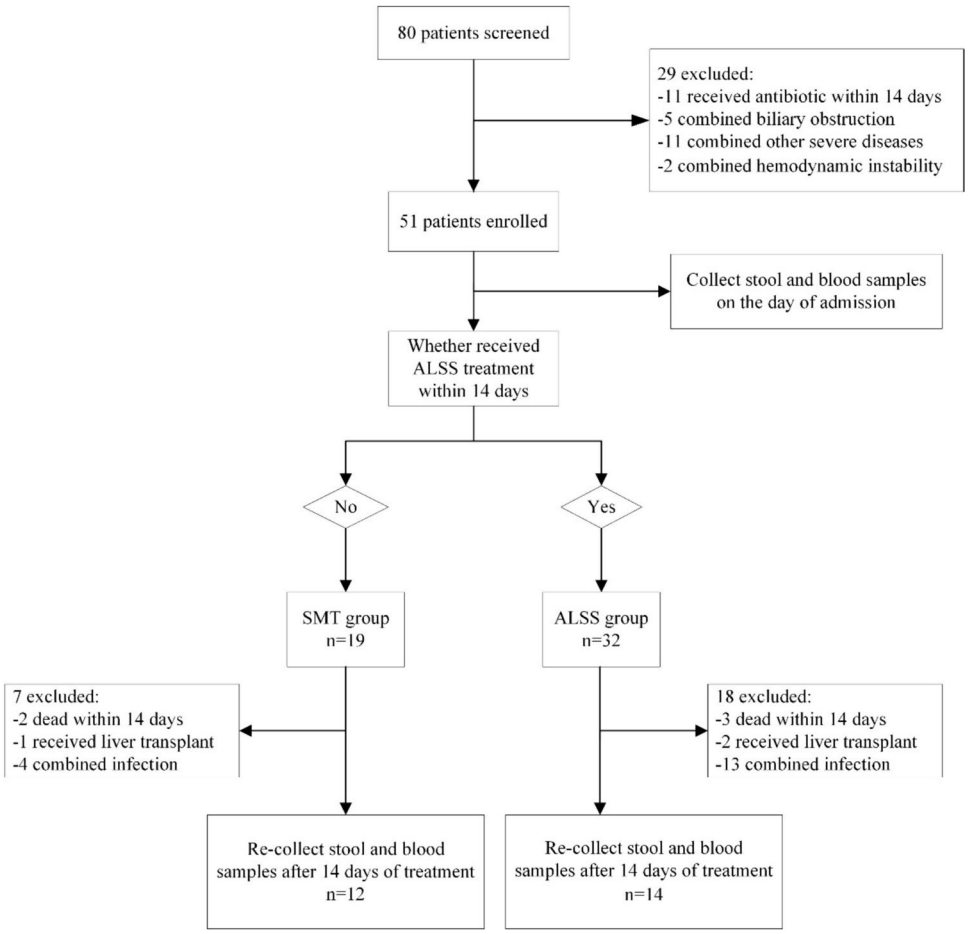


Fig. 1 Flowchart of the study

Table 1 Comparison of clinical characteristics between the SMT and ALSS group

	SMT ($n=19$)	ALSS ($n=32$)	p value
Gender (male, n [%])	16 (84.8)	28 (87.5)	1.000
Age (year, [X ± S])	44.3 ± 13.4	48.9 ± 15.2	0.287
Hemoglobin (g/L, [X ± S])	125.8 ± 22.9	125.7 ± 24.9	0.988
Platelet (10^9/L, M[P_{25} ~ P_{75}])	152 (85.3, 197.8)	114 (76.5, 144.0)	0.156
ALT (U/L, M[P_{25} ~ P_{75}])	127.1 (40.4, 598.1)	405.1 (206.7, 1030.2)	0.009
AST (U/L, M[P_{25} ~ P_{75}])	91.5 (56.5, 308.4)	222.7 (131.5, 787.7)	0.004
Total bilirubin(μmol/L, M[P_{25} ~ P_{75}])	219.3 (179.2, 290.9)	282.6 (233.3, 359.8)	0.206
Albumin (g/L, [X ± S])	33.7 ± 4.0	32.6 ± 5.4	0.448
BUN (mmol/L, M[P_{25} ~ P_{75}])	3.9 (3.4, 5.2)	3.5 (3.1, 5.6)	0.496
Creatinine (μmol/L, M[P_{25} ~ P_{75}])	68.3 (44.0, 115.4)	70.3 (38.4, 243.1)	0.535
INR (M[P_{25} ~ P_{75}])	1.9 (1.7, 2.2)	1.9 (1.7, 2.4)	0.460
PTA (%, M[P_{25} ~ P_{75}])	34.0 (29.5, 36.5)	32.0 (25.5, 37.0)	0.587
MELD score (M[P_{25} ~ P_{75}])	20.6 (19.3, 22.8)	20.5 (18.2, 24.9)	0.807

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BUN* blood urea nitrogen, *INR* international normalized ratio, *PTA* prothrombin activity

was some separation on the PCo2 axis. In conclusion, the α-diversity and β-diversity analyses demonstrated that the abundance and diversity of the intestinal microbiota in ACLF patients decreased, and ALSS could improve the intestinal microbiota imbalance in ACLF patients to a certain extent.

In the ASV/OTU analysis (Fig. 2e), the SMT group found 989 OTUs, while the ALSS group had a slightly

Table 2 Comparison of clinical outcomes between the SMT and ALSS group

Group	The number of patients (*n*)	Clinical improvement (*n*, [%])
ALSS	32	24 (75.0)
SMT	19	8 (42.1)
χ^2-value		5.519
p value		0.019

higher number of 1069 OTUs. Notably, 300 OTUs co-owned. To further compare the differences in species composition between samples, a heatmap was constructed using abundance correlation data of the top 20 genera based on their mean abundances. As shown in Fig. 2f, the ALSS group demonstrated a relatively higher abundance of beneficial bacterial genera, such as *Parabacteroides_B*, *Ligilactobacillus*, *Lactobacillus*, and *Phocaeicola_A*.

Subsequently, we conducted a further analysis of the differences in the relative abundance of species at the genus level among the four groups (Fig. 3g). The results indicated that, compared to the HC group, the ACLF group exhibited increased relative abundances of *Escherichia* ($p < 0.05$) (Fig. 3a) and *Streptococcus* ($p < 0.05$) (Fig. 3c), along with decreased relative abundances of *Collinsella* ($p < 0.05$) (Fig. 3b). These findings suggest alterations in the intestinal microbiota composition of ACLF patients, characterized by an overall increase in harmful microbiota and a decrease in beneficial microbiota. Interestingly, when comparing the ALSS group to the SMT group, we observed higher relative abundances of *Phocaeicola_A* ($p < 0.05$) (Fig. 3d) and *Ligilactobacillus* ($p < 0.05$) (Fig. 3f) in the ALSS group, along with lower relative abundances of *Escherichia* ($p < 0.05$) (Fig. 3a). These findings suggest that ALSS exerts a regulatory effect on the intestinal microbiota composition.

The impact of ALSS on the BA profiles of ACLF patients

Before treatment, serum samples were collected from 51 patients with ACLF (Pre-treat = 51). Fourteen days after treatment, serum samples were collected from 26 of these patients (Post-treat = 26). In this study, the concentrations of 18 BAs in the serum were quantified both pre- and post-treatment. Based on above the concentrations of 18 BAs, the levels of total BAs, total unconjugated and conjugated BAs, total primary and secondary BAs, as well as total hydrophobic and hydrophilic BAs were calculated. Among the four groups, the top five most abundant BAs were consistently glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), and glycoursodeoxycholic acid (GUDCA), accounting for over 90% of the concentration of total BAs (Fig. 4a). Subsequent Principal Component Analysis (PCA) of the four groups revealed that samples within the Post-treat group were more separated compared to the Pre-treat group. Additionally, samples within the ALSS group were more clustered compared to the SMT group (Fig. 5a).

Unconjugated and conjugated BAs

Serum BAs can be classified as unconjugated and conjugated forms based on whether they are bound to glycine or taurine. Compared to the Pre-treat group, the concentrations of CA, CDCA, and UDCA of the Post-treat group increased significantly ($p < 0.05$) (Fig. 4b,

Table 3 Comparison of clinical baseline characteristics among patients with available second samples

	SMT (*n* = 12)	ALSS (*n* = 14)	*p* value
Gender (male, *n* [%])	11 (91.7)	12 (85.7)	1.000
Age (year, [X ± S])	43.9 ± 14.8	44.6 ± 15.6	0.905
Hemoglobin (g/L, [X ± S])	119.5 ± 22.9	127.1 ± 25.6	0.433
Platelet (10^9/L, M[P_{25} ~ P_{75}])	125 ± 65.4	125.6 ± 52	0.978
ALT (U/L, M[P_{25} ~ P_{75}])	99 (20.7, 2863.8)	596.1 (91, 5549.2)	0.031
AST (U/L, M[P_{25} ~ P_{75}])	81.6 (29.1, 1737.2)	269.4 (90.9, 2088.3)	0.008
Total bilirubin(μmol/L, M[P_{25} ~ P_{75}])	222.8 (166.7, 620.9)	272.7 (125, 579.2)	0.176
Albumin (g/L, [X ± S])	32.8 ± 4.5	33.9 ± 4	0.525
BUN (mmol/L, M[P_{25} ~ P_{75}])	3.7 (2.1, 5.7)	3.5 (1.8, 10.6)	0.940
Creatinine (μmol/L, M[P_{25} ~ P_{75}])	55.6 (44.0, 91.7)	57.3 (38.4, 131.9)	0.677
INR (M[P_{25} ~ P_{75}])	2.1 (1.5, 3.1)	1.9 (1.6, 3.8)	0.929
PTA (%, M[P_{25} ~ P_{75}])	32.5 ± 5.1	31.5 ± 9.1	0.738
MELD score (M[P_{25} ~ P_{75}])	20.7 ± 2.9	22.2 ± 5.4	0.383

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BUN* blood urea nitrogen, *INR* international normalized ratio, *PTA* prothrombin activity

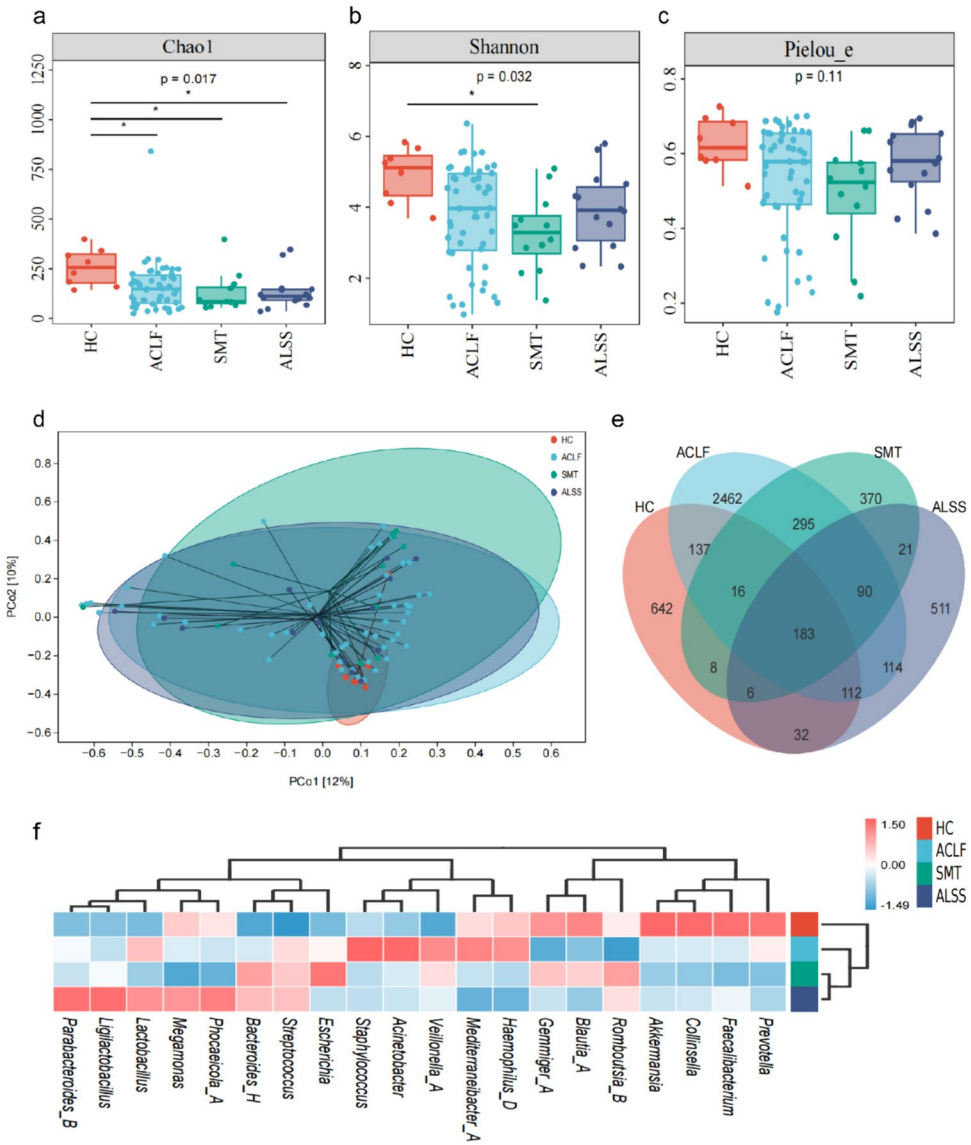


Fig. 2 The impact of ALSS on the intestinal microbiota richness and diversity of ACLF patients. **a** The Chao1 index. **b** The Shannon index. **c** The Pielou_e index. **d** PCoA of β-diversity index. **e** ASV/OTU Venn diagram. **f** The clustered heat map of microbiota abundance correlation. *HC* healthy control, *ACLF* Intestinal microbiota of ACLF patients on admission, *SMT* Intestinal microbiota in the SMT group after 14 days of treatment, *ALSS* Intestinal microbiota in the ALSS group after 14 days of treatment. $*p < 0.05$

c, f), while the concentrations of TCDCA and GCDCA decreased ($p < 0.05$) (Fig. 5b, c). No significant differences were observed in the concentrations of the remaining unconjugated and conjugated BAs, as well as the total unconjugated and total conjugated BAs. Compared to the SMT group, the concentrations of UDCA, GUDCA, and total unconjugated BAs were significantly higher in the ALSS group ($p < 0.05$) (Figs. 4f, 5d, f). However, there were no significant differences in the concentrations of the remaining unconjugated and conjugated BAs, as well as total conjugated BAs.

Primary and secondary BAs

Primary BAs undergo transformation into secondary BAs via the action of intestinal microbiota. Specifically, DCA is typically derived from the conversion of CA, whereas LCA is derived from CDCA. Upon comparison with the Pre-treat group, no significant differences were observed in the concentrations of total primary BAs or total secondary BAs in the Post-treat group. Similarly, a comparison between the ALSS and SMT groups revealed no significant differences in the concentrations of total primary and secondary BAs. These findings suggest that ALSS did not significantly impact the overall composition of primary and secondary BAs.

Hydrophobic and hydrophilic BAs

The hydrophobic BAs include CDCA, GCDCA, DCA, and LCA, while the hydrophilic BAs include CA, UDCA, hyodeoxycholic acid (HDCA) and their conjugated forms. Compared to the Pre-treat group, the concentrations of total hydrophilic BAs and total hydrophobic BAs in the

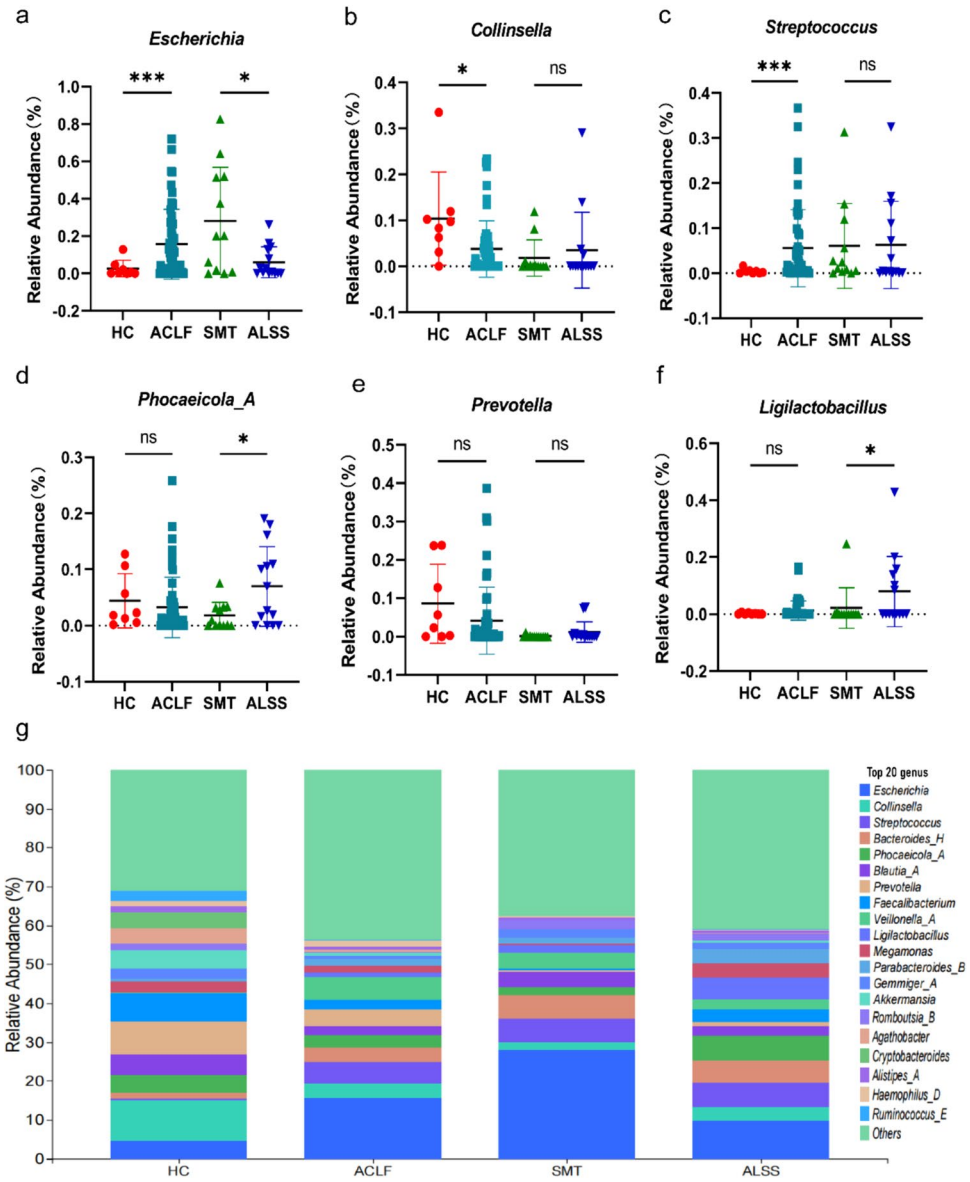


Fig. 3 The impact of ALSS on species composition of intestinal microbiota in ACLF patients. **a–f** The relative abundance of Escherichia, Collinsella, Streptococcus, Phocaeicola_A, Prevotella and Ligilactobacillus. **g** The species composition analysis of HC, ACLF, SMT and ALSS group. *HC* healthy control, *ACLF* Intestinal microbiota of ACLF patients on admission, *SMT* Intestinal microbiota in the SMT group after 14 days of treatment, *ALSS* Intestinal microbiota in the ALSS group after 14 days of treatment. *$p < 0.05$, *** $p < 0.001$, and ns indicating not significant

Post-treat group were significantly reduced ($p < 0.05$) (Fig. 5i, j). However, the ratio of total hydrophilic to total hydrophobic BAs increased ($p < 0.05$) (Fig. 5k). Additionally, when comparing the ALSS group to the SMT group, no significant differences were observed in the concentrations of total hydrophobic BAs or total hydrophilic BAs.

In summary, the serum BA profiles of patients with ACLF are rich in GCA, GCDCA, TCDCA, TCA, and GUDCA. After treatment, significant differences occurred in the concentrations of some unconjugated and conjugated BAs, as well as hydrophobic and hydrophilic BAs, while no significant differences were observed in the concentrations of total primary and secondary BAs. Compared to SMT, ALSS was effective in increasing the concentrations of UDCA and GUDCA.

Correlation analysis of intestinal microbiota, serum BAs, and clinical improvement

We used the spearman correlations analysis to investigate the correlation among the intestinal microbiota genera (top 20), serum BAs, and clinical improvement in ACLF patients. As shown in Fig. 6, various BAs generally exhibited a positive correlation, especially between the glycine- and taurine-conjugate forms of CA, CDCA, DCA, LCA, UDCA, and HDCA, which showed strong positive correlations ($p < 0.05$). Interestingly, this suggests that an increase in the synthesis of glycine-conjugated BAs promotes the synthesis of taurine-conjugated BAs, and vice versa. Additionally, except for *Prevotella*, *Megamonas* and *Ligilactobacillus*, *Streptococcus* and *Staphylococcus*, there were overall positive correlations among the other

genera, such as *Bacteroides_H*, *Collinsella*, *Blautia_A*, and *Faecalibacterium*.

However, we observed an overall negative correlation between intestinal microbiota genera and BAs, such as total primary BAs, total conjugated BAs, total hydrophobic BAs, total hydrophilic BAs, and most of the intestinal microbiota genera. Conversely, total secondary BAs exhibited a positive correlation with *Gemmiger*, *Mediterraneibacter_A*, and *Romboutsia_B* ($p < 0.05$). Additionally, Fig. 6 demonstrates a positive correlation between DCA along with its conjugated forms and *Ligilactobacillus* and a negative correlation with *Bacteroides_H* ($p < 0.05$). TCDCA and GCDCA showed a positive correlation with *Megamonas* ($p < 0.05$), and UDCA exhibited positive correlation with *Veillonella_A* ($p < 0.05$).

Interestingly, we also observed a positive correlation between *Faecalibacterium* and *Gemmiger* with clinical improvement ($p < 0.05$), whereas TCDCA exhibited a negative correlation with clinical improvement ($p < 0.05$).

Study on intestinal microbiota, serum BAs, and prognosis of ACLF patients

Firstly, we conducted the correlation analysis between the relative changes in *Escherichia* and UDCA before and after treatment in the SMT group and the ALSS group, and the clinical improvement of ACLF. In the SMT group, the correlation coefficient between the relative change in *Escherichia* before and after treatment and clinical improvement was − 0.205, with a p value of 0.523. The correlation coefficient between the relative change in UDCA before and after treatment and clinical improvement was 0.512, with a p value of 0.049(Supplementary Table 1). In the ALSS group, the correlation coefficient between the relative change in *Escherichia* before and after treatment and clinical improvement was − 0.497, with a p value of 0.071. The correlation coefficient between the relative change in UDCA before and after treatment and clinical improvement was 0.022, with a p value of 0.942(Supplementary Table 1).

Secondly, we performed the univariate logistic regression analyses of 18 BAs, the top 20 intestinal microbiota at the genus level, and clinical prognosis. Then, we included statistically significant indicators including TCA, TCDCA, and TLCA into the multivariate regression analyses (Supplementary Table 2). Finally, through multivariate regression analyses, we failed to find intestinal microbiota or BAs that contributed to clinical improvement ($p > 0.05$) (Supplementary Table 2).

Lastly, we presented the 90-day survival status of the increased and decreased groups of *Phocaeicola_A*, *Ligilactobacillus* and *Escherichia* using Kaplan–Meier survival curves, with all p values > 0.05 (Supplementary Fig. 1).

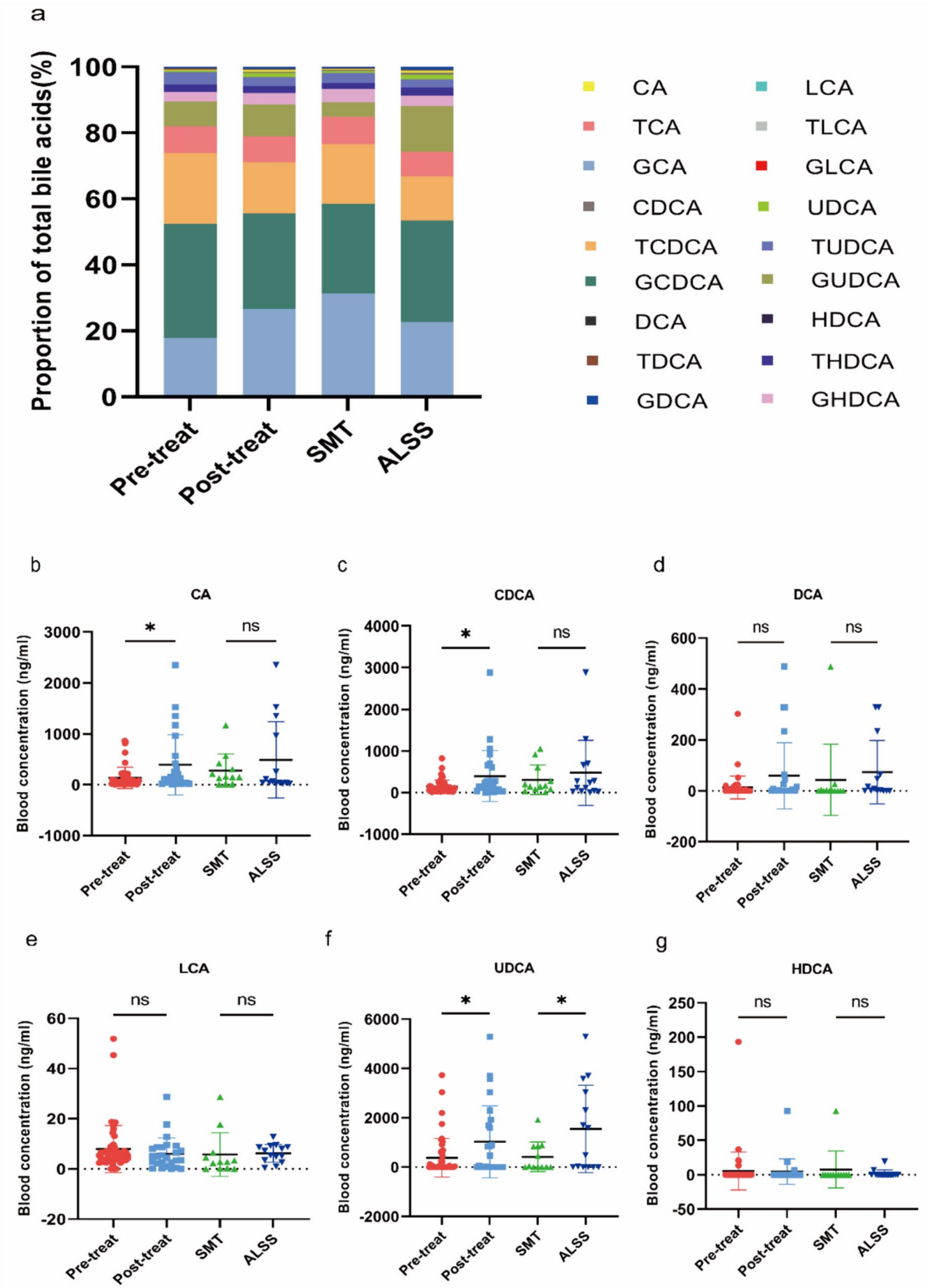
Discussion

In this study, we performed a comprehensive analysis of the intestinal microbiota and serum BA profiles in ACLF patients before treatment and after 14 days of treatment. Initially, the compositional characteristics of the intestinal microbiota and serum BA profiles in ACLF patients were described. Subsequently, significant changes in the intestinal microbiota and serum BA profiles were observed in ACLF patients after 14 days of treatment. Finally, through spearman correlation analysis, it was found that there was a significant correlation between the intestinal microbiota and serum BA profiles. Furthermore, ACLF patients were further divided into SMT and ALSS groups to observe the impact of ALSS on the intestinal microbiota and serum BA profile.

Using 16S rRNA sequencing, our study found that the abundance and diversity of the intestinal microbiota in ACLF patients were significantly reduced. Additionally, there was an increase in the relative abundance of opportunistic pathogens such as *Escherichia* and *Streptococcus*, while the relative abundance of the beneficial microbiota *Collinsella* decreased. These findings indicated the presence of intestinal microbiota dysbiosis in ACLF patients, consistent with previous reports [13, 14].

Escherichia is a commensal bacteria in the human intestine but often acts as an opportunistic pathogen involved in various opportunistic infections, such as spontaneous bacterial peritonitis in cirrhosis or urinary tract infections [16]. A recent study has shown that *Escherichia* promotes the occurrence of metabolic associated fatty liver disease (MAFLD) through flagellin [17]. *Streptococcus* is a common bacterium among pyogenic cocci, mostly opportunistic pathogens. Studies have shown that the relative abundance of *Streptococcus* is highly enriched in patients with alcoholic cirrhosis, and the relative abundance of *Streptococcus* can be used to predict the severity of liver injury in patients with alcoholic hepatitis [18]. *Collinsella* is a probiotic bacterium that plays an important role in maintaining intestinal health, promoting nutrient absorption, and reducing inflammatory responses [19]. In summary, ACLF patients have reduced abundance of beneficial bacteria and increased abundance of pathogenic bacteria, indicating intestinal microbiota dysbiosis.

Furthermore, by comparing the SMT group and the ALSS group, it was found that the relative abundances of *Phocaeicola_A* and *Ligilactobacillus* were significantly higher in the ALSS group ($p < 0.05$) (Fig. 3d, f), while the relative abundance of *Escherichia* was lower ($p < 0.05$) (Fig. 3a). This discovery suggests that ALSS can influence


a
Proportion of total bile acids(%)
100
80
60
40
20
0
Pre-treat
Post-treat
SMT
ALSS
CA
TCA
GCA
CDCA
TCDCA
GCDCA
DCA
TDCA
GDCA
LCA
TLCA
GLCA
UDCA
TUDCA
GUDCA
HDCA
THDCA
GHDCA
b
CA
c
CDCA
d
DCA
e
LCA
f
UDCA
g
HDCA
Blood concentration (ng/ml)
*
ns

◂**Fig. 4** The impact of ALSS on serum unconjugated BAs in ACLF patients. **a** Changes in serum BA composition before and after treatment. **b–g** The serum concentrations of CA, CDCA, DCA, LCA, UDCA and HDCA. Pre-treat: The blood concentration of BAs before treatment; Post-treat: The blood concentration of BAs after 14 days of treatment; *SMT* The blood concentration of BAs after 14 days of treatment in SMT group, *ALSS* The blood concentration of BAs after 14 days of treatment in ALSS group. *$p < 0.05$, and ns indicating not significant

the composition of the intestinal microbiota in ACLF patients. Both *Phocaeicola_A* and *Ligilactobacillus* are beneficial bacteria in the intestine. A recent study reported that *Phocaeicola_A* can attenuate the progression of MAFLD in mice by downregulating histone acetylation levels [20]. Other studies have shown that *Phocaeicola_A* can help alleviate atherosclerosis and colitis [21, 22]. *Ligilactobacillus* is a probiotic with significant potential that is widely involved in the intestinal metabolism and immune activities of the host [23]. Therefore, it can be seen that ALSS has a significant positive regulatory effect on the intestinal microbiota of ACLF patients, which is consistent with the conclusion of a previously reported study [24]. However, the reasons behind the increase in beneficial bacterial genera and decrease in harmful bacterial genera are not fully understood and require further investigation. This could involve conducting viability assays to identify the molecular factors driving their proliferation. In future studies, we still need to strive to investigate the causes of the aforementioned phenomena.

In this study, we observed a decrease in the serum concentrations of total hydrophilic BAs and total hydrophobic BAs, whereas an increase in the ratio of total hydrophilic BAs to total hydrophobic BAs in ACLF patients after 14 days of treatment. This indicates that the serum BA profiles became increasingly hydrophilic after treatment. The enhancement of the hydrophilic environment is beneficial in alleviate liver toxicity caused by the accumulation of certain BAs. Another important finding in our study is that compared with the SMT group, the concentrations of UDCA and GUDCA in the ALSS group increased significantly ($p < 0.05$). This indicates that ALSS contributes to increasing the concentrations of UDCA and its glycine-conjugate form. Consistent with our findings, Yu et al. also found that the serum UDCA concentration increased significantly after ALSS [15]. UDCA is converted from CDCA or HDCA endogenously. UDCA has diverse biological functions including the inhibition of hepatocyte apoptosis, antagonism against BA toxicity, and regulating immunity. It is widely used in the treatment of various chronic liver diseases and is a first-line drug for cholestatic liver diseases [25, 26]. Studies have shown that GUDCA has cytoprotective, anti-inflammatory, and antioxidant effects [27].

As depicted in Fig. 6, we also conducted an analysis of the correlation between the intestinal microbiota and serum BA profiles in patients with ACLF. The hydrolysis of primary conjugated BAs synthesized in the liver is the first crucial step in their conversion to secondary BAs in the intestine, a process that requires the involvement of BSH. BSH dissociates the glycine and taurine groups from primary conjugated BAs, and the released glycine and taurine residues serve as important nutritional sources for the intestinal microbiota [28]. *Bacteroides_H* has been confirmed to possess BSH activity [28], playing a significant role in the dissociation process. As shown in Fig. 6, *Bacteroides_H* exhibits a negative correlation with total conjugated BAs ($p < 0.05$). An increase in *Bacteroides_H* is accompanied by a decrease in total conjugated BAs and a corresponding increase in total unconjugated BAs. The hydroxylation of primary unconjugated BAs is the second crucial step in their conversion to secondary BAs in the intestine, a process that requires the involvement of 7α-hydroxylase. In addition to BSH activity, the *Bacteroides_H* also possesses 7α-hydroxylase activity [29]. Figure 6 demonstrates a negative correlation between *Bacteroides_H* and total primary BAs ($p < 0.05$). When *Bacteroides_H* increases, primary BAs are converted into secondary BAs through hydroxylation, resulting in a decrease in primary BAs and an increase in secondary BAs. In addition to *Bacteroides_H*, *Blautia*, *Eubacterium*, *Clostridium*, and *Roseburia* also possess BSH activity [28]. As depicted in Fig. 6, *Blautia* exhibits an inverse correlation with total conjugated BAs, although this correlation is not statistically significant ($p > 0.05$). However, in our study, the relative abundances of the other three bacteria exhibiting BSH activity were quite low, limiting their significance in this study.

Through further analysis of the clinical outcomes of ACLF patients in the SMT group and the ALSS group, it was found that ALSS is an effective treatment for ACLF, contributing to improved patient prognosis. Our study founded that compared to the SMT group, the relative abundance of beneficial intestinal microbiota such as *Phocaeicola* and *Ligilactobacillus* increased ($p < 0.05$) while the relative abundance of opportunistic pathogens such as *Escherichia* decreased ($p < 0.05$) after ALSS treatment. Furthermore, ALSS was found to increase the concentrations of UDCA and GUDCA in serum. Based on these findings, we speculate that the regulatory effects of ALSS on the intestinal microbiota and serum BA profiles may be additional mechanisms contributing to its effectiveness in treating ACLF. However, given the limited available research in this area, further intricate mechanistic studies are necessary to corroborate this speculation. Additionally,

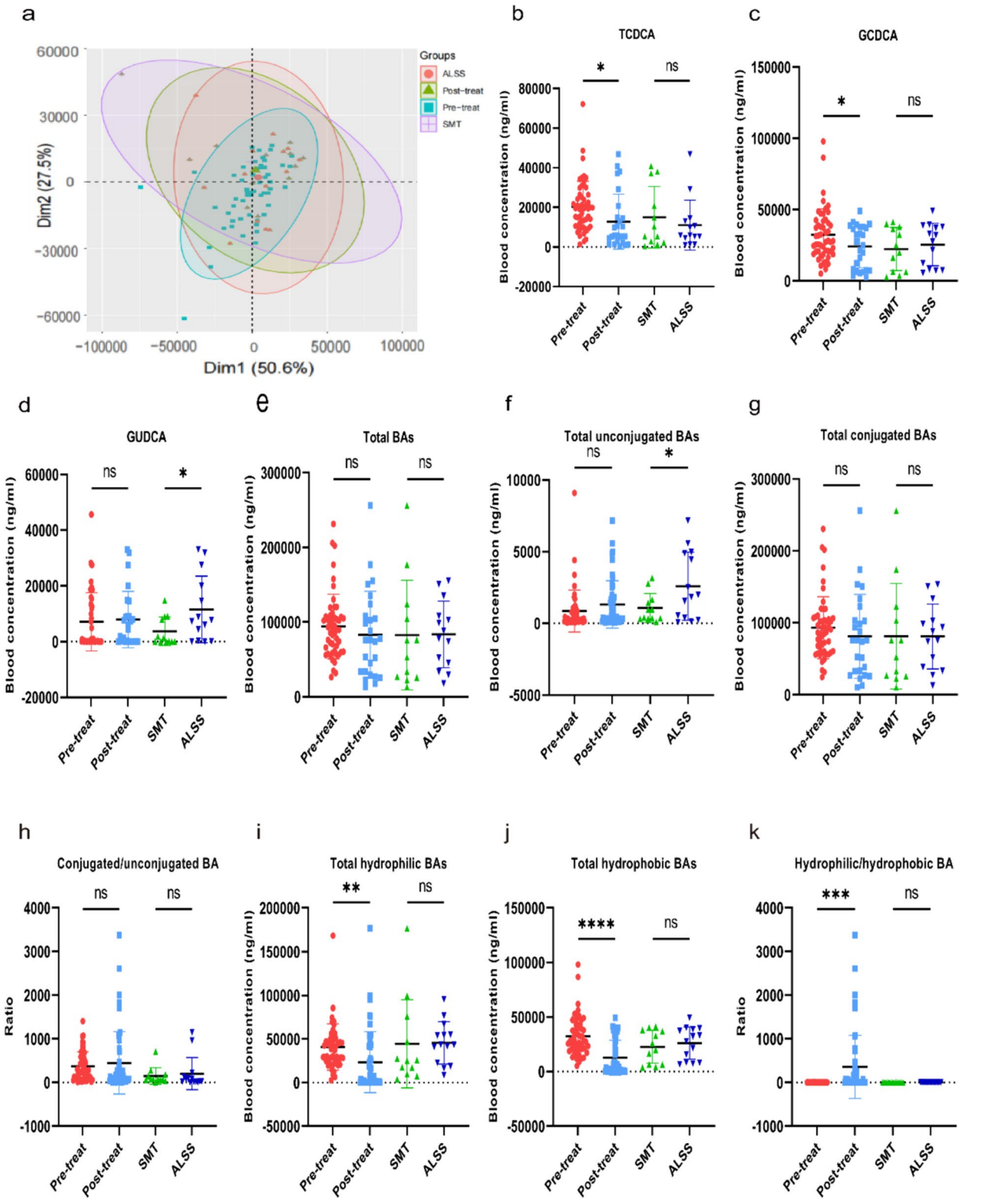


Fig. 5 The impact of ALSS on serum conjugated BAs and various combinations of BAs in ACLF patients. **a** The PCoA analysis of serum BAs after 14 days of treatment. **b–g** The blood concentrations of TCDCA, GCDCA, GUDCA, total BAs, total unconjugated BAs and total conjugated BAs. **h** The ratio of conjugated to unconjugated BAs. **i, j** The blood concentrations of total hydrophilic BAs and total hydrophobic BAs. **k** The ratio of hydrophilic to hydrophobic BAs. Pre-treat: The blood concentration of BAs before treatment; Post-treat: The blood concentration of BAs after 14 days of treatment; *SMT* The blood concentration of BAs after 14 days of treatment in SMT group, *ALSS* The blood concentration of BAs after 14 days of treatment in ALSS group. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicating not significant

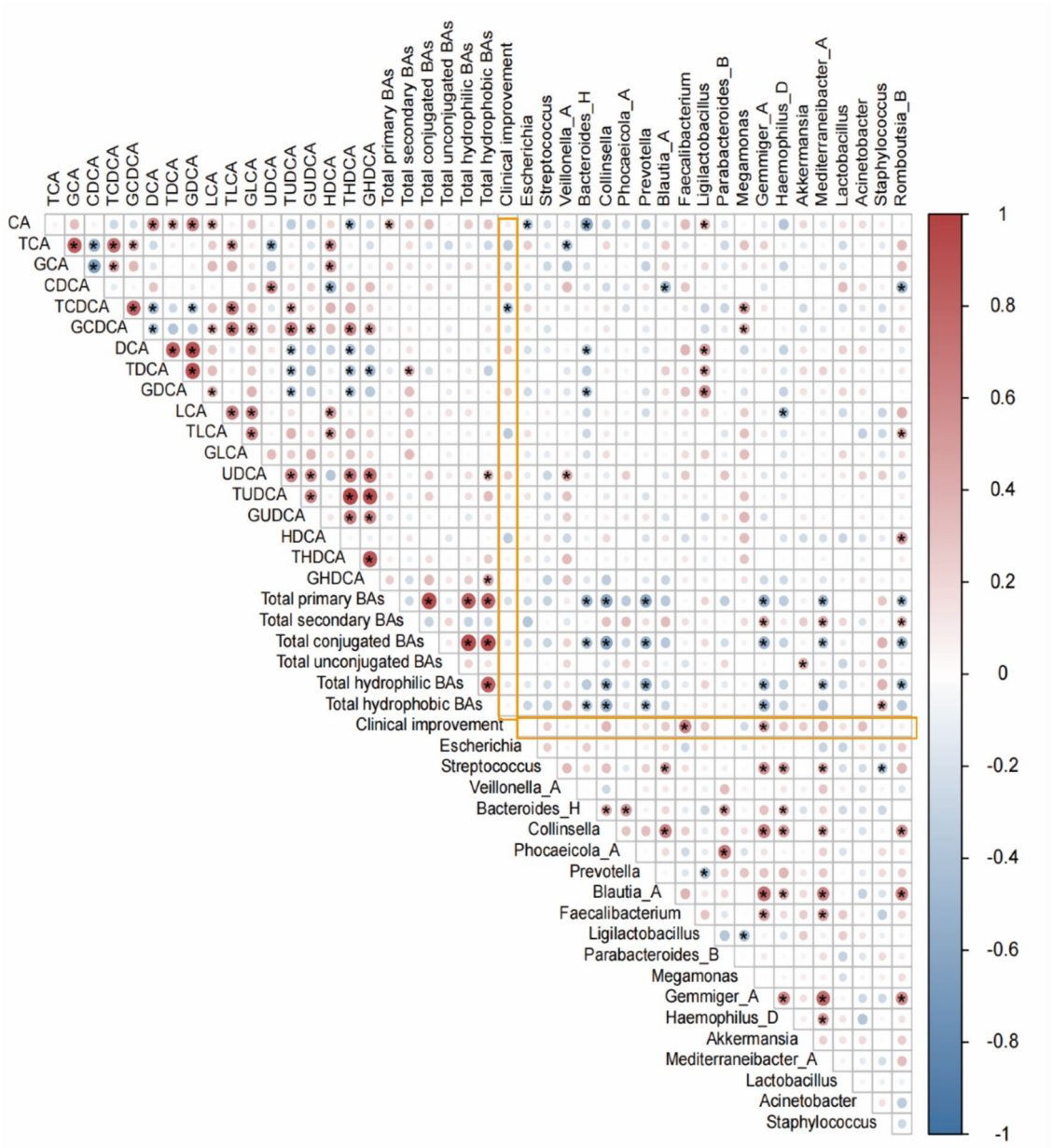


Fig. 6 Spearman correlation analysis of intestinal microbiota (Top 20), BA profiles, and clinical prognosis. *p* values are represented in red and blue, where blue indicates a negative correlation and red indicates a positive correlation. $*p < 0.05$

through analyzing the correlations between intestinal microbiota, serum BA profiles, and clinical outcomes in ACLF patients, we observed a positive correlation between *Faecalibacterium* and *Gemmiger* with clinical improvement ($p < 0.05$), and an inverse correlation between TCDCA and clinical improvement ($p < 0.05$), consistent with previous research findings [14, 30].

This study has some limitations. Firstly, this study is based on a small number of cases from one center, and the subgroups were not balanced in sample size, this may breed out some bias. Secondly, the absence of female patients in the study may introduce a bias that limits the generalizability of the findings. Lastly, the challenges faced in sample collection, including infection, liver transplant rejection, and mortality, can significantly impact the sample size and may lead to biased results.

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Author contributions All authors contributed to the study's conception and design. Yuyu Zeng: writing the original draft and methodology.

Dakai Gan: writing the original draft and data curation. Kaige Zhang: software and data curation. Tao Long: supervision. Yan He, Rui Zhou and Shuanglan Liu: validation and investigation. Molong Xiong: conceptualization and supervision. All authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability The datasets presented in this study can be found in online repositories. The names of the repositories and accession numbers can be found in the article.

Declarations

Conflict of interest Yuyu Zeng, Dakai Gan, Kaige Zhang, Tao Long, Yan He, Rui Zhou, Shuanglan Liu and Molong Xiong declare that they have no conflict of interest. The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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